A Combination Antioxidant Therapy in the Treatment and Prevention of Age Related Hearing Loss

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Dedication

Commit everything you do to the Lord. Trust him to help you do it and he will.

– Psalm 37:5

Commit your work to the Lord, then it will succeed.

– Proverbs 16:3

Whatever you do, work at it with all your heart, as working for the Lord, not for men.

– Colossians 3:23

Now unto him that is able to keep you from falling, and to present you faultless before the presence of his glory with exceeding joy,

To the only wise God our Savior, be glory and majesty, dominion and power, both now and ever.

– Jude 1: 24-25

Abstract

Presbycusis, or age-related hearing loss, is one of the most common conditions impacting the aging population, characterized by progressive sensorineural hearing loss and impairment in speech discrimination capability. Current management is limited to auditory rehabilitation with the assistance of hearing aids or cochlear implants in advanced cases. There currently exists no effective treatments available for the prevention or attenuation of hearing loss in association with presbycusis. The purpose of this study was to evaluate the potential of a combination antioxidant therapy in the prevention and treatment of presbycusis. The objectives of this randomized controlled animal study were to: identify an anesthetic which would allow for repeated reliable longitudinal auditory brainstem response (ABR) testing in a mouse model, identify a combination antioxidant which targets multiple sites within the oxidative pathway and assess its ability to prevent age-related threshold shifts and to arrest further age-related threshold shifts in C57BL6 mice with documented age-related threshold shifts. Mice were randomized to one of three groups: an early treatment group, a late treatment group, or a control group. The treatment groups of mice were fed with a combination agent comprised 6 antioxidant agents. ABRs were performed comparing Avertin to ketamine/xylazine with no significant difference in test thresholds. ABR thresholds were recorded at baseline and every 3 months following initiation of treatment in the 3 randomized groups. Threshold shifts from baseline were decreased in the treatment groups when compared to the control group at all tested frequencies and time points.

iii

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction and Research Objectives

Presbycusis (or age-related hearing loss) is a common condition affecting the aging population. According to the United States Census Bureau, the population age 65 and older will more than double between the years of 2012 and 2060. In 2060, it is estimated that approximately 92 million Americans will be within this age range (United States Census Bureau, 2012). With the rapidly increasing aging population, presbycusis is becoming an increasingly important public health concern.

Age-related hearing loss represents the sum of cumulative damage to the auditory system that progresses with aging. As such, its etiology is multifactorial including oxidative injury associated with aging, noise exposure, ototoxin exposure, environmental toxin exposures, otologic disease, and injury from systemic disease. Various histopathologic changes have been noted in association with presbycusis including diffuse cellular losses, evidence of oxidative injury within the cochlea and evidence of atrophy of vascular supply within the stria vascularis. The typical clinical presentation of presbycusis is one of high frequency sensorineural hearing loss. Difficulty with speech understanding in ambient conditions and tinnitus are also commonly associated. Preventive actions should be employed to thwart otologic insult throughout life. However, clinical management options are limited; they include environmental modifications, hearing assistive devices, hearing aids and cochlear implants of

which only cochlear implants typically are covered my health insurance providers in cases of severe sensorineural hearing loss. There are no medical therapies currently available for the prevention or treatment of age-related hearing loss. Given the lack of support provided by health insurance providers for hearing assistive devices and hearing aids as well as the increase in the absolute number and percentage of the geriatric population, identifying a low cost, non-surgical therapy for the treatment or prevention of age-related hearing loss presents an opportunity to have a significant public impact and positively influence the lives of many individuals. This was the goal of this dissertation.

The objective of this study was to explore the possibility of preventing and attenuating age-related hearing loss by administration of a combination antioxidant therapy which targets multiple sites within the oxidative pathway. Within the cochlea, oxidative damage caused by free-radicals and reactive oxidative species has been widely demonstrated to contribute to the development of age-related hearing loss. The consequence of such cumulative oxidative stress is depletion of glutathione, an endogenous antioxidant, in cochlear cells which results in lack of protection from oxidants, loss of cochlear outer hair cells, alterations in biophysiology in the cochlear lateral wall and diminution in high frequency hearing acuity. To study the potential for preventing oxidative damage in the cochlea as related to aging a combination antioxidant was developed. C57BL/6 mice were used as an animal model. It was hypothesized that a combination antioxidant could be developed for oral

administration which targets multiple sites within the oxidative pathway and is safe for oral consumption in C57BL/6 mice. It was further hypothesized that age-related hearing loss would be slowed or arrested by the administration of a combination agents that prevent or reduce this oxidative damage. Longitudinal analysis was required for assessment of progression of hearing loss in the C57BL/6 mice. The following specific aims were designed to address these issues:

Specific Aim 1. To validate 2,2,2 tribromoethanol as an anesthetic for mouse auditory brainstem response (ABR) testing using the C57BL/6 mouse model.

To study the impact of the combination antioxidant, longitudinal analysis was required with ABR assessments performed on the mice at various time points following treatment. Preliminary investigation revealed a significant rate of mortality in C57BL/6 mice following administration of standard doses of ketamine and xylazine anesthesia for the procedure. This high rate of mortality complicated the ability to perform longitudinal analysis. An alternative anesthetic was sought to provide adequate anesthesia for a period of approximately 60 minutes to complete the ABR testing with a low associated rate of mortality. Avertin, or 2,2,2 tribromoethanol was chosen as an alternative to ketamine and xylazine because of its low morbidity and mortality profile reported within the literature. We hypothesized that 2,2,2 tribromoethanol would effectively provide adequate anesthesia for repeated reliable longitudinal auditory brainstem response (ABR)

testing in a mouse model without impacting otophysiological response. To test this hypothesis, we measured ABRs from mice anesthetized with both 2,2,2 tribromoethanol and ketamine/xylazine, respectively.

<u>Specific Aim 2. To study the effects of daily, oral administration of a combination antioxidant targeting multiple sites within the oxidative pathway on the prevention of the onset of age-related hearing loss</u>.

We hypothesized that administration of a combination antioxidant targeting multiple site within the oxidative pathway can prevent the onset of age-related hearing loss in C57BL/6 mice. This was tested by performing longitudinal ABR analysis in C57BL/6 mice that received antioxidant therapy prior to onset of age-related threshold shifts.

<u>Specific Aim 3. To study the effects of daily, oral administration of a combination antioxidant targeting multiple sites within the oxidative pathway on the prevention of the progression of age-related hearing loss</u>.

We hypothesized that administration of a combination antioxidant targeting multiple site within the oxidative pathway can prevent the progression of age-related hearing loss in C57BL/6 mice. This was tested by performing longitudinal ABR analysis in C57BL/6 mice that received antioxidant therapy following onset of age-related threshold shifts.

Chapter 2: Background and Significance

Presbycusis is one of the most common conditions affecting the aging population. Presbycusis, also commonly referred to as age-related hearing loss (ARHL), may be defined as progressive sensorineural hearing loss that occurs as a function of a variety of contributing factors accumulated throughout life within the geriatric population. The hearing loss associated with ARHL is characteristically high frequency with accompanying deficits in speech discrimination. The prevalence of age-related hearing loss increases with age. The etiology of age-related hearing loss is multifactorial with oxidative injury, noise exposure, heredity and otologic injury (i.e. ototoxins and otologic disease) representing major contributing factors. Presbycusis has a wide potential impact not only on the individual, but also on society as a whole making prevention, early diagnosis and treatment of critical importance in circumventing this potential problem. This chapter provides an overview of the current state of knowledge regarding etiology, diagnosis, treatment and prevention of age-related hearing loss. This chapter also reviews the relevant histopathology and pathophysiology as related to presbycusis. A review of the literature regarding cochlear oxidative injury associated with age-related hearing loss and previously attempted antioxidants in the prevention of age related hearing loss is provided. Finally, a description of the C57BL/6 mouse model of age-related hearing loss as well as a review of mouse anesthetic for research procedures is presented.

Section 2.1: Clinical Perspective and Public Health Impact of ARHL

Definition of Presbycusis

Presbycusis may be defined as any sum of conditions and exposures that lead to the development and progression of hearing loss with aging. Age-related hearing loss is characterized by high frequency sensorineural hearing loss that progressively increases with aging. Figure 1 depicts an audiogram characteristic of moderately advanced age-related hearing loss. As can be seen in Figure 1, individuals with age-related hearing loss tend to develop losses within the frequencies at and above 2000 Hz earlier in the course of disease. As such, individuals tend to experience difficulty with understanding the voiceless consonants of speech (i.e. c (k), ch (tʃ), f (f), k (k), p (p), s (s), sh (ʃ), t (t), th (θ)). For example, patients with presbycusis may experience difficulty discriminating the words "bash," "bath," "bat," "back," "batch," and "bass". Because of this, patients often experience difficulty with speech understanding particularly in ambient conditions. The complaints that patients present with regarding this speech discernment in noise is often seemingly out of proportion to the degree of hearing loss noted on traditional audiologic testing in quiet. In fact, older adults with normal hearing have been demonstrated to perform worse on speech discrimination tasks particularly in noise when compared to young normal hearing adults (Gordon-Salant et al. 2005; Humes & Dubno, 2009; [a]Gordon-Salant et al. 2010; [b]Gordon-Salant et al. 2010). These findings are dramatically

increased with pure-tone shifts in age-related hearing loss (Gordon-Salant et al. 2005; [b]Gordon-Salant et al. 2010). This can lead to significant deficits in communication ability in social, recreational and professional situations precipitating a sense of angst and frustration.

Epidemiology

Presbycusis or age-related hearing loss is one of the most common conditions affecting the aging population. Within the United States, presbycusis is among the top three most common condition affecting the aging population ([a]Frisina et al. 2006; Yueh et al. 2003). It is the most common communication disorder affecting the geriatric population ([a]Frisina et al. 2006). Table 1 presents recently published prevalence rates for age-related hearing loss (Seidman 2000, Yueh et al., 2003; Parham et al. 2011; Mao et al. 2013). Within the United States, for individuals over the age of 65 years, the prevalence of age-related hearing loss ranges from 35% to 50%(Seidman 2000, Yueh et al., 2003; Parham et al. 2011). By age 75 years, the prevalence increases to 40% to 65%. By age 80 years, the prevalence increases to approximately 80%, and at 100 years, the prevalence approximates 90% (Seidman 2000, Yueh et al., 2003; Parham et al. 2011; Mao et al. 2013). Similar prevalence rates have been reported internationally.

In a review of 42 population-based studies in 2012, the World Health Organization (WHO) released estimates of disabling hearing loss rates in

individuals over the age of 65. The WHO defined disabling hearing loss as hearing loss greater than 40 dB in the better hearing ear (WHO, 2012). The estimated prevalence of disabling hearing loss in individuals age 64 years and older by international region is presented in Figure 2. With the exception of the Middle East and North Africa regions, all estimated prevalence rates fall between 30% and 50% for individuals 65 years of age and over. The highest prevalence rates for disabling hearing loss in adults over 65 according to this review were noted in South Asia. Interestingly on review of United States prevalence alone, racial differences in prevalence have been reported ([b]Lin et al., 2011). When comparing individuals who classify themselves as black and white, statistically significant differences in the rates of hearing loss defines as pure tone average in speech frequencies of greater than 25 dB in the better hearing ear were noted. Overall prevalence of hearing loss in black males age 70 and older was noted as 48.3% (95% confidence interval: 36.3-60.3) versus 71.5% (95% confidence interval: 64.8-78.3) in white males ($p = 0.002$). Similar, prevalence differences were found among females age 70 years and older ($p = 0.03$): black females 39.8% (95% confidence interval: 20.6-59.1) versus white females 59.0% (95% confidence interval: 51.3-66.8). These findings are consistent with previous reports within the literature regarding a decreased prevalence of age-related hearing loss in black individuals (Cooper, 1994; [b]Helzner et al., 2005; Agrawal et al., 2008). This is believed by some to be secondary to a hypothesized protective

effect of increased melanin particularly within the stria vascularis (Riley, 1997; Ohlemiller et al., 2009; Murillo-Cuesta et al., 2010).

Economic status has been noted to be correlated with the presentation of age-related hearing loss. This may be related to the multifactorial etiology of age-related hearing loss and the fact that undertreated otologic disease and systemic disease may contribute to hearing loss. Thus, individuals with higher socioeconomic status may have access to more preventive care and therapeutic treatment averting the onset or progression of hearing loss. According to the WHO study, individuals with an overall increased income of age 65 years and greater were correlated with a decreased prevalence of age-related hearing loss (WHO, 2012). Similar findings were reported by Lin et al. in a review of presbycusis prevalence in the US ([b]Lin et al., 2011).

Gender has been found to impact the presentation of presbycusis. The male population appears to be more affected by age-related hearing loss as compared to their female counterparts. This has been identified in a number studies based both on self-reported subjective measures and objective audiometric data (Garstecki & Erler, 1999; Uchida et al., 2003; Gordon-Salant, 2005; Stevens et al., 2011). In a data analysis of the National Health and Nutritional Examination Survey 2005-2006 cycle, hearing assessments among 717 adults age 70 and older were reviewed. A statistically significant difference in the rate of presbycusis (defined as speech frequency pure tone average of

greater than 25 dB in the better hearing ear) was noted in males as compared to females, 69.8% and 58.2%, respectively ([b]Lin et al., 2011).

Various epidemiological factors have been associated with the presentation of age-related hearing loss. As described above, international regional association may play a role in the rates of presentation. Socioeconomic status has been correlated with rates of age-related hearing loss likely secondary to healthcare disparities. Additionally, race and gender have been found to have a strong correlation with the presentation of presbycusis.

Etiology

As presbycusis represents the sum of life influences that precipitation the presentation of hearing loss with aging, there are a number of factors that have been linked to the development of presbycusis. Gates poignantly describes presbycusis as "a mixture of acquired auditory stresses, trauma, and otologic diseases superimposed upon an intrinsic, genetically controlled, aging process" (Gates, 2005). The etiology of presbycusis is influenced by genetics (up to 50% have significant family history), cardiovascular health (in turn influenced by smoking and diabetes), history of noise exposure as well as, ototoxic exposure and otologic disorders (Parham et al, 2013).

Aging and oxidative injury. In a landmark article by Denham Harman in 1956, the free-radical theory of aging was introduced (Harman, 1956). Since that time, free radical damage has been implicated as an etiologic factor in various

organ systems including the ophthalmologic, integumentary, and hepatic systems (Beatty et al., 2000; Justilien et al., 2007; Lu et al., 1999; Sanz et al. 1997). Similarly, oxidative stress has been hypothesized to be an integrally involved etiological factor in the development of presbycusis via free radical associated mitochondrial dysfunction presenting in the form of reactive oxygen species and reactive nitrogen species (Kidd & Bao, 2012). Various findings have been identified in animal studies to support this theory. Markers of oxidative stress have been noted to be increased in the cochlea of aged CBA/J mice (Jiang et al., 2007). Mice missing the gene encoding copper/zinc superoxide dismutase (Cu/Zn superoxide dismutase), a critical enzyme in the reduction of reactive oxygen species and maintenance of oxidative balance, show premature presbycusis (McFadden et al., 1999; Keithley et al., 2005). Similarly, overexpression of mitochondria-localized catalase which eliminates reactive oxygen species has been demonstrated to be protective against age-related threshold shift (Someya et al., 2009). It is postulated that with the accumulation of mitochondrial DNA mutation, oxidative phosphorylation is impaired and expression of antioxidant enzymes is altered leading to further increase in reactive oxygen species in the cochlea (Wang et al., 2013).

Human temporal bone studies support these findings. Various deletions within the mitochondrial genome have been noted in human temporal bone tissue (Bai et al. 1997; Fischel-Ghadsian et al., 1997; Maykaryan et al. 2008; Maykaryan et al. 2009; Maykaryan et al. 2010). A correlation has been identified

between the extent of age-related threshold shifts and mitochondrial DNA deletions (Bai et al. 1997; Maykaryan et al. 2009). The role of oxidative injury in the pathophysiology of age-related hearing loss will be further reviewed in Chapter 3.

Noise. Noise exposure is a well-established risk factor for hearing loss (Agrawal et al. 2008). Noise exposure contributes to oxidative stress and the production of reactive oxygen species which mediates short-term injury, but also may contribute to long-term damage. Hearing loss from noise exposure, also known as noise induced hearing loss, may take the form of transient threshold shifts or long-term acoustic changes. Everyday noise exposure can cause hearing loss and may accelerate the process which leading to presbycusis. Table 2 presents the current National Institute for Occupational Safety and Health (NIOSH) and Occupational Safety and Health Administration (OSHA) guidelines for noise exposure. To put these noise exposure levels into perspective, Table 3 provides a list of commonly and less commonly encountered noises with associated noise levels. It is interesting to note that listening to high power headphones on a near maximum level for a period of one hour in the gym or on a long commute, attending a concert, or using the snow blower for over 30 minutes may exceed the current NIOSH recommendations for noise exposure. It is easy to see how simple life experiences may contribute to noise damage over time.

Remarkably, recent studies have demonstrated that the effect of noise induced hearing loss may extends long after the exposure has ceased (Gates et

al. 2000). Noise damage occurs even with only temporary hearing loss or even with no immediate hearing changes noted, but this damage is believed to contribute to accelerated presbycusis. This is supported by findings in animal models of hearing loss demonstrating permanent neuronal losses in the spiral ganglion which has been correlated with accelerated age-related hearing loss (Kujawa & Liberman, 2006; Kujawa & Liberman, 2009; [c]Lin et al., 2011) and loss of synaptic terminal between inner hair cells and spiral ganglion neurons (Kujawa & Liberman, 2009; Bao & Ohlemiller, 2010). In clinical studies, presbycusis has been found to be more severe in individuals thought to have suffered cochlear damage in their youth from noise exposure (Gates et al., 2000).

It is important to note that presbycusis can develop in patients without a history of excessive noise exposure. Additionally, the shape and progression of hearing loss may differ in individuals with significant noise exposure. Gates et al. (2000) found that in subjects with noise induced threshold shifts, there is a reduced progression of hearing loss at 3, 4, and 6 kHz and accelerated hearing loss at the surrounding frequencies, particularly 2 kHz, with age which is the reverse of that seen in individuals without previous significant noise exposure. Not only does noise exposure contribute to the development of presbycusis, it also may impacts the character of threshold shifts associated with presbycusis.

Hereditary Factors. Heredity is an important factor in age-related hearing loss. A strong familial association has been implicated in presbycusis. This familial association suggests a potential genetic susceptibility to age-related

13

hearing loss (Christensen et al., 2001; McMahon et al., 2008). Approximately, 30 to 50% of variance in presbycusis is attributed to the effects of genes (McMahon et al. 2008; Gates et al. 1999). Various candidate genes have been proposed; however, there currently is no widely accepted genetic etiology that has been identified. The multifactorial nature of the etiology of presbycusis poses a challenge in the identification of the genetic contribution to this disease process in clinical studies.

Additional Etiological Factors. As presbycusis represents the progression of hearing loss with aging, various additional factors have been identified that affect the development and progression of presbycusis. Table 4 describes additional etiological factors that have been proposed to contribute to the development of age-related hearing loss (Fowler & Jones, 1999; Hultcrantz et al., 2006; Bao & Ohlemiller 2010; Uchida et al. 2010; Cruickshank et al. 2010; Kujawa & Liberman, 2006; Kujawa & Liberman, 2009; [c]Lin et al., 2011; Poortinga, 2007; Seidman, 2000; Torre et al., 2004; Iwai et al., 2003; Danielidis et al., 2007; [a]Helzner et al., 2005; Picciotti et al., 2004; Torres et al., 2005; [b]Frisina et al., 2006; Antonelli et al., 1990; Johnson & Nylen, 1995; Fuente & McPherson, 2006; Fuente et al., 2006; Morata et al., 2002; Brant et al., 1996; Itoh et al., 2001; Stypulkowski, 1990; Mills et al., 1999; Chen et al., 2007; Lee et al., 1998; Rybak et al., 2007; Selimoglu, 2007). One major contributing factor is otologic disease. In theory, any otologic condition that precipitates hearing loss throughout life will contribute to the progression of age-related hearing loss.

14

Studies in the literature have highlighted the etiologic contribution of otosclerosis, chronic otitis, Meniere's and temporal bone trauma (head trauma) to the presentation of age-related hearing loss (Howarth & Shone, 2006; Danielidis et al., 2007; Rosenhall et al., 2011). In addition to otologic disease, ototoxins have been described as a contributing factor in the etiology of age-related hearing loss, namely aminoglycosides (e.g. gentamicin and streptomycin), platinum-based chemotherapeutic agents (e.g. cisplatin and carboplatin), high dose loop diuretics (e.g. ethacrynic acid and furosemide), and salicylate and phosphodiesterase type 5 inhibitors (e.g. sildenafil, vardenafil, and tadalafil) (Stypulkowski, 1990; Mills et al., 1999; Chen et al., 2007; Lee et al., 1998; Rybak et al., 2007; Selimoglu, 2007; Snodgrass et al., 2010; Barreto & Bahmad, 2013). Toxic exposures such as heavy metals (e.g. lead and mercury) and solvents (e.g.toluene, styrene and xylene) also have been implicated (Johnson & Nylen, 1995; Morata et al., 2002; Fuente & McPherson, 2006; Fuente et al., 2006). Tobacco and alcohol abuse have been proposed as etiologic factors in presbycusis; however there are conflicting reports within the literature with this regard (Brant et al., 1996; Itoh et al., 2001). In multiple studies, cardiovascular disease and diabetes have been associated with the progression of presbycusis (Picciotti et al., 2004; Torre et al., 2005; Fowler & Jones, 1999; [b]Frisina et al., 2006; Diniz & Guida, 2009; Ren et al., 2009; Uchida et al. 2010;). Additionally renal failure and impaired immune function may play a contributing role (Anotelli et al., 1990; Iwai et al., 2003).

