PUDRITH, CHARLES BRIAN, PhD. Association of Pigmentation and Melanocortin-one Receptor Genotype with Susceptibility to Noise-induced Hearing Loss in College-aged Music Students. (2017)
Directed by Dr. Susan Phillips. 99 pp.

Approximately 13% of the American population between the ages of 20-69 have signs of noise-induced hearing loss. Noise exposure at least partially causes hearing loss by generating free radicals in the ear, which damage cells. Eumelanin is an antioxidant that scavenges the free radicals and may protect the ear from noise-induced damage. Eumelanin is also a polymer that depicts hair and eye color and sensitivity to sunlight. Production of eumelanin is partially regulated by the gene, melanocortin-one receptor; individuals with single-nucleotide polymorphisms in this gene have reduced eumelanin expression. The purpose of this study was to measure the extent to which pigmentation, e.g. hair and eye color and sunlight sensitivity, is associated with noise-induced hearing loss, and measure the extent to which single nucleotide polymorphisms in the melanocortin-one receptor gene are associated with noise-induced hearing loss. To accomplish this goal, we used a phased approach design and first evaluated hearing loss and pigmentation in 155 student musicians. This data was used to measure the association of pigmentation and noise-induced hearing loss. Then, buccal cells were collected in 111 student musicians with low to moderate levels of sunlight sensitivity so that we could measure single nucleotide polymorphisms

in the melanocortin-one receptor gene. According to two multifactor analyses of variance, no association was found between noise-induced hearing loss and pigmentation ($F_{(82,72)} = 0.707$, $p = 0.936$), nor melanocortin-one receptor genotype ($F_{(79,39)} = 0.488$, $p = 0.996$). Despite our statistically insignificant results, we were able to detect a trend of increased thresholds in individuals with pigmentation indicating decreased levels of eumelanin. Also, one single nucleotide polymorphism, rs2228479, did show enough of an association with noise-induced hearing loss to warrant further investigation. Our inability to detect significant effects may have been due to an unexpected decrease in audiometric thresholds compared to previous measurements in this population. In this study, we used ER-3A inserts to evaluate hearing, which are more reliable for measuring thresholds between 4000 and 8000 Hz compare to TDH-39's, which were used in previous analyses. It is also possible that we would have detected a stronger association of melanocortin-one receptor genotype and noise-induced hearing loss if we had sequenced the entire gene. Therefore, further research is required to evaluate the effects of noise exposure on student musicians using more sensitive audiometric criteria. Also, the association of melanocortin-one receptor genotype and noise-induced hearing loss should be evaluated with the entire melanocortin-one receptor sequence.

ASSOCIATION OF PIGMENTATION AND MELANOCORTIN-ONE RECEPTOR GENOTYPE WITH SUSCEPTIBILITY TO NOISE-INDUCED HEARING LOSS IN COLLEGE-AGED MUSIC STUDENTS

by

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A Dissertation Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro
2017

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ACKNOWLEDGEMENTS

I would like to express my deep gratitude to Susan Phillips for her constant guidance and dedication. I would like to thank my committee members, Vincent Henrich, Scott Richter, and Denise Tucker for their support. I would also like to thank my previous mentors, Bharti Katbamna, Timothy Jung, Derek Wildman, and Paul Coussens for all that they have taught me. And, a special thanks goes to the School of Heath and Human Services Theodore and Loreta Williams Research Fund in Arts Health and the American Speech and Hearing Association Foundation for their financial support for this project.

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CHAPTER I

INTRODUCTION

Approximately 13% of the American population between the ages of 20-69 have a hearing loss with a notched audiometric profile in at least one ear, indicating a possible noise-induced threshold shift (Mahboubi et al., 2013). Prolonged exposure to noise can increase these threshold shifts until they lead to noise-induced hearing loss. Major factors associated with noise-induced hearing loss include, occupation, age, genetics, and veteran status. Minor factors include gender, education level, marital status, smoking history, and diabetes.

Individuals who suffer from hearing loss struggle to communicate, which puts a strain on relationships and can lead to depression and anxiety. Hearing loss can increase the risk of injury (Cantley et al., 2015), and recent research has also shown that hearing loss may reduce cognitive function in the elderly (Taljaard, Olaithe, Brennan-Jones, Eikelboom, & Bucks, 2016). Modern hearing aids alleviate some of the communication difficulties caused by hearing loss, but they still have limitations. For instance, Kuk, Lau, Korhonen, & Crose et al., (2015) have recently shown that individuals with moderate to severe sensorineural hearing loss wearing modern hearing aids perform worse on an auditory perception task than normal hearing individuals.

The effect that noise has on hearing can be predicted from the volume of the noise and the length of exposure, but this prediction only explains a small percent of the variability in hearing loss within noise-exposed individuals (American National Standards Institute, 1996). This variability may at least partially be explained by the characteristics of the noise and the genetics of exposed individuals (Phillips et al., 2015; Tufts, Weathersby, & Marshall, 2009).

In an attempt to measure the role of genetics in susceptibility to noise exposure, (Demeester et al., 2010) measured bulge depths, another indicator of noise-induced hearing loss, in a Flemish sibling study and found that 23-30% of the variance of noise-induced hearing loss is regulated by genetics. More work is needed to confirm these results by repeating the study in another population and using a measurement of noise-induced hearing loss that has been validated.

Research into noise-induced hearing loss has been hindered by the inconsistency in techniques for measuring the effect of noise on hearing across studies. Clinicians typically use audiometric notches and case history to diagnose noise-induced hearing loss. It is difficult to combine these factors; therefore, the diagnosis is typically based on judgment (Rabinowitz et al., 2006). But, despite the likely low type I error rate of notches, they are not useful population studies because they correlate poorly with reports of noise exposure. According to unpublished results in our laboratory, the mean bilateral pure-tone average at 4000 and 6000 Hertz has a stronger correlation with reports of noise exposure than notch definitions. This may explain why many studies focusing on the

genetics of noise-induced hearing loss use threshold measures as a diagnostic measurement (Sliwinska-Kowalska & Pawelczyk, 2013).

Music students are susceptible to noise-induced hearing loss. In a study by Phillips, Henrich, & Mace, (2010), 45% of student musicians had an audiometric notch of at least 15 dB in at least one ear, which is an indicator of noise-induced hearing loss. These students are at a high risk for NIHL because they are exposed to loud levels of sound and are reluctant to wear hearing protection (McIlvaine, 2012). Students often report that they do not wear these devices because they believe that the devices disrupt the quality of music (Killion & Stewart, 1988).

Noise at least partially damages the cochlea by generating reactive oxygen species, a set of immunoregulatory free radicals that at high concentrations, can lead to DNA damage and can cause lipid peroxidation (Henderson, Bielefeld, Harris, & Hu, 2006). The extent to which reactive oxygen species leads to noise-induced hearing loss is still not fully understood.

Antioxidants are any type of chemical that inhibit oxidation. These chemicals can also scavenge free radicals, such as reactive oxygen species. There are many endogenous antioxidants in the ear that may protect the ear from noise-induced reactive oxygen species. Eumelanin, a type of melanin, is one antioxidant that may protect the ear from noise-induced reactive oxygen species.

Population studies have supported the role of eumelanin in hearing loss. Individuals with indicators of high melanin levels, including hair, race, and sun-sensitive skin type, are more resistant to hearing loss than those with indicators of low levels of eumelanin (Da Costa, Castro, & Macedo, 2008; Ghazizadeh, Bakhshaee, Mahdavi, & Movahhed, 2012; Ishii & Talbot, 1998; F. R. Lin et al., 2012). Post-mortem studies have found an association between race and eumelanin levels in the cochlea, but more work is needed to evaluate the extent to which pigmentation indicators predict cochlear eumelanin levels (Sun et al., 2014).

Along with pigmentation, genotype can be also used to estimate cochlear eumelanin levels. Melanocortin-one receptor is the gene with the strongest association with eumelanin production (Sturm et al., 2003). Some single DNA sites that are on this gene and vary throughout the human population, which are called single-nucleotide polymorphisms, have a strong association with eumelanin production. These sites are labeled as ‘R’ alleles. Single-nucleotide polymorphisms with a weak association with eumelanin production are labeled as ‘r’ alleles. To date, no studies have measured the association of noise-induced hearing loss with single-nucleotide polymorphisms on the melanocortin-one receptor gene.

The purposes of this study are to 1) measure the extent to which pigmentation characteristics are associated with susceptibility to noise-induced hearing loss within college aged student musicians, and 2) measure the extent to

which single-nucleotide polymorphisms on the melanocortin-one receptor gene are associated with susceptibility to noise-induced hearing loss in college aged student musicians with moderate to low levels of eumelanin.

As seen in Figure 1., noise generates a particular reactive oxygen species called super oxide. The fate of this chemical depends on the concentration of antioxidants, such as eumelanin, in the surrounding area. If the antioxidant levels are low, it is more likely to be metabolized to the hydroxyl radical, which can cause cell damage and noise-induced hearing loss. Alternatively, if the antioxidant levels are high, it becomes more likely that super oxide will be converted back to oxygen.

The ability to produce eumelanin depends partly on the genotype of melanocortin-one receptor. The wild type version of this gene produces eumelanin at standard rates; individuals with particular single-nucleotide polymorphisms in this gene have reduced rates of eumelanin production. Therefore, individuals with the single-nucleotide polymorphisms may struggle to produce enough eumelanin to prevent the production of the hazardous hydroxyl radical, leading to susceptibility to noise exposure.

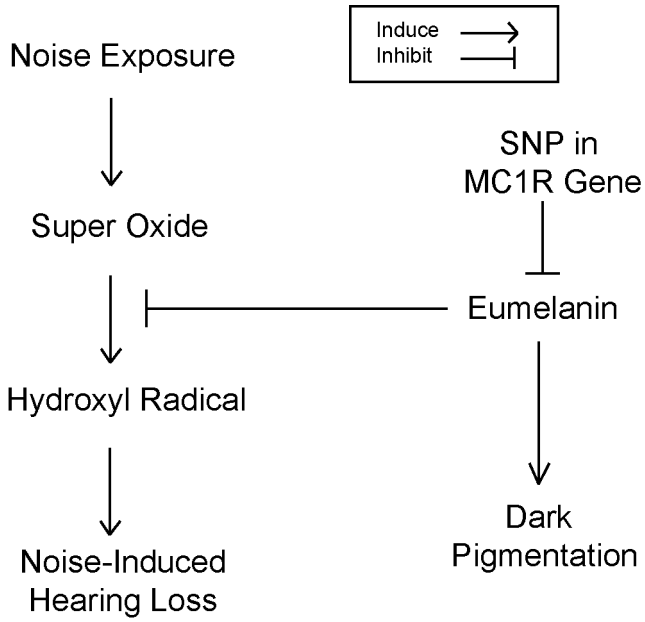

Figure 1. Theoretical Model Demonstrating the Effects of Melanocortin-one Receptor on Noise-induced Hearing Loss. SNP, Single-nucleotide Polymorphism; MC1R, Melanocortin-one Receptor

Pigment characteristics, such as hair and eye color, sunlight sensitivity, and ethnicity, are markers for eumelanin production. Consequently, these characteristics may be associated with super oxide metabolism rates, and therefore, susceptibility to noise-induced hearing loss.

This has led me to two hypotheses.

- Music students with pigment characteristics indicating low levels of eumelanin production will have higher noise-exposure adjusted audiometric thresholds than music students with pigment characteristics indicating high levels of eumelanin

- Music students with moderate to high sun-sensitive skin types and specific single-nucleotide polymorphisms in the melanocortin-one receptor gene will have higher noise-exposure adjusted audiometric thresholds than music students with moderate to high sun-sensitive skin types and a wild-type melanocortin-one receptor gene.

This project will improve upon the status quo by examining the extent to which individual pigmentation groups are associated with noise-induced hearing loss. To the best of our knowledge, all studies measuring the association of pigmentation and hearing loss in noise-exposed populations have used binary pigmentation groups (Da Costa et al., 2008; Ghazizadeh et al., 2012; Ishii & Talbot, 1998). In one study of the general population, Lin et al., (2012) measured the association of hearing loss with multiple ethnicities and skin type group. The authors were only able to detect an association of skin-type and hearing loss within those of Hispanic descent. This project may improve upon Lin et al., (2012) by including interaction effects with other pigment indicators and melanocortin-one receptor genotype.

This project will improve upon the status quo by providing the first association study of melanocortin-one receptor genotype and susceptibility to noise-induced hearing loss. Although previous studies have examined the relationship between noise-induced hearing loss and genotypes of other genes related to reactive oxygen species metabolism, to the best of our knowledge, no

study has measured the association of noise-induced hearing loss with this particular gene.

This project will also improve upon the status quo by providing the first association study of eumelanin indictors and susceptibility to noise-induced hearing loss in young adults. Previous studies in this area have examined hearing loss in older adults because they have more years of noise exposure (Da Costa et al., 2008; Ghazizadeh et al., 2012; Ishii & Talbot, 1998). It may be easier to detect predictors of noise-induced hearing loss in younger populations because this population has lower incidences of other factors associated with noise-induced hearing loss, such as diabetes, cardiovascular issues, and exposures to ototoxins. Student musicians are an ideal population because previous studies have shown that these individuals have noise-induced hearing loss (Phillips et al., 2010; Phillips & Mace, 2008).

Detecting an association of pigmentation characteristics and single-nucleotide polymorphism within the melanocortin-one receptor gene with susceptibility to noise-induced hearing loss within college aged student musicians will provide evidence to support the role of eumelanin in the reduction of noise-induced reactive oxygen species damage to the cochlea.

This work may have three primary benefits to society.

- This project may lay the groundwork for future research comparing the protective effects of endogenous antioxidants found in the ear, including

melanin agonists, which can assist in the development of pharmaceuticals to prevent and treat noise-induced hearing loss.

- This project may lead to clinical studies on the effectiveness of genetic screenings for individuals either participating in noisy activities or showing early signs of noise-induced hearing loss.
- This project may also help improve the field of pharmacogenetics, where genotypes are used to evaluate which individuals are likely to benefit from pharmaceutical therapy.

CHAPTER II

REVIEW OF THE LITERATURE

Cellular and Molecular Biology of Noise-induced Hearing Loss

Reactive Oxygen Species

Noise-induced cochlear injury can be mechanical or metabolic. In mechanical injury, high volumes break apart stereocilia and disrupt the membranes that separate cochlear fluids, where metabolic injuries pull the cells away from homeostasis, leading to cell death if the cell cannot restore equilibrium. Oxidative equilibrium is one homeostatic state affected by noise. In oxidative equilibrium, reactive oxygen species are balanced with antioxidants. When exposed to noise, the ear increases adenosine triphosphate production (Ohlemiller, Wright, & Dugan, 1999). When cells generate too much adenosine triphosphate, reactive oxygen species are generated as a by-product. Noise has been shown to increase reactive oxygen species levels four fold in the perilymph. These chemicals have also been found in mice 2.2 hours after noise exposure and have been shown to persist for up to 10 days (Daisuke Yamashita, Jiang, Schacht, & Miller, 2004).

Free radicals are any chemicals with an unpaired electron. Reactive oxygen species are free radicals that specifically have an unpaired electron attached to an oxygen atom that readily destroys important biological chemicals,

including lipids and DNA. Reactive oxygen species are primarily generated in the mitochondria. Here, superoxide ions are formed as a result of an incomplete reduction of oxygen in the electron transfer chain. Other reactive oxygen species producers include NADPH and arachidonic acid.

High levels of reactive oxygen species can lead to cell death. Although it is difficult to measure reactive oxygen species during cell death, chemical markers have helped to identify the effects of these chemicals on cells. For example, it has been shown that noise increases levels of Isoprostane, an indicator of oxidative damage, in outer hair cells (Ohinata, Miller, Altshuler, & Schacht, 2000). Therefore, it is likely that reactive oxygen species caused the noise-induced death of these hair cells.

Reactive oxygen species agonists and antagonists have also helped to support the role of these chemicals in noise-induced hearing loss. For instance, Bielefeld, Hu, Harris, & Henderson, (2005) showed that placing paraquat, a chemical that generates reactive oxygen species, near the round window membrane of chinchillas produces more hearing loss than saline controls. Also, iron, a reactive oxygen species agonist and acidifying agents have been shown to exacerbate hearing loss when the ear is stimulated with reactive oxygen species activating drugs (Song, Sha, & Schacht, 1998; Tanaka, Whitworth, & Rybak, 2004).