A number of factors have been found to be protective against the progression of age-related threshold shift. Hormonal function including aldosterone and estrogen have been found to be protective against age-related changes in hearing (Tadros et al., 2005; Hultcrantz et al., 2006). Higher levels of bone mineral density have been correlated with decreased rate of change in hearing with age ([a]Helzner et al., 2005). Additionally, caloric restriction may play a preventative role (Seidman, 2000; Torre et al., 2004). Further study is required to fully elucidate these effects.

Impact of Presbycusis

The impact of age-related hearing loss is not limited to merely the ability of an individual to hearing and comprehend speech. The impact of presbycusis is far reaching. It not only impacts the individual from a psychosocial perspective and potentially increases the progression of dementia, but it also has a significant public health impact internationally.

Psychosocial Impact. The psychosocial impact of presbycusis has been well established. With progression, presbycusis typically precipitated difficulty with speech discrimination and word understanding particularly in ambient situations. This translates to difficulty participating in conversation at a busy restaurant or understanding the punch line of a joke at a family gathering. Although this may seem minor, for many individual this can produce significant

embarrassment and frustration. Social isolation and anxiety in social situation have been described in association (Cacciatore et al., 1999; Bernabei et al., 2011). In countless patients, this progresses to decreased autonomy and depression significantly impacting their activity level and social interconnectivity (Uhlmann et al., 1989; Kalayam et al. 1995; Heine & Browning, 2002; Dalton et al., 2003; Kramer et al. 2008; Mohlman, 2009; Bernabei et al., 2011). This has been associated with a significant decline in overall quality of life reported by individuals affected with presbycusis (Li-Korotky, 2012; Ciobra et al., 2012).

    Association with dementia. The association between dementia and age-related hearing loss has been described for decades within the literature. However, our understanding of the impact that presbycusis plays on the progression of dementia has evolved significantly. Within the mid-1980s, various publications were produces revealing the significant correlation between hearing impairment in the elderly and the progression of dementia (Uhlmann et al., 1989; Uhlmann et al., 1986; Weinstein & Amsel, 1986). Global functional decline also has been noted in association with age-related hearing loss shy of the clinical diagnosis of dementia within this population (Herbst & Humprey, 1980; LaForge et al., 1992; Cacciatore et al., 1999). A direct positive correlation between degree of hearing loss and cognitive decline has been noted on both verbal and non-verbal cognitive testing within numerous reports in the literature (Ohta et al., 1981; Peters et al., 1988; Uhlmann et al. 1989; Lindenberger & Baltes, 1994; Gussekloo et al., 2005; Tay et al., 2006; Valentijn et al., 2005). More recently,

this association has been further quantified. Among individuals free of dementia or mild cognitive impairment, the impact of the degree of hearing loss was quantitatively associated with decline in cognitive function. It was noted that a 25 dB hearing loss produces a decline equivalent to an age difference of 6.8 years on test of executive function (Lin, 2011; [a]Lin et al., 2011). Additional study is require to further investigate this potential causative relationship.

 Public Health Impact. Presbycusis poses a significant public health concern internationally. The percentage of the population over the age of 65 is projected to grow internationally over the ensuing decades (Kinsella & Wan, 2008). As such, the prevalence of age relating hearing loss will increase precipitously. According to the World Health Organization (WHO) hearing loss is one of the six leading contributors to the burden of disease in industrialized countries (Mathers et al., 2000). Based upon the WHO 2012 Hearing Loss Estimate, adults make up 91% of individuals internationally with disabling hearing loss, when defined as greater than or equal to 40 dB of hearing loss, accounting for 328 million of 360 million total individuals internationally affected (WHO, 2012). Adults over the age of 64 years make up more than half of all adults with hearing loss (Schiller et al., 2012). It is projected by 2025 that there will be 1.2 billion people over 60 years of age worldwide, with more than 500 million individuals who will suffer significant hearing impairment from presbycusis (Sprinzl & Riechelmann, 2010). Additionally, presbycusis may contribute to early

undesired retirement and decreased workforce participation creating a significant economic impact.

Schuknecht Classification

Pathophysiological changes have been noted in various components of the auditory system in association with age-related hearing loss. Traditionally, presbycusis was believed to be a disorder of the peripheral auditory system, namely the cochlea. Schuknecht developed a classification system which highlights the cochlear changes that have been noted in association with age-related hearing loss (Schuknecht, 1964; Ramadan & Schuknecht, 1989). Figures 3 to 6 depicts audiograms that would accompany four of the classic classifications of presbycusis proposed by Schuknect. Sensory presbycusis is characterized by slowly progressive, bilateral steep down-sloping high frequency hearing loss associated with loss of cochlear hair cells particularly at the basal turn of the cochlea (Figure 3). Neural presbycusis is characterized by a decline in speech discrimination associated with spiral ganglion cell loss (Figure 4). Metabolic presbycusis is characterized by flat hearing loss attributed to atrophy within the stria vascularis (Figure 5). Mechanical presbycusis is associated with a gradual descending pattern of hearing loss presumed to occur as a function of stiffening of the basilar membrane (Figure 6). The most common form encountered is mixed presbycusis which encompasses flat, sloping or high frequency hearing loss and is associated with a combination of hair cell, spiral

ganglion, and strial losses (Schuknecht & Gacek, 1993). In addition to the

histopathologic changes described within the Schuknecht classification system,

various cochlear changes have been described in association with aging

including loss of outer hair cells and support cells, changes in hair cell stereocilia,

vascular changes in the stria vascularis, decreased synapses particularly at the

basal turn, and loss of nerve fibers and ganglion cells (Nadol, 1979; Suga &

Lindsay, 1976; Felder & Schrott-Fischer, 1995; Scholtz et al., 2001; Nelson &

Hinojosa, 2003; Nelson & Hinojosa, 2006). The histopathology and molecular

pathophysiology of the peripheral pathway in presbycusis will be further reviewed

in Section 2.2.

Clinical Evaluation

The clinical evaluation of patients present with age-related hearing loss

often begins with the primary care provider. Primary care providers including

gerontologists are typically charged with the responsibility of coordinating the

care of elderly patients. Referral for audiometric evaluation should be including in

the routine evaluation of the geriatric patients. This appears as one of the goals

of United States National Institutes of Health (NIH) Healthy People 2020 (ENT-

VSL-5: increase the number of persons who are referred by their primary care

physician or other health care provider for hearing evaluation and treatment)

(NIH 2014). It is essential to realize that individuals with early presbycusis may

minimize their symptoms. Patients with presbycusis may initially be reluctant to admit that they are experiencing hearing loss. Alternatively, some individuals view hearing loss as a normal process of aging "like wrinkles" or perceive it as something that they must suffer through. Therefore, regardless of the patient report among the geriatric population, referral for audiometric evaluation should be made at least every 5 years. This is in accordance with the NIH Healthy People 2020 objective ENT-VSL-4 which aspires to increase the proportion of persons who have had hearing examination on schedule which is suggested as within a 5 year period (NIH 2014).

On clinical presentation, patients typically present with high frequency sensorineural hearing loss accompanied by decline in speech discrimination and speech understanding. Patients may report particular difficulty understanding conversation in ambient conditions. They may report complaints or their family may report that they complain that others tend to mumble or slur their words. Individuals with presbycusis often report that men's voices are easier to hear and understand than women's and children's voices.

A major related complaint of patients with presbycusis is its associated tinnitus. As the hearing loss progresses, tinnitus tends to progress as well. The tinnitus may present as intermittent, but typically becomes constant with progression of hearing loss.

The clinical evaluation of patient should include a complete head and neck examination with cranial nerve evaluation and otologic evaluation. Audiometric

evaluation should be performed including pure tone audiometry, speech discrimination, tympanometry and acoustic reflexes. Typical findings are of symmetric high frequency sensorineural hearing loss. Imaging of the temporal bone or skull base is typically not indicated with normal otologic examination and characteristic audiometric findings. If asymmetric hearing loss or otologic abnormalities are noted, clinical discretion should direct imaging.

Prevention

The key to prevention of age-related hearing loss is avoidance of factors that can promote or accelerate its progression. Adequate treatment of potentially contributing otologic disease should be employed. Ototoxins should be avoided when possible. Potential contributing comorbidities should actively and aggressively be managed to prevent associated threshold shifts.

Prevention of noise trauma is a major component of prevention of age-related hearing loss. Patient should avoid excessive noise exposure. When noise exposure is unavoidable (e.g. occupational requirements or recreation), adequate noise protective equipment should be utilized to dampen noise exposures. Insert ear plugs provide approximately 15 to 25 dB of sound attenuation. Alternatively, noise protection ear muffs provide approximately 20 to 30 dB of sound attenuation. For individuals with high acoustic professional demand with significant noise exposure (i.e. musicians, music teachers,

recording engineers or sound crew members), high fidelity hearing protection or musicians ear plugs are recommended. These customized ear plugs reduce sound level presented to the ear while maintaining clear and nature sound without muffled character as experienced with traditional ear plugs. The level of attenuation too is customizable.

Management

The mainstay of management of age-related hearing loss is environmental optimization and auditory rehabilitation. Auditory rehabilitation takes many forms ranging from simple assistive devices to cochlear implantation. There currently are no medical therapies available for the treatment or prevention of age-related hearing loss. This presents a significant opportunity for future research and development.

Environmental Optimization. Patient with presbycusis, whenever possible, should make modifications to their environment to optimize their auditory milieu. This may take the form of turning off televisions or decreasing the volume of background music during dinner conversations or sitting in a favorable location to facilitate face to face conversation to not only allow for lip reading, but also use of nonverbal cues (i.e. facial expression and gestures) to augment speech understanding. If comfortable, individuals may request speakers to speak more

slowly as comprehension of rapid speakers is commonly impaired in individuals with presbycusis.

Auditory Assistive Devices. Hearing assistive devices are non-prescription, non-personalized aids that augment environmental sounds or provide alternatives to challenging acoustic situation. These devices include such devices as telephone amplifiers, television amplifiers, alarm clocks with vibrators, frequency-modulation transmitters, doorbell signalers, and modified smoke detectors. These devices are of great benefit to patient with age-related hearing loss, but are limited in their ability to assist in the most challenging of communication situations.

Hearing Aids. Hearing aids represent the mainstay of treatment for age-related hearing loss. Hearing aids are typically recommended for sound amplification in individuals with hearing thresholds within the speech frequencies of 40 dB or greater. In selective cases where employment or educational demands are unusually demanding, individuals with losses of less than 40 dB may derive benefit from amplification.

Currently available hearing aids are mainly digital, although analogue devices remain on the market. Recent advances in hearing aid technology have leant to the development of enhanced features to improve sound appreciation and functionality including noise suppression technology, telephone coils, and multiple programming modes optimized for quiet, noise, or music appreciation.

These features are accessible manually or in some cases with automatic smart technologies.

Unfortunately, hearing aids are not covered by many healthcare plans and for countless patients the cost of the devices is prohibitive. Within the United States, fewer than 25% of patients affected by presbycusis who would benefit from hearing aids use them (Kochkin & MarkeTrak, 2009; Popelka et al., 1998). Amongst individuals with presbycusis who do not use hearing aids, 76% indicate that the cost is a significant reason for not using hearing aids and 64% simply state that they cannot afford hearing aids (Henkel, 2009). Conversely, in health systems where hearing aids are a covered benefit, elder hearing-impaired hearing aid use is greater than 60% (Corna et al., 2009). As hearing aid use has been reported to improve quality of life (Tsakiropoulou et al, 2007), increasing hearing aid availability provides a significant opportunity to improve public health internationally.

Hearing aid use does require a period of cognitive adaptation. Unfortunately, for some patients intermittent use precipitates dissatisfaction secondary to lack of complete adaptation to the hearing aid device. In a study of hearing aid use amongst individuals 70 years and older, use of 5 hours or greater was reported in only 19.1% of individuals ([b]Lin et al. 2011). The rate of use was noted to be dependent upon the degree of hearing loss with reported use rates of 3.4% for individuals with mild hearing loss, 40% for individuals with moderate hearing loss, and 76% for individuals with severe hearing loss ([b]Lin et al. 2011).

This data is similar to previous reports within the literature of underuse or abandonment of hearing aids of 25% and 40% underuse or abandon (Gates et al., 1990; Hanratty & Lawlor, 2000). The most commonly reported reasons for abandoning hearing aid use or underusing the technology are problems with fit or understanding the operation of the device as well as unmet expectation (Gates et al. 1990; Kochkin & Marke Trak, 2000; Ovegard & Ramstrom, 1994; Popelka et al., 1998; Smeeth et al., 2002; McCormack & Fornum, 2013). Thus, adequate education and establishment of realistic expectations are critical to hearing aid acceptance, adaptation and use.

Cochlear Implants. For patients with severe to profound bilateral sensorineural hearing loss, cochlear implantation may be an option. Cochlear implantation is recommended for patients who do not derive benefit from hearing aid use and meet certain criteria for speech discrimination testing (i.e. score 50% or less on sentence recognition testing in their worse hearing ear and 60% or less in the bilateral best aided conditions). However, criteria for cochlear implantation is continually evolving and broadening to encompass individuals with a greater degree of residual hearing. Within the geriatric population, the procedure is typically performed in the outpatient setting unless precluded by medical comorbidities. It is well tolerated with low surgical morbidity and high rates of success with significant improvement in quality of life (Poissant et al., 2008; Coelho et al., 2009; Budenz et al., 2011; Sanchez-Cuadro et al., 2013;

Lenarz et al., 2012; Lin et al., 2012). Interestingly, unlike hearing aids, cochlear

implantation is covered by most healthcare plans.

Section 2.2: Histopathology and Pathophysiology of ARHL

Various histopathologic finding have been associated with age-related hearing loss. These changes have been noted throughout the cochlea with some central changes reported within the recent literature. The cellular losses and histopathologic changes are believed to be instrumental in the clinical presentation of presbycusis.

With regard to cellular changes, loss of sensory hair cells and spiral ganglion cells have been reported; additionally, accumulation of lipofuscin, collection of lysosomal granules and absence of hair cell stereocilia have been associated (Nadol, 1979; Bohner et al, 1990; Schuknecht and Gacek, 1993; Mizuta et al, 1993; Adams et al, 1997; Ingham et al, 1999; Meyer zum Gottesberge et al, 2001; Scholtz et al, 2001; Juhn et al 2005). In addition to the age-related degeneration of the hair cells within the auditory pathway, degeneration of the cochlear lateral wall is considered to be another important mechanism of presbycusis (Ichimiya et al., 2000; Hequembourg and Lieberman, 2001). Tang et al (2014) illustrated that the lateral wall structures are crucial in the maintenance of endolymphatic potassium levels, endocochlear potentials and ion homeostasis within the cochlea. In studies of aging animals, increased apoptosis and atrophy have been noted within the lateral wall (Thomopoulos et al 1997; Suzuki et al, 2006, Juhn et al., 2005).

Studies indicate that the cochlear lateral wall may play a larger role in the pathogenesis of age-related hearing loss than previously acknowledged. A

number of vascular changes have been noted in the cochlear lateral wall. Thickening of the basement membrane of capillaries in the stria vascularis has been observed (Thomopoulos et al 1997; Juhn et al., 2005; Suzuki et al, 2006). These reports in the literature have suggested that age-related changes in the lateral wall of the cochlea, namely, observed thickening of the basement membrane of capillaries in the stria vascularis, may contribute to degenerative changes within the strial epithelial cells (Thomopoulos et al 1997; Suzuki et al, 2006). Additionally, thickening of the lamina densa within the stria vascularis and spiral ligament of aged rats when compared to young rats has been described. Immunohistochemical studies of the basement membrane (BM) have demonstrated increased deposition of laminins and immunoglobins (Sakaguchi et al, 1997a; Sakaguchi et al, 1997b). Immunohistochemical analysis of C57BL/6J mouse temporal bones demonstrated expression of vascular endothelial growth factor (VEGF) in the stria vascularis, spiral ligament and spiral ganglion cells of young mice (Picciotti et al., 2004). However, aged mice demonstrated a significant decrease in immunoreactivity in all of the aforementioned areas. Specific cochlear VEGF receptor, Flt-1 and Flk-1, expression was also evaluated. The Flt-1 receptor was found to be present in the organ of Corti, stria vascularis and spiral ligament in young mice, while aged mice had little to no immunoreactivity present in the organ of Corti, with expression in the stria vascularis and spiral ligament virtually unchanged. However, the Flk-1 receptor was present in the stria vascularis, spiral ganglion cells, modiolus, and the basilar

membrane and was virtually unchanged in the aged animals. Similar results were reported by Clinkard et al (Clinkard et al. 2013). Within the C57BL/6 mouse model, significant decreases in strial area, blood vessel number, luminal size, and luminal area were noted when comparing mice age 4 weeks to age 32-36 weeks of age. Additionally, significant up-regulation of Flt-1 was noted in aged mice with no change noted in Flk-1 receptor expression. However within Swiss Webster mice, these findings were not replicated (Kandasamy et al., 2012). No changes in VEGF, Flt-1 or Flk-1 were noted in aged Swiss Webster mice as compared to younger mice. None the less, this finding in the majority of reports in the literature implies decreased permeability, which may cause slowed diffusion of ions, metabolites, and oxygen through capillaries. This may ultimately result in disturbance of ion homeostasis.

Changes have been observed within the fibrocytes of the lateral wall in association with the aging process. Fibrocytes play a critical role in potassium ions recycling and the maintenance of the endocochlear potential; potassium is transported via a network of gap junctions between the fibrocytes within the lateral wall to be re-released in to the endolymph. Hequembourg and Liberman (2001) found age-related widespread degeneration of fibrocytes in the spiral ligament, especially among the type IV cell class within the C57BL/6 mice. These changes were found to precede inner and outer hair cell loss in the organ of Corti.

Kusunoki et al. (2004) studied the temporal bones of individuals aged 1 day to 86 years and identified a number of histopathologic changes with aging. In addition to loss of hair cells and spiral ganglion cell, they identified a statistically significant increase in the area of the stria vascularis in the lower basal turn of temporal bones of infants when compared to elderly patients. There was a significant correlation between advancing age and loss of fibrocytes in the spiral ligament. A loss of type II and IV fibrocytes was found to precede the loss of type I and III fibrocytes. Additionally, a greater loss was observed of type II and IV fibrocytes when compare to type I and III fibrocytes. Loss of fibrocytes implies impair potassium transport and maintenance of endocochlear potential.

A number of additional findings on immunohistochemical analysis of aging animals indicate impaired potassium ion transport as a crucial factor in presbycusis. Decreased expression of connexins has been observed with aging in atrophic endothelium and brain astrocytes (Yeh et al., 2000; Cotrina et al., 2001). Similarly, connexins, within the cochlear lateral wall, form gap junctions between fibrocytes allowing for the transport of potassium ion to be recycled into the endolymph. Ichimiya et al. (2000) observed a decrease in expression of connexin 26 in the cochlear lateral wall of the C57BL/6 mice as well as disorganization of the organ of Corti and a decrease in the number of spiral ganglion cells. Na^+-K^+-ATPase immunolabeling was also increased in the spiral ligament. Spicer et al. (1997) examined the temporal bones of young and aged gerbils and found alterations in Na^+-K^+-ATPase, carbonic anhydrase or creatine

kinase level by immunohistochemical analysis within the stria vascularis and spiral ligament. Age-dependent degeneration and loss of Na^+-K^+-ATPase in the stria vascularis was found to occur predominantly in the apex, lower base and hook of the cochlea. Na^+-K^+-ATPase, carbonic anhydrase and creatine kinase immunostaining intensity in fibrocytes changed in relation to the decline in strial marginal cell Na^+-K^+-ATPase activity, revealing up-regulation followed by down-regulation. These finding indicate that strial changes may precede changes within the spiral ligament, but support the notion that ion homeostasis is crucial in the pathogenesis of presbycusis. Spiess et al. (2002) also observed vacuolization and degeneration of type II and IV fibrocytes which was found to be more pronounced in areas of advanced strial degeneration. They attributed this vacuolization and degeneration in ATPase-rich fibrocytes and the associated intercellular edema to a secondary responses, possibly as a result of impaired diffusion of potassium through downstream marginal cells. Changes in marginal cell morphology have also been identified with the aging process, namely alterations in primary and secondary processes (Spicer & Schulte, 2005).

These findings within the lateral wall imply the possibility of decreased potassium recycling, impaired ion homeostasis and altered endocochlear potentials as a probable contributing etiology of age-related changes. In fact, Schmiedt (1996) reported decreased endocochlear potentials in a gerbil model in association with similar finding within the cochlear lateral wall. Additionally, Wu and Markus (2003) found reduced potassium concentrations by 30% in the basal

and apical turn of aged CD-1 mice, an accelerated model of hearing loss. The endocochlear potentials were also found to be reduced in magnitude. Severe loss of cells within the region of type IV fibrocytes was also observed in the basal and apical turns; type II fibrocytes loss was observed in the basal turn. Histopathologic changes in the lateral wall appear to be essential in potassium homeostasis derangement and alteration of endocochlear potential.

In addition to the previously proposed peripheral histopathology related to age-related hearing loss, recent evidence supports a central component to age-related hearing loss citing the fact that auditory performance in elderly individuals is largely impacted by decreased spiral ganglion cells, decreased central plasticity, central auditory processing disorder, as well as increased incidence of central nervous system disease and cognitive decline (Gates et al., 2010; Parham et al., 2013). Central presbycusis likely represents a component of the overall presentation of presbycusis rather than an isolated entity in and of itself (Humes et al. 2012). Further study is require to better characterize the central component of presbycusis.