Reactive oxygen species can kill cells through multiple mechanisms. For instance, these chemicals can lead to lipid peroxidation, which breaks down

cellular membranes. Unfortunately, as the membranes break down, more reactive oxygen species leak out and damage other cells, causing a negative feedback loop (Ohinata et al., 2000). Cell membrane deterioration leads to cell death through a process called necrosis. Ohinata, Miller, & Schacht, (2003) supported the role of lipid peroxidation in noise-induced hearing loss by showing that chemicals that that preserve cell membranes reduce noise-induced damage.

Reactive oxygen species can also kill cells through a process of cell-mediated suicide known as apoptosis. Several studies have demonstrated the activation of different apoptotic pathways in animals exposed to noise. For instance, noise exposure leads outer hair cells to activate c-Jun N-terminal kinase-signaling pathway, which is known to mediate apoptosis with reactive oxygen species (Kamogashira, Fujimoto, & Yamasoba, 2015). The role of reactive oxygen species in apoptosis was also supported when Huang et al., (2000) demonstrated that in cisplatin-induced ototoxicity, reactive oxygen species activates the apoptotic mediator 4-hydroxynonenal. Furthermore, Baker & Staecker (2012) showed that applying hydrogen peroxide, a pro-oxidant, to ex-vivo cochlear cells leads an upregulation of the pro-apoptotic gene, caspase, and eventually, cell death. In combination, these studies support the effect noise-induced reactive oxygen species may have on apoptosis in the cochlea.

Antioxidants

Antioxidants are chemicals that prevent the damaging effects of reactive oxygen species. As seen in Figure 2., super oxide, a reactive oxygen species,

may either cause cell damage or become metabolized into an inert chemical like oxygen or water. Antioxidants are the metabolizers that convert super oxide into these inert chemicals. Therefore, the fate of super oxide depends on the concentration of antioxidants. Due to the high metabolic activity in the cochlea, both the organ of Corti and the stria vascularis have high concentrations of various types of antioxidants, including superoxide dismutase, catalase, and glutathione peroxidase.

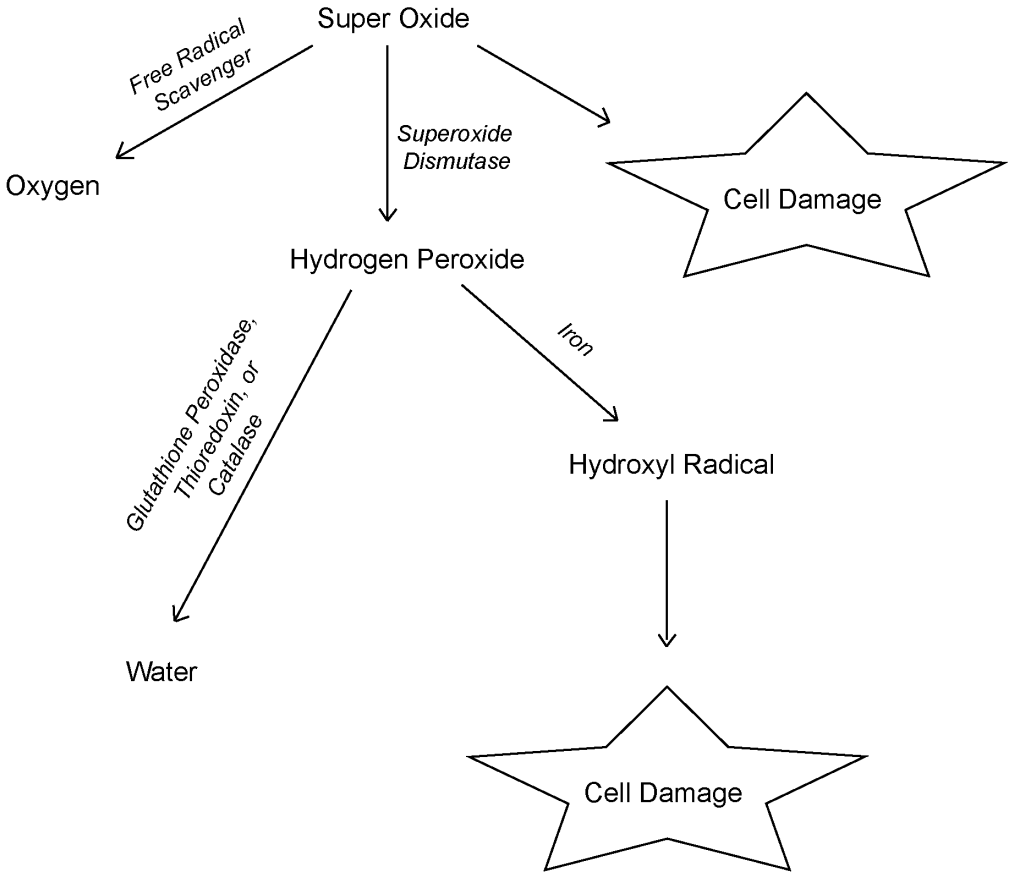

Figure 2. Reactive Oxygen Species metabolism

The use of pharmaceutically administered antioxidants has supported the role of reactive oxygen species in noise-induced hearing loss. For instance, Yamashita, Jiang, Le Prell, Schacht, & Miller, (2005) have demonstrated that antioxidants can reduce noise-induced hearing loss for up to three days after noise exposure. Specific antioxidants shown to decrease NIHL include the glutathione precursors, glutathione modoethyl, N-acetylcysteine, and ascorbic acid (Hight, McFadden, Henderson, Burkard, & Nicotera, 2003; Lorito, Giordano, Petruccelli, Martini, & Hatzopoulos, 2008). N-acetyl-cysteine also has some effect in preventing NIHL when administered shortly after noise exposure.

Although antioxidants have been shown to protect against NIHL in animals, there is currently little evidence that these drugs are effective in human clinical trials. Recently, a randomized clinical trial in military personnel measured the protective effects of N-acetylcysteine (Kopke et al., 2015). Although no statistically significant effects were found, the difference in NIHL between treated and untreated groups approached significance. The authors attributed their lack of significant findings to suboptimal dosing.

Endogenous antioxidants may also play an important role in regulating susceptibility to NIHL. A recent mouse genome wide association study found an association between NIHL and the antioxidant, NADPH oxidase-3 (Lavinsky et al., 2015). Knocking out production of the antioxidant, superoxide dismutase increases susceptibility to noise (Ohlemiller, Rybak, Rice, Lett, & Gagnon, 2009). In humans, measuring polymorphisms in genes that code for antioxidants has

supported the role of these proteins, and reactive oxygen species, in noise-induced hearing loss. For instance, Konings et al. (2007) demonstrated that polymorphisms in the antioxidant catalase may be associated with noise-induced hearing loss. The association of polymorphisms in other antioxidants have been measured in noise-induced hearing loss, but these results are less convincing (Sliwinska-Kowalska & Pawelczyk, 2013).

Eumelanin

Melanin depicts pigmentation characteristics, particularly in hair, eyes, and skin tone. There are two types of melanin, eumelanin and pheomelanin. Eumelanin is brown, yellow, or black where pheomelanin, is red or orange.

Eumelanin is a specific antioxidant called a free radical scavenger. As depicted in Figure 2., free radical scavengers remove extra electrons, converting radical chemicals, such as super oxide, back to their original inert structure. Eumelanin is able to scavenge free radicals because it has a high ionizing radiation, which gives it strong electron accepting and donating properties (Meredith & Sarna, 2006). Although it is commonly accepted that eumelanin can neutralize reactive oxygen species, the extent to which it protects the cochlea from noise-induced hearing loss is still not understood.

Individuals with low eumelanin levels tend to have higher pheomelanin levels, which is why those with fair skin and low pigmentation also tend to have red hair. Pheomelanin is not as beneficial as eumelanin because it does not scavenge reactive oxygen species as it has an aromatic ring that lowers the

ionizing potential (Morgan, Lo, & Fisher, 2013). Production of pheomelanin produces more reactive oxygen species byproducts than eumelanin, and requires the metabolism of a number of reactive oxygen species scavengers, including glutathione.

Melanin is made in melanosomes, which are organelles found inside a specific cell called a melanocyte. Each melanosome commits to either eumelanin or pheomelanin production, but one melanocyte can contain both eumelanin and pheomelanin producing melanosomes (Slominski, 2004). Eumelanin production is initiated when alpha-melanocortin stimulating hormone binds to the membrane bound melanocortin-one receptor. The binding of these two proteins produces cyclic-AMP, which initiates the production of eumelanin synthesizing enzymes such as tyrosinase, the main enzyme linked with dictating the eumelanin/pheomelanin ratio. Eumelanin production requires more of this enzyme than pheomelanin production; therefore, a higher concentration of tyrosinase favors eumelanin synthesis.

Melanocortin-one Receptor

The melanocortin-one receptor gene has several single-nucleotide polymorphisms associated with reduced eumelanin production and pigmentation characteristics that indicate lower level eumelanin levels such as red or brown hair or blue eyes (Sulem et al., 2007). Single-nucleotide polymorphisms in melanocortin-one receptor genotype account for 67% of the variability of eumelanin levels in hair (Naysmith et al., 2004). These variants are more

prevalent in populations that live where there is little direct sunlight, likely because this skin type favors the penetration of UV into the skin to increase vitamin D synthesis.

As seen in Figure 3., melanocortin-one receptor variants fall into two phenotypic groups. The group with a strong association with pigmentation are called ‘R’ alleles; the group with a weak association with pigmentation are called ‘r’ alleles (Sturm et al., 2003). Kanetsky et al., (2010) measured the prevalence of nine non-synonymous melanocortin-one receptor single-nucleotide polymorphisms in a population of 325 Caucasians individuals from the northeast region of The United States. In control subjects from this study, ‘R’ variants had an overall frequency of about 11% and ‘r’ variants had an overall frequency of about 35%, Figure 3.

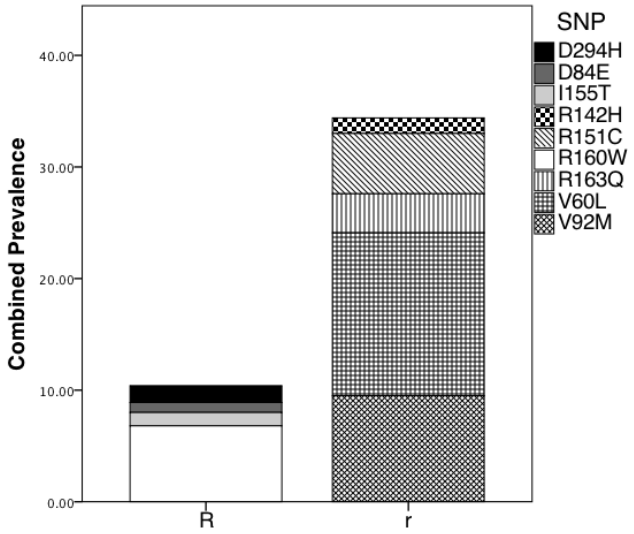

Figure 3. Prevalence of ‘R’ and ‘r’ Non-synonomous Single-nucleotide Polymorphims in a Population of 325 Caucasian Individuals (Kantesky, 2010)

Support for Eumelanin as an Otoprotectant in Noise-induced Hearing Loss

Individuals with pigmentation indicating low eumelanin levels are more susceptible to noise-induced hearing loss. As seen in Table 1., population studies have measured audiometric thresholds in noise exposed individuals and found decreased thresholds in individuals with pigment indicators that indicate low eumelanin levels (Da Costa et al., 2008; Ghazizadeh et al., 2012; Ishii & Talbot, 1998). Specifically, those with low eumelanin indicators have audiometric thresholds that are about 8 - 15 dB higher in the high frequency region compared to those with high eumelanin indicators. The pigment indicators used in these studies include hair and eye color, and ethnicity.

Table 1. Association Studies Supporting a Decrease in Audiometric Thresholds for Those with low Eumelanin Levels.

Eumelanin Indicator	***Population***	***High Eumelanin Hearing (SD)***	***Low Eumelanin Hearing (SD)***	***P Value***	***Reference***
Hair Color (Light/Dark)	*Military*	*14.7 (13.6)*	*29.5 (17.3)*	*0.008*	*Ghazizadeh, 2012*
Race (White/ Non-White)	*Metal Workers*	*17.7 (12.1)*	*26.0 (13.6)*	*<0.001*	*Ishii, 1998*
Race (White/ Non-White)	*General Population*	*14*	*22*	*<0.001*	*Lin, 2012*
Iris Color (Light/Dark)	*Metal Workers*	*17.2 (13.1)*	*26.0 (15.0)*	*<0.01*	*Da Costa, 2008*

Lin et al. (2012) also measured audiometric thresholds in individuals without noise exposure to determine the association of skin type and ethnicity with high frequency hearing loss. The authors found that among Caucasians,

Hispanics, and African Americans, skin type is only associated with hearing loss within Hispanics, likely because this race has a larger range of skin types compared to Caucasians and African Americans.

The role of eumelanin as an otoprotectant from noise exposure has been demonstrated in guinea pigs. Xiong, He, Lai, & Wang, (2011) have shown that noise exposure leads to more reactive oxygen species in the ear and hearing loss in animals with light coats compared to animals with dark coats. Animals with dark coats likely have more eumelanin in the cochlea, which reduces reactive oxygen species concentrations, thereby protecting the animal form noise-induced hearing loss. Also, pigmented mouse strains have a thicker stria vascularis than genetically similar albino strains (Ohlemiller et al., 2009). This thicker stria vascularis may permit the transmission of nutrient supplements and inflammatory mediators to the organ of Corti.

To date, no study has demonstrated an association between melanocortin-one receptor single-nucleotide polymorphisms and noise-induced hearing loss; however, our group has found preliminary evidence to support this association. In a recent study, we used a case control analysis to measure the association of 205 variants and notched audiograms, an indicator of noise-induced hearing loss, in students musicians (Phillips et al., 2015). To do this, we selected students from a population of 640 to create three case control groups of 81: group one had no audiometric notches, group two had a unilateral notch, and group three had bilateral notches. Of the individuals with melanocortin-one

receptor single-nucleotide polymorphisms, more students had bilateral notches compared to those with no notches, indicating that melanocortin-one receptor may be associated with noise-induced hearing loss (Table 2.).

Table 2. Distribution of Music Students with no Notches (NN), Unilateral Notches (UN), and Bilateral Notches (BN), for five Melanocortin-one Receptor Single-nucleotide Polymorphisms (Phillips, 2015). SNP, Single-nucleotide Polymorphism; AA, amino acid; synonymous indicates that the nucleotide substitution does not affect the protein secondary structure

SNP	DNA Substitution	AA Substitution	Students		
			NN	UN	BN
rs1110400	C/T	I155T	0	2	3
rs117952079	C/T	A57V	0	2	4
rs2228478	A/G	synonymous	0	0	2
rs2229617	G/A	G104S	1	3	4
rs34090186	G/A	R67Q	0	0	4

Monogenetic disorders with pigment phenotypes also indicate that eumelanin may play a role in NIHL. Genetic disorders such as Waardenberg syndrome and Vitiligo lead to hearing loss and affect melanin production (Angrisani, de Azevedo, Pereira, Lopes, & Garcia, 2009). The combination of hearing loss and changes in pigmentation from a single mutation demonstrate that sensitivity to noise exposure can be caused by a gene that causes changes in pigmentation.

Potential Pathophysiology of Eumelanin Protection in Noise-induced Hearing Loss

Protection of Spiral Ganglion Neurons

Noise leads to the formation of reactive oxygen species in spiral ganglion neurons (Xiong et al., 2011). These cells are housed in Rosenthal's canals, which, as indicated by recent studies have supported, also has eumelanin (Sun et al., 2014). It is currently unknown if the melanin in Rosenthal's canals protect spiral ganglion neurons; however, African Americans, who are more resistant to noise-induced hearing loss, do have more eumelanin in their Rosenthal's canals than Caucasians. This physiological difference in eumelanin levels between races may explain their differences in sensitivity to noise, but more research is needed to support this claim.

Protection of the Organ of Corti

Hair cells in the organ of Corti are more sensitive to noise exposure than any other cell in the cochlea (Liberman & Dodds, 1984). Although there is no eumelanin in the organ of Corti, eumelanin may protect these cells indirectly by protecting the stria vascularis, which houses melanin-synthesizing melanocytes. Extra-cochlear chemicals enter the cochlea through the capillaries in the stria vascularis. These chemicals permeate to the endolymph and then to the rest of the cochlea. It stands to reason that if these chemicals are unable to exit the stria vascularis, then they may not reach the organ of Corti and provide protection

from noise-induced damage. Although this mechanism has not been validated, many studies have provided evidence to support this pathway.

Noise may disrupt stria vascular permeability through many different methods. For instance, noise may disrupt permeability by interfering with the blood-labyrinth barrier. This barrier is formed by endothelial cells that form tight junctions around capillaries. These junctions increase and decrease permeability in response to regulatory agents (Zhang et al., 2012). When noise damages the stria vascularis, it first swells up and then shrinks to a width below the starting width (Wang, Hirose, & Liberman, 2002). This swelling and shrinking may disrupt the tight junction regulation of capillary permeability. Another mechanism that noise may use to disrupt stria vascular permeability is to reduce cochlear blood flow (Scheibe, Haupt, & Ludwig, 1993). Any decrease in blood flow may reduce the transmission of supplements to the organ of Corti. Noise may also disrupt permeability by depleting the supply of the ubiquitous active transport secondary messenger, ATP (Yang et al., 2011). As cells lose their ATP, they also lose their ability to actively transport secondary messengers across membranes. Collectively, noise-induced reactive oxygen species may decrease permeability in the stria vascularis by interrupting tight-junction regulation, disrupting blood flow, and depleting ATP. Increases in eumelanin may protect the stria vascularis, allowing it to continue to provide supplements through the endolymph to the organ of Corti.

It is difficult to determine which chemicals are transmitted through the stria vascularis, and of these, which are necessary supplements for the organ of Corti to repair itself from noise-induced damage. However, recent developments have allowed researchers to predict the chemicals in the cochlea by examining proteins that actively transport chemicals across the membranes involved in the blood-labyrinth barrier. Uetsuka et al., (2015) has helped to identify 25 novel ion channels and 79 novel transporter channels in this region that can transport a host of secondary messengers, many of which are important in the immune response, including phospholipids, myo-inositol, thyroid hormones, fatty acids, and riboflavin.

Secondary messengers can help the organ of Corti repair itself from noise induced damage by either providing nutrients to support repair or providing inflammatory mediators to induce a series of pathways that lead to cell mediated suicide, which is known as apoptosis. The role of nutrient support is poorly understood; however, extrinsic inflammatory mediators that regulate apoptosis have been well characterized (Furness, 2015). Mediators known to bind to hair cells include FAS, FADD, caspase-8, TNF-alpha, and TRAIL. Once bound, these messengers induce apoptosis, which is important for health of the organ of Corti.

Apoptosis is important to the cochlea because it prevents cells from killing neighboring cells when they die. This is because when these pathways are initiated, the cell membrane collapses in and traps hazardous chemicals into small compartments that are ingested by immune cells (Furness, 2015). Once

trapped, the hazardous chemicals are neutralized and destroyed so that the dead cell can be absorbed by immune cells and do not cause any harm to cell in the surrounding environment. If extrinsic apoptotic messengers are cut off, then the cell will become increasingly unhealthy until it dies from necrosis. Unlike apoptosis, necrosis is dangerous to surrounding cells because it leads to the release of hazardous contents into the environment (Szondy, Garabuczi, Joos, Tsay, & Sarang, 2014). This is especially dangerous in the organ of Corti because it is an enclosed space with poor fluid movement, and many of these cells form tight membranes that maintain ionic balance. Specifically, the lateral membrane of outer hair cells make up the reticular lamina, and unlike apoptosis, necrosis is more likely to leave a hole in this membrane. This would lead to a strong influx and efflux of endolymph and perilymph, thereby disrupting the electrochemical potential of outer hair cells. This disruption may lead to the '3rd death pathway,' where the basilar end of outer hair cells are ruptured, possibly due to a rapid exposure to high levels of potassium that enter through a hole in the reticular lamina (Bohne, Harding, & Lee, 2007).

Eumelanin may protect the organ of Corti by preventing noise-induced damage to the stria vascularis, thereby maintaining transmission of extracochlear signals for cellular repair or apoptosis. However, more research is needed to validate these pathways.

Justification of Technical Aspects

Measuring Noise-induced Hearing Loss

There are no universal standards for assessing the effect of noise on hearing. This leads to inconsistencies across research studies on this topic. Although there are many different ways to assess noise-induced hearing loss, the measurements fall into two main groups, binary and continuous.

In clinical research, authors will often compare the results obtained among different audiometric notches, which are binary criteria that assess the relative decrease in hearing somewhere between 3000 to 6000 Hz (Nondahl et al., 2009). The specific criteria for each of these notches varies based on the thresholds used and the relative threshold differences required to constitute a noise-induce hearing loss. Currently, no single definition is universally accepted.

Despite the strong clinical support for audiometric notches, many researchers use high frequency thresholds to evaluate the effect of noise on hearing. As seen in Table 1., all four studies that have measured the association of noise-induced hearing loss and eumelanin markers used high frequency thresholds to diagnose noise-induced hearing loss. This practice extends to genetic research where in a recent review article of nine genetic association studies of noise-induced hearing loss, all nine studies used similar high-frequency threshold measurements (Sliwinska-Kowalska & Pawelczyk, 2013). Researchers likely use these measurements because continuous measurements can increase power and accuracy of a study by accounting for magnitude,

especially when the continuous indicator is normally distributed within a population. For example, Kizilkaya, Fernando, & Garrick, (2014) showed that in a genetic association study, a categorical variables needs to increase the sample size by 2.25 fold to have the same power as a continuous variable.

Despite the benefits of high threshold measurements for assessing noise-induced hearing loss, the main drawback to this technique is that these unadjusted thresholds are more strongly affected by age-related hearing loss than notch definitions (Ali, Morgan, & Ali, 2015). Because of this, the association between age and high frequency thresholds may mask the association between noise and thresholds, making it difficult to detect the effect of noise on hearing.

In order to determine which method was best for evaluating noise-induced hearing loss, I measured the Pearson's correlations of reported noise exposure and hearing loss by using several different indicators of noise-induced hearing loss. However, because it is difficult to compare binary indicators, such as notch definitions, with continuous indicators, we first developed a continuous indicator that measures the likelihood of a notch called the slope adjusted notch depth.

As seen in Figure 4., the slope-adjusted notch depth is calculated as follows: First, the low and high frequency anchors for the slope adjustment line are located. Pure-tone threshold average at 500, 1000 and 2000 Hertz serves as the low frequency anchor (point A in Figure 4.) and is placed at 1000 Hz. Pure-tone threshold average at 6000 and 8000 Hertz serves as the high frequency anchor (point B in Figure 4.), and is marked at 8000 Hz. With both anchors

identified, the linear slope adjustment line is then drawn to connect the two anchors. After the slope adjustment line is calculated, the highest threshold from 3000 to 6000 Hz is marked as the upper intensity limit (point C in Figure 4.). If the highest threshold is the same at multiple frequencies then the lowest frequency is used to calculate the slope-adjusted notch depth. Next, the lower intensity limit (point D in Figure 4.) is marked. This limit is the point where the slope adjustment line crosses the frequency line of the upper intensity limit. Finally, the slope-adjusted notch depth is calculated by subtracting the lower intensity limit from the upper intensity limit.

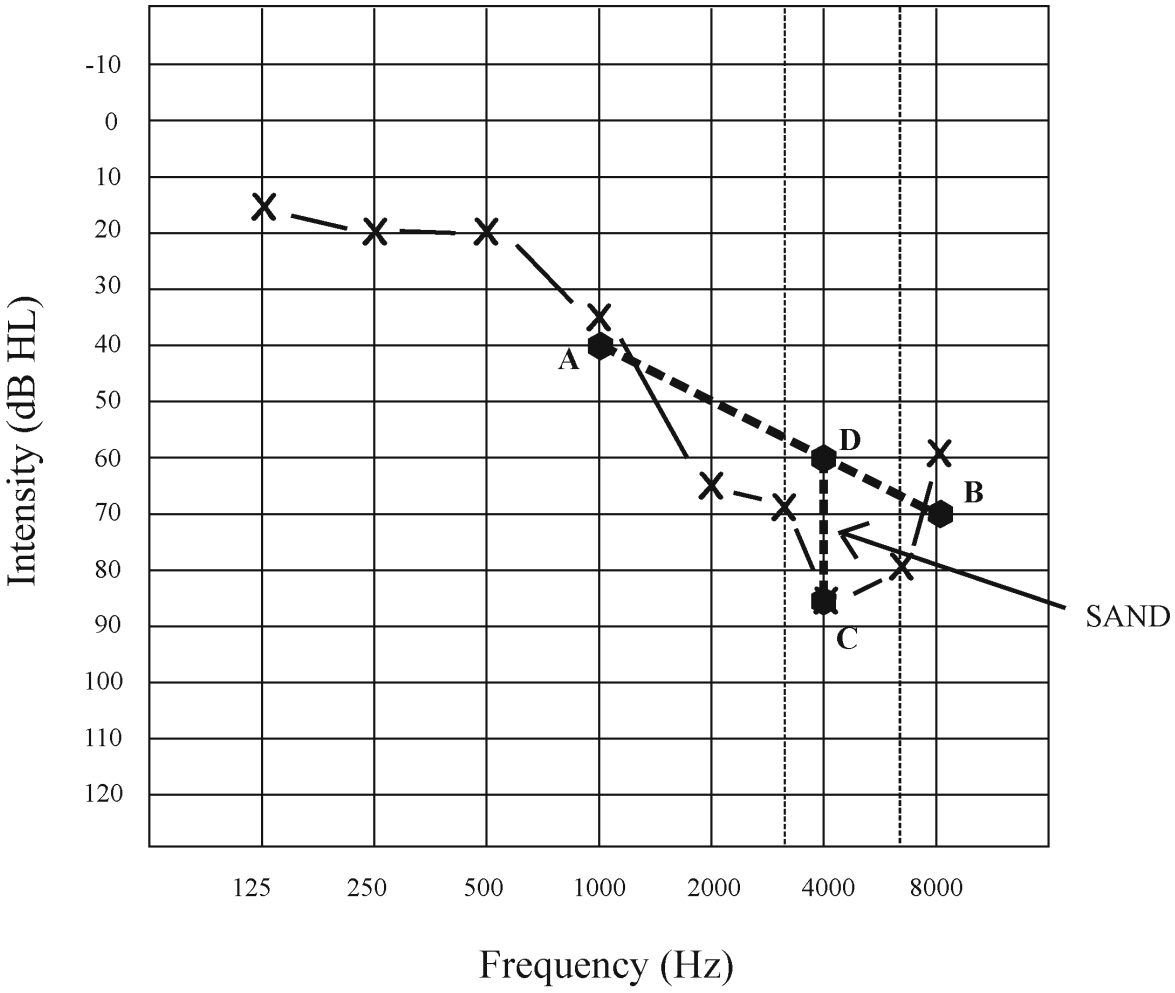

Figure 4. Diagram Showing the key Points used to Calculate the Slope-adjusted Notch Depth in a Sample Audiogram. A) Lower anchor for slope adjustment. B) Upper anchor for slope adjustment. C) High intensity threshold for Slope-adjusted Notch Depth. D) Low intensity threshold for Slope-adjusted Notch Depth. SAND, Slope-adjusted Notch Depth.

With a continuous notch measurement, we were able to compare the association of noise exposure and hearing loss using different measurements of noise-induced hearing loss, including high frequency thresholds, the slope-adjusted notch depth, and two other algorithms designed to measure noise induced

hearing loss, the notch index and bulge depth (Demeester et al., 2010; Rabinowitz et al., 2006). First we measured Pearson's correlations between hearing loss and reports of high levels of noise exposure, in months. This data was collected from the National Health and Nutrition Examination Survey database where 304 individuals reported at least one month of loud occupational noise exposure and met the inclusion criteria of the study (National Center for Health Statistics (U.S.), 2013). As seen in Figure 5., both high frequency threshold measurements were significantly (p<0.05) correlated with reports of noise exposure. Hearing loss measured with the slope adjusted notch depth, notch index, and bulge index did not significantly correlate with noise exposure.

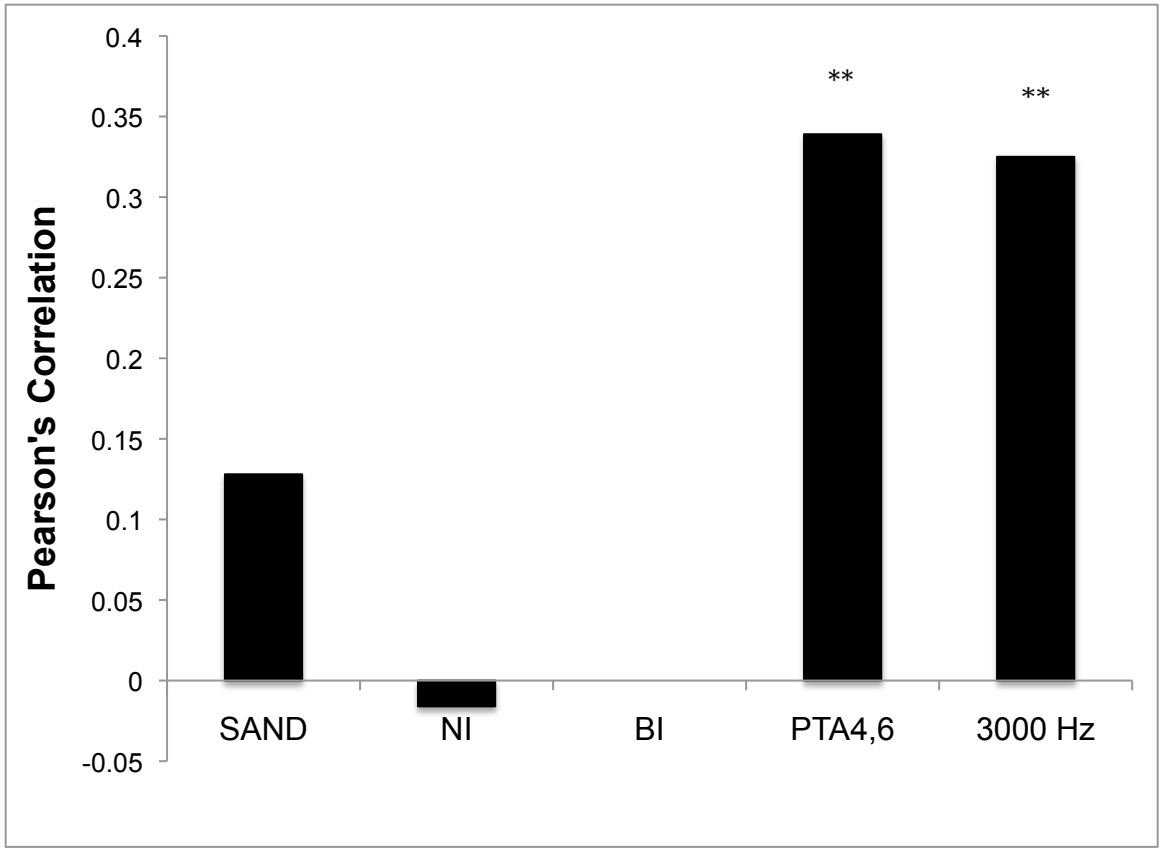

Figure 5. Pearson's Correlation of Noise Exposure and Hearing Loss with five Different Indicators of Noise Exposure. SAND, Slope Adjusted Notch Depth; NI, notch index; BI, bulge index; PTA4,6, pure-tone threshold average at 4000 and 6000 Hertz.

To further evaluate the association of noise exposure and hearing loss, the effects of hearing protection and age were measured in a multiple linear regression model for each indicator. Noise was measured in months exposed to very loud levels, age was measured in years, and hearing protection was measured in reported percent of time it is used when exposed to loud sounds. All values were mean centered to evaluate their average affects. This model also included the interaction effects of hearing protection and noise exposure, and

age and noise exposure. The multiple linear regression models were run for all five diagnostic indicators, but the overall model was only significant ($p<0.05$) for the slope-adjusted notch depth, the pure-tone threshold average for 4000 and 6000 Hertz, and the 3000 Hertz threshold.

The unstandardized coefficient (B) for the factors of the three significant linear regression models are listed in Table 3. All three noise-induced hearing loss measurements were significantly associated with age, after accounting for effects of the other four factors. However, none of the indicators were significantly associated with noise, after accounting for effects of the other four factors. The pure-tone threshold average at 4000 and 6000 Hertz was also significantly associated with the interaction between noise exposure and hearing protection, and noise exposure and age. These interaction effects indicate that hearing protection affects the association of noise and pure-tone threshold average at 4000 and 6000 Hertz, and that the relationship between noise and this threshold average is inconsistent across age groups.