Section 2.3: Oxidative Injury and Antioxidants in Age-Related Hearing Loss

The diversity of histopathologic changes identified within the cochlea in association with presbycusis as well as the variability in onset and severity encountered clinically with presbycusis implies a multifactorial etiology. A number of factors have been proposed to contribute to the presentation of presbycusis. These factors include oxidative stress, decreased stress defense mechanism, exogenous factors and genetics. Age-related changes in the cochlea are proposed to be a function of both genetics and environmental stresses, which lead to alterations in electrolyte homeostasis and cellular metabolism (Petropoulou et al, 2000). These alterations may affect various pathways leading to loss of membrane integrity, changes in ion permeability, damage to cellular structures, generation of toxic oxygen species and free radicals, and dysfunction of intracellular transport mechanisms.

Oxidative injury has been well classified as an etiologic factor in the process of aging. Oxidative injury caused by free radical damage is perhaps the most fundamental cause of age-related pathology in the biological aging of cells and may be an important intrinsic factor in presbycusis. Increased concentrations of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are implicated as a mediator of oxidative stress, inflammatory and infection-related damage in the central nervous system (CNS) and other tissues. ROS comprise superoxide free-radicals, hydrogen peroxides and hydroxyl

radicals. Concurrent with the increase in ROS is a decreased production of

function of the endogenous enzymes that protect the cell against ROS damage

including superoxide dismutase (SOD), catalase and glutathione peroxidase.

These ROS have been shown to contribute in part to producing mitochondrial

DNA (mtDNA) damage by causing deletions in the mitochondrial genome

(Seidman et al., 2004). The main contributor to RNS is the gaseous transmitter,

nitric oxide (NO). NO and its oxidation products, such as peroxynitrite, have

been shown to be potent cellular mutagens and cytotoxic agents which may

inflict accumulating damage to normal cells (Cooney and Mordan, 1995). The

increased release of NO may cause pathological conditions of the stria

vascularis. ROS may contribute to presbycusis by damaging metabolically active

tissues of the inner ear structure (e.g. stria vascularis).

Nitric oxide may play a critical role in the progression of disease in

presbycusis. Nitric oxide is a highly diffusible cellular mediator with potent

mutagenic and cytotoxic potential, which is capable of causing accumulated

damage in normal cells (Cooney and Mordan, 1995). Additionally, nitric oxide,

when overproduced, is a pro-apoptotic modulator that functions by activating the

caspase family of proteases within the cochlea (Chung et al, 2001). For these

reasons, nitric oxide may be a critical component of cochlear damage in age-

related hearing loss.

Within the cochlear, nitric oxide is produce by i-nitric oxide synthase (iNOS).

Preliminary data revealed elevation in the expression of iNOS in the lateral wall

of aged animal when compared to young animals. NOS inhibitors, such as NW-nitro-L-arginine-methyl ester (L-NAME), may slow or prevent the production of nitric oxide within the cochlea and its subsequent injury. L-NAME has been reported to block vestibular hair cell degeneration (Takumida and Anniko, 2000). Thus, iNOS inhibitor, like L-NAME, could potentially prevent the oxidative damage created by the presence of nitric oxide in the cochlea in ARHL.

Glutathione is an important ubiquitous antioxidant found in the inner ear. Lautermann et al. (1997) found reduced levels of glutathione in the inner ear of aged Fisher 344 rats when compared to young rats. We have observed inducible nitric oxide synthase (iNOS) immunoreactivity in the stria vascularis of aged animals while no iNOS expression was detected in that of young rats. Moreover, cultured marginal cells of the stria vascularis exposed to NO with sodium nitroprusside (SNP) as an NO donor exhibited elevation of intracellular calcium. This may be related to the NO-induced cellular apoptosis identified in the aging lateral wall (Juhn et al., 2006).

Deletions in the mitochondrial genome have been reported in association with presbycusis. The high incidence of mitochondrial (mt)DNA[4977] deletion in the temporal bones of presbycusis patients suggests a correlation between the mtDNA[4977] deletion and presbycusis (Bai et al., 1997). Dai et al. (2004) reported that the mtDNA[4977] deletion was accompanied by a narrowing of the inner ear blood vessels. They speculated that the narrowing of those vessels would cause hypoxia of the inner ear and initiate a chain of episodes including ischemia in the

inner ear, malfunction of oxygen metabolism, reduction in the level of oxidative phosphorylation, elevation of free radicals, damage of mtDNA, and accumulation of mtDNA[4977] deletion and other mtDNA mutants. Resultant malfunction of the mitochondria and impaired oxidative phosphorylation ultimately accelerates the degeneration and death of cells in the organ of Corti and the nerve fibers of the inner ear (Dai et al., 2004; Seidman et al., 2004; Fischel-Ghodsian et al., 2004).

Another aspect of the aging process can be represented by decreased ability of the body to defend itself against environmental insults. The inner ear and its associated structures sustain damage throughout life. Heat shock proteins (HSPs) are involved in major defense mechanisms within the inner ear. HSPs are proteins that are involved in protecting cells from a variety of stress by binding to denatured proteins and assisting in proper refolding. It has been reported that oxidative stress can induce the expression of HSPs, and pre-treatment increases the protection of cells for subsequent oxidative stress (Luna et al., 2000; Grosslau and Rensing, 2000; Smolka et al., 2000). Therefore, if the level of HSPs decreases with aging, it will indirectly accelerate age-related changes by not protecting cells effectively against various cellular stresses including oxidative stress. It has been reported that HSP70 mRNA level decreased with aging (Bernstein et al., 2000; Fargnoli et al., 1990). Investigation of HSP70 immunoreactivity in the stria vascularis of young and old rats has been reported to reveal reduced expression of HSP70 in old rats as compared to young rats (Juhn et al., 2002). This finding was found to correlate with elevations

in ABR thresholds at all frequencies in old rats as compared to young rats (Juhn et al., 2002).

Additionally, antioxidants also may prevent or retard oxidative damage that occurs in the cochlear lateral wall in association with age-related hearing loss. Antioxidants have been demonstrated to improve high frequency auditory sensitivities with human and animal models (Seidman, 2000; Takumida and Anniko, 2005). Reactive oxygen species (ROS) is considered to be involved in the mechanism of hearing loss. Many intrinsic enzymes, such as superoxide dismutase (Fridovich, 1981), catalase (Warholm et al., 1981) and glutathione transferase (Flohe, 1985) have been reported to protect cells from oxidative damage. It has been reported that antioxidant mechanisms require the action of a variety of small molecules in the diet, such as vitamin E (tocopherol) and vitamin C, which trap radicals in lipid and water-soluble membranes and reduce oxidative stress (Bieri et al., 1983). Seidman (2000) demonstrated that intervention designed to reduce reactive oxygen metabolites damage using antioxidants, such as vitamin E, vitamin C, and melatonin, can protect against age-related hearing loss. Takumida and Anniko (2005) also demonstrated that daily oral treatment with rebamipide and vitamin C in patients with age-associated hearing loss significantly improved pure tone hearing level thresholds at 125, 250, 500 and 8,000 Hz, but with no significant changes at 1, 2 and 4 kHz. Recently, a new prodrug, L-CySSG, was shown to abrogate hepatotoxicity induced by the ROS (Berkeley et al., 2003). Durga et al. (2007) reported slowing

of hearing decline in elderly patients following a 3-year dietary supplementation of folic acid. Derin et al. (2004) found improvement of age-related hearing deterioration in Wistar rats with administration of L-carnitine. Le et al. (2007) reported decreased cochlear degeneration in a group of mice treated with a combination lipoic acid and l-carnitine diet. Most recently, Seidman and colleagues (2008) a reduction in progression of ARHL and decreased expression of cycloxygenase-2 and 5-lipoxygenase in Fisher 344 rats with the administration of resveratrol, an antioxidant derived from the extract of grapes and red wine. Mikuriya et al (2008) reported that the administration of geranylgeranylacetone (GGA), which is a heat shock protein inducer, suppresses age-related hearing loss in DBA/2J mice.

In addition to nitric oxide related damage, hydrogen peroxide and reactive oxygen species have been known to produce toxicity in the cochlea. Superoxide dismutase, catalase and glutathione normally detoxify these free-radicals within the cochlea (Staecker et al 2001). Glutathione (GSH), an endogenous cellular antioxidant present in the cochlea, is a tripeptide consisting of L-cysteine (Cys), L-glutamic acid (Glu), and glycine (Gly). It is biosynthesized in a two-step, catalyzed by the enzymes gamma-glutamylcysteine synthetase and glutathione synthetase. Herbert T. Nagasawa, Professor of Medicinal Chemistry and Toxicology at the University of Minnesota, has developed a diverse series of L-Cys and GSH prodrug antioxidants for the treatment of drug-induced hepatotoxicity which is mediated by oxidative damage. Recently, he has

identified L-cysteine-glutathione mixed disulfide (L-CySSG), a mixed disulfide of

Cys and GSH, and he has shown that this sufhydryl-modified GSH prodrug is

highly effective in protecting the liver from hepatotoxicity in a mouse model of

oxidative stress induced by acetaminophen (Berkely et al, 2003). The

consequence of cumulative oxidative stress is depletion of the cellular antioxidant

GSH in cochlear cells, resulting in lack of protection from oxidants, loss of

cochlear outer hair cells and diminution in high frequency hearing acuity. Thus, L-

CySSG may be a potential protector against oxidative stress in the cochlea as it

has been described to be in the liver.

Section 2.4: C57BL/6 Mice as an Animal Model of ARHL

The clinical study of ARHL is limited by the capability to perform longitudinal study given the protracted time course of onset and progression of age-related hearing loss in the patient population. For this reason, animal models have commonly been utilized for the study of the histopathology, pathophysiology and potential treatment of ARHL. One of the most common animal models used for the study of presbycusis is the C57BL/6 mouse model.

The C57BL/6 mouse model is an accepted model of ARHL which has extensively been studied for decades (Mikaelian, 1979; Henry & Chole, 1980; Shone et el., 1991; Willott, 1991; Kazee et al., 1995; Spongr et al., 1997; Hequembourg & Liberman, 2001). The hearing loss described in this strain of mice has been well characterized and documented within the literature. As in presbycusis, age-related threshold shifts in C57BL/6 mice begin at the high frequencies. Also similarly, hearing loss begins well after the mice have reached sexual maturity (sexual maturity between 6 to 8 weeks of age; the onset of hearing loss approximately 5 to 6 months of age). These mice experience progressive high frequency sensorineural hearing loss as in the human population thereafter in addition to later onset low frequency sensorineural hearing loss. Complete deafness typically occurs by 18 months of age. In addition to changes in auditory physiology which mirror that of clinical presbycusis, histopathological changes have been documented within the C57BL/6 mice to mirror those noted in human temporal bone studies with aging.

Chapter 2.5: Rodent Anesthesia for Auditory Brainstem Response Testing

Ketamine and Xylazine

Ketamine is a controlled substance which is classified as a dissociative agent. Ketamine alone is known to provide analgesia, but lacks the ability to provide relaxation (Smith, 1993; Erhardt et al., 1984; Arras et al. 2001). A major adverse response to ketamine in the rodent population is life-threatening tremor and tonic-clonic convulsions (Green et al., 1981).

Xylazine is an α_2-agonist commonly used in veterinary and research anesthesia. Unlike ketamine, xylazine provides both analgesia and sedation. Xylazine is known to be a potent hypnotic with powerful central muscular relaxant properties (Green et al., 1981). Xylazine is has been documented to precipitate cardiac arrhythmia (Green et al. 1981).

Together, ketamine and xylazine are one of the mostly utilized combination anesthetics utilized for rodent anesthesia (Buitrago et al., 2008; Arras et al., 2001; Chaves et al., 2003; Deschepper et al., 2004; Flecknell, 1996; Furukawa et al., 1998; Hart et al., 2001; Janssen et al., 2004; Kawahara et al. 2005; Lorenz, 2002; Roth et al., 2002; Yang et al., 1999; Zuurbier et al., 2002). The recommended injectable dose of anesthetic for mice varies substantially in the literature secondary to differences in operators, mouse strains and a combined effect (Buitrago et al., 2008; Arras et al., 2001). The published dosage ranges from 80 to 200 mg/kg for ketamine and 0.5 to 10 mg/kg for xylazine

(Buitrago et al., 2008; Arras et al., 2001; Chaves et al., 2003; Deschepper et al., 2004; Flecknell, 1996; Furukawa et al., 1998; Hart et al., 2001; Janssen et al., 2004; Kawahara et al. 2005; Lorenz, 2002; Roth et al., 2002; Yang et al., 1999; Zuurbier et al., 2002). Potential adverse effects that have been reported in the literature with ketamine and xylazine administration in mice include profound bradycardia (Chaves et al., 2001; Fuentes et al., 2006; Hoit et al., 1997; Ishizaka et al., 2004; Roth et al., 2002; Hart et al., 2001), cardiac depression (Hoit et al., 1997; Kawahara et al., 2005; Roth et al., 2002), hypotension (Fuentes et al., 2006; Hoit et al., 1997; Ishizaka et al., 2004; Kawahara et al., 2005; Roth et al., 2002), and death (Arras et al., 2001). Of importance for longitudinal studies in which mice will be administered multiple dosages of anesthesia, a death rate as high as 40% has been reported in association with ketamine and xylazine administration within the literature (Arras et al., 2001).

Ketamine and xylazine has become the gold standard for mouse anesthesia in auditory brainstem response testing (van Looij et al., 2004; Henry 2002; Huang et al., 1995; Jero et al., 2001; Miller et al., 1998; Ou et al., 2000). Interestingly, ketamine and xylazine has been documented to alter waveform structure in auditory brainstem response testing, increase wave latencies by as much as 1ms and decrease wave amplitudes up to 20% (Smith & Mills, 1989; van Looij et al., 2004).

2, 2, 2 Tribromoethanol

Avertin (also known as 2, 2, 2 tribromoethanol or tribromoethanol) is a non-controlled anesthetic agent used for rodent anesthesia. Tribromoethanol is a safe and rapid onset anesthetic with a primary benefit of ease of preparation (Maheras & Gow, 2013; Papaioannou & Fox, 1993; Weiss & Zimmermann, 1999). The main advantages of 2,2,2 tribromoethanol for mouse anesthesia are the rapid rate of induction and recovery, the fact that it provides adequate surgical plane anesthesia, and has a relative lack of complications (Papaioannou & Fox, 1993). The dose range is quite wide with a low risk for overdose and lethality. Within the literature, the reported dosage range has been reported to be 125 to 800 mg/kg with dosages of 200 to 400 mg/kg most commonly administered (Cho et al., 2010; Flecknell, 2005; Fish & Meyer, 2008; Hedenqvist et al., 2003). In a study of 30 mice administered tribromoethanol with adequate surgical anesthesia attained, a death rate of 0% was observed (Cho et al., 2010). Similarly, the morbidity and mortality rate of tribromoethanol has been reported to be less than 1% (Papaioannou & Fox, 1993). The main adverse effect reported in the literature in association with tribromoethanol is peritoneal inflammation (Zeller et al., 1998; Maheras & Gow, 2013). Tribromoethanol was reportedly used in the literature for ABR assessment (Zheng et al., 1999; Gow et al., 2004; Maheras & Gow, 2013). However, there is no report of validation of tribromoethanol compared to rodent ABR anesthetic gold standard of ketamine-xylazine.

Chapter 3: Methods

Animal Model

      C57BL/6 mice were chosen as the animal model for this study. As reviewed in section 2.4, these mice are an accepted model of age-related hearing loss. The hearing loss experienced by this strain has been well characterized and documented within the literature (Hequembourg & Liberman, 2001). These mice experience onset of hearing loss around 5-6 months of age with near complete deafness by 18 months of age. The mice were housed within the RAR facilities at the University of Minnesota under conditions in accordance with the NIH guidelines for animal care, PHS Policy on Use of Laboratory Animals and the Animal Welfare Act. Care was taken to ensure that discomfort, pain, distress and injury were limited in the mice included in this study. Mice were given food and drinking water ad libitum. No operative procedures were performed on the animals. During ABRs, mice were maintained at a comfortably body temperature. Prior to sacrifice, mice were anesthetized with ketamine (30mg/kg) and sacrificed by decapitated. This method of sacrifice is consistent with the recommendation of the Panel of Euthanasia of American Veterinary Medicine Association.

Auditory Brainstem Response (ABR) Testing Technique

      During the ABR testing, the body temperature of each mouse was actively maintained at 37°C by placing the animal on a thermal blanket. ABRs were

collected by differentially amplifying voltages presented at subdermal needle electrodes using a standard vertex positive, ipsilateral mastoid-negative montage. ABR recordings, in response to calibrated acoustic signals, were obtained in the mice to verify auditory sensitivity to rarefaction clicks and tone bursts over a 1 to 32 kHz range. Total tone durations were 1 to 4 ms and consisted of Blackman rise/fall times and no plateau. The polarity of the signals were altered during a trial run to identify and reduce signal artifacts and thus to minimize the acquisition of spurious signals. The sampling rates were 50 kHz and durations were 10 ms for clicks and tone-pips. Five-hundred twelve presentations were averaged at each intensity. The gain of the physiological amplifier were set to 100,000x for ABRs. Threshold were determined in 5 dB steps of decreasing stimulus intensity, until waveforms lose reproducible morphology.

Combination Antioxidant

Given the contribution of oxidative damage to the pathogenesis of presbycusis (Figure 7), antioxidants may prevent the onset or progression of disease. A number of antioxidants have been reported in the literature to attenuate the progression of presbycusis. Some agents previously investigated include vitamin E, vitamin C, melatonin, folate, α-lipoic acid, L-carnitine, rebamipide, and resveratrol (Seidman, 2000; Takumida & Anniko, 2005; Durga et al., 2007; Le & Keithley, 2007; Derin et al., 2004; Seidman et al., 2008). The

46

associated results reported within the literature have been modest. Interestingly, most previous studies targeted one or two sites within the oxidative pathway. This study endeavored to create a combination antioxidant which targets multiple sites within the oxidative pathway.

To study the potential for preventing oxidative damage in the cochlea as related to age-related a combination antioxidant was developed. Figure 7 depicts the oxidative pathway within the cochlea. The goal of the combination antioxidant agent was to target multiple sites within the oxidative pathway. Six agents were chosen to be included with in the combination antioxidant based upon previous reports with in the literature or based upon site of action within the oxidative pathway. These agents included vitamin B12, folate, vitamin C (ascorbic acid), NW-nitro-L-arginine methyl ester (L-NAME), L-cysteine-glutathione mixed disulfide (L-CySSG), and ribose-cysteine.

DNA synthesis and repair is targeted by both vitamin B12 (Figure 8) and folate (Figure 9) which may function in the repair of mitochondrial genomic deletions. Clinical studies have revealed a correlation between vitamin B12 and folate deficiency with the presentation of presbycusis (Houston et al., 1999; Park et al., 2006). Additionally, within a clinical trial of 728 aged individuals in the Netherlands, folate supplementation administered over 3 years was found to lead to a statistically significant ($p = 0.02$) decrease in age-related threshold shift (Durga et al., 2007). These findings, however, were not clinically significant in that the difference in threshold shift between the folate and control groups was only 0.7 dB (Durga et al., 2007). There, however, have been reports within the

literature that challenge the correlation between folate and vitamin B12 with age-related hearing loss (Berner et al., 2000).

Vitamin C, also known as ascorbic acid, is a free radical scavenger that specifically targets reactive oxygen species (Figure 10). Level 1 evidence of ascorbic acid supplementation in Fischer 344 rats revealed an attenuation of age related hearing loss of 10-25 dB over the course of 22.5 months of treatment (Seidman, 2000). Similarly, clinical trials have revealed an approximate 5 dB attenuation of hearing loss at low frequencies with the administration of vitamin C and rebamipide over 8 to 52 weeks (Takumida & Anniko, 2005). With the addition of α-lipoid acid the hearing result was improved at both low and high frequencies. Takumida & Anniko, 2009).

Inducible nitric oxide synthase (iNOS) is a crucial enzyme in the production of nitric oxide, a potent reactive nitrogen species (RNS). Within the cochlea, nitric oxide is produced by inducible nitric oxide synthase (iNOS). Increased expression of iNOS has been demonstrated in the cochlea of aged Fischer 344 rats when compared to young rats (Juhn et al., 2002). NOS inhibitors may slow or prevent the production of nitric oxide within the labyrinth and its subsequent injury. L-NAME is a nitric oxide synthase inhibitor with cochlear bioavailability (Figure 11). L-NAME has been demonstrated to be protective against noise-induced hearing loss in an animal model and to decrease the production of nitric oxide within the perilymph (Diao et al., 2007; Shi et al., 2002). L-NAME has been reported to block vestibular hair cell degeneration (Takumida

& Anniko, 2000). Thus, L-NAME could potentially prevent the oxidative damage created by the presence of nitric oxide in the cochlea in ARHL.

Glutathione is an important ubiquitous, endogenous antioxidant found in the inner ear. Glutathione is a tripeptide consisting of L-cysteine (Cys), L-glutamic acid (Glu), and glycine (Gly). It is biosynthesized in two-steps catalyzed by the enzymes gamma-glutamylcysteine synthetase and glutathione synthetase. Reduced levels of glutathione were found in the inner ear of aged Fisher 344 rats when compared to young rats (Lautermann et al., 1997). L-CySSG and ribose-cysteine are both pro-drugs for glutathione (Figure 12 and Figure 13). L-CySSG is a mixed disulfide of L-cysteine and L-glutamic acid which has been found to be highly effective in protecting the liver from hepatotoxicity in a mouse model of oxidative stress induced by reactive oxygen species (Berkeley et al., 2003). Ribose-cysteine is a L-cysteine pro-drug which in turn is critical in the biosynthesis of GSH. Ribose-cysteine has been demonstrated to reduce hepatic and renal toxicity induced by reactive oxygen species (Lucas et al., 2000; Slitt et al., 2005). Neither L-CySSG nor ribose-cysteine has previously been investigated in the management of ARHL.

Together these antioxidants target four specific sites within the oxidative pathway: preventing mitochondrial genomic deletions, reducing nitric oxide synthase, reducing reactive oxygen species and enhancing glutathione production (Figure 14). The antioxidant dosages administered are depicted in Table 5. The daily dosages were determined based upon current data regarding maximal daily dose and previous reported dosages in the literature for auditory

pathologies. The combination antioxidant agent was administered orally via the mouse feed. Mice were fed with a milled powder formation of the Teklad NIH-07 rodent diet. The antioxidants were mixed within the powdered feed. The daily dose of antioxidants was mixed with the powdered feed based upon an average daily consumption of 5 g of feed daily per 30 g body weight (Bachmanov et al, 2002). This rate of food consumption is relatively consistent between the ages of 3 months and 12 months of age (Starr & Saito, 2012).

Statistical Analysis

Standard statistical analysis was performed using Excel software. For comparison of threshold means and threshold shift means comparing study groups two tailed t-tests were performed with a $p<0.05$. Additionally, comparing the treatment groups and control group Krustal-Wallis analysis of variance (ANOVA) was performed to assess for statistically significant differences across groups. With $p<0.05$, post-hoc Tukey method analysis was performed to confirm statistical significance as well.

Specific Aim 1. To validate 2,2,2 tribromoethanol as an anesthetic for mouse auditory brainstem response (ABR) testing using the C57BL/6 mouse model.

Six C57BL/6 mice were assigned for this portion of the study. Each of the mice were assessed bilaterally in a cross-over study design. Mice were assessed at age 3 months. In three of the six mice, 2,2,2 tribromoethanol was used for ABR assessment on day one followed by ketamine and xylazine on day three. In the remaining three of six mice ketamine and xylazine was used for ABR assessment on day one followed by 2,2,2 tribromoethanol on day three. The following techniques were used for the respective anesthetics.

Avertin (2, 2, 2, tribromoethanol) was administered intraperitoneal. A dose of 50 mg/kg 2, 2, 2, tribromoethanol was utilized. Approximately 5 minutes were allowed for the anesthetic effect of the 2, 2, 2 tribromoethanol. The appropriate level of anesthesia was confirmed with toe pinch. ABR testing was performed per the protocol stated above. Thresholds were recorded bilaterally. Statistical analysis was performed comparing the ABR thresholds obtained using ketamine and xylazine as an anesthetic versus 2, 2, 2 tribromoethanol as an anesthetic.

The standard dose of ketamine and xylazine was utilized as a control: ketamine 100 mg/kg and xylazine 5 mg/kg. The ketamine and xylazine was administered intraperitoneal. Approximately 5 minutes was allowed for the anesthetic effect of the ketamine and xylazine. The appropriate level of anesthesia was confirmed with toe pinch. ABR testing was then performed as reported above. The thresholds were recorded for bilateral assessment.

Specific Aim 2. To study the effects of daily, oral administration of a combination antioxidant targeting multiple sites within the oxidative pathway on the prevention of the onset of age-related hearing loss.

To assess the impact of oral administration of the combination antioxidant on the prevention of onset of age-related hearing loss, C57BL/6 mice were randomized to one of two experimental groups: a control group which received normal mouse diet and an early treatment group which received initiation of antioxidant therapy at 3 months of age (prior to onset of presbycusis as documented in the literature). The sample size was determined based on a desired power of 0.8, a desired alpha of 0.05 and an estimated size affect delta of approximately 1.4 for preliminary data utilizing L-CySSG alone. The size effect delta for this study was estimated to be approximately 1.8. Therefore a minimum of six animals would need to be assigned to each group for assessment. A total of twelve mice were randomized to the control group and twelve mice were randomized to early treatment group. The timeline for auditory brainstem response testing is presented in Figure 15. Auditory brainstem response testing was performed on the mice in both groups at 3 months of age upon initiation of the study. Subsequently auditory brainstem response testing was performed every 3 months until the mice were aged 12 months for a total of 9 months of serial auditory assessments. All data was recorded within an Excel file. Statistical analysis was performed using Microsoft Excel.

Specific Aim 3. To study the effects of daily, oral administration of a combination antioxidant targeting multiple sites within the oxidative pathway on the prevention of the progression of age-related hearing loss.

To assess the impact of oral administration of the combination antioxidant on the prevention of the progression of age-related hearing loss, C57BL/6 mice were randomized to one of two experimental groups: a control group which received normal mouse diet and a delayed treatment group which received initiation of antioxidant therapy at 6 months of age (3 months following the onset of presbycusis as documented in the literature). The sample size was determined as above based on a desired power of 0.8, a desired alpha of 0.05 and an estimated to be approximately 1.8. Therefore a minimum of six animals would need to be assigned to each group for assessment. A total of twelve mice were randomized to the control group and twelve mice were randomized to the delayed treatment group. The timeline for auditory brainstem response testing is presented in Figure 16. Auditory brainstem response testing was performed on the mice in both groups at 3 months of age upon initiation of the study. Additionally, ABR was performed at 6 months of age to confirm onset of age-related threshold changes. The mice were initiated on antioxidant therapy at 6 months of age. Subsequently auditory brainstem response testing was performed at 9 months and 12 months. All data was recorded within an Excel file. Statistical analysis was performed using Microsoft Excel.

Chapter 4: Results

Section 4.1: Avertin versus Ketamine/Xylazine for ABR Testing

A total of 6 mice were selected to assess the impact of Avertin on ABR physiology utilizing the technique described above. Within the Avertin then ketamine/xylazine group three of three mice survived the complete assessment. One mouse did seize and expire after completion of ABR assessment with ketamine/xylazine. Within the ketamine/xylazine then Avertin group, one mouse expired following ketamine/xylazine administration; therefore, it could not be assess in the Avertin condition. Thus, assessments were performed in a total of 5 mice in a cross-over study design for assessment of Avertin versus ketamine/xylazine as anesthetic for ABR. Of note two mortalities among 6 mice may be attributed to anesthesia with ketamine and xylazine. There were no mortalities attributed to Avertin administration.

Figure 17 provides the mean ABR thresholds by frequency obtained while the mice were anesthetized with ketamine/xylazine (presented in green) and mean ABR thresholds by frequency obtained while the mice were anesthetized with Avertin (presented in blue). Additionally, the raw ABR threshold data is presented in Table 6. A two tailed t-test was performed of the ABR thresholds by frequency, overall in total and as mean thresholds. No statistically significant difference was noted for Avertin versus ketamine/xylazine (p>0.05).

Section 4.2   Early Antioxidant Treatment

A total of 12 mice were assigned to the control group, and a total of 12 mice were assigned to the early antioxidant treatment group. Nine mice within the control group survived for the duration of the study. The three mice that expired during the course of the study were apparently independent of the study assessments (i.e. death did not occur within 48 hours of anesthesia for ABR). A total of 9 control mice and 12 early antioxidant treatment mice were assess longitudinally.

ABRs were performed at the baseline time period of 3 months (prior to onset of antioxidant therapy within the early antioxidant treatment group). The ABR threshold data per study animal ear is presented in Figure 18. The control mice data are presented in blue, and the early antioxidant treatment mice data are presented in yellow. The raw data for most mice within both groups as depicted in Figure 18 is relatively similar. This graph does highlight that there does appear to be one relative outlier animal (presented as two relative outliers plots for right and left ears on the graph) within the early antioxidant treatment group with ABR thresholds 10 to 20 dB greater than the mean for either group. The mean baseline thresholds by frequency for the control group as compared to the early antioxidant treatment group is presented in Figure 19. There was no statistically significant difference between the mean thresholds by frequency at baseline for the control group versus the early antioxidant treatment group (p>0.05 with two-tailed *t*-test).

ABRs were then performed at the 6 month time period. At this time point, the early antioxidant treatment group had received 3 months of antioxidant therapy. The ABR threshold data per study animal is presented in Figure 20. This graph again highlights the presence of two relative outliers within the early antioxidant treatment group. Despite the presence of these outliers, a statistically significant difference (p < 0.001) was noted between the mean threshold shift from the 3 month baseline ABR assessment to the 6 month time point. At the 6 month time point, the mean ABR threshold shift for the control group was 5.6 dB as compared to 0.76 dB within the early antioxidant treatment group. There was, however, no statistically significant difference (p > 0.05) on two-tailed *t*-test when comparing the threshold means for the control group as compared to the early antioxidant treatment group at 6 months with the exception of at 32 kHz. This is partially secondary to the impact of the two relative outliers in the mean threshold analysis. With exclusion of these two outlier data points, a statistically significant difference was noted between the control ABR mean thresholds and early antioxidant treatment ABR mean thresholds at 4 kHz, 16 kHz, and 32 kHz.

The statistical significance of the early administration of antioxidants in this animal model becomes more evident at the 9 month time point. At the 9 month ABR assessment time point, the early antioxidant treatment group has received 6 months of antioxidant therapy. Figure 21 presented the ABR threshold data per study animal within the control group and early antioxidant treatment group for the 9 month time point. As is apparent by Figure 21, the ABR thresholds for the control group overall are greater than that of the early antioxidant treatment

group. Figure 22 presents the mean ABR threshold by frequency for the control group as compared to the early antioxidant treatment group. These threshold differences were statistically significant at all frequency with a *p*-value of <0.001. On assessment of ABR threshold shift from the 3 month baseline to the 9 month time point, the mean overall ABR threshold shift for the control group was 23 dB as compared to a 4 dB mean overall ABR threshold shift for the early antioxidant treatment group. This difference was statistically significant with a *p*-value of <0.001. On analysis of the mean ABR threshold shift by frequency, the mean threshold shift for the control group ranges from 19 to 25 dB as compared to that of the early antioxidant treatment group which ranges from 3 to 6 dB. The mean threshold shift by frequency as well as associated *p*-values are presented in Table 10. Statistical significance was reached at all frequencies when comparing ABR threshold shift from 3 months to 9 months for the control group versus the early antioxidant treatment group.

The impact of early antioxidant therapy become even more substantial on evaluation at the 12 month time point of the study. By the 12 month ABR assessment time point, the early antioxidant treatment group has received 9 months of antioxidant therapy. Figure 23 presented the ABR threshold data per study animal within the control group and early antioxidant treatment group for the 12 month time point. The gap between the ABR thresholds by frequency for individual mice in the control group as compare to the early antioxidant treatment group as depicted in Figure 23 is quite pronounced (with the exception of the outlier data which has been note throughout the study in the early antioxidant

treatment group). Figure 24 presents the mean ABR threshold by frequency for the control group as compared to the early antioxidant treatment group. A statistical significance of $p <0.001$ was obtained for all mean threshold frequencies when comparing control versus early antioxidant treatment group. Statistical significance ($p <0.001$) was also noted for the mean overall ABR threshold shifts from the 3 month baseline assessment to the 12 month ABR assessment for the control versus early antioxidant treatment group which was found to be 29 dB and 6 dB, respectively. Table 11 depicts the mean ABR threshold shift by frequency for the control group versus the early antioxidant treatment group at 12 months with associated p-value. The mean threshold shift was noted to range from 29 to 38 dB within the control group and 6 to 10 dB in the early antioxidant treatment group.

Finally, the pure tone average over the course of the study was graphically evaluated. The pure tone average for each animal was calculated as the sum of the threshold at 4 kHz, 8 kHz, 12 kHz, 16 kHz and 32 kHz divided by 5. Figure 26 graphically depicts the shift in pure tone average by mouse at each of the study time points for the control versus early antioxidant treatment groups. This figure provides a graphical progression of the impact of early antioxidant therapy on ABR thresholds.

Section 4.3   Delayed Antioxidant Treatment

A total of 12 mice were assigned to the control group and a total of 12 mice were assigned to the control group. Nine mice within the control group and nine mice within the delayed treatment group survived for the duration of the study. The three mice that expired during the course of the study within each group were apparently independent of the study assessment and independent of ABR analysis (i.e. death did not occur within 48 hours of anesthesia for ABR). A total of 9 control mice and 9 early treatment mice were assess longitudinally.

ABRs were performed at the baseline time period of 3 months (prior to onset of sensorineural hearing loss in the control and delayed antioxidant treatment group). The baseline 3 month ABR threshold data per study animal ear is presented in Figure 27. The control mice data are presented in blue, and the delayed antioxidant treatment mice data are presented in pink. The raw data for most mice within both groups as depicted in Figure 27 is similar for both groups. The mean ABR baseline thresholds by frequency for the control group as compared to the delayed antioxidant treatment group is presented in Figure 28. There was no statistically significant difference between the mean thresholds by frequency at baseline for the control group versus the early treatment group (p>0.05 with two-tailed t-test).

ABRs were then performed at the 6 month time period to confirm onset of sensorineural hearing loss in the delayed antioxidant treatment group. The ABR threshold data per study animal is presented in Figure 29. As expected, the ABR thresholds for the control group and the delayed antioxidant treatment groups are

similar at the 6 month time period as the delayed antioxidant treatment group has yet to receive antioxidant therapy. Figure 30 provides the mean ABR thresholds by frequency for the control group and the delayed antioxidant treatment groups. No statistically significant difference is noted on two tailed t-test between the overall thresholds or the threshold by frequency for these two groups (p >0.05). Following the 6 months ABR assessment, the mice within the delayed antioxidant treatment group were initiated on the combination antioxidant therapy.

ABR assessment was then performed 3 months following initiation of antioxidant therapy within the delayed antioxidant treatment group at the 9 month time point. Figure 31 presented the ABR threshold data per study animal within the control group and delayed antioxidant treatment group for the 9 month time point. As is apparent by Figure 31, the ABR thresholds for the control group have progressed over the course of 3 months to be overall greater than that of the delayed antioxidant treatment group with only 3 months of antioxidant therapy even after onset of hearing loss in the delayed antioxidant treatment group. Figure 32 presents the mean ABR threshold by frequency for the control group as compared to the delayed antioxidant treatment group. These threshold differences were statistically significant at all frequency with a p-value of <0.001. On assessment of ABR threshold shift from the 3 month baseline to the 9 month time point, the mean overall ABR threshold shift for the control group was 23 dB as compared to a 4 dB mean overall ABR threshold shift for the delayed antioxidant treatment group. This difference was statistically significant with a p-value of <0.001. On analysis of the mean ABR threshold shift by frequency, the

mean threshold shift for the control group ranges from 19 to 25 dB as compared to that of the delayed antioxidant treatment group which ranges from 2 to 6 dB. The mean threshold shift by frequency as well as associated *p*-values are presented in Table 13. Statistical significance was reached at all frequencies when comparing ABR threshold shift from 3 months to 9 months for the control group versus the delayed antioxidant treatment group.

The threshold differences between the control and delayed antioxidant treatment groups became more marked at the 12 month time point of the study. By the 12 month ABR assessment time point, the delayed antioxidant treatment group has received 6 months of antioxidant therapy. Figure 33 presents the ABR threshold data per study animal within the control group and delayed antioxidant treatment group for the 12 month time point. The chiasm between the ABR thresholds by frequency between individual mice in the control group as compare to the delayed antioxidant treatment group as depicted in Figure 23 is more pronounced that that observed at the 9 month time point. Figure 34 presents the mean ABR threshold by frequency for the control group as compared to the delayed antioxidant treatment group at 12 months. A statistical significance of *p* <0.001 was obtained for all mean threshold frequencies when comparing control versus delayed antioxidant treatment group. Statistical significance (*p* <0.001) was also noted for the mean overall ABR threshold shifts from the 3 month baseline assessment to the 12 month ABR assessment for the control versus delayed antioxidant treatment group which was found to be 29 dB and 9 dB, respectively. Table 14 depicts the mean ABR threshold shift by frequency for the

control group versus the delayed antioxidant treatment group at 12 months with associated *p*-value. The mean threshold shift was noted to range from 29 to 38 dB within the control group and 6 to 11 dB in the delayed antioxidant treatment group.

Finally, the pure tone average over the course of the study was graphically represented. The pure tone average for each animal was calculated as the sum of the threshold at 4 kHz, 8 kHz, 12 kHz, 16 kHz and 32 kHz divided by 5. Figure 36 graphically depicts the shift in pure tone average by mouse at each of the study time points for the control versus delayed antioxidant treatment groups. This figure provides a graphical progression of the impact of delayed antioxidant therapy on ABR thresholds.

Chapter 5: Discussion

Section 5.1: Summary of Findings

Auditory brainstem response testing has been used extensively in the auditory physiology literature as a means of characterizing hearing within various strains of mice and as a means of assessing the impact of various therapeutic modalities on hearing in animal models. Traditionally, within the mouse model ketamine and xylazine have been used as a mouse anesthetic as it has been characterized to produce consistently reliable results with minimal morbidity for single assessment with only minor changes noted in wave latency and amplitude (Smith & Mills, 1989; Huang et al., 1995; Miller et al., 1998; Ou et al., 2000; Jero et al., 2001; Henry 2002; van Looij et al., 2004). However, with multiple dosages administered, a significant mortality rate for ketamine and xylazine has been documented in the literature (Arras et al., 2001). Within specific aim 1 of this study, Avertin was compared with the gold standard ketamine and xylazine for anesthetic during auditory brainstem response testing in a mouse model. Avertin was found to have equivalent anesthetic properties without noted significant alteration in auditory brainstem responses in an animal model. The rate of mortality associated with these mice was noted to be decreased with Avertin as compared to ketamine and xylazine with 2 deaths attributable to ketamine and xylazine versus none attributed to Avertin. Similar results were recently reported by Maheras and Gow regarding the use of Avertin alone and Avertin plus chloral hydrate for auditory brainstem response testing in mice. Although necropsy was

63

not performed in this current study for specific aim 1, necropsy was performed by Maheras and Gow which revealed no significant intraperitoneal changes with the exception of mild inflammation following intraperitoneal injection of Avertin.

The combination antioxidant was investigated in specific aims 2 and 3 to determine whether this combination antioxidant has the potential for preventing age related threshold shifts in an animal model (specific aim 2) and/or treat age related threshold shifts in an animal model (specific aim 3). With both early treatment and delayed treatment, a statistically significant difference was noted in ABR threshold shift. The combination antioxidant which was designed to target 4 specific sites within the oxidative pathway appears to have prevented age related threshold shifts within the animal model of the C57BL6 mouse. Amongst the study groups there were no apparent toxicities or mortalities that can be attributed to the administration of the oral antioxidant.

These findings are suggestive of the potential for developing a combination antioxidant agent which targets multiple sites within the oxidative pathway for treatment or prevention of age-related hearing loss in the clinical setting. However, further study would be necessary to make the transition from bench-top to bedside. The data presented within this document has been published by *Otolaryngology – Head and Neck Surgery* (Appendix A), and has since been cited by various authors internationally (Appendix B). There are a number of limitations within this study that would need to be evaluated further via investigation or review of the literature prior to embarking upon clinical trials. However, these results are promising in the potential for developing a drug

therapy for the treatment and/or prevention of age-related hearing loss in a

clinical setting which will become an increasing public health concern.

Section 5.2: Study Limitation

As with any study, there are a number of limitations which must be acknowledged. First, although the sample size is small, it is adequate to attain statistically significant analysis. However, the power of the study would be increased with a larger sample size. Second, although the C57BL6 has been a well-established model commonly used for the investigation of age-related hearing loss, some controversy has arisen within recent literature regarding the pathogenesis of age-related thresholds changes in C57BL6 and its association with the pathogenesis of age-related hearing loss in the clinical setting. Within the clinical setting, age-related hearing loss is believed to have a genetic component, but it is believed to largely present as a function of various other environmental and physiological factors (DeStefano et al., 2003; Kane et al., 2012). However, within the C57BL6 mouse model a mutation in the *ahl* gene at the *Cdh23^{ahl}* allele has been established to be the pathophysiologic agent for accelerated hearing loss. Histological and immunohistological assessment of the C57BL6 mice with aging has been noted to parallel changes noted in presbycusis. Third, physiological data is provided without histological or immunohistological sections. This was unfortunately due to the lack of availability of these services at the time of sacrifice. Fourth, the goal of the study was to identify an agent that targets multiple sites within the oxidative pathway; however, previously within the age-related hearing loss literature, each of these agents had not individually be examined thoroughly, namely ribose cysteine and L-NAME. Fifth, although no deaths or mortality within this study can be attributed

to the administration or consumption of the combination antioxidant agent, formal

toxicity analysis was not performed. The evidence suggests that the combination

antioxidant did not produce any toxic effects in that within the group that received

the longest duration of treatment, there were no deaths over the 6 month

treatment period while 3 deaths respectively were experienced in the control and

delayed treatment groups. However, toxicity of the combination antioxidant was

not performed. Individual toxicity data for the agents included in the combination

antioxidant were reviewed from the literature and utilized for designation of

dosage within the combination agent. A detailed review is provided in Appendix

C.

Section 5.3: Future Studies

In light of the limitations within this current study and to further investigate the potential of this combination antioxidant as a potential clinical therapy for the prevention or treatment of age-related hearing loss, various further studies are of interest. First, given the concern regarding the pathogenesis of accelerated age-related threshold shifts in the C57BL6 mouse model, the study could be repeated within an animal model lacking the $Cdh23^{ahl}$ allele mutation of the ahl gene. Alternatively, toxicity analysis could be performed within any animal model to assess for potential toxicities in association with this combination high dosage antioxidant followed by moving to clinical trials if no significant toxicity is established.

It may also be of interest to investigate the two drug components not previously investigated for their potential impact on age-related hearing loss. Theoretically it is possible, but less likely, that the decrease in threshold shift noted within this study with age is attributable to one of these single agents not previously investigated. This theory is contrary to the premise of this study that targeting multiple sites within the oxidative pathway prevents therapeutic resistance and activation of alternative pathways within the oxidative system. A potential future study would investigate each of these agents individually as well as in various permutations as combination agents to assess for the optimal agent to prevent or treat age-related threshold shift.