Table 3. Unstandardized Coefficients of mean Centered Factors for Three Multiple Regression Analysis with Different Dependent Variables. Significant factors are indicated with an asterisk. SAND, slope adjusted notch depth; PTA4,6, pure-tone threshold averages at 4000 and 6000 Hertz; 3000 Hz, pure-tone threshold at 3000 Hertz.

	Indicator		
	SAND	**$PTA_{4,6}$**	**3000 Hz**
Age	0.107*	0.686*	0.548*
Noise	0.003	0.009	0.015
HP	-0.658	-2.32	-3.000
Noise*HP	-0.020	-0.048*	-0.043
Age*Noise	0.000	0.002*	0.001

In this study, we will use the pure-tone average at 4000 and 6000 Hertz to measure noise-induced hearing loss because it has the strongest Pearson's correlation with noise exposure. The interaction between age and noise found with measurement will not affect this study because our population will have a very narrow range of ages.

Pigmentation Groups

In this study, we measured the association of noise-induced hearing loss and pigmentation for four different indicators of pigmentation, each containing four to six groups. This is in contrast to previous studies measuring the association of noise-induced hearing loss and pigmentation groups, which only used two groups for each indicator (Da Costa et al., 2008; Ghazizadeh et al., 2012; Ishii & Talbot, 1998).

Measuring the association of noise-induced hearing loss and pigmentation with only two groups does not produce conclusive results because it is difficult to

determine if the hearing loss is associated with pigmentation, or genetic differences across races between the groups. As stated in the innovation section, this study improved upon previous research by measuring hearing loss within four to six pigmentation groups. This type of an analysis may have allowed us to evaluate the association of pigmentation and noise-induced hearing loss, independent of genetic differences across groups.

Genetic Selection

In this study, we analyzed the association of noise-induced hearing loss with seven single nucleotide polymorphisms on the melanocortin-one receptor gene. We analyzed the melanocortin-one receptor gene because it has a biological relevance to NIHL, there is phenotypic evidence for this association with pigment studies, and we have preliminary data supporting this association (Phillips et al., 2015). Including more genes into this study would have reduced our ability to detect an association between noise-induced hearing loss and melanocortin-one receptor.

When analyzing the melanocortin-one receptor genotype, we specifically measured the association of noise-induced hearing loss with seven of the nine single-nucleotide polymorphisms listed in Figure 3. (Two single-nucleotide polymorphism, I155T, and R160W, were not commercially available). These single-nucleotide polymorphism were studied in Kanetsky et al., (2010), who genotyped 60 melanocortin-one receptor single-nucleotide polymorphism in 396 control subjects from the Mid-Atlantic region of the US, and only found these

nine. In addition, (Latreille et al., 2009) sequenced codons 60 to 265 and 294 of MC1R in 488 Caucasian women from Paris and only found 6 individuals (1.2%) that had heterozygous substitutions that we would have missed with this 9 variant panel. From these studies, we concluded that it would be more cost effective to measure these single-nucleotide polymorphisms before expanding this project to analyze the entire gene.

Population Age

In this study, we only measured the association of noise-induced hearing loss with pigmentation characteristics and melanocortin-one receptor single-nucleotide polymorphisms in college age student musicians. This is because it is difficult to separating out the effects of age and noise, (see the section entitled “Measuring noise induced hearing loss”. Therefore, the most accurate way to evaluate the effects of noise exposure is to use a group of individuals with similar ages. Furthermore, a younger population is less exposed to many secondary factors associated with noise-induced hearing loss, such as smoking, diabetes, exposure to organic solvents, are associated with noise-induced hearing loss (Mahboubi et al., 2013). This technique is rare in noise-induced hearing loss association studies because older populations are much larger and have more noise exposure; however, we believe that this method is more effective because it increases our ability to detect those susceptible to noise-induced hearing loss.

And despite their age, college-aged music students are still exposed to enough noise to cause noise-induced hearing (Phillips, Shoemaker, Mace, & Hodges, 2008).

CHAPTER III

OUTLINE OF PROCEDURES

Research Design

The purpose of this study was to determine the extent to which pigmentation and melanocortin-1 receptor genotype are associated with susceptibility to noise-induced hearing loss in college-aged music students. To achieve this goal, we measured the association of hair and eye color, sunlight sensitivity, ethnicity, and MC1R genotype groups with mean bilateral pure-tone threshold averages at 4000 and 6000 Hz. We hypothesized that individuals with pigment and genotypes indicating lower cochlear eumelanin levels would have more noise-induced hearing loss than those with pigment and genotypes indicating higher eumelanin levels.

For subjects, we recruited music students from the College of Visual and Performing Arts at the University of North Carolina at Greensboro. Specifically, we recruited students in the fall semester of 2016, when all first and fourth year instrumentalists had their hearing tested as a part of a school-based Hearing Conservation Program. Recruiting students while they participated in this program increased the likelihood that they would participate in this study because we could use data that was already being collected.

Pigment and genotype association measurements were collected in two phases. For the first phase, which included the pigment association measurement, we estimated that we would collect data from 200 participants, Figure 6. This estimate came from data collected in previous years where about 250 students have received hearing tests within this time frame. We anticipated that about 10% would decline to participate, bringing our estimated sample size to 225. In addition, we anticipated excluding another 10% of the students based on these criteria, bringing our estimated sample size to 200. Exclusion criteria for the first phase included a hearing loss with a specific etiology, ototoxic medications, or an outer or middle ear pathology as determined by otoscopy or tympanometry. All students within the age range of 18 to 25 years were allowed to participate.

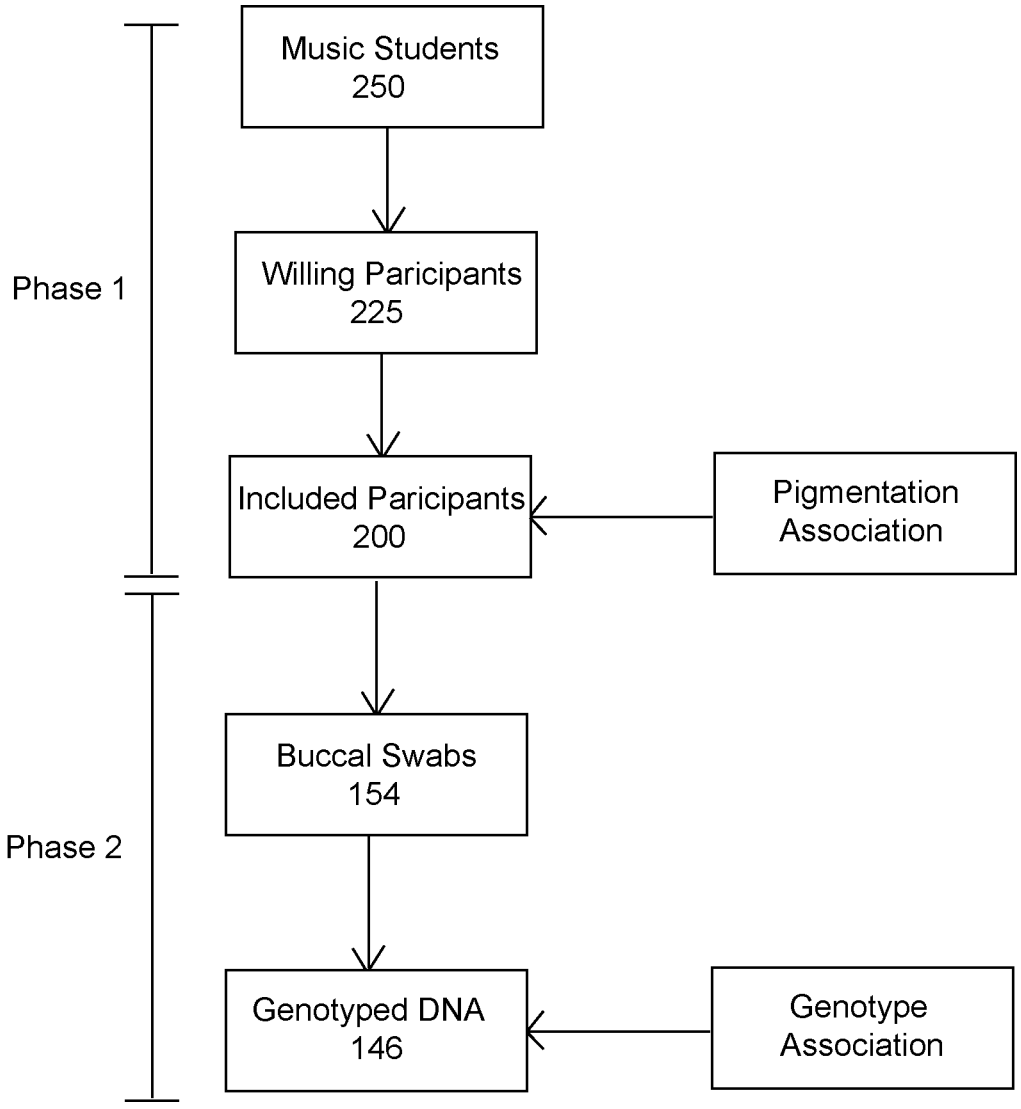

Figure 6. Anticipated Consort Diagram of this Phased Approach Study. This diagram gives the estimated number of participants or samples at each step of the study. The first three steps indicate how many samples we estimated to lose at each step of phase one, the Pigmentation association study. The next two steps indicate the estimated number of samples lost at each step of phase two, the Genotype association.

For the second phase, which included the genotype association measurement, we anticipated collecting data from 146 individuals. The second

phase included an additional exclusion criterion where DNA samples were only collected from individuals with skin that reacts to sunlight exposure, as indicated by the Fitzpatrick Skin Test, because the prevalence of melanocortin-one receptor single nucleotide polymorphism is too low in sunlight-resistant individuals to be cost effective. We estimated that we would collect DNA samples from 154 individuals because nearly all Caucasians react to sunlight exposure (Eilers et al., 2013); and, in data from previous studies of this population, 77% of these subjects were Caucasian (Phillips et al., 2015). Also, based on data from the same study, we anticipated that about 5% of the DNA samples would be lost in genotyping analyses, yielding an expected 146 DNA samples for the genomic analyses.

Before measuring associations, we assessed the need for adjusting the dependent variable, hearing loss. To do this, we measured the effect of demographics (e.g. age, and gender), hearing health (e.g. a family history of hearing loss), and noise exposure groups, (e.g. instrumentation and ensemble participation) on hearing loss. All factors from this analysis of variance with a medium or large effect size ($\eta^2_p \geq 0.06$) were included in another multifactor analysis of variance to obtain an adjusted mean bilateral pure-tone threshold average at 4000 and 6000 Hertz based on residuals.

After the dependent variable was calculated, two additional multifactor analyses of variance were used to measure the association of pigment, such as hair and eye color, and genotype with hearing loss. Bonferroni corrections were

applied to account for the two omnibus tests. A post-hoc power analysis was also included to determine if our sample size was large enough to detect significant differences among groups (Ranganathan, Buyse, & Pramesh, 2015).

For the first analysis, the association of hair and eye color, skin type, and ethnicity with the adjusted mean bilateral pure-tone threshold average at 4000 and 6000 Hertz was measured in all students meeting the inclusion criteria. All two, three, and four way interaction components were also assessed, and Tukey's post-hoc measurements were also used to assess the relationship within groups of any significant interactions.

For the second multifactor analysis of variance, we measured the association of melanocortin-one receptor genotype groups with the adjusted mean bilateral pure-tone threshold average at 4000 and 6000 Hertz of all participants with sunlight reactive skin types (skin-types I-IV). Again, two, three, and four-way interactions with pigmentation factors were included and Tukey's post-hoc measurements were used to assess the groups within any significant interactions.

Power Analysis

A power analysis was conducted with data from a similar study to determine the number of subjects needed to detect a significant difference in noise-induced hearing loss between two groups with different pigmentation characteristics. To date, no study has examined the association of these melanocortin-one receptor single-nucleotide polymorphisms and noise-induced

hearing loss; therefore, the power of both omnibus tests were estimated with this analysis. The data for the power analyses came from Da Costa et al. (2008), which found that workers with dark eyes had 9.1 dB lower hearing thresholds compared to workers with light eyes. With a pooled standard deviation of 15.2 and an α level of 0.05, a one-sided power analysis indicated that 26 individuals per a group were needed to reach a power of 0.8. This power analysis was conducted with an online calculator (Rosner, 2010). In this study of music students, five pigment and genotype factors had groups ranging from three to six groups per a factor. With an anticipated 200 individuals for the pigmentation analysis and 146 individuals for the genotype analysis, we expected a range of 33 to 51 individuals per group, which would be enough to obtain a power of 0.8 for all factors.

Procedures

Subjects

We recruited first and fourth year music students between the ages of 18 and 25 when they have their hearing tested for the hearing conservation program. Recruitment and testing occurred within the Music Building at the University of North Carolina at Greensboro. This recruitment was authorized by the manager of the Hearing Protection Program, Appendix A; and, all procedures were approved by the Institutional Review Board at the University of North Carolina at Greensboro, Appendix B.

Participation in the hearing conservation program was required for all instrumentalists participating ensembles in College of Visual and Performing Arts at the UNCG. Recruiting students as they participated in the conservation program increased the likelihood that students would be willing to participate because we were asking for data that was already being collected. We only recruited from first and fourth year students because these are the only students participating in the hearing conservation program in the fall; however, all students who participated in the Hearing Conservation Program during the fall semester of 2016 were included in the study.

All recruiting and testing was performed in the morning to reduce the possibility of a temporary noise-induced hearing loss. Any students reporting noise exposure less than 12 hours before testing were asked to return for testing on another day. All students were informed to avoid noise exposure on the night before testing through the Hearing Conservation Program.

Students agreeing to participate in the study were asked to read and sign a consent form. Then, a sticker was place on the form to identify each participant with a four-digit code. This code was used to label all samples and data collected. The consent form was stored in a locked cabinet, separate from the data.

Survey

Once each student consented to the study, they filled out a survey that was used to collect data on demographics (such as age and gender), hearing

health (such as smoking status), noise exposure history (such as instrumentation), and pigmentation (such as hair color), Appendix C. This survey was also used to determine which students met the inclusion criteria for the first phase of the study, as indicated in the *Research Design* section, and determine which students were able to participate in the second phase of the study, the genotyping analysis. The inclusion questions asked students if they had a congenital hearing loss, a hearing loss with a specific etiology, a history of ear surgery, or were taking ototoxic medications. Individuals who answer yes to any of these questions were excluded from the study. The demographic questions asked about age, gender, and year in the program. The hearing health questions asked about family history of hearing loss and history of smoking. The sound exposure questions were open ended and ask students to list their primary instrument, major, and have them list their history of ensemble participation. These open-ended questions were grouped once all of the data is collected. The pigment questions ask students to list their natural hair color, eye color, ethnicity, and skin-type. Skin-type will was assessed with the Fitzpatrick Skin Test, as described in the Literature Review.

The survey was coded and stored in a locked cabinet separate from the consent forms. All data from the survey was entered into an SPSS file with the codes listed in Appendix D. This file was be stored on the UNCG Box server, which is encrypted and password protected.

Outer and Middle Ear Screening

After completing the survey, all participants received otoscopy (visual inspection of the outer ear canal) and a middle ear screening. For otoscopy, an audiologist will verified that the ear canal was at least 25% clear of earwax. Participants with unexplained abnormalities were excluded from the study and referred to a physician. Participants with wax occlusion were rescheduled for an appointment after the wax was removed.

Next, a middle ear screener, called a tympanometer (Maico MI 24; See Appendix E for calibration), was used to assess the health of the middle ear cavity. This instrument applied positive and negative pressure into the ear canal and measured the reflection of a 226 Hz tone to evaluate the movement of the eardrum; the tone would reflect abnormally for participants with unhealthy middle ears. Participants who failed the middle ear screener were rescheduled for at least two weeks after the appointment so that any infection inside the middle ear could clear up. If a student's middle ear problems persisted, then they were excluded from the study and referred to a physician.

Hearing Test

For students who met the inclusion criteria, we measured thresholds at 500, 1000, 2000, 3000, 4000, 6000 and 8000 Hz in both ears by performing pure-tone audiometry with a calibrated audiometer (Interacoustics AC-40; Eden Prairie, MN; see Appendix F for calibration). The test will be conducted in an MDL 4242 sound booth (Whisper Room, Inc; Knoxville, TN) that met ANSI

standards using the ER-3A insert headphones (Etymotic Research; Elk Grove Village, IL). The hearing tests were conducted by certified audiologists with clinical experience. The audiologists used the modified Hughson Westlake (Carhart and Jerger, 1959) technique where they presented pure-tones to the participants and asked them to raise their hand when they heard the sound. The recorded thresholds were the quietest presentation levels that produced a reliable response.