This current study focuses on electrophysiologic data to support the potential impact of this combination antioxidant on the progression of age-related

changes within the cochlea. Further study including histological analysis of characteristic changes with aging with the cochlea or the lack of such changes with the combination antioxidant therapy would further support the electrophysiological findings. Similarly, immunohistochemical analysis for changes in various protein and enzymatic expression that is characteristic of cochlear aging (i.e. connexin, Na^+-K^+-ATPase, carbonic anhydrase and creatine kinase expression).

Additionally, given the fact that this combination antioxidant agent targets the oxidative-reductive pathway within the inner ear, this agent could be investigated for the prevention or treatment of alternative disease processes with a similar mechanism of injury of oxidative stress and damage. One major concern particularly within the military and police force is the associated occupational hazard of noise induce hearing loss. Chronic administration of a combination antioxidant could prove beneficial in attenuating or preventing the commonly associated noise induced threshold shifts and association tinnitus which is most bothersome to patients within this population. Similarly, chemotherapy and radiation therapy are believed to incite sensorineural hearing loss through mechanisms partially mediated by oxidative damage. This combination agent could prove instrumental in preventing ototoxicity in chemotherapy and radiation therapy which could greatly impact the landscape of cancer treatment globally. Finally, intratympanic administration of this agent could be investigated as an alternative means of drug delivery.

Section 5.4: Potential Clinical Application

Although this represents a single animal case-controlled trial of a combination antioxidant which targets 4 main sites within the oxidative pathway, the data presented within this study provides promising results for the potential of the development of a medical therapy for the treatment and prevention of age-related hearing loss clinically. This combination agent, because of its composition, could be administered in a number of formats including as a vitamin tablet (akin to Ocuvite for vision health with aging), as a gummy or chewable, or in a beverage form as a dietary supplement for the prevention or treatment of age-related hearing loss. Additionally, with investigation of it use in alternative disease processes mediated by oxidative damage, this agent potentially could be used to prevent or treat various forms of oxidative stress mediated cochlear damage with associated hearing loss.

Interestingly, with regard to the United States Food and Drug Administration (FDA), relative to vitamins and natural substances, the FDA does not require nor does it provide approval for vitamins and natural substances in claims of treatment for disease processes. The FDA only certifies safety of vitamins and natural substances, but does not require efficacy testing, nor does it support claims of efficacy for treatment. Thus, there are relatively few barriers from a regulatory perspective in bringing a combination antioxidant such as the one described within this manuscript to market for clinical usage aside from investigation of agent safety.

Table 1. Prevalence of Presbycusis with Increasing Age

Age of Population	Prevalence
65 years of age or greater	35 to 50%
75 years of age or greater	40 to 65%
85 years of age or greater	over 80%
100 years of age or greater	approximately 90%

Table 2. National Institute for Occupational Safety and Health (NIOSH) and Occupational Safety and Health Administration (OSHA) Recommended Permissible Noise Exposure Levels

Permissible Noise Level Exposure		
Hours per day	Sound Level	
	NIOSH	OSHA
8	85 dB	90 dB
6	86 dB	92 dB
4	88 dB	95 dB
3	89 dB	97 dB
2	90 dB	100 dB
1.5	92 dB	102 dB
1	94 dB	105 dB
0.5	97 dB	110 dB
0.25	100 dB	115 dB*
0	112 dB	

Table 3. Commonly and Less Commonly Encountered Environmental Sounds

Environmental Sounds	
Stimulus	Sound Level
Rustling leaves	20 dB
Whisper at 6 feet	30 dB
Residential area at night	40 dB
Quiet office	50 dB
Normal Conversation	60 - 65 dB
Hair dryer	60-95 dB
Car noise	70 dB
Traditional vacuum cleaner	70 dB
Door bell	80 dB
Blender	80-90 dB
City Traffic	85 dB
Subway	90 - 115 dB
Hand Drill	95 - 100 dB
Gas lawnmower	95 - 110 dB
Snowblower	95 - 110 dB
Snowmobile	100 dB
Motorcycle	100 dB
Chain saw	110 dB
Sandblaster	115 dB
Maximum volume ear buds	85 - 115 dB
Ambulance siren	120 dB
Loud Concert	100 - 125 dB
Pneumatic riveter	125 dB
Jet engine	140 dB
0.22 Caliber riffle	145 dB
Fireworks	150 dB
12 Gauge Shotgun	165 dB
0.357 Caliber revolver	170 - 175 dB

Table 4. Factors Contributing to Presbycusis

Factor	Exposures
Environmental:	Noise
	Low socioeconomic status
Chemical Exposures:	Toluene
	Trichloroethylene
	Styrene
Otologic Disease:	Otosclerosis
	Chronic otomastoiditis
	Temporal bone trauma
	Meniere's
Medications:	Aminoglycosides
	Platinum-based chemotherapeutic agents
	Loop diuretics
	phosphodiesterase type 5 inhibitors
	Salicylate
Habitual:	Tobacco*
	Alcohol abuse*
Systemic Disease:	Renal Failure
	Diabetes
	Cardiovascular disease
	Immunodeficiency
Protective Factors:	Estrogen
	High bone mineral density
	Caloric Restriction*
	Aldosterone

*conflicting reports in the literature

Table 5. Combination Antioxidant Component Daily Dosage

Antioxidant	Daily Dose
Vitamin B12	80 mcg/kg
Folate	100 mcg/kg
Ascorbic Acid	200 mg/kg
L-NAME	250 mg/kg
L-CySSG	200 mg/kg
Ribose-cysteine	200 mg/kg

Table 6. Avertin versus Ketamine/Xylazine Case-Control Raw ABR Data

		Avertin ABR								Ketamine/Xylazine ABR							
		Click	1kHz	2kHz	4kHz	8kHZ	12kHz	16kHz	32kHz	Click	1kHz	2kHz	4kHz	8kHZ	12kHz	16kHz	32kHz
1	R	30	75	70	40	10	10	15	30	30	75	70	35	10	10	15	30
	L	35	75	70	45	15	20	20	40	35	75	70	50	15	20	20	35
2	R	30	70	70	30	15	15	15	20	30	70	70	30	10	15	20	20
	L	30	75	70	40	15	15	20	25	30	75	70	40	10	15	20	25
3	R	35	75	70	50	20	15	30	40	35	75	70	50	20	15	30	40
	L	30	70	60	40	15	15	30	40	30	70	60	40	15	15	30	40
4	R	30	80	70	35	15	10	20	25	30	80	70	40	15	10	20	30
	L	30	80	70	30	10	10	20	40	30	80	70	30	10	15	20	35
5	R	30	80	75	40	10	10	20	30	30	80	75	40	10	10	20	30
	L	30	80	75	40	10	10	20	30	30	80	75	40	10	10	20	30

Table 7. Control Cohort Longitudinal Raw ABR

	ID #	Side	3 months ABR (dB)					6 months ABR (dB)					9 months ABR (dB)					12 months ABR (dB)				
			4kHz	8kHz	12kHz	16kHz	32kHz	4kHz	8kHZ	12kHz	16kHz	32kHz	4kHz	8kHZ	12kHz	16kHz	32kHz	4kHz	8kHZ	12kHz	16kHz	32kHz
Control	23	R	20	10	10	20	30	40	20	20	30	40	60	40	40	55	60	70	50	60	60	70
Control	23	L	35	15	15	20	40	40	20	20	30	40	60	40	40	50	60	70	50	50	60	70
Control	24	R	35	10	10	15	35	50	20	20	30	50	60	40	40	45	60	70	50	55	60	65
Control	24	L	30	15	15	20	35	40	15	15	20	40	55	30	40	50	60	60	45	55	60	70
Control	25	R	40	10	15	15	30	50	20	20	30	50	60	40	40	50	60	70	50	60	65	70
Control	25	L	30	10	10	15	30	40	15	20	30	40	50	30	30	40	60	60	50	50	55	70
Control	26	R	40	10	10	20	40	40	20	15	25	40	55	40	40	50	60	70	60	60	65	70
Control	26	L	40	20	10	20	40	40	20	15	20	40	50	40	30	45	55	65	55	40	50	70
Control	28	R	50	20	20	30	40	50	20	20	30	40	60	40	40	50	60	70	50	55	60	65
Control	28	L	40	15	15	20	35	50	15	20	30	40	55	40	40	50	60	70	55	50	60	70
Control	31	R	40	15	20	25	30	40	20	20	30	40	60	40	35	40	55	70	55	50	50	65
Control	31	L	40	15	15	25	30	40	20	20	30	40	50	40	35	40	60	60	50	45	55	65
Control	32	R	40	15	20	25	55	60	20	30	40	60	70	40	40	50	70	80	55	60	65	75
Control	32	L	45	15	15	20	40	45	15	15	20	40	60	35	30	40	60	70	50	40	50	70
Control	43	R	30	15	15	20	30	40	15	20	30	50	60	40	40	40	70	70	50	55	50	75
Control	43	L	50	15	15	20	30	50	20	15	30	50	55	45	40	45	70	60	60	55	50	75
Control	44	R	50	15	15	30	40	50	20	20	30	50	60	40	35	45	60	70	55	40	60	70
Control	44	L	50	15	15	30	40	50	20	15	30	40	60	40	40	40	65	70	50	50	55	75

Table 8. Early Treatment Cohort Longitudinal Raw ABR

	ID #	Side	3 months ABR (dB)					6 months ABR (dB)					9 months ABR (dB)					12 months ABR (dB)				
			4kHz	8kHz	12kHz	16kHz	32kHz	4kHz	8kHZ	12kHz	16kHz	32kHz	4kHz	8kHZ	12kHz	16kHz	32kHz	4kHz	8kHZ	12kHz	16kHz	32kHz
Early	56	R	60	30	30	40	30	60	30	30	40	30	65	40	30	40	45	70	40	40	40	50
Early	56	L	65	40	40	60	70	65	40	40	60	70	70	40	40	60	70	70	40	40	60	70
Early	57	R	30	15	15	20	30	30	15	15	20	30	40	20	20	30	30	50	20	25	30	35
Early	57	L	30	15	15	20	25	30	15	15	20	30	35	20	15	20	35	35	20	20	30	40
Early	60	R	35	25	20	25	30	35	25	20	25	30	40	30	30	30	40	40	30	30	40	45
Early	60	L	40	10	15	20	25	40	15	20	20	30	40	15	20	25	30	40	20	20	30	35
Early	71	R	40	20	20	30	35	40	20	20	30	35	40	20	20	30	40	40	20	20	30	40
Early	71	L	40	20	20	30	35	40	20	20	30	35	50	20	25	30	40	50	25	30	35	40
Early	75	R	30	15	20	20	25	30	15	20	20	30	30	20	20	25	30	30	20	20	30	35
Early	75	L	30	15	15	20	25	30	15	20	20	30	30	15	20	30	35	30	15	20	30	35
Early	62	R	50	20	20	25	30	50	20	20	25	30	50	20	20	30	35	55	20	25	35	40
Early	62	L	50	20	15	25	30	50	20	20	25	30	55	20	20	30	40	55	30	25	35	40
Early	65	R	30	10	10	15	30	35	15	15	20	30	40	15	20	20	35	40	20	20	25	40
Early	65	L	30	10	10	15	30	35	15	15	20	30	40	20	15	20	35	40	20	20	20	40
Early	66	R	40	15	10	20	30	40	15	10	20	30	45	15	15	20	30	50	20	15	20	35
Early	66	L	40	15	15	20	30	40	15	15	20	30	50	20	15	25	35	50	20	20	30	40
Early	67	R	40	20	20	30	40	40	20	20	30	40	40	20	20	35	50	45	20	20	40	50
Early	67	L	30	15	15	20	40	30	15	15	20	40	30	15	20	25	40	35	29	25	30	50
Early	68	R	30	10	10	20	30	35	10	10	20	30	40	15	15	30	35	45	20	20	30	40
Early	68	L	50	15	10	20	25	50	15	10	20	25	50	20	15	20	30	50	30	20	30	40
Early	69	R	30	20	20	20	35	30	20	20	20	35	30	20	20	40	40	35	20	25	40	45
Early	69	L	40	15	20	20	35	40	15	20	20	35	50	20	25	40	45	50	20	30	40	50
Early	70	R	50	20	20	30	40	50	20	20	30	40	50	20	30	30	45	55	25	30	30	50
Early	70	L	40	10	15	20	30	40	15	15	20	30	40	15	20	20	30	40	20	20	25	40

Table 9. 6 Month Control versus Early Antioxidant Treatment Impact of Outliers

Frequency (kHz)	Control Mean Threshold (dB)	Early Treatment		Early Treatment Less Outliers	
		Mean Threshold (dB)	p - value	Mean Threshold (dB)	p - value
4	45	40	0.06	38	0.002
8	19	18	0.85	17	0.06
12	19	19	0.83	17	0.12
16	29	25	0.12	23	<0.001
32	44	34	<0.001	32	<0.001

Table 10. 9 Month Mean ABR Threshold Shift by Frequency Control versus Early Antioxidant Treatment Group

Frequency (kHz)	Control	Early Treatment	p - value
4	19	4	<0.001
8	25	3	<0.001
12	23	4	<0.001
16	24	5	<0.001
32	25	6	<0.001

Table 11. 12 Month Mean ABR Threshold Shift by Frequency Control versus Early Antioxidant Treatment Group

Frequency (kHz)	Control	Early Treatment	p - value
4	29	6	<0.001
8	38	6	<0.001
12	37	7	<0.001
16	36	8	<0.001
32	34	10	<0.001

Table 12. Delayed Antioxidant Treatment Cohort Longitudinal Raw ABR

	ID #	Side	3 months ABR (dB)					6 months ABR (dB)					9 months ABR (dB)					12 months ABR (dB)				
			4kHz	8kHz	12kHz	16kHz	32kHz	4kHz	8kHZ	12kHz	16kHz	32kHz	4kHz	8kHZ	12kHz	16kHz	32kHz	4kHz	8kHZ	12kHz	16kHz	32kHz
Delayed	33	R	65	30	30	40	50	70	30	30	40	50	60	30	30	40	50	60	30	35	40	60
Delayed	33	L	50	25	35	50	65	50	30	30	50	60	50	30	30	50	60	55	30	30	55	60
Delayed	47	R	40	20	20	30	40	40	20	20	30	40	40	25	15	30	50	40	30	20	30	55
Delayed	47	L	40	20	20	30	40	40	20	20	30	40	50	20	20	20	50	50	30	20	25	60
Delayed	48	R	30	15	15	20	25	30	20	20	30	35	35	20	20	30	50	40	25	30	40	50
Delayed	48	L	30	15	10	15	20	30	20	15	20	30	30	25	20	20	50	35	30	25	30	50
Delayed	41	R	50	15	20	20	40	50	20	20	30	40	50	20	15	25	40	50	20	20	30	40
Delayed	41	L	50	20	15	20	40	50	20	20	30	40	50	20	20	20	40	55	30	20	30	45
Delayed	42	R	40	20	20	30	50	40	30	30	40	50	40	20	20	30	40	50	30	20	30	40
Delayed	42	L	40	20	25	30	40	40	30	30	40	50	40	30	20	30	50	45	40	30	35	50
Delayed	52	R	50	15	20	20	30	50	20	20	30	40	50	20	30	30	50	50	30	30	40	50
Delayed	52	L	40	10	10	20	30	40	15	20	30	40	50	20	20	20	35	55	30	30	35	40
Delayed	54	R	40	20	15	30	40	40	20	20	30	40	40	20	20	30	40	45	30	30	40	55
Delayed	54	L	30	15	15	30	45	40	20	20	30	50	45	20	20	35	50	50	30	30	40	50
Delayed	55	R	30	10	10	15	20	40	20	15	20	30	40	20	20	20	35	45	20	20	30	40
Delayed	55	L	50	15	20	25	35	50	20	20	30	40	50	20	20	30	35	50	20	30	35	40
Delayed	45	R	60	15	15	30	40	60	20	20	30	40	60	20	20	30	40	65	30	30	40	50
Delayed	45	L	50	15	15	25	40	50	20	20	30	40	50	20	20	30	40	55	30	30	40	55

Table 13. 9 Month Mean ABR Threshold Shift by Frequency Control versus Delayed Antioxidant Treatment Group

Frequency (kHz)	Control	Delayed Treatment	p - value
4	19	3	<0.001
8	25	5	<0.001
12	23	3	<0.001
16	24	2	<0.001
32	25	6	<0.001

Table 14. 12 Month Mean ABR Threshold Shift by Frequency Control versus Delayed Antioxidant Treatment Group

Frequency (kHz)	Control	Delayed Treatment	p - value
4	29	6	<0.001
8	38	11	<0.001
12	37	7	<0.001
16	36	8	<0.001
32	34	11	<0.001

Figure 1. Pure Tone Audiogram Characteristic of Presbycusis
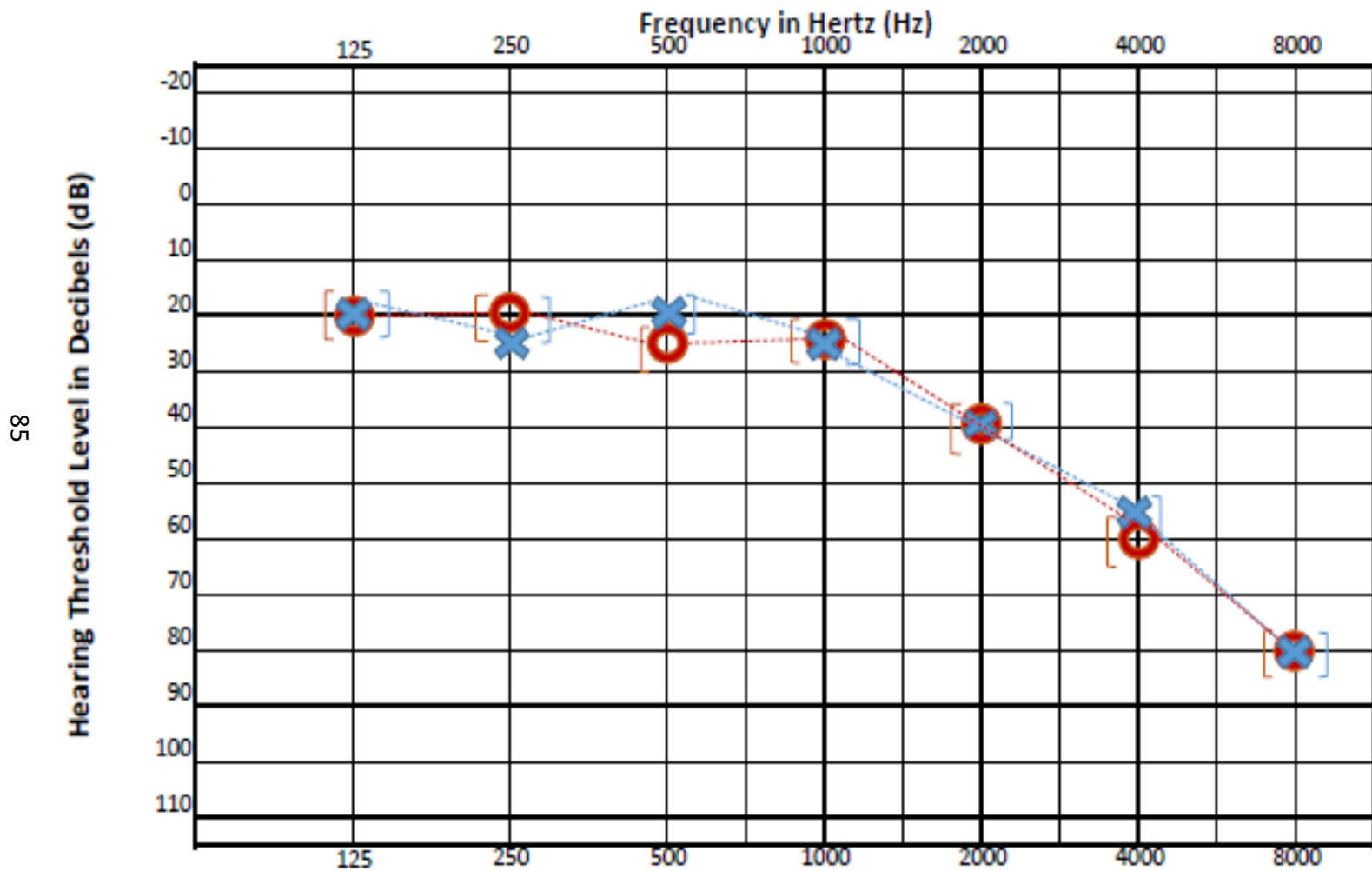
Figure 2. WHO 2012 Estimates of Disabling Hearing Loss in Individuals 65 Years and Over by Region

98
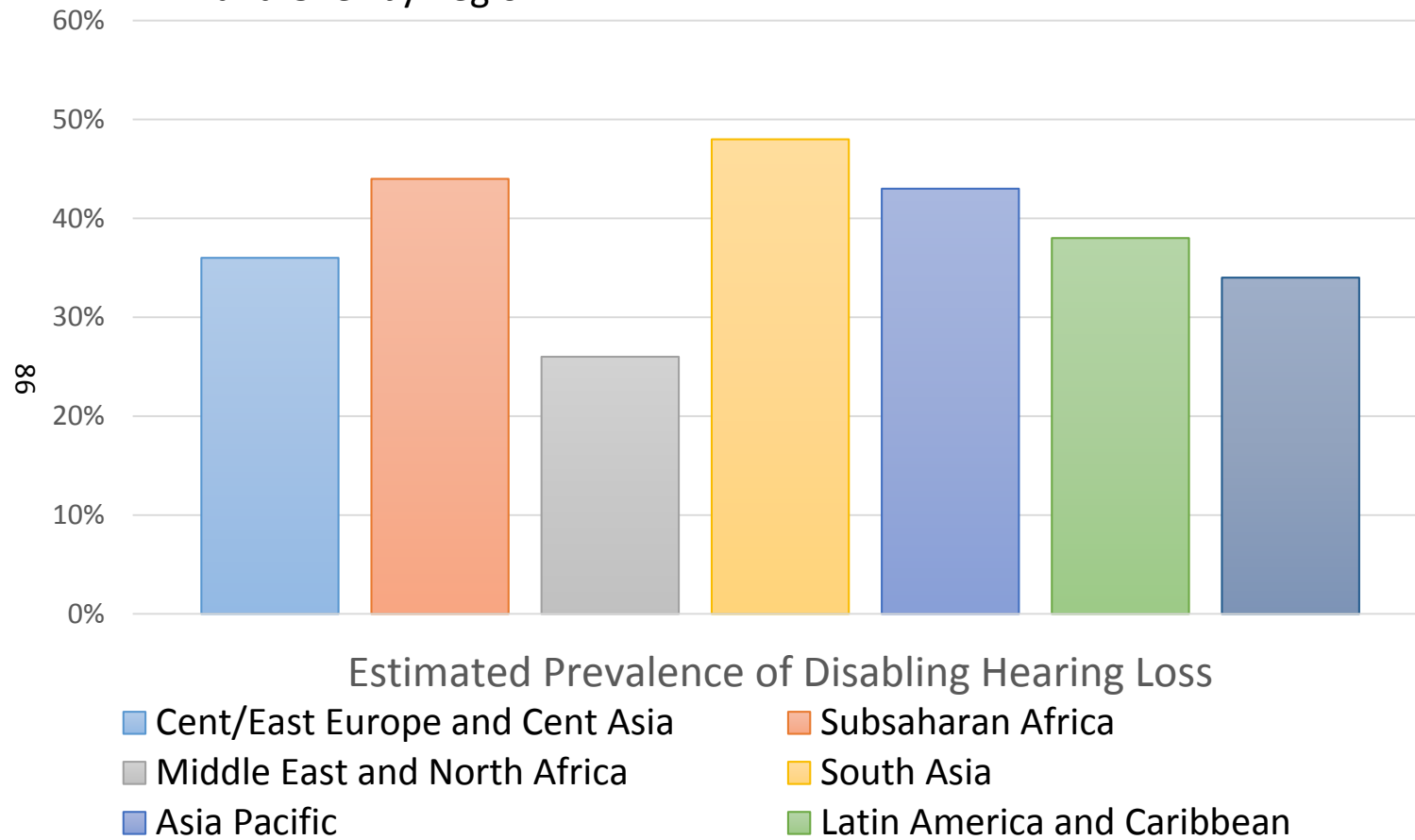
Figure 3. Sensory Presbycusis
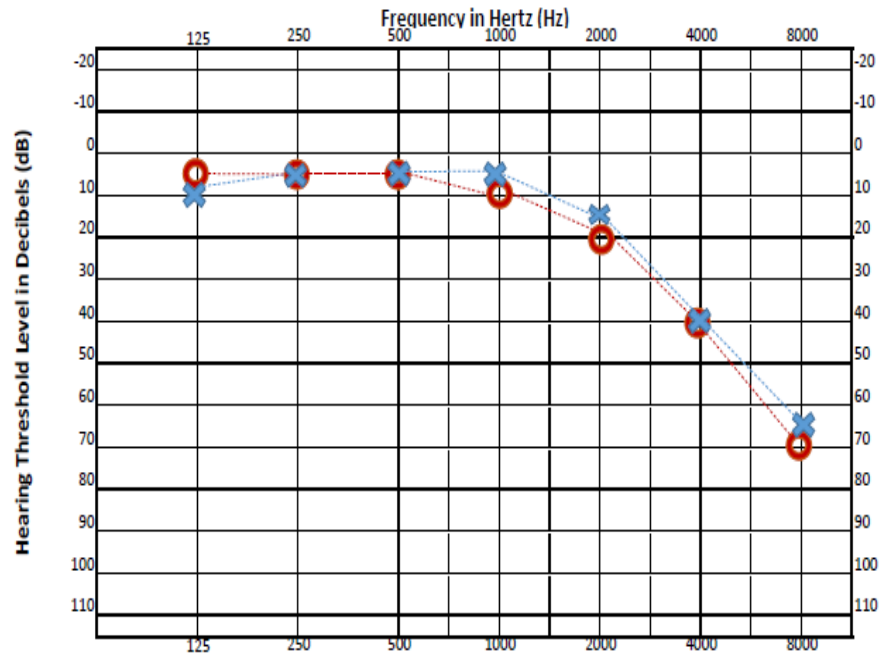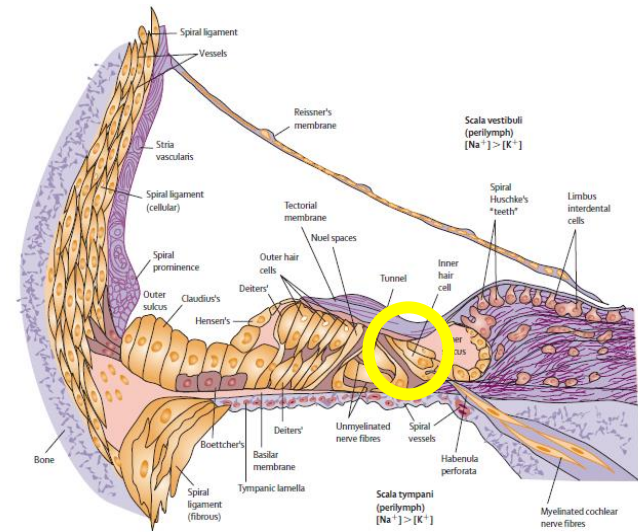
Figure 4. Neural Presbycusis
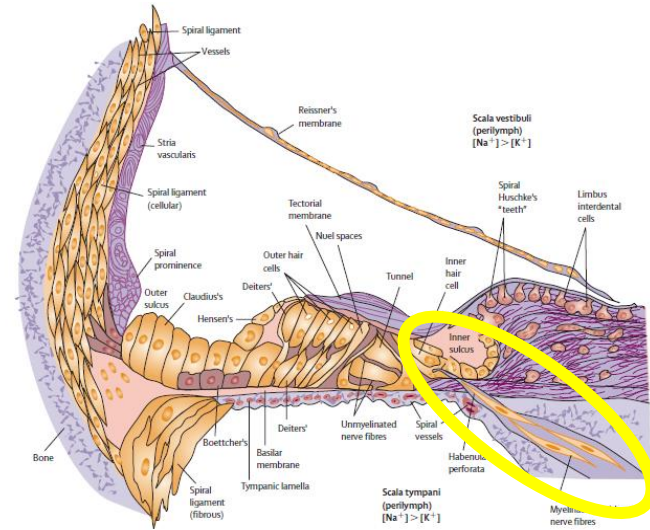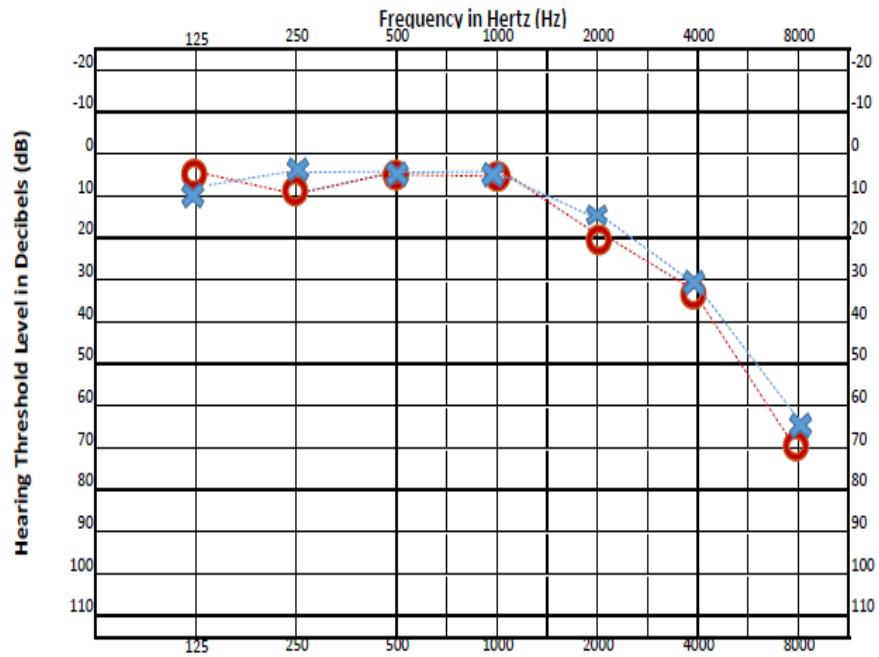
Figure 5. Metabolic Presbycusis
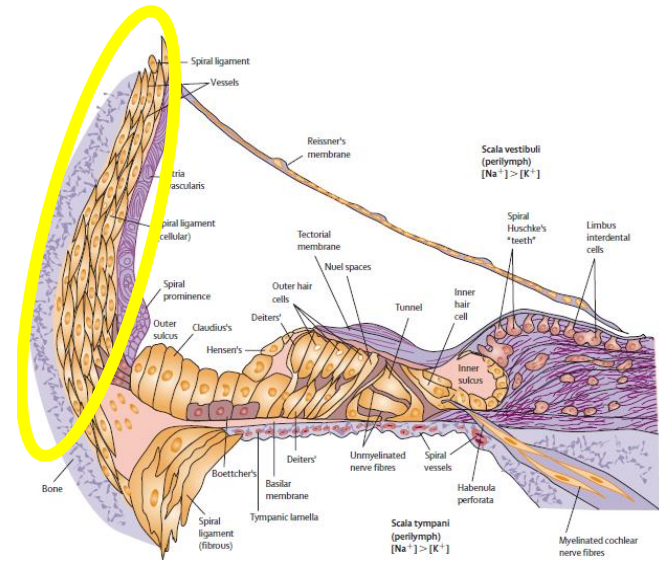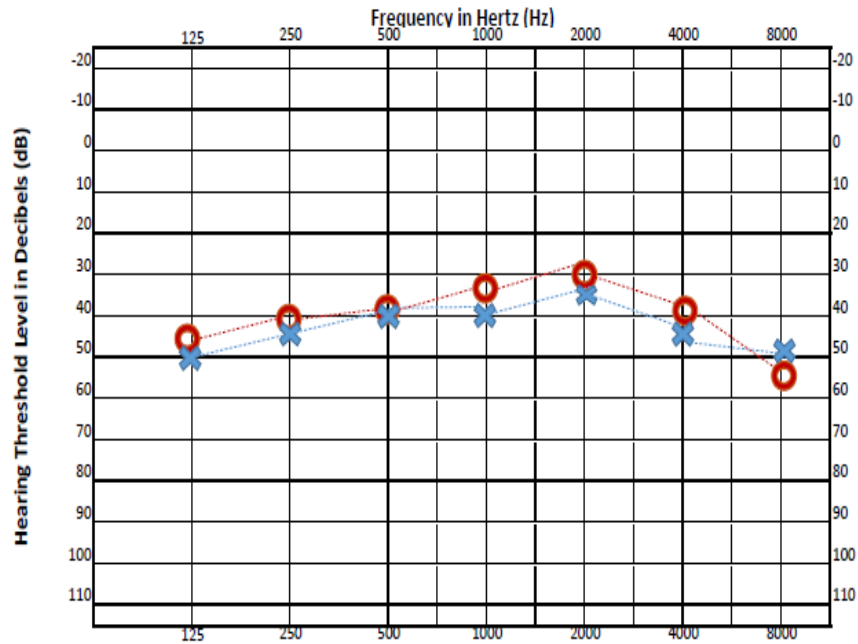
Figure 6. Mechanical Presbycusis
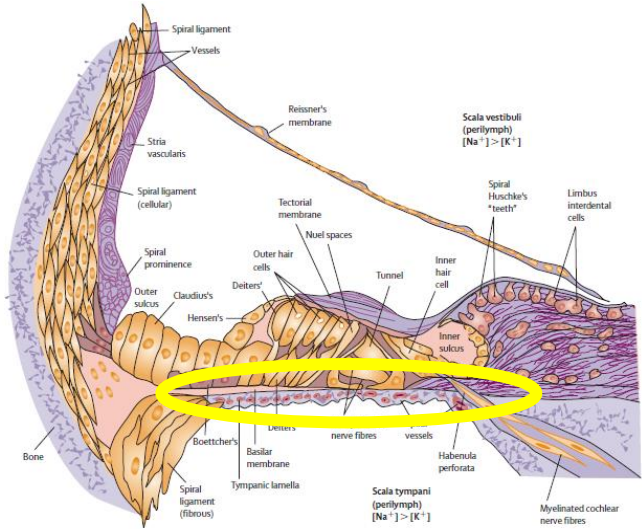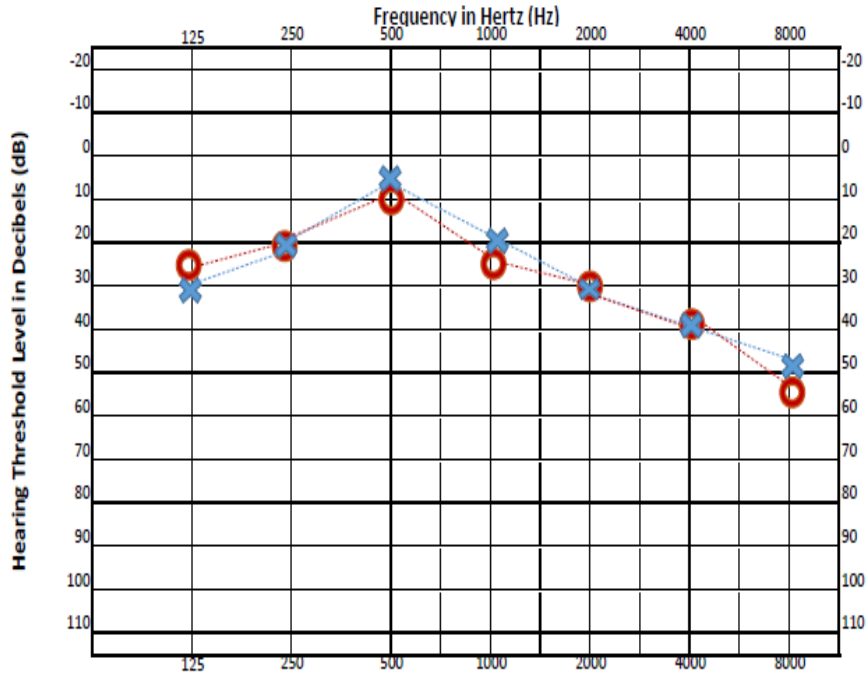
Figure 7. Oxidative Pathway within the Cochlea

91
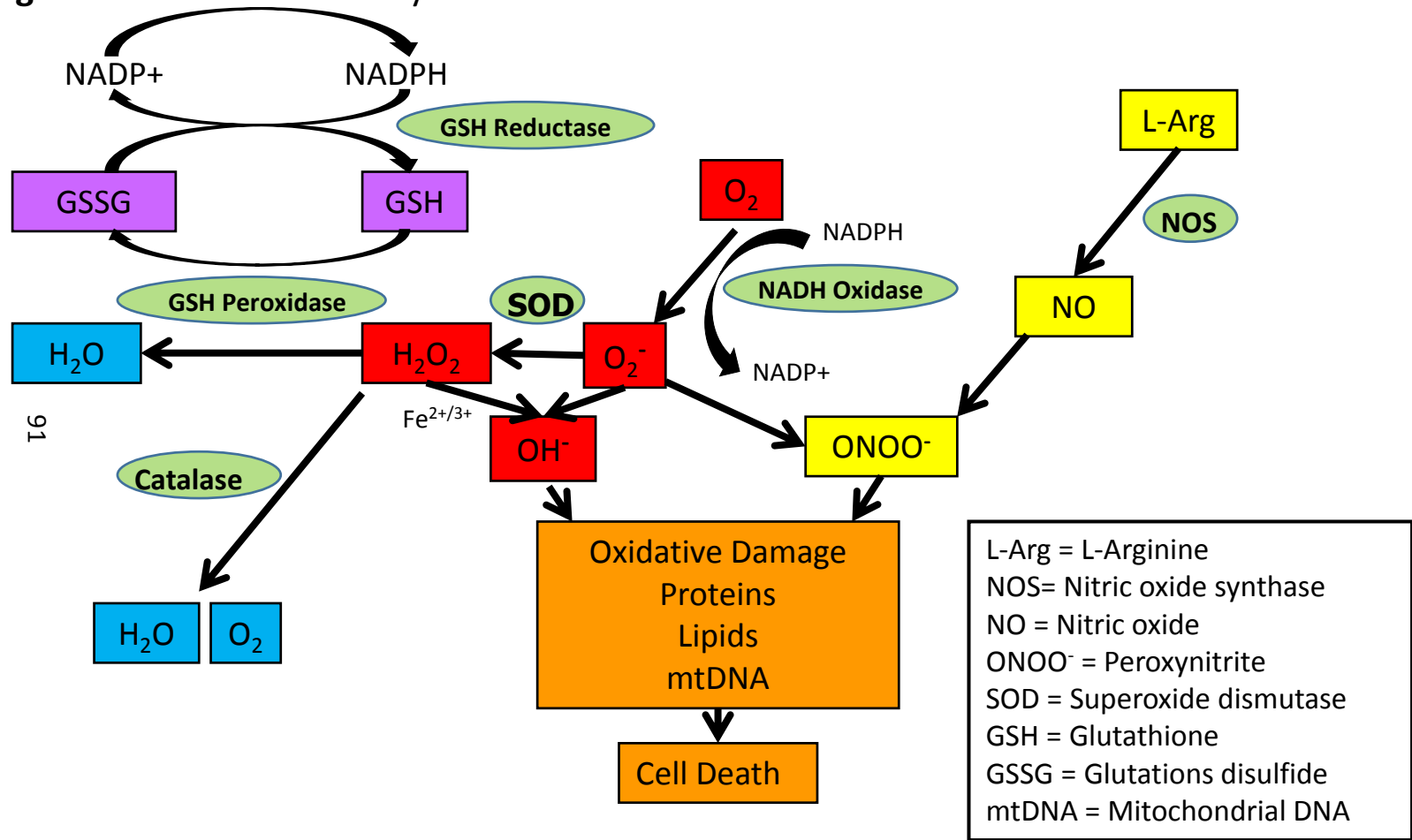
Figure 8. Vitamin B12 Site of Action within the Oxidative Pathway

92
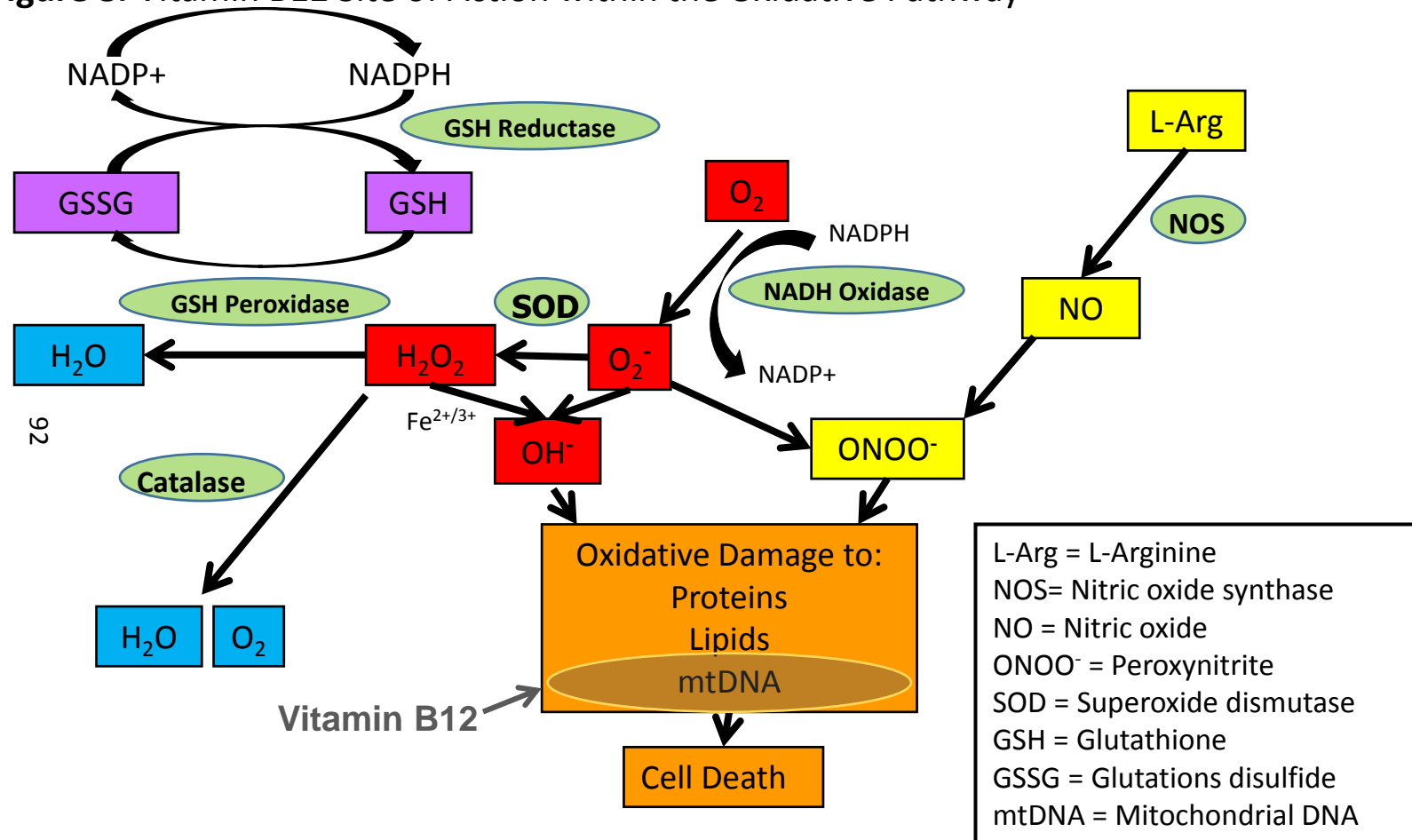
Figure 9. Folate Site of Action within the Oxidative Pathway