All thresholds were recorded on the same hard copy form as the survey. Again, this form was stored in a locked cabinet, separate from the consent form, and then transferred to the same SPSS file as the survey data.

Genetic Tests

We used the Fitzpatrick Skin Test, a single question on the survey, to determine which participants were included in the genotyping analysis. For this question, participants reported their sensitivity to sunlight exposure on a scale ranging from I, highly sensitive, to VI, no reaction. We only genotyped individuals with skin types I – IV because the prevalence of MC1R variants are too low in individuals with skin types V and VI for the test to be cost effective.

To perform the genotyping analysis, we first collected DNA from all included participants by rubbing a buccal swab (Isohelix; Harrietsham, UK) against the inside of each cheek 30 times before placing it into a sealed Eppendorf tube. This tube was labeled with the participant's code and stored in a standard 4°C mini-refrigerator in the testing room.

After all buccal samples were collected, we moved them to the UNCG Core Molecular Biological Laboratory where they were stored inside of a -20°C freezer, processed and genotyped. This facility was located in a locked room within the Biology Building. To process the samples, we first isolated the DNA using the DNeasy Blood & Tissue Kit (QIAGEN; Germantown, MD). This kit provided the solutions to break down cell membranes and protein, while preserving the DNA. It also provided buffers and filters to clean and collect the DNA using centrifugation at room temperature so that it could be eluted into an eppendorf tube with water. All procedures followed manufacturer specifications. Briefly, cells were broken down with lysis buffer at 56°C for 10 minutes. Then the lysate was collected in micro-filters spun at 8000xg designed to collect DNA while eluting cell debris. This lysate was washed with a series of buffers at room temperature until it was eluted with water into separate tube. After isolation, we measured sample concentrations and purity of DNA samples with Nanodrop spectrophotometry (Thermo Fisher, Waltham, MA). These samples were then stored in the same -20°C freezer until DNA was isolated from all of the samples.

Genotyping was performed with the Applied Biosystems SNP Genotyping TaqMan Assays, the TaqMan Gene Expression Master Mix, the Quantitative Real-Time PCR and the TaqMan Genotyper Software (Thermo Fisher; Waltham, MA). The samples, genotyping assays, and master mix were added into 96 well plates. These plates were arranged to genotype seven non-synonymous SNPs (see table 2.) in all estimated samples. Most sample/SNP combinations were run

once; however, we re-ran questionable samples and a minimum of 25% of all samples to ensure repeatability. Each plate also had four negative controls of each SNP genotyping assay on that plate to ensure that the solutions were not contaminated. Once all samples were genotyped, the data was transferred to the same SPSS file that had the survey and audiometric data.

Table 4. Single-nucleotide Polymorphism Number, Amino acid Change, Allele, and Minimum Allele Frequency. 'r' indicates alleles with a weak effect on phenotype; 'R' indicates alleles with a strong effect on phenotype. The minimum allele frequency comes from Kantesky, et al., 2010, which measured the frequencies in a population of American Caucasians.

SNP Number	AA Change	Allele	MAF (%)
rs1805005	V60L	r	14.6
rs1805006	D84E	R	0.9
rs2228479	V92M	r	9.5
rs11547464	R142H	r	1.4
rs1805007	R151C	R	5.4
rs885479	R163Q	r	3.5
rs1805009	D294H	R	1.5

Statistical Analyses

Noise-induced hearing loss was estimated by calculating the average pure-tone thresholds at 4000 and 6000 Hz in both ears. As stated in the *Review of the Literature*, this technique was similar to the methods used in previous studies (Konings et al., 2007; Van Eyken et al., 2007). The distribution of pure-tone thresholds at 4000 and 6000 Hz collected in our data set were compared to thresholds collected in a previous data set of student musicians at The University of North Carolina at Greensboro in paneled histograms (Phillips et al., 2015). All

measurements were run with the Statistical Package for the Social Sciences software (*IBM SPSS Statistics*, 2016).

Once the mean bilateral pure-tone thresholds at 4000 and 6000 Hz were calculated and compared with previous measurements, we assessed the effects of demographic, noise exposure, and experimental (tester) factors on hearing loss. To accomplish this, we first counted the number of individuals in each noise exposure group. These groups are listed in Appendix B and described in the *Survey* subsection. Only factors with at least two groups with greater than 10 individuals were included in the analysis; any factor that did not meet this criteria were assumed to not have the statistical power to make any claims regarding an effect on hearing loss. Next, we used a multifactorial analysis of variance to measure the effect, partial-eta squared (η^2_p), that each factor had on hearing loss. Any factors that had a medium or greater effect size, ($\eta^2_p \geq 0.06$), on hearing loss, were used on another multifactorial analysis of variance to calculate the residuals, thereby obtaining an adjusted mean bilateral pure-tone thresholds at 4000 and 6000 Hz. The means of factors with medium or large effects were also tabulated to analyze group differences.

To assess the effect of pigmentation on the adjusted mean bilateral thresholds, we used a single direction multifactorial analysis of variance with hair and eye color, ethnicity, and skin type. Again, the codes for these factors are listed in appendix B. This analysis included all participants meeting the inclusion criteria along with all three and four way interactions among the factors. The

omnibus test was adjusted with a Bonferroni correction factor to account for the two multifactorial analyses of variance that were run in this study.

Next, we grouped the genotype data based on each allele's effect on eumelanin production. As described in the *Literature Review* section, we grouped all participants into one of three genotyping groups. Individuals with at least 'R' alleles or at least two 'r' or 'R' alleles were placed into the strong effect group; individuals with no 'R' alleles and only one 'r' allele were placed into the weak effect group; individuals with no 'R' or 'r' alleles were placed into the wild type group. The effect of each individual SNP is listed in table 2. Estimates from previous reports indicated that individuals with skin-types I-IV will be relatively evenly distributed among these three groups (Kanetsky et al., 2010). Specifically, from the 146 participants that we anticipate to recruit for this study, we believe that the genotyping analysis will yield 31 individuals for group 1, 47 for group 2, and 68 for group 3. The number of individuals in each group was compared to these group numbers.

Once the genotype groups were established, we assessed the association of these groups with the adjusted mean bilateral pure-tone threshold averages, with a single direction multifactorial analysis of variance. This model included a bivariate interaction with genotype group, and three-way interactions with genotype group and each pigment indicator. Again, the omnibus test was adjusted with a Bonferroni correction factor to account for the two tests.

CHAPTER IV

RESULTS

Population and Demographics

We collected sound exposure, pigmentation, and hearing data from 168 music students, which was only 75% of the number of students that we expected to recruit Figure 7. Of these students, 155 were included for the first phase of the study, the pigmentation analysis, yielding 77% of our expected number of participants. After completing the Fitzpatrick Skin Test, we collected buccal samples from 119 students. Only one DNA sample was lost during genetic processing, leading to an analysis of 118, or 82% of the estimated number of DNA samples for the analysis of melanocortin-one receptor single nucleotide polymorphisms.

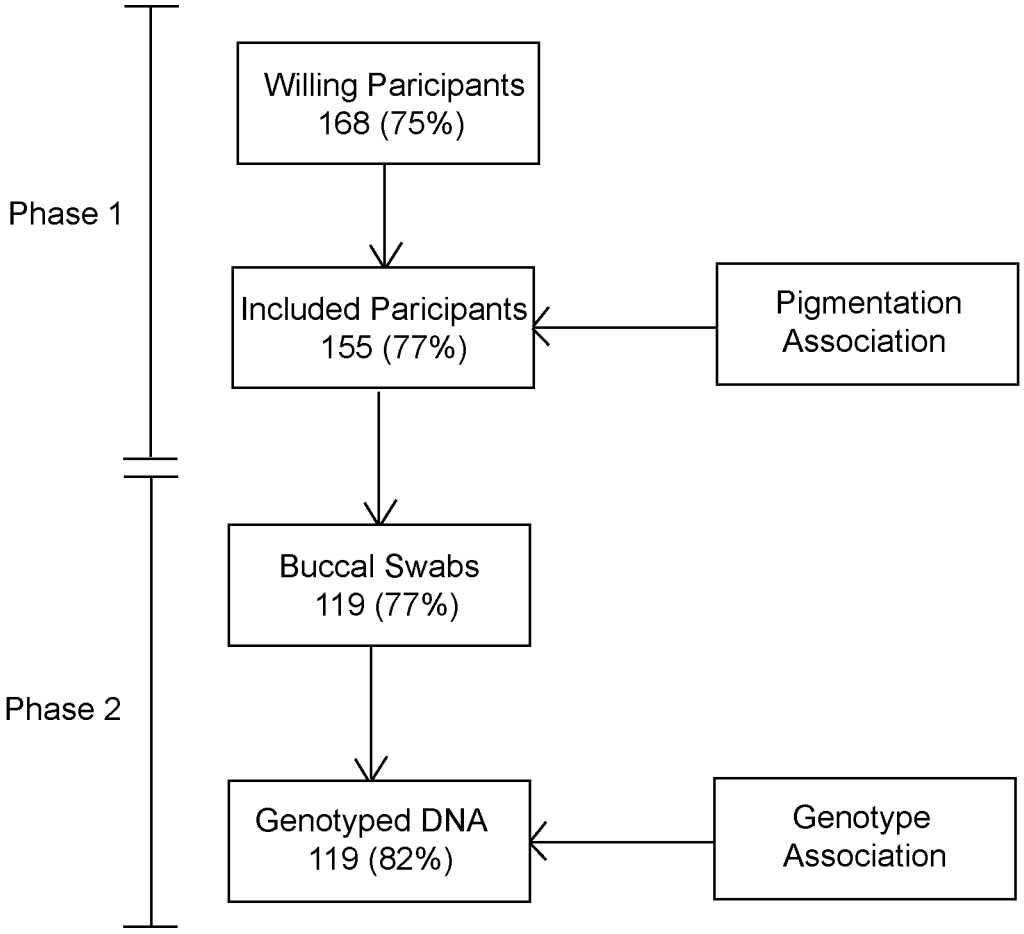

Figure 7. Consort Diagram with the Number of Individuals or Samples in each step. The percent of individuals or samples relative to the expected amount is listed in parentheses.

There were slightly more females than males in the population, Table 5. The average age was 19.4 (SD 2.0). The students were unevenly distributed among years in the program because the Hearing Conservation Program was designed to test first and fourth year students in the semester that we collected data; however, some students from other classes were included in this study. Very few participants indicated a history of smoking; therefore, this variable was

not included in the noise exposure analysis. About a third of the students reported a family history of hearing loss. Of these students, most of them reported that their family member did not lose their hearing until well after retirement ages; however, a small percent of students did report young family members with hearing loss.

Table 5. The Number of Individuals for each Demographics Group is Listed Below. The Ct Total is the count for all subjects, and the Ct GA is the count for participants in the genetic analyses.

Demographics		
Variable	**Ct (%) Total**	**Ct (%) GA**
Gender		
Male	69 (44.5)	51 (42.9)
Female	81 (52.3)	63 (52.9)
Other	5 (3.2)	5 (4.2)
Year		
1st	97 (62.6)	71 (59.7)
2nd	6 (3.9)	5 (4.2)
3rd	3 (1.9)	3 (2.5)
4th	41 (26.5)	34 (28.6)
5th	7 (4.5)	5 (4.2)
6th	6 (0.6)	1 (0.8)
Nicotine		
No	146 (94.2)	111 (93.3)
Yes	9 (5.8)	8 (6.7)
Family HX		
None	106 (68.4)	80 (67.2)
Elderly	44 (28.4)	35 (29.4)
Young	5 (3.2)	4 (3.4)

Noise-induced Hearing Loss

The mean bilateral pure-tone thresholds at 4000 and 6000 Hertz in the 155 students included in this study had an overall mean value of 1.49 with a

standard deviation of 5.09. As seen in Figure 8., the values were within normal limits (<20 dB HL) for all but two participants, who were extreme (>3 IQR) outliers. These data points were not normally distributed, (t = 0.939, p < 0.001) according to the Shaipro-Wilk test, likely because the distribution reaches the lower limits of the audiometer is -10 dB HL.

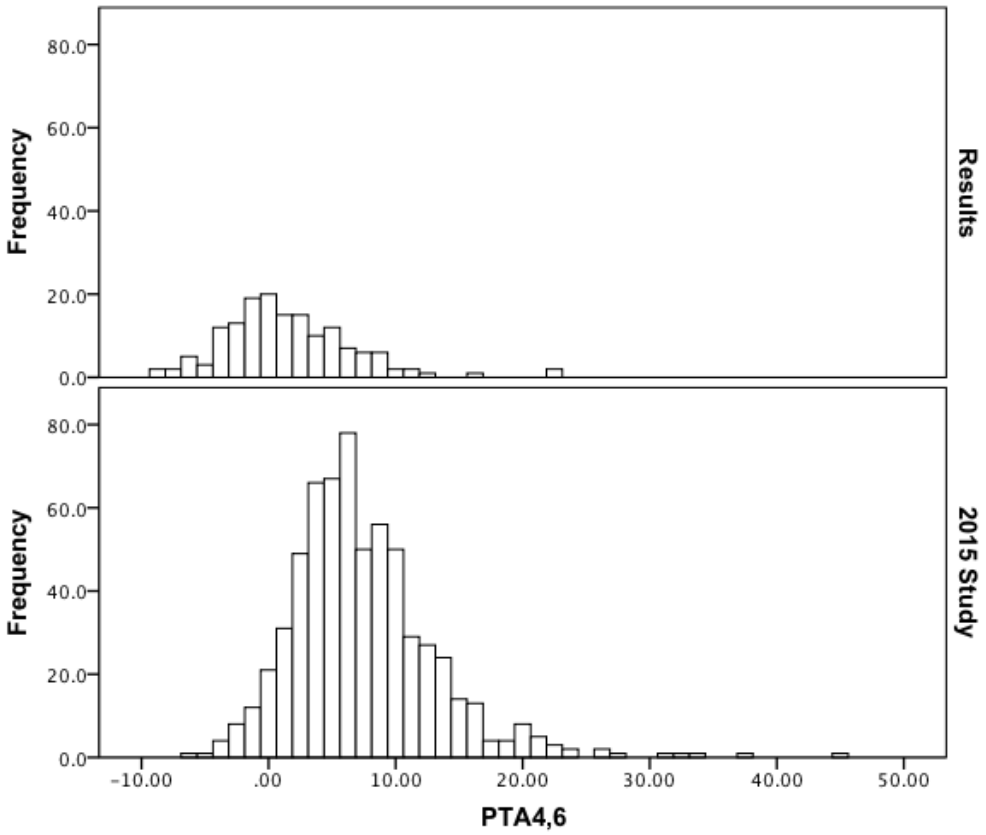

Figure 8. Histogram of mean Bilateral Pure-tone Threshold Averages at 4000 and 6000 Hertz ($PTA_{4,6}$) for all Participants. The total number of individual in each bin are given for data collected in this study (above) and data collected in Phillips, (2015) (Below). (Two outliers from Phillips, (2015), one at 66 dBHL and one at 85 dbHL, were deleted to improve clarity of the figure. These outliers are included in mean and median calculations.)

A histogram displaying the distribution of mean bilateral pure-tone thresholds at 4000 and 6000 Hertz among 636 college aged music students at The University of Greensboro School of Music is shown below the histogram of thresholds collected for this study. As seen in the histogram, the median thresholds were higher for the students in the previous study compared to the students in this study.

Sound Exposure Factors and Noise-induced Hearing Loss

The total number of individuals in each sound exposure group are listed in Table 6. All factors have a fairly even distribution of individuals among the groups, except for participation in a rock band, which was not included in the noise exposure analyses.