L-Arg = L-Arginine
NOS= Nitric oxide synthase
NO = Nitric oxide
ONOO⁻ = Peroxynitrite
SOD = Superoxide dismutase
GSH = Glutathione
GSSG = Glutations disulfide
mtDNA = Mitochondrial DNA

93
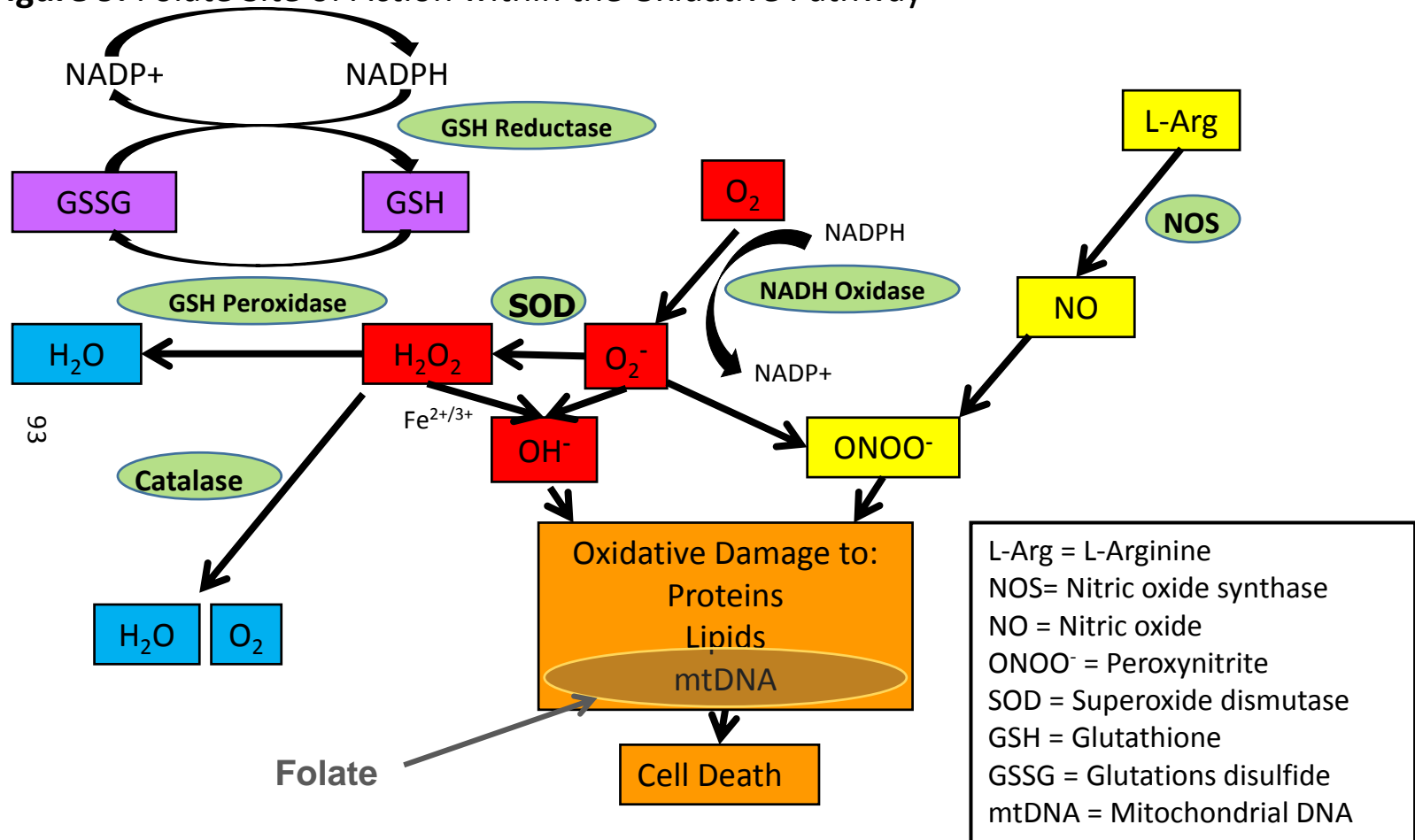
Figure 10. Ascorbic Acid Site of Action within the Oxidative Pathway

94

L-Arg = L-Arginine
NOS= Nitric oxide synthase
NO = Nitric oxide
ONOO⁻ = Peroxynitrite
SOD = Superoxide dismutase
GSH = Glutathione
GSSG = Glutations disulfide
mtDNA = Mitochondrial DNA
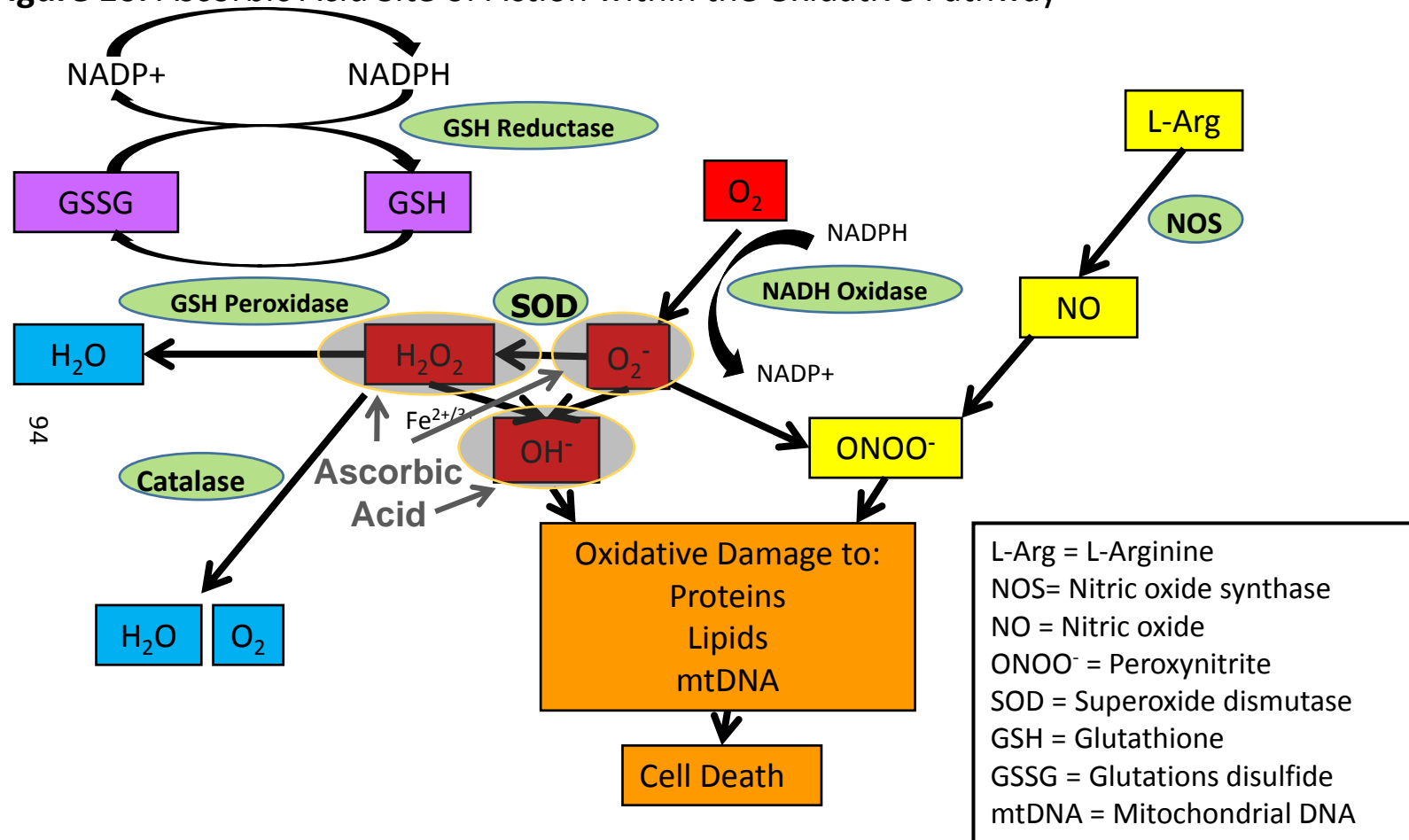
Figure 11. L-NAME Site of Action within the Oxidative Pathway

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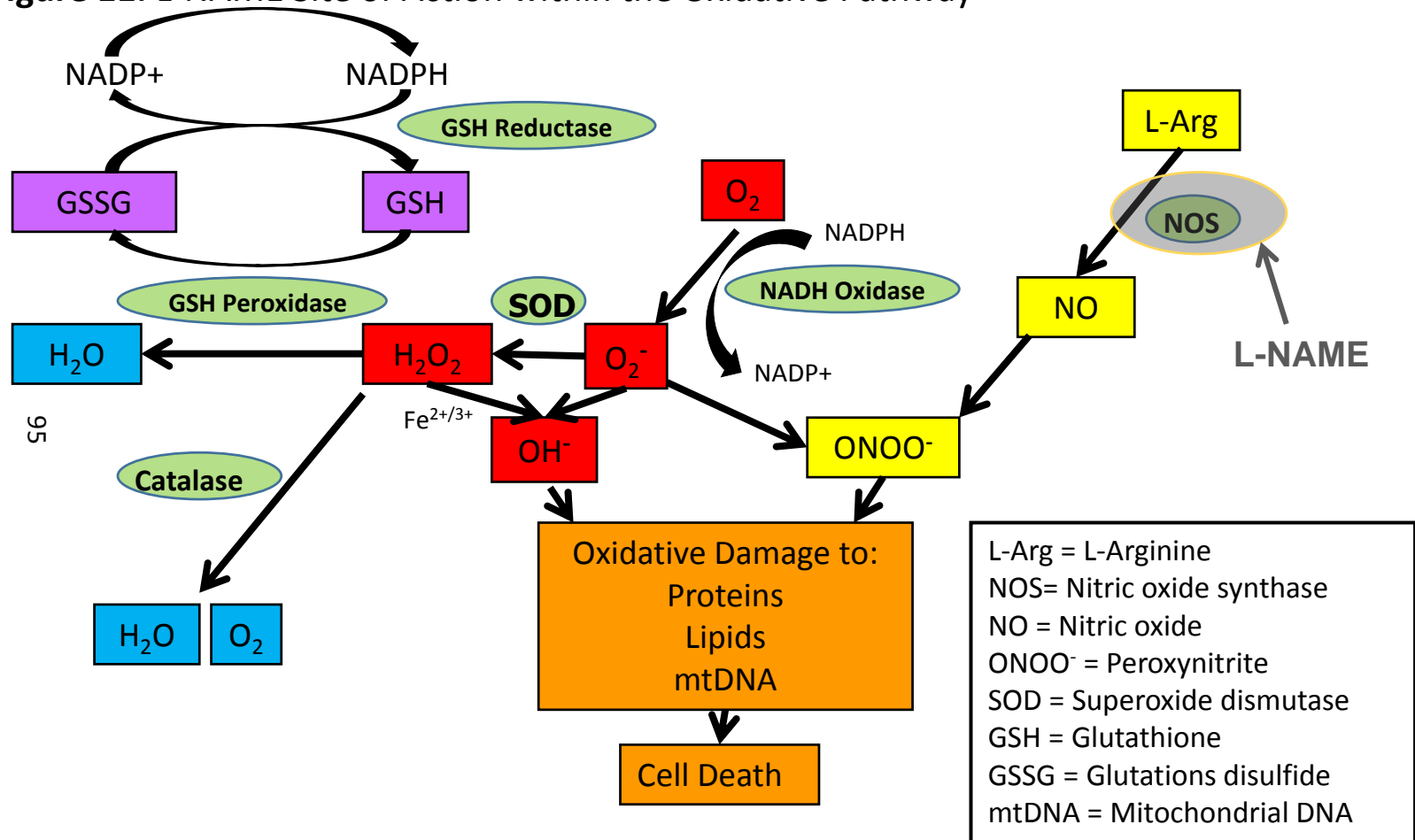
Figure 12. L-CySSG Site of Action within the Oxidative Pathway
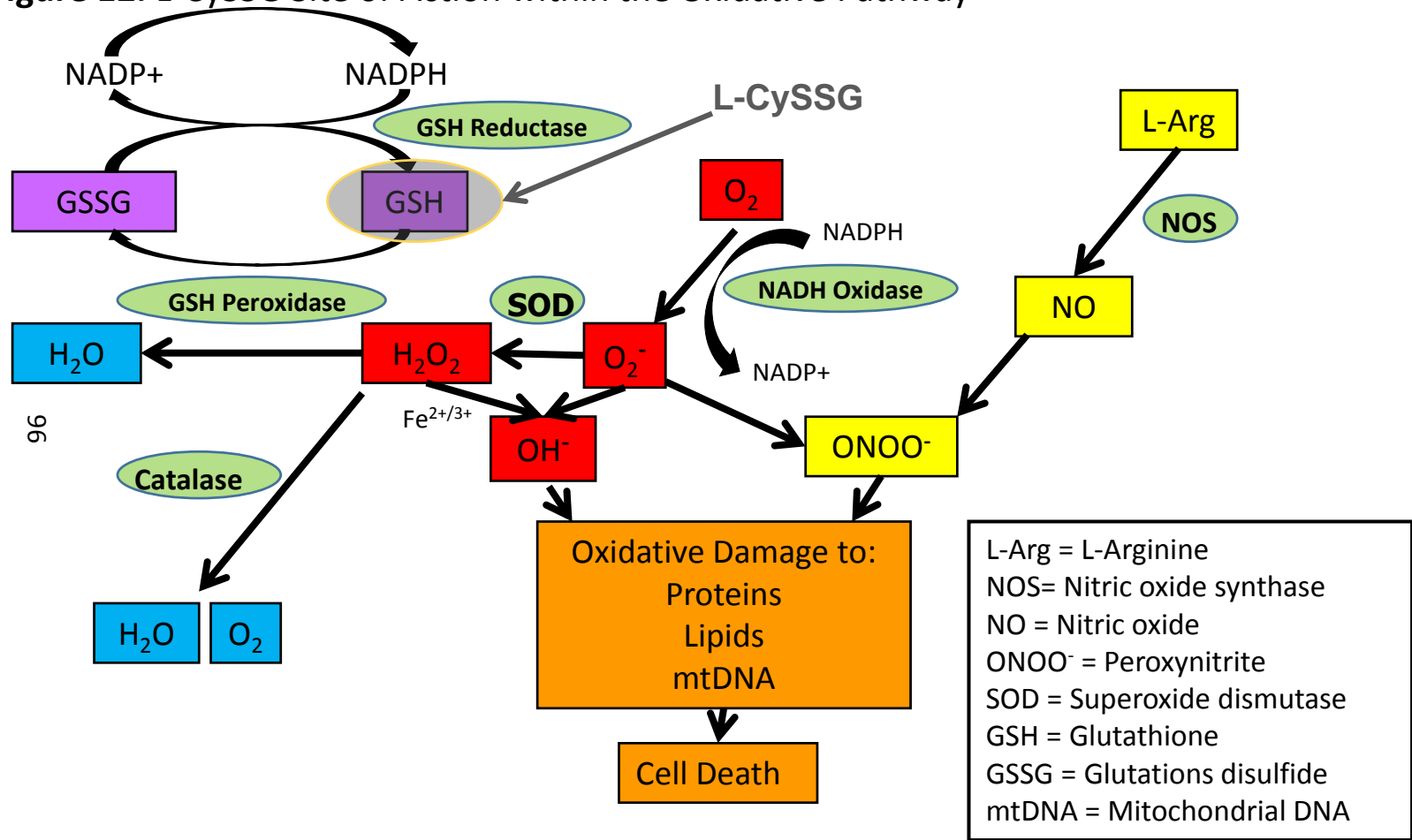
Figure 13. Ribose-Cysteine Site of Action within the Oxidative Pathway
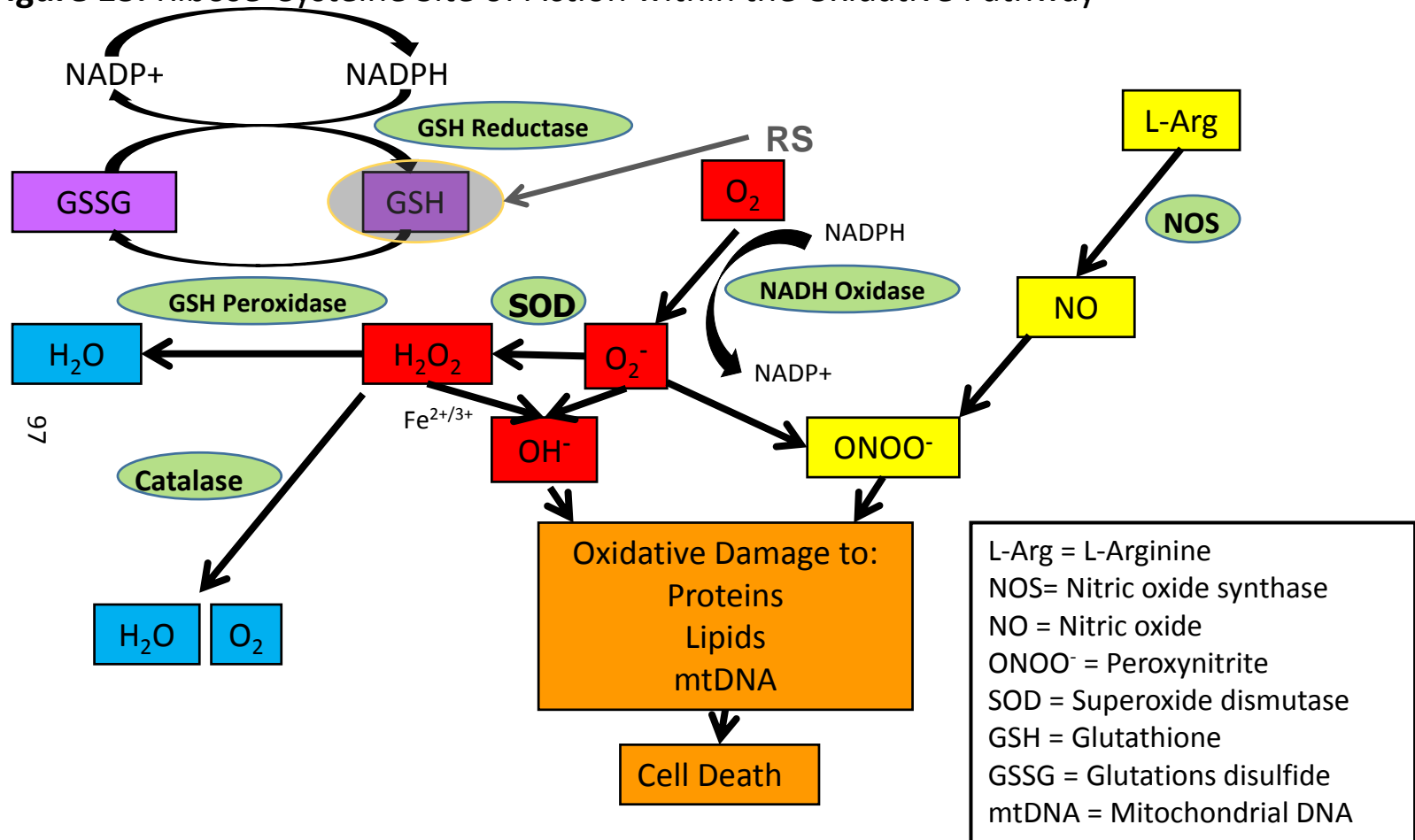
Figure 14. Combination Antioxidant Therapy Site of Action within the Oxidative Pathway