Table 6. Distribution of Students in Music Groups, Instrumentation, and Major. Ct, count; GA, genomic analysis

			Music Exposure		
Variable	**Ct (%) Total**	**Ct (%) GA**	**Variable**	**Ct (%) Total**	**Ct (%) GA**
Symphonic			**Inst. Group**		
No	71 (45.8)	54 (45.4)	Saxophone	13 (8.4)	11 (9.2)
Yes	84 (54.2)	65 (54.6)	Tuba/Euphonium	4 (2.6)	3 (2.5)
Orchestra			Double Bass	5 (3.2)	4 (3.4)
No	119 (76.8)	88 (73.9)	Viola	8 (5.2)	4 (3.4)
Yes	36 (23.3)	31 (26.1)	Cello	5 (3.2)	4 (3.4)
Small Ens			Horn	6 (3.9)	5 (4.2)
No	67 (43.2)	47 (39.5)	Guitar/Harp	4 (2.6)	4 (3.4)
Yes	88 (56.8)	72 (60.5)	Trumpet	10 (6.5)	8 (6.7)
Choir			Trombone	10 (6.5)	8 (6.7)
No	120 (77.4)	92 (77.3)	Voice	35 (22.6)	23 (19.3)
Yes	35 (22.6)	27 (22.7)	Cla/Oboe/Bassoon	13 (8.4)	9 (7.6)
Marching			Flute/Piccolo	11 (7.1)	9 (7.6)
No	116 (74.8)	88 (73.9)	Percussion	10 (6.5)	8 (6.7)
Yes	39 (25.2)	31 (26.1)	Piano/Organ	14 (9.0	12 (10.1)
Rock Band			Violin	8 (5.2)	7 (5.9)
No	151 (97.4)	115 (96.6)			
Yes	4 (2.6)	4 (3.4)	**Major**		
Jazz Band			Performance	36 (23.3)	26 (21.8)
No	122 (78.7)	93 (78.2)	Education	62 (40.0)	47 (39.5)
Yes	33 (21.3)	26 (21.8)	Other	57 (36.8)	46 (38.7)

A multifactorial analysis of variance was used to determine the effect, as measured by partial-eta squared (η^2_p), of each sound exposure factor on the

mean bilateral pure-tone average threshold at 4000 and 6000 Hertz. Also, a tester effect was included in this analysis to determine if the differences in threshold obtained by different audiologists affected the results. As seen in Table 7., 12 of the 13 factors explaining sound exposure had less than a medium effect ($\eta^2_p \geq 0.06$) on NIHL. Instrumentation group, on the other hand, had a medium effect of ($\eta^2_p = 0.087$). Therefore, the thresholds were adjusted based on instrumentation for each individual.

Table 7. The Partial-eta Squared Values are Listed for each Sound Exposure Factor. *Instrumentation is the only factor with a medium effect on mean bilateral pure-tone threshold averages at 4000 and 6000 Hertz. $^{+}$Tester is not a sound exposure factor, but it was included in this analysis to demonstrate that there were no effects from differences in thresholds obtained among the audiologists.

Variable	η^2_p
Year	0.005
Age	0.006
Gender	0.003
Family Hx	0.013
Major	0.006
Instrumentation	**0.087***
Tester^{+}	0.004
Ensemble Participation	
Symphonic	0.057
Orchestra	<0.001
Small Ensemble	0.002
Choir	<0.001
March	0.027
Jazz	0.002

As seen in Table 8., group mean pure-tone threshold averages for 6000 and 8000 Hz ranged from -0.63 db HL in the violin group to 4.33 dB HL in the Alto/Tenor Saxophone group. There was no clear trend relating broader instrument groups, such as brass or woodwinds, with noise-induced hearing loss.

Table 8. Mean PTA_{46} and Standard Deviation for each Instrument Group

	Mean PTA_{46}	**St Dev**
Saxophone	4.33	8.05
Tuba/Euphonium	4.06	6.40
Double Bass	4.00	11.30
Viola	2.86	3.04
Cello	2.75	4.09
Horn	2.29	2.90
Guitar/Harp	1.88	8.26
Trumpet	1.88	3.64
Trombone	1.63	3.12
Voice	1.42	4.74
Clarinet/Oboe/Bassoon	0.29	4.95
Flute/Piccolo	0.11	4.20
Percussion	0.00	2.50
Piano/Organ	-0.09	4.69
Violin	-0.63	3.78

Pigmentation and Noise-induced Hearing Loss

Pigmentation characteristics, such as hair and eye color, were collected from all subjects using the survey. As see in Table 9., there was a fairly even distribution of individuals in all four indicators. The over and under representation of groups were similar to what was expected considering previous ethnicity

distributions of this population and pigmentation distributions within each ethnicity.

Table 9. Total Number of Individuals for each Pigmentation Group. The Ct total lists the number of total students in each group (Phase 1); the Ct GA lists the number of students in the genotyping analyses for each group (Phase 2).

Eumelanin Indicators		
Variable	**Ct (%) Total**	**Ct (%) GA**
Hair Color		
Red	7 (4.5)	7 (5.9)
Blonde	21 (13.5)	21 (17.6)
Light Brown	13 (8.4)	12 (10.1)
Brown	78 (50.3)	65 (54.6)
Dark Brown	7 (4.5)	6 (5.0)
Black	29 (18.7)	8 (6.7)
Eye Color		
Blue	37 (23.9)	35 (29.4)
Green	16 (10.3)	15 (12.6)
Hazel	29 (18.7)	27 (22.7)
Brown	66 (42.6)	39 (32.8)
Dark Brown/Black	7 (4.5)	3 (2.5)
Skin Type		
I	11 (7.1)	11 (9.2)
II	28 (18.1)	28 (23.5)
III	45 (29.0)	45 (37.5)
IV	35 (22.6)	35 (29.4)
V	22 (14.2)	0
VI	14 (9.0)	0
Ethnicity		
Caucasian - Main	91 (58.7)	85 (71.4)
Caucasian - North	26 (16.8)	23 (19.3)
African	24 (15.5)	3 (2.5)
North Asian	5 (3.2)	3 (2.5)
South Asian	1 (0.6)	0
Hispanic	4 (2.6)	2 (1.7)
Other	4 (2.6)	3 (2.5)

A multifactorial analysis of variance was run to measure the association of all four pigmentation groups and all two, three, and four-way interactions with the adjusted mean bilateral pure-tone thresholds averages at 4000 and 6000 Hertz. The overall model was not significant, ($F_{(82,72)} = 0.707$, $p = 0.936$). All bivariate and interaction effects were also not significant ($p < 0.05$). The post-hoc power was 0.763.

Despite the lack of significance, all pigmentation indicators followed a trend of decreased thresholds with increased eumelanin production (Figure 9.). For hair color, those with blonde hair, which is the hair color with the second least amount of eumelanin, had the largest adjusted mean bilateral pure-tone threshold averages at 3000 and 4000 hertz (Figure 9A.). Also, those with dark brown hair, which is the hair color with the second most amount of eumelanin, had the lowest thresholds. For eye color, the thresholds were highest in individuals with blue eyes, who have the least eumelanin in their eyes, and continued to decrease for each color category indicating increases in eumelanin (Figure 9B.). With skin-type, the negative relationship between adjusted mean bilateral pure-tone threshold averages and increases in predicted eumelanin levels was not as apparent as other pigmentation indicators, but the overall relationship is still observed (Figure 9C.). As for ethnicity, there was no consistent relationship between estimated melanin levels and $PTA_{4,6}$.

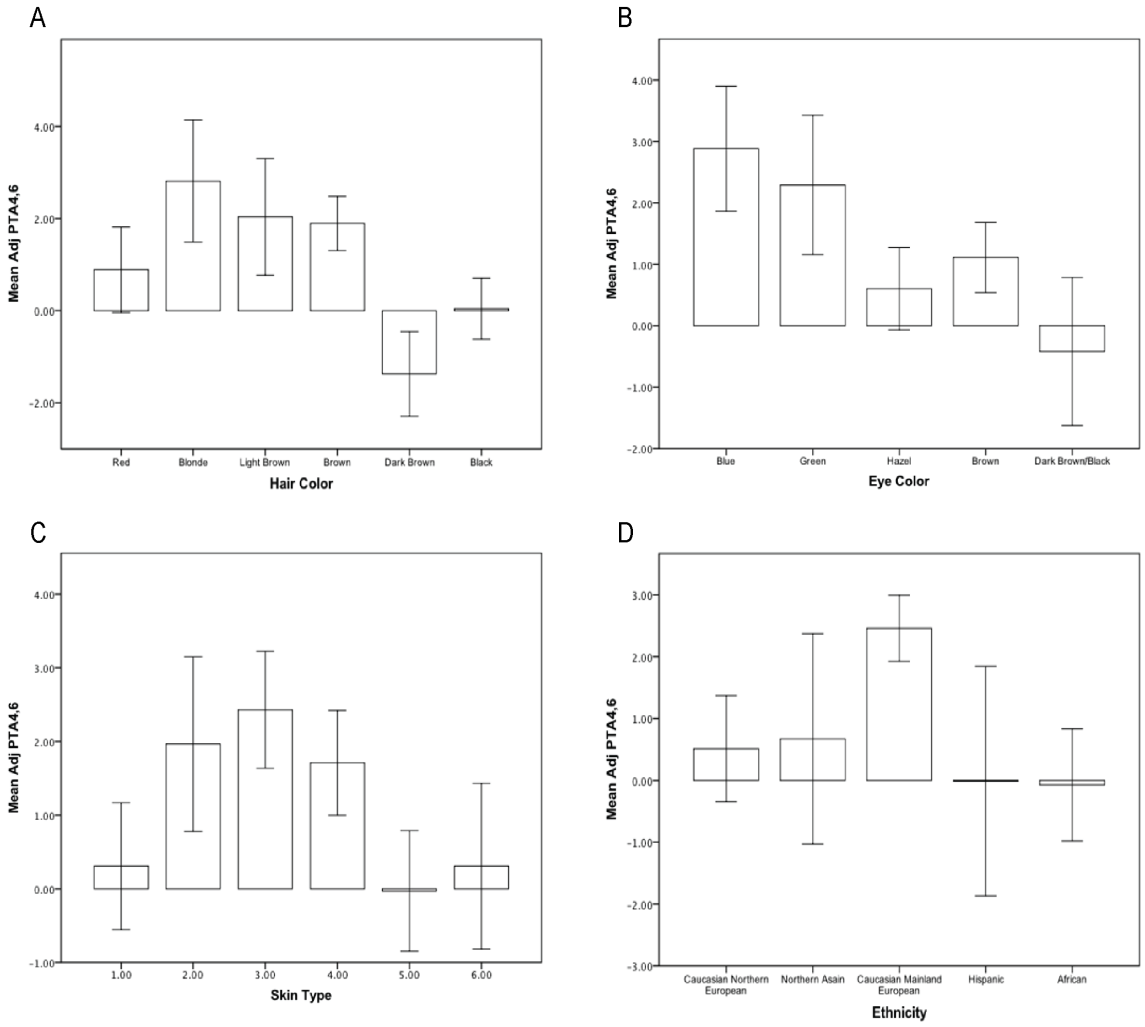

Figure 9. Adjusted mean Bilateral Pure-tone Threshold Averages at 3000 and 4000 Hertz of all Student Musician Groups by A) six hair Colors, B) five eye Colors, C) six skin Types, and D) five Ethnicity Groups. All Eumelanin indicators are listed from lowest levels of estimated Eumelanin to the left (e.g. red hair, blue eyes, Skin-Type 1, and Northern Europeans) and highest levels of estimated eumelanin (e.g. Black hair, Black eyes, Skin-Type 6, and African) to the right. Error bars indicate one standard error.

Genotype Groups and Noise-induced Hearing Loss

Buccal cells were collected from 119 music students, but genomic analyses were only run on 118 DNA samples because one sample could not be

genotyped. The number of participants in each group that were included in this phase of the study are listed in Table 5. for demographics, Table 6. for noise exposure, and Table 9. for pigmentation. As expected, the percent of individuals in demographic and noise exposure groups were similar between the cohort of 155 students who participated in the first phase of the study and the cohort of 118 students who participated in the second phase of the study. The percent of individuals in pigmentation groups were different between phases because the individuals in the second phase were selected based off of skin-type, an indicator of eumelanin levels.

The seven single-nucleotide polymorphisms from melanocortin-one receptor listed in Table 4. were genotyped in 118 students. The individual allele frequencies measured in this study were similar to the frequencies obtained in Kanetsky et al., (2010). As for genotype groups, 29 individuals had either one ‘R’ allele or two or more ‘r’ alleles, indicating a strong association with reduced eumelanin production, 15 individual had 1 ‘r’ allele and no ‘R’ alleles, indicating a weak association of reduced eumelanin production, and 75 individuals had no ‘R’ or ‘r’ alleles, indicating no association with eumelanin production.

The association between genotype group and all four pigment indicators was measured to evaluate the effects of the single nucleotide polymorphisms on eumelanin production. As seen in Figure 10A., individuals with hair color indicating less eumelanin production were more likely to be in genotype groups associated with reduced eumalain production (R or r/r genotype); and individuals

with hair color indicating more eumelanin production were more likely to be in wild type groups. However, this relationship was not as apparent for eye color, skin type, or ethnicity; the relative prevalence of genotype groups was consistent across all pigmentation groups within each of these three indicators (Figure 10B.-D.).

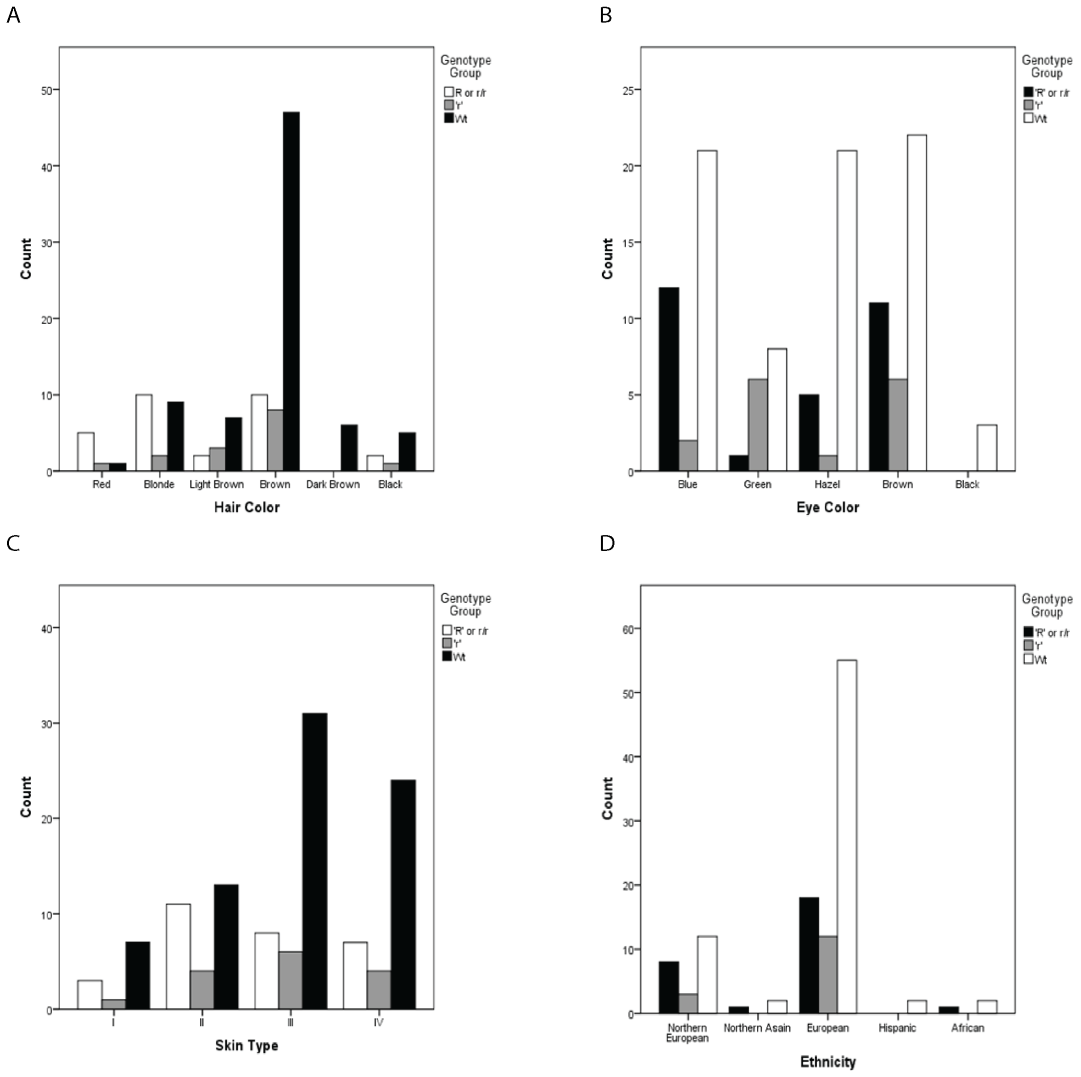

Figure 10. Distribution of Individuals in Genotype Groups Across Pigment Groups. Total number of individuals in each of the three Genotype Groups compared across A) hair color, B) eye color, C) skin-type, and D) Ethnicity. Groups associated with low Eumelanin levels are listed to the left and groups associated with high Eumelanin levels are listed to the right.

A multifactorial analysis of variance was run to evaluate the association of genotype groups and mean adjusted pure-tone threshold averages at 4000 and 6000 Hertz. Interaction affects between genotype group and pigmentation

indicators were included in this model. The overall omnibus test was not significant ($F_{(79,39)}$ = 0.488, p = 0.996). No significant effects for interaction components were detected. The post-hoc observed power was 0.368. As expected, the group of individuals with genotypes indicating a weak reduction in eumelain production, (one ‘r’ allele and no ‘R’ alleles) had a greater mean threshold than the group of individuals with the wild type alleles; however, individuals in genotype groups indicating a strong association with reduced eumelanin production (‘R’ or r/r alleles) had mean thresholds than were nearly equal to the wild-type group (Figure 11.).

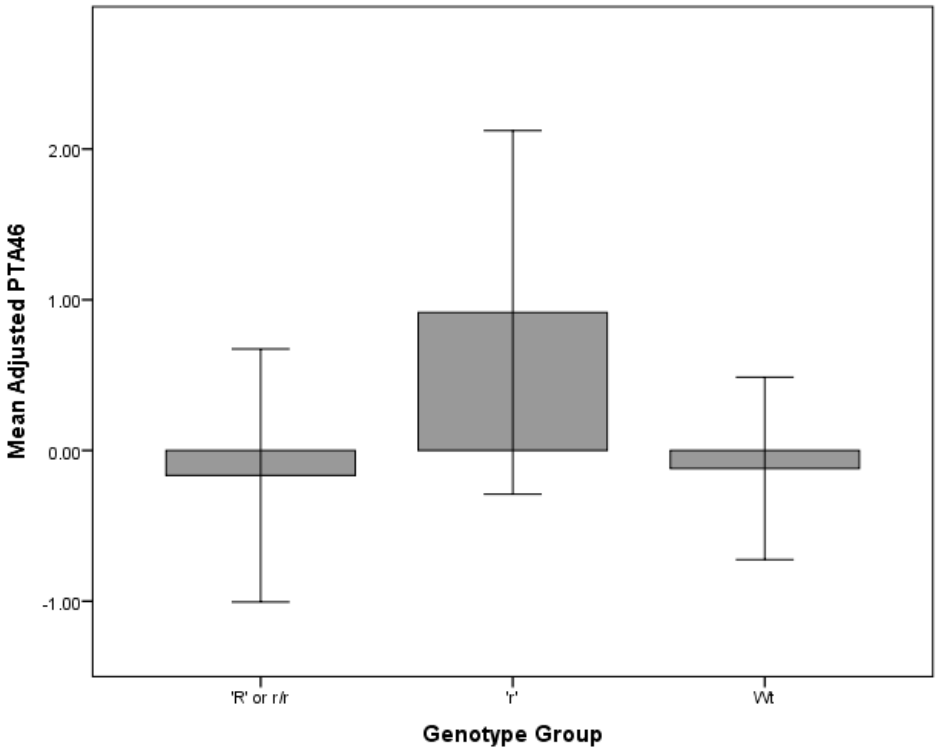

Figure 11. Adjusted mean Bilateral Pure-tone Threshold Averages at 4000 and 6000 Hertz Across the Three Genotype Groups. The Genotype Group associated with strong reduction in Eumelanin is listed to the left; the Genotype Group associated with no reduction in Eumelanin is listed to the right.

The mean adjusted pure-tone threshold average at 4000 and 6000 Hertz was also examined for each individual SNP (Table 10.). Individuals with single-nucleotide polymorphisms rs1805006, an ‘R’ allele, and rs2228479, an ‘r’ allele, had higher mean thresholds compared to those with wild type, or homozygous major, alleles, which supports our hypothesis. However, individuals with single-nucleotide polymorphisms rs1805007 and rs1805009, ‘R’ alleles, and rs11547464, an ‘r’ allele, had lower mean thresholds compared to those with wild type, or homozygous major, alleles.

Table 10. Mean Adjusted Pure-tone Threshold Average at 4000 and 6000 Hertz for each Single-nucleotide Polymorphism Group. The standard error is listed in parentheses.
Homozygous Minor (Hmi), Heterozygous (Het), and Homozygous Major (Hma)

	Mean Adj $PTA_{4,6}$ (SE)		
SNP	**Hmi**	**Het**	**Hma**
R			
rs1805006	-	1.68 (5.11)	-0.743 (1.19)
rs1805007	-2.19 (3.61)	-1.46 (2.62)	0.026 (1.43)
rs1805009	-	-0.443 (2.55)	-0.513 (1.29)
r			
rs11547464	-	-2.68 (5.11)	-0.249 (1.19)
rs2228479	-1.86 (3.61)	2.29 (2.64)	-1.09 (1.43)

CHAPTER V

DISCUSSION

The purpose of this study was to evaluate the extent to which pigmentation and melanocortin-one receptor genotype are associated with sensitivity to noise-induced hearing loss in college-aged music students. The ominibus multifactor analysis of variance was not significant for pigmentation ($F_{(82,72)}$ = 0.707, p = 0.936, η^2 = 0.763); however, the groups of pigmentation did follow the expected trends, i.e. those with low levels of pigmentation had higher hearing thresholds than those with lower levels of pigmentation. Furthermore, the second omnibus multifactor analysis of variance significant for melanocortin-one receptor genotype groups was also not significant ($F_{(79,39)}$ = 0.488, p = 0.996, η^2 = 0.368). However, one single-nucleotide polymorphism, rs2228479, did trend towards an association with susceptibility to noise-induced hearing loss. Unfortunately, the hearing thresholds of student musicians were lower than expected. This finding limited our ability to detect significant effects of pigmentation and genotype.

Correspondence of Findings with Previous Literature

Participant Recruitment

In this study, we used a phased approached design where we recruited student musicians from the UNCG school of Music for the pigmentation study,

then selected those with possible single-nucleotide polymorphisms in the melanocortin-one receptor gene for the genotype analysis. Unfortunately, we only were able to recruit 77% of the estimated number of subject for pigmentation analysis. This was likely because our estimates were based on the total number of music students, and we did not take into account the large number of Freshman that were younger than 18 years old at the time of testing. We were, however, able to test 82% of the expected number of samples for the genotype analysis because we did not lose as many samples as expected in the genotyping analyses.

Hearing Loss

The mean pure-tone average threshold at 4000 and 6000 hertz was 1.477 dB HL (SD 5.1), which was less than the same measurement taken in this population from a previous study, 7.67 dB HL (SD 6.69) (Phillips et al., 2015). Lüders, Gonçalves, de Moreira Lacerda, Ribas, & de Conto, (2014) also measured hearing in student musicians and found a mean pure-tone average threshold at 4000 and 6000 Hertz of 6.8 dB HL. Interestingly, the authors of this study were comparing student musicians with non-musicians and found that non-musicians had higher mean thresholds. The decrease in thresholds from this study compared to Phillips et al., (2015) and may be explained by the difference in sample size because thresholds have right tailed distribution from bottoming out effects of the audiometer; the larger sample size likely shifted the mean thresholds upward. However, the median thresholds in this study were also less

than the median thresholds in Phillips et al., (2015) and Lüders et al., (2014); this difference cannot be explained by sample size. It is also important to note that Phillips et al., (2015) reported that 14.8% of the student musicians had audiometric notches, but when the same criteria were applied to the current population, only 1 of 155 students had an audiometric notch.

The thresholds measured in this study may have been lower than the thresholds measured in Phillips et al., (2015) and Lüders et al., (2014) because ER-3A inserts were used in this study, where the previous studies used TDH-39 headsets. Previous reports have indicated that supra-aural headsets, such as the TDH-39, are unreliable for detecting small degrees of high-frequency hearing loss. For instance, Serpanos, Senzer, Renne, Langer, & Hoffman, (2015) found that after testing hearing with supra-aural headsets, retesting with inserts reduced referral rates by 13 to 16%. False positives of noise-induced hearing loss were also found by Schlauch & Carney, (2011) in a study of the hearing tests, which were conducted with TDH-39's, from the 2003 cohort of the National Health and Nutrition Examination Survey. In this study, the authors found an increase in thresholds at 6000 Hz in young adults, as previously described in Niskar et al., (2001), and in younger children ages 12 to 19, and 6 to 11 years old. Niskar et al., (2001) claimed that the hearing loss in the young adults was likely caused by noise. However, in Schlauch & Carney, (2011), the authors argued that the hearing loss was more likely due to measurement errors from the supra-aural headphones because the children ages 6 to 11 were too young to

experience significant noise exposure. It is not currently understood why supra-aural transducers may have increased thresholds in high frequencies, but some authors have speculated that it is because these frequencies form standing waves in the ear canal. Lutman & Qasem, (1998) have provided evidence for variations of up to 5 decibels among different couplers that are used to calibrate audiometers. If the couplers are inconsistent, then it is reasonable to assume that this inconsistency would transfer into audiometric testing, especially when averaged across populations.

Noise Exposure

Differences in mean bilateral pure-tone threshold average at 4000 and 6000 hertz were evaluated across demographic and noise exposure groups with a multifactorial analysis of variance to determine if these thresholds needed to be adjusted for co-variants. Instrumentation was the only factor to affect thresholds enough to meet the cut off for effect size ($\eta^2_p \geq 0.06$); therefore, adjusted thresholds were used for this study that accounted for differences across instrumentation group. It should also be noted that those who participated in the symphonic ensemble did have lower thresholds and those who had participated in a marching band did have higher thresholds. Both of these factors warrant further investigation in future studies.

As for instrumentation, we did compare the mean thresholds among instrument groups to see if any trends among broader instrument groups, e.g. brass, woodwinds, could explain differences among smaller groups.

Unfortunately, we were not able to find any of these trends (Table 8.). However, the high thresholds for saxophone players may be explained by high levels of noise exposure found in a previous study (Washnik, Phillips, & Teglas, 2016). It should also be noted that these differences in groups may be caused by their associations in ensemble participation. Although we did measure ensemble participation, we did not measure interaction effects among instruments and ensembles. Further research into these interactions may explain the effect of instrument group on audiometric thresholds.

Pigmentation and Hearing Loss

Due to the low variability of thresholds, we were unable to detect a significant association between hearing loss and pigmentation. Furthermore, we collected data from enough individuals to achieve a post-hoc power of 0.763. Therefore, with our high p-value of 0.936, it is unlikely that increasing the sample size would lead to significant differences among groups.

Despite these negative findings, the statistical effects (eta squared, η^2_p) of hearing loss within pigmentation groups, which is unaffected by variation in the dependent variable, was similar to the effects found in the literature. For instance, with hair color, Ghazizadeh et al., (2012) found a 15.1 decibel difference in hearing thresholds between noise-exposed workers with dark and light hair. With a standard deviation of 16.3, this led to an effect of 0.92. In our results, the largest group differences were between those with blond hair and those with dark brown hair, with a difference of 4.18 decibels. With an average

standard deviation of only 4.25, the statistical effect in our study was 0.98. This same phenomenon occurred with eye color. For instance, Da Costa et al. (2008) found an 8.8 decibel difference between those with dark colored eyes and those with light colored eyes in a noise exposed population. With a mean standard deviation of 14.05, this lead to an effect of 0.63. In this study, our largest group difference was only 3.03 decibels, which was between those with blue eye and those with brown/black eyes. With a mean standard deviation of 4.69, our results yielded a statistical effect of 0.65.

This study was the first to measure the association of hearing loss and skin-type in a noise-exposed population. (Lin et al., 2012) used multiple linear regression to measure the association of high frequency pure tone threshold average (3000 to 8000 Hertz) and skin-type within adults with Hispanic decent, but this was not in a noise-exposed population. In this study, the authors found that those in lower skin-type groups (i.e. low eumelanin levels) had thresholds that were 3.0 decibels lower than those in high skin-type groups. Unfortunately, the standard deviations of their data set were not reported. Therefore, we cannot compare effect sizes. However, these results were similar to the results that we obtained where the largest group differences were between skin-type groups I and V and those in skin-type group III had mean pure tone threshold average at 4000 and 6000 Hertz that were 2.46 decibels higher than those in skin-type group five. However, unlike the hair and eye color data, the skin-type data did not follow the expected trends; we did not measure a consistent decrease in

thresholds from high skin-type groups to low skin-type groups. I believe that this is because the Fitzpatrick Skin Test is designed to be administered in as an interview, where in this study, we used a questionnaire (Eilers et al., 2013). It is possible that students were confused about the wording of the question, particularly those who do not have a history of high levels of sun exposure, or are resistant to the effects of this exposure. It is also possible that some students were hesitant to indicate that their reaction to sunlight was on the extreme ends of the scale. Repeating this measure by administering the questions for the Fitzpatrick Skin Test in a questionnaire may yield different results.

For ethnicity, we only collected enough individuals to compare those of Northern European, Mainland European, and African descent. The difference between those of Mainland European and African descent were as expected; those of Mainland European decent had thresholds that were 2.53 dB higher than those of African descent. In comparison, Ishii & Talbot (1998) found that white workers had hearing loss that was 8.28 decibels higher than non-white workers. Unfortunately, again, the standard deviations of their data set were not reported. Therefore, we cannot compare effect sizes.

One consistent anomaly in these results is the lower than expected thresholds in the lowest eumelanin group, eg red hair, skin-type I, and Caucasians of Northern European decent. It is possible that many students fall into all three of these groups, and we simply do not have the statistical power to make any claims. However, it is also interesting to note that individuals with the

more common genetic disorders that suppress eumelanin production (i.e. albinism) often do not show any susceptibility to noise exposure (Montoliu et al., 2014). The most likely explanation for this phenomenon is a compensatory mechanism where cells use other pathways to produce antioxidants at an accelerated rate to make up for the lack of eumelanin. Therefore, it is also possible that even within the general population, individuals who produce very low levels of eumelanin may also rely on the same compensatory mechanism to maintain homeostasis among free radicals. Of course, more research is required to validate this theory.

Pigmentation and Melanocortin-one Receptor Single-nucleotide Polymorphisms

The distribution of individuals based on pigmentation among melanocortin receptor genotype groups were as expected; individuals with pigmentation indicating low eumelanin levels were more likely to have an R or r/r melanocortin-one receptor genotype than individuals with hair or eye color indicating high eumelanin levels (Figure 10.). These results correspond with previous studies that have demonstrated a relationship between pigmentation and melanocortin-one receptor genotype (Branicki, Brudnik, & Wojas-Pelc, 2009; Lin et al., 2015; Sulem et al., 2007).

Melanocortin-one Receptor Genotype Groups and Susceptibility to Noise-induced Hearing Loss

Melanocortin-one receptor genotype groups were not significantly associated with mean adjusted pure-tone thresholds at 4000 and 6000 Hertz

($F_{(79,39)}$ = 0.488, p = 0.996). We only collected data from enough individuals to achieve a post-hoc power of 0.368, indicating that increasing the sample size may increase our ability to detect differences among groups.

As seen in Figure 11., the thresholds follow the similar trend seen in the pigmentation study where individuals with some indications of reduced melanin production have higher thresholds that those with high levels of melanin, but for those with indicators of very low levels of eumelanin, the thresholds are actually reduced. Again, this may be due to a compensatory mechanism for those with very low levels of melanin; however, it is also possible that there are other genes on the melanin pathway that explain the relationship between pigmentation indicators and susceptibility to noise-induced hearing loss.

One single nucleotide polymorphism, rs2228479, did show a trend towards association with noise-induced hearing loss. Although this effect was insignificant, it does warrant further investigation.

Limitations of the Study

The low number of DNA sites analyzed was a limitation in this study. As seen in Table 2., many of the single nucleotide polymorphisms identified in Phillips et al., (2015) were not included in this study because they have very low minor allele frequencies. In this analysis, we measured the association of hearing loss with single nucleotide polymorphisms that are more prevalent in the population. This may explain our lack of significant findings because, as discussed in the genetics selection section of the literature review, the role of

rare variants is increasingly being accepted as a major factor explaining phenotypes.

The technique used to assess noise-induced hearing loss was another limitation in this study. We used thresholds because previous studies have indicated that college-aged student musicians have audiometric thresholds that are worse than non-musicians (Barlow, 2011). Unfortunately, we did not include a control population of non-music student musicians in our data set; therefore, we cannot draw any conclusions about music and non-music student. But, as seen in Figure 8., nearly all of the thresholds measured in this study were within normal limits, and over 80% were below 10 dB HL.

Future Directions

Despite the negative findings of this study, the results have supported future research in both hearing loss in student musicians and the genetics of sensitivity to noise exposure. For hearing loss in student musicians, it would be useful to measure audiometric thresholds in students who participate in university marching bands because our multifactorial analysis of music groups indicated that students who participated in marching bands were more likely to have noise-induced hearing loss. The students in this cohort only participated in marching band at the high school level.

It is also important to assess other indicators of hearing loss in student musicians, such as high frequency audiometry, otoacoustic emissions, and electrophysiology. There is some evidence that music students have higher

thresholds within the high frequency range and reduced otoacoustic emissions, but more work is needed to determine the best method for evaluating susceptibility to noise in this population (da Silva, de Oliveira, Tauil, de Castro Silva, & Sampaio, 2017; Lüders et al., 2014). Also, the slope-adjusted notch depth, as describe in the Justification of Technical Aspects section, may help to identify those with susceptibility to noise-induced hearing loss. This measurement was not used in this study because it has a lower correlation with noise exposure compared to pure-tone thresholds. However, the slope-adjusted notch depth may have a lower type one error rate than threshold measurements, making it useful when used in combination with other measurements.

Other future directions include investigating more variants within the melanocortin-one receptor gene. In this study, we examined the association of noise-induced hearing loss with several single nucleotide polymorphisms. The melanocortin-one receptor is a small single exon gene; therefore, it is possible to repeat this test with sequencing the entire gene. This will help to measure the effect of rare variants within the gene.

To date, no study has measured the association of the melanocortin-one receptor and susceptibility to noise-induced hearing loss; furthermore, only one study has measured the association genes and hearing loss in college-aged music students. It is possible that this study introduced too many techniques, making it difficult to interpret the findings. In the future, after further characterizing the study population, I would like to measure the association of noise-induced

hearing loss with genes that have been previously measured in this area, such as Catalase (Konings et al., 2007). Retesting this gene in college-aged music students has the potential to further support these findings while justifying the use of this population for genetic association studies. Once this is complete, it will be possible to expand into other genes with evidence to support an association with susceptibility to noise-induced hearing loss, such as melanocortin-one receptor, especially rs2228479, and NADPH Oxidase 3 (Lavinsky et al., 2015; Phillips et al., 2015).

Conclusions

Noise-induced hearing loss was not significantly associated with pigmentation or melanocortin-one receptor genotype; however, those with pigmentation indicating low levels of eumelanin were more likely to have higher mean pure-tone threshold averages at 4000 and 6000 Hertz than those with pigmentation indicating high eumelanin levels. Also, one single-nucleotide polymorphism, rs2228479, did show a trend toward an association with susceptibility to noise-induced hearing loss. We may have failed to detect significant group differences because the audiometric thresholds were lower than we expected based on previous studies (Phillips et al., 2015). Further work is needed to evaluate hearing loss among college-aged student musicians.

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APPENDIX A

APPROVAL LETTERS

August 22nd, 2016

To: Institutional Review Board (IRB)
The University of North Carolina at Greensboro

Re: Study #16-0242
Effects of melanin on susceptibility to noise-induced hearing loss

To Whom It May Concern,

As the Program Associate charged with the management of the Hearing Protection Program in the UNCG School of Music, I consent to use of student information for the purposes of collecting data toward the completion of research study #16-0242 with the following conditions:

(1) All participating students sign a consent form
(2) All protected health information is stored in a locked cabinet

This consent is contingent upon completion of the study as described. Any changes to study design are subject to my approval prior to implementation.

Sincerely,

Rebecca Libera, DMA
Program Associate, Hearing Protection Program
UNCG School of Music

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To: Charles Pudrith
Comm Science and Disord
Comm Science and Disord

From: UNCG IRB

Authorized signature on behalf of IRB

Approval Date: 8/31/2016
Expiration Date of Approval: 8/30/2017

RE: Notice of IRB Approval by Expedited Review (under 45 CFR 46.110)
Submission Type: Initial
Expedited Category: 3.Noninvasive bio-specimens,4.Noninvasive clinical data,5.Existing or non-research data,7.Surveys/interviews/focus groups
Study #: 16-0242
Study Title: Effects of melanin on susceptibility to noise-induce hearing loss

This submission has been approved by the IRB for the period indicated. It has been determined that the risk involved in this research is no more than minimal.

Study Description:

Hazardous levels of noise can cause noise-induced hearing loss. Noise damages the cochlea at least partially by initiating the production of reactive oxygen species. Antioxidants, such as melanin, can protect against reactive oxygen species. The extent to which melanin protects agains noise-induced hearing loss is unknown. Cochlear melanin levels can be estimated with surveys to measure skin type and genotyping the melanin regulating gene, melanocortin receptor-one. The purpose of this study is to determine if skin type and melanocortin receptor-one genotype predict susceptibility to noise-induced hearing loss.

Investigator's Responsibilities

Federal regulations require that all research be reviewed at least annually. It is the Principal Investigator's responsibility to submit for renewal and obtain approval before the expiration date. You may not continue any research activity beyond the expiration date without IRB approval. Failure to receive approval for continuation before the expiration date will result in automatic termination of the approval for this study on the expiration date.

Signed letters, along with stamped copies of consent forms and other recruitment materials will be scanned to you in a separate email. **Stamped consent forms must be used unless the IRB has given you approval to waive this requirement.** Please notify the ORI office immediately if you have an issue with the stamped consents forms.

You are required to obtain IRB approval for any changes to any aspect of this study before they can be implemented (use the modification application available at http://integrity.uncg.edu/institutional-review-board/). Should any adverse event or unanticipated problem involving risks to subjects or others occur it must be reported immediately to the IRB using the "Unanticipated Problem-Adverse Event Form" at the same website.

Please be aware that valid human subjects training and signed statements of confidentiality for all members of research team need to be kept on file with the lead investigator. Please note that you will also need to remain in compliance with the university "Access To and Retention of Research Data" Policy which can be found at http://policy.uncg.edu/university-policies/research_data/.

APPENDIX B

SURVEY

Student Survey

Research Project: Effects of melanin on susceptibility to noise-induce hearing loss
PI: Charles Pudrith AuD

Year in school: _____ Gender: (Circle one) M / F / Other Age:____ Date of Test:_____

Hearing Health History (Circle one)

1. Were you born with hearing loss? Yes / No
2. Do you have a hearing loss with a specific cause? Yes / No
3. Have you ever used nicotine products continuously? Yes / No
3. Do you have any family members who wear hearing aids? Yes / No

If yes, please indicate whom and cause of loss: _____

4. Have you ever had surgery on either ear? Yes / No

If yes, please describe: _____

5. Please list any medications that you take daily (Do not include vitamins and birth control):

_____

Sound Exposure

1. What is your primary instrument? _____ 2. What is your major? _____

3. Please list the type of ensembles that you have performed in: _____

_____

Pigmentation

1. Hair Color? _____ 2. Eye Color? _____
3. Please indicate your primary ethnicity: (Circle one)

A) Caucasian – Mainland European B) Caucasian – Northern European C) African
D) Northern Asian (Including China) E) Southern Asian F) Hispanic
G) Native American H) Middle Eastern I) Other

4. If after several months of not being in the sun, you stayed outdoors for about **1 hour** at noon for the first time of summer without sunscreen, what would happen to your skin **within 24 hours, and then after 7 days**? (Circle one)

A) Always burn and never tan C) Burn moderately, then light tan E) No burn, then dark tan
B) Burn easily, then slight tan D) Burn minimally, then moderate tan F) No burn, no change

---------- For office use only ----------

Otoscopy (RE): Normal / Abnormal, If abnormal, describe: _____
Otoscopy (LE): Normal / Abnormal, If abnormal, describe: _____
Tympanogram (RE): A B C Tympanogram (LE): A B C

	250Hz	500Hz	1kHz	2kHz	3kHz	4kHz	6kHz	8kHz
RE								
LE								

Code:_____ Complete _____ Tested by: _____ Recorded in SPSS:_____

Coding used for each variable

Question	Code
Year in school	
Freshman	1
Sophomore	2
Junior	3
Senior	4
5th Year Senior/1st Year Masters	5
2nd Year Masters	6
Gender	
Male	0
Female	1
Other	2
Nicotine Use	
No	0
Yes	1
Family Hx of Hearing loss	
No	0
Elderly	1
Younger	2
Primary Instrument	
Voice	0
Piano/Organ	1
Cello	2
Viola	3
Violin	4
Tuba/Euphonium	5
Horn	6
Trumpet	7
Trombone	8
Alto/Tenor Sax	9
Guitar/Harp	10
Clainet/Oboe/Bassoon	11
Flute/Piccolo	12
Percussion	13
Major	
Performance	1
Education	2
Other	3
Participation in Symphonic Band	
No	0
Yes	1

Participation in Orchestra	
No	0
Yes	1
Participation in a Small Ensemble	
No	0
Yes	1
Participation in a Choir	
No	0
Yes	1
Participation in Marching/Pep Band	
No	0
Yes	1
Participation in Jazz Ensemble	
No	0
Yes	1
Participation in Rock Band	
No	0
Yes	1
Hair Color	
Brown	0
Light Brown	1
Dark Brown	2
Blonde	3
Black	4
Eye Color	
Brown	0
Blue	1
Green	2
Hazel	3
Dark Brown/Black	4
Primary Ethnicity	
Caucasian-Mainland Europe	1
Caucasian-Northern Europe	2
African	3
Northern Asian	4
Southern Asian	5
Hispanic	6
Native American	7
Middle Eastern	8
Other	9
Fitzpatrick Skin Test	
Always burn, never tan	1
Burn easily, then slight tan	2

Burn moderately, then light tan	3
Burn minimally, then moderate tan	4
No burn, then dark tan	5
No burn, no change	6

APPENDIX C

CALLIBRATION SHEETS

OWNER Unc Greensboro MAKE MAICO MODEL MI34 SERIAL NO. 755936

COMPLIANCE SECTION

Probe Tone		
Actual	Freq. Hz	SPL
226 Hz	226	85
1000 Hz	-	-

Immittance Scale Linearity, ml						
Actual	.5	1	2	3	4	5
226 Hz	0.5	-	2	-	-	5
1000 Hz	-	-	-	-	-	-

MANOMETER SECTION

Pressure Readings, daPa									
Actual	+200	+100	0	-100	-200	-300	-400	-500	-600
Indication	200	100	0	-100	-200	-300	-400	-	-
Deviation	0	0	0	0	0	0	0	-	-

Reflex Scale Linearity Pass

REFLEX SECTION / AUDIOMETER

Attenuator Linearity, dB Deviation																									
Dial Indication	125	120	115	110	105	100	95	90	85	80	75	70	65	60	55	50	45	40	35	30	25	20	15	10	5	0
Phone	-	-	-	-	-	-	-	-	-	-	-	0 REF	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Frequency, Hz		Reflex*		Audiometer*	
Indicated	Actual	Contra	IPSI	Left	Right
125	N/A	N/A	N/A	-	-
250	-	-	N/A	-	-
500	500	-	0	-	-
1000	1000	-	0	-	-
2000	2000	-	0	-	-
3000	-	-	-	-	-
4000	4000	-	0	-	-
6000	-	-	N/A	-	-
8000	-	-	N/A	-	-

* Deviation from dB HL (ANSI), SPL or Manufacturer Specifications

	Pass	Fail
Distortion	X	
Rise/Decay - O.S.	X	
Off Ratio	X	
Shock Hazard	X	
Noise Stimulus	NA	
Mechanical and Electrical Integrity	X	
Pressure Leakage	X	
Battery Check	NA	

Earphone Type	TDH 39

Acoustic Immittance: Refers collectively to acoustic impedance, acoustic admittance and acoustic compliance units.

Units calibrated to manufacturer specifications or to current ANSI S3.6 specifications where it may apply to the reflex section. Manufacturer specifications may supercede ANSI S3.6 Audiometer or ANSI S3.39 Immittance Standards.

Date of Calibration 06/27/2016

Certified By Rob Griggs

480 Hillsboro St Ste 100 Pittsboro, NC (800) 776-9046

SLM LD 824 # A4287 12/24/2015
LS 2575 # 1357
LD AMC493B, CA-250 #1253

COMMENTS:

Install new pump and annual cal done

CASS 1017 05/13

OWNER Unc Greensboro MAKE INTERACOUSTICS MODEL AC40 SERIAL NO. 762074

Frequency Hz		Earphone Transducers - SPL dB Deviation @ 70 dB HL For Transducer Type - See Lower Right of Certificate						Masking dB Deviation		Distortion THD, - dB Down or %			Bone dB Deviation		Sound Field - Warble dB Dev. 45°	
		Primary* - Channel 1		Primary* - Channel 2		Secondary Transducers*										
Indicated	Actual	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Bone	Channel 1	Channel 2	Left	Right
125	125	0.2	-0.2	-0.3	-0.7	0.6	-0.7	-4.3	-5.6	N/A	N/A	N/A	N/A	N/A	-	-
250	250	1	0.9	0.5	0.2	-0.3	0.1	-5	-3.6	0.3	0.3	-	-	-	-	-
500	500	0.6	0	0.1	-0.1	-0.3	-0.8	-5.2	-4.3	0.2	0.2	-	-	-	-	-
750	750	0.7	0.2	0.2	-0.4	-0.3	-0.5	-2.1	-2.2	N/A	N/A	N/A	-	-	-	-
1000	1000	0.9	0.8	0.4	0.1	0.2	0.1	-3.2	-3.1	0.3	0.3	-	-	-	-	-
1500	1500	0.4	0.3	0	-0.3	-0.2	0.1	-1.9	-2.6	N/A	N/A	N/A	-	-	-	-
2000	2000	0.4	0.4	-0.1	-0.3	0	-0.3	-3.7	-4.3	0.6	0.7	-	-	-	-	-
3000	3000	0.5	0.3	0	-0.3	0.2	0.8	-4.1	-3.3	N/A	N/A	N/A	-	-	-	-
4000	4000	1.1	0.6	0.6	-0.1	-0.5	-0.1	-3.6	-2.7	0.6	0.6	-	-	-	-	-
6000	6000	0.7	0.1	0.1	-0.5	-0.5	-0.3	-2.2	-1.8	N/A	N/A	N/A	-	-	-	-
8000	8004	-0.8	-1	-1.2	-1.6	0.2	0.6	-7	-7	N/A	N/A	N/A	-	-	-	-
10000	-	-	-	-	-	-	-	-	-	N/A	N/A	N/A	N/A	N/A	-	-
12000	-	-	-	-	-	-	-	-	-	N/A	N/A	N/A	N/A	N/A	-	-
Speech	N/A	-0.2	-1.1	-0.2	-0.6	-1.9	-0.9			0.6	0.6					

Attenuator Linearity, dB Deviation																							Off Level	Cross Talk
Dial Indication	110	105	100	95	90	85	80	75	70	65	60	55	50	45	40	35	30	25	20	15	10	5	0		
Channel 1 Left	-0.1	-0.1	-0.1	-0.1	-0.1	0	0	0	0 Ref.	0	0	-0.1	0	-0.1	-0.1	-0.1	-0.1	-0.2	0.1	0.4	-0.1	0.9	0	87	82
Channel 2 Right	-0.1	-0.1	-0.1	-0.1	-0.1	0	0	0	0 Ref.	0	0	0	0	0	0.1	0	-0.1	-0.1	0.1	0.7	0.5	1	-0.4	83	88

	Channel 1	Channel 2
Fall Time, ms	30	15.75
Rise Time, ms	30	25.25
Overshoot, dB	0.14	0.22
Aux./Ext. dB Deviation	0	0
Channel Mix Error, dB Deviation	0	0
White Noise - SPL @ 70 dB HL	-	-

	Pass	Fail
Attenuator Extended Range	X	
Shock Hazard	X	
Other Unwanted Sound	X	
Mechanical Integrity	X	
Warble/FM	X	
Speech S/N	X	

*Primary Transducer Type	TDH 39
*Secondary Transducer Type	3A

Bone Transducer Type	B71
Note: Masking and Distortion Values Based On Primary Transducer	

Date of Calibration 06/27/2016 Certified By Rob Griggs

Comments:

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SLM L/D 824 # A4267 12/24/2015
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CASS 1016 05/13