L-Arg = L-Arginine
NOS= Nitric oxide synthase
NO = Nitric oxide
ONOO⁻ = Peroxynitrite
SOD = Superoxide dismutase
GSH = Glutathione
GSSG = Glutations disulfide
mtDNA = Mitochondrial DNA
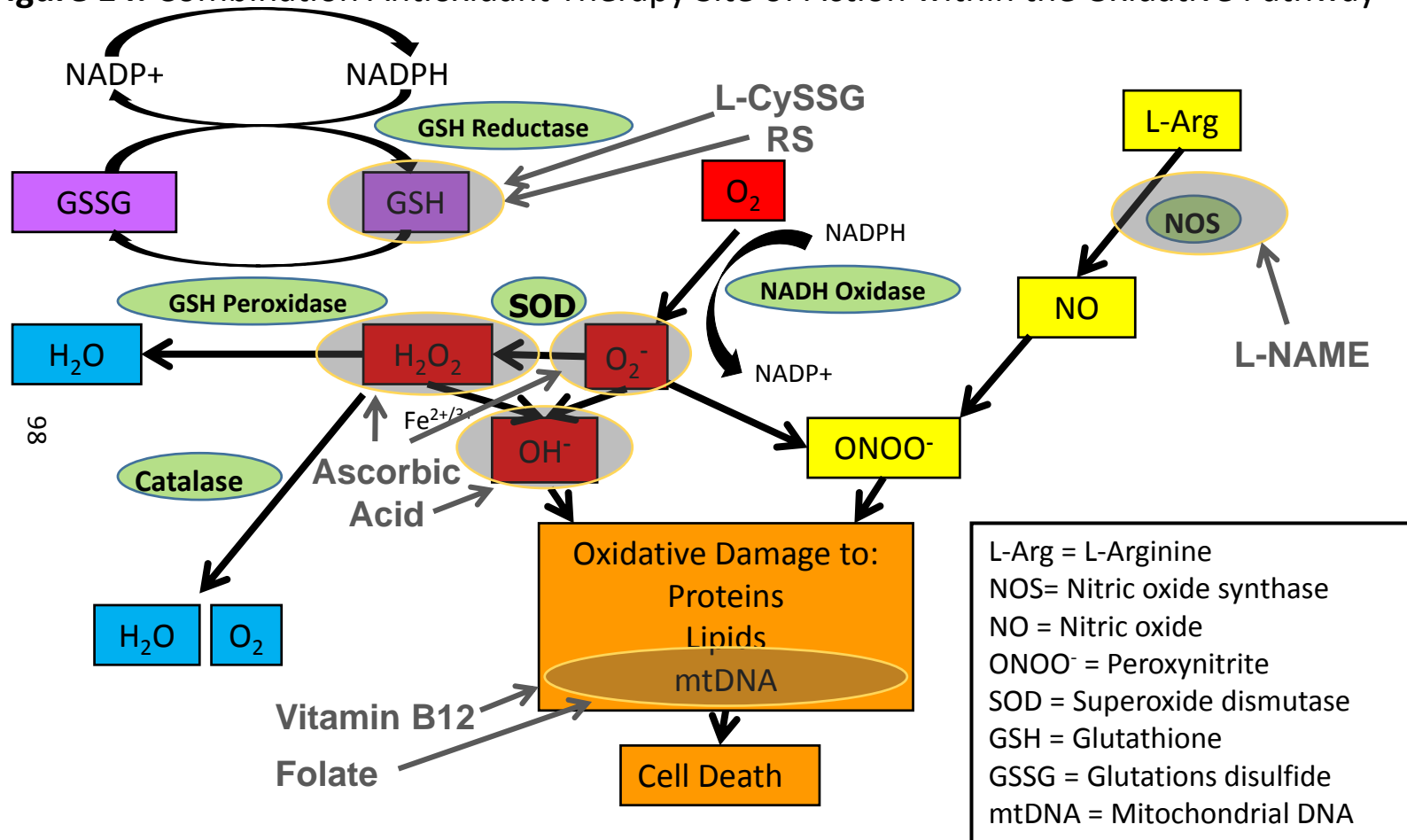
Figure 15. Specific Aim 2: Timeline for Early Antioxidant Treatment Protocol (in Months)

66
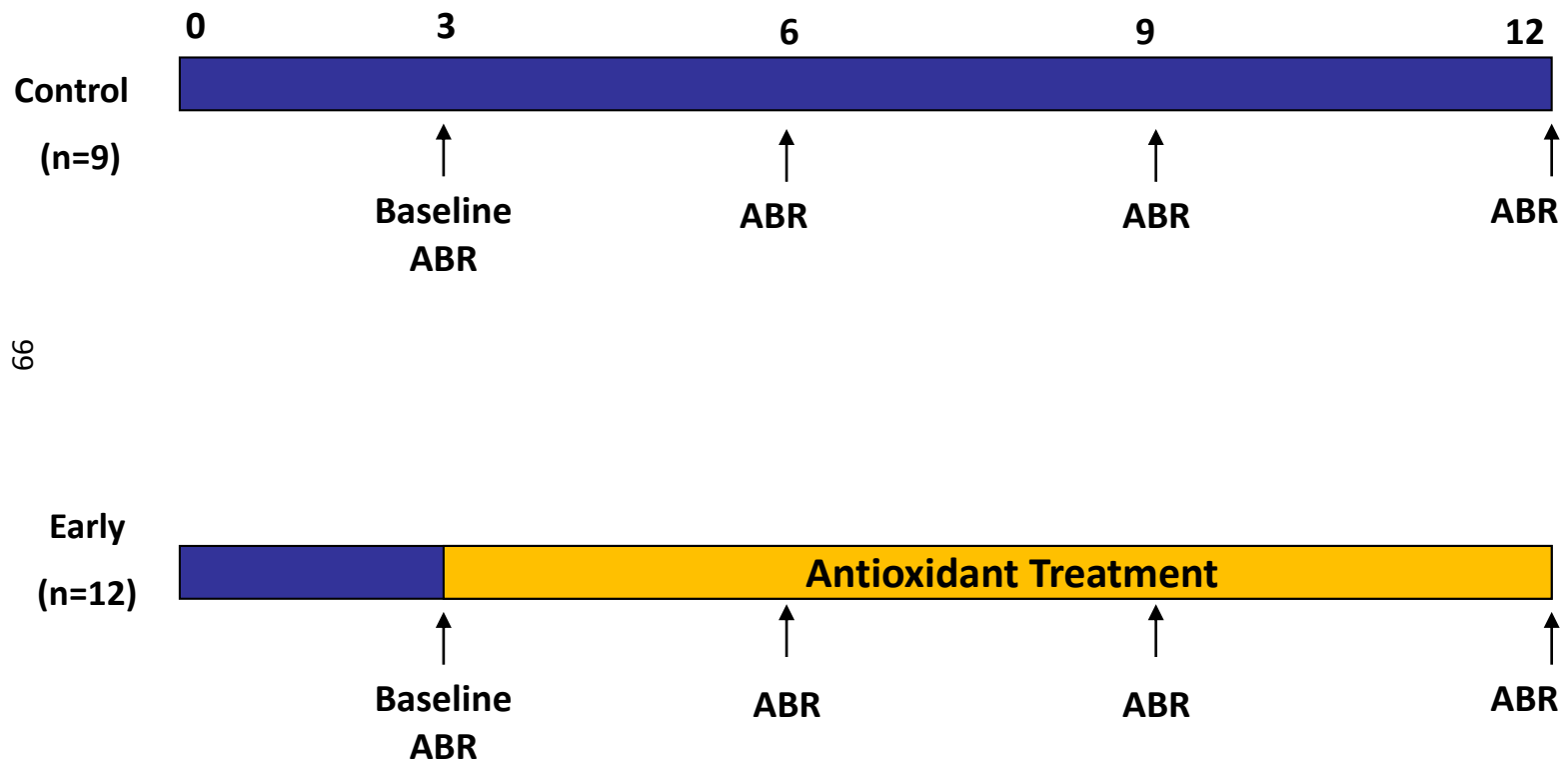
Figure 16. Specific Aim 3: Timeline for Delayed Antioxidant Treatment Protocol (in Months)

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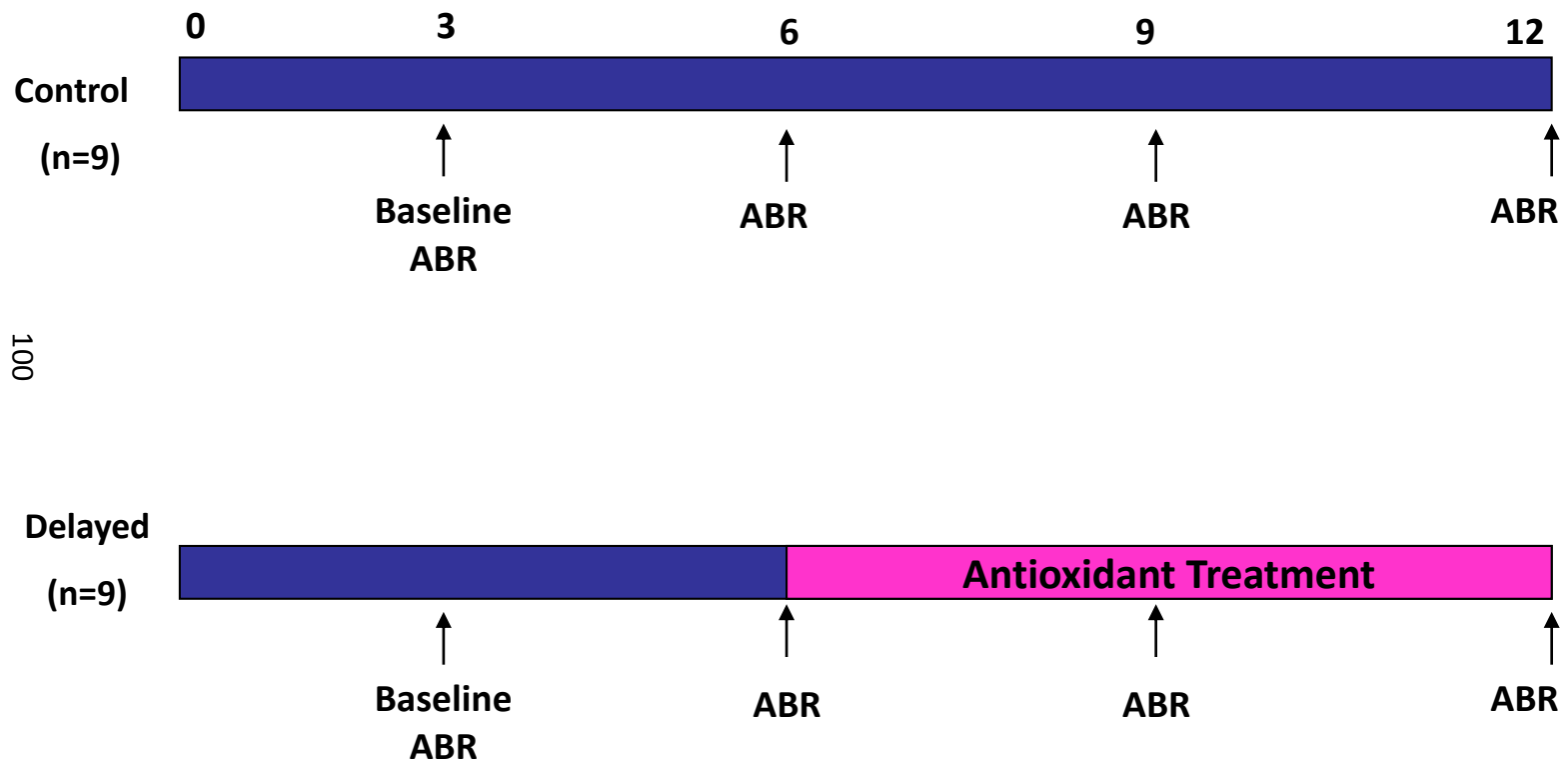
Figure 17. Specific Aim 1: 2,2,2 Tribromoethanol versus Ketamine/Xylazine for ABR in C57BL/6 Mice

101
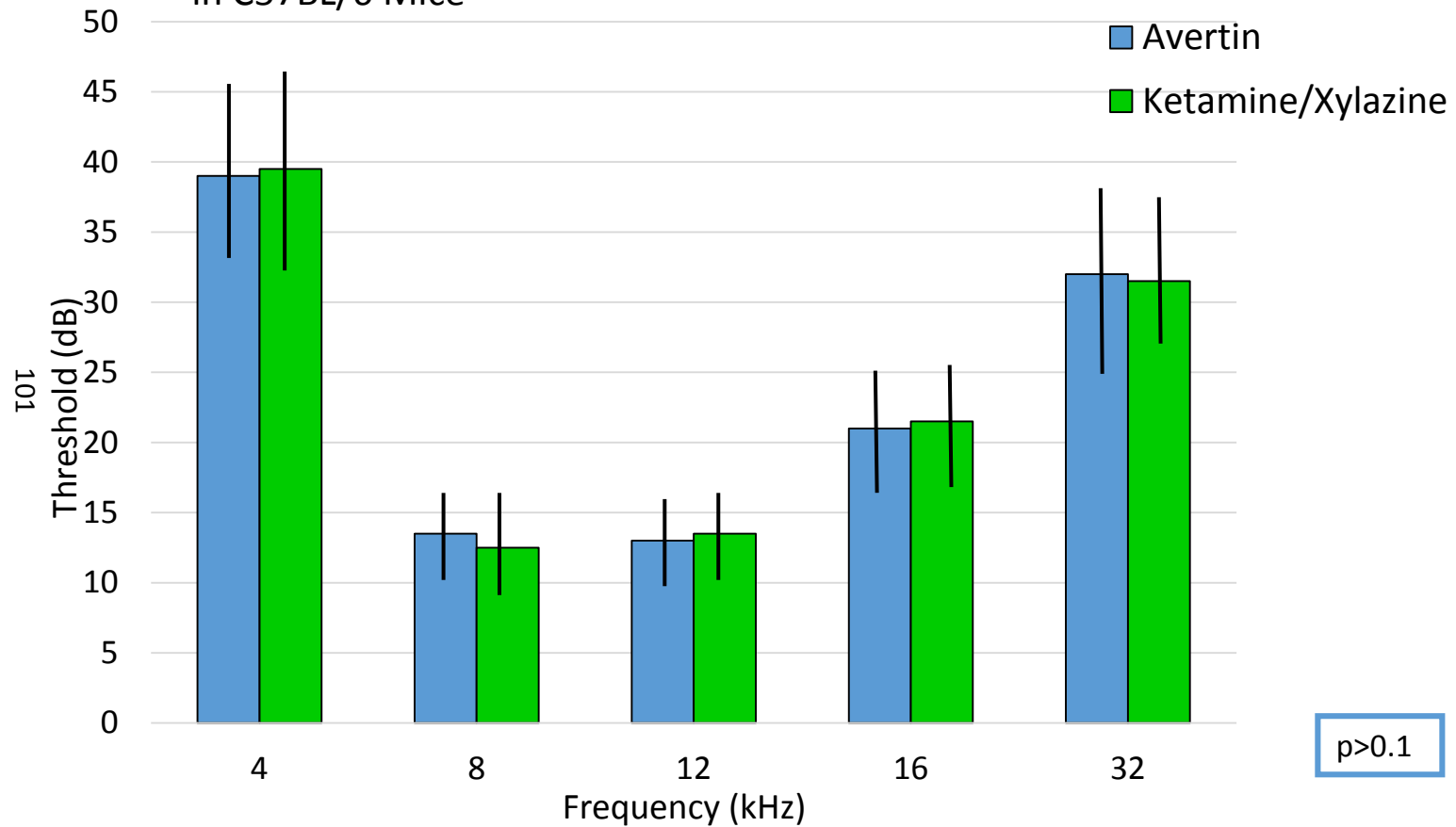
Figure 18. Specific Aim 2: 3 Month Baseline Individual ABR Data Early Antioxidant Treatment Versus Control Groups
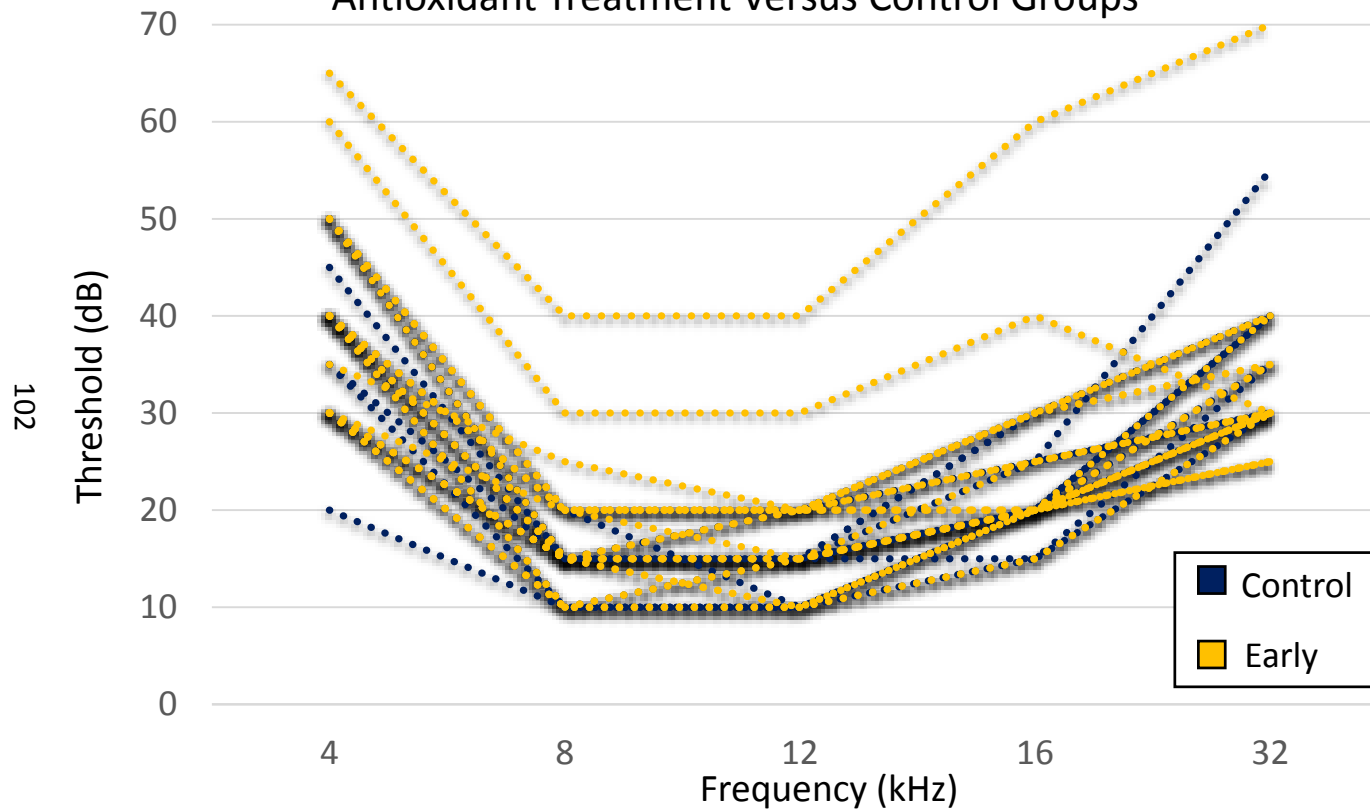
Figure 19. Specific Aim 2: 3 Month Baseline Mean ABR Thresholds Early Antioxidant Treatment Versus Control Groups
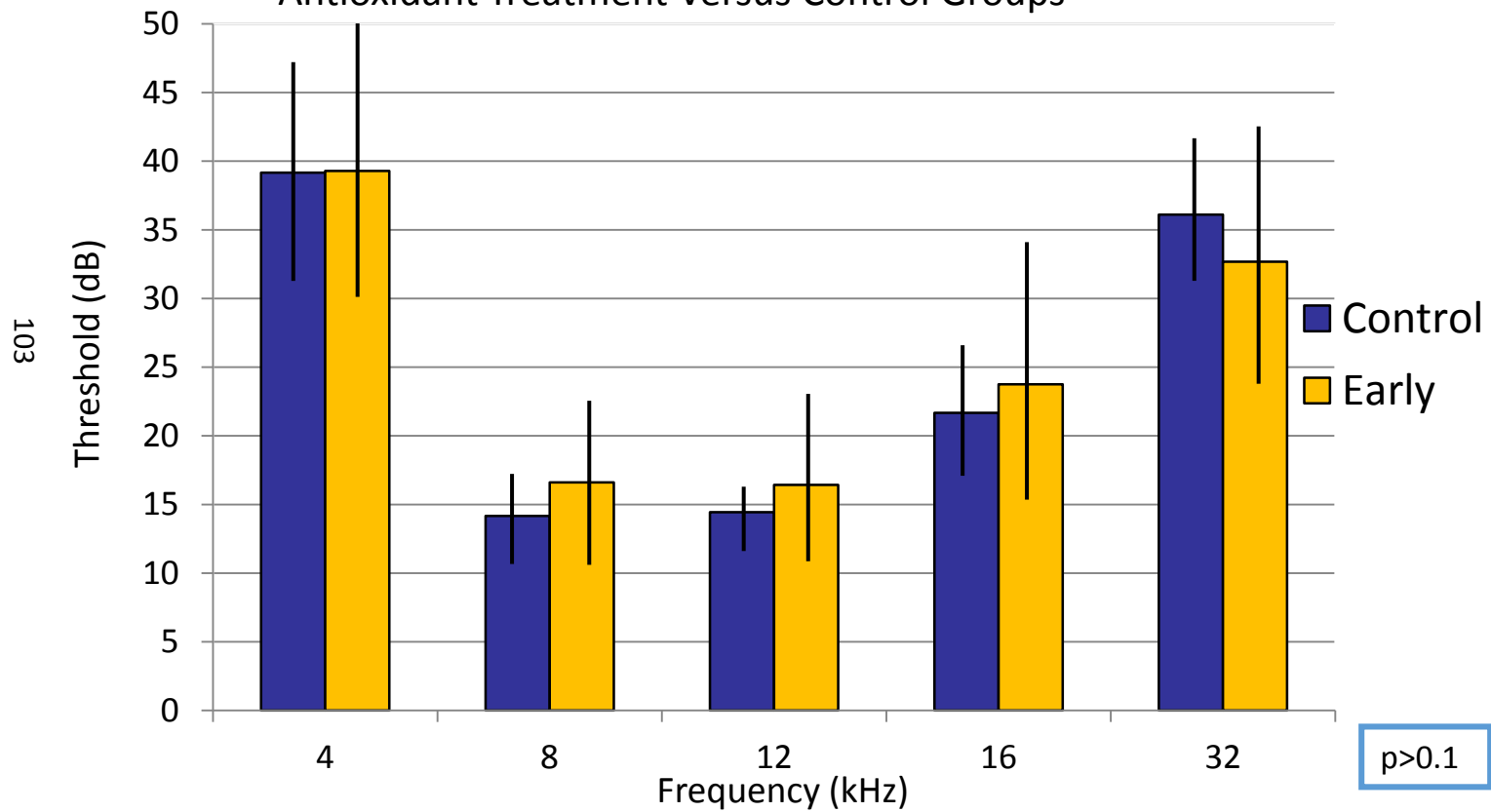
Figure 20. Specific Aim 2: 6 Month Individual ABR Data Early Antioxidant Treatment Versus Control Groups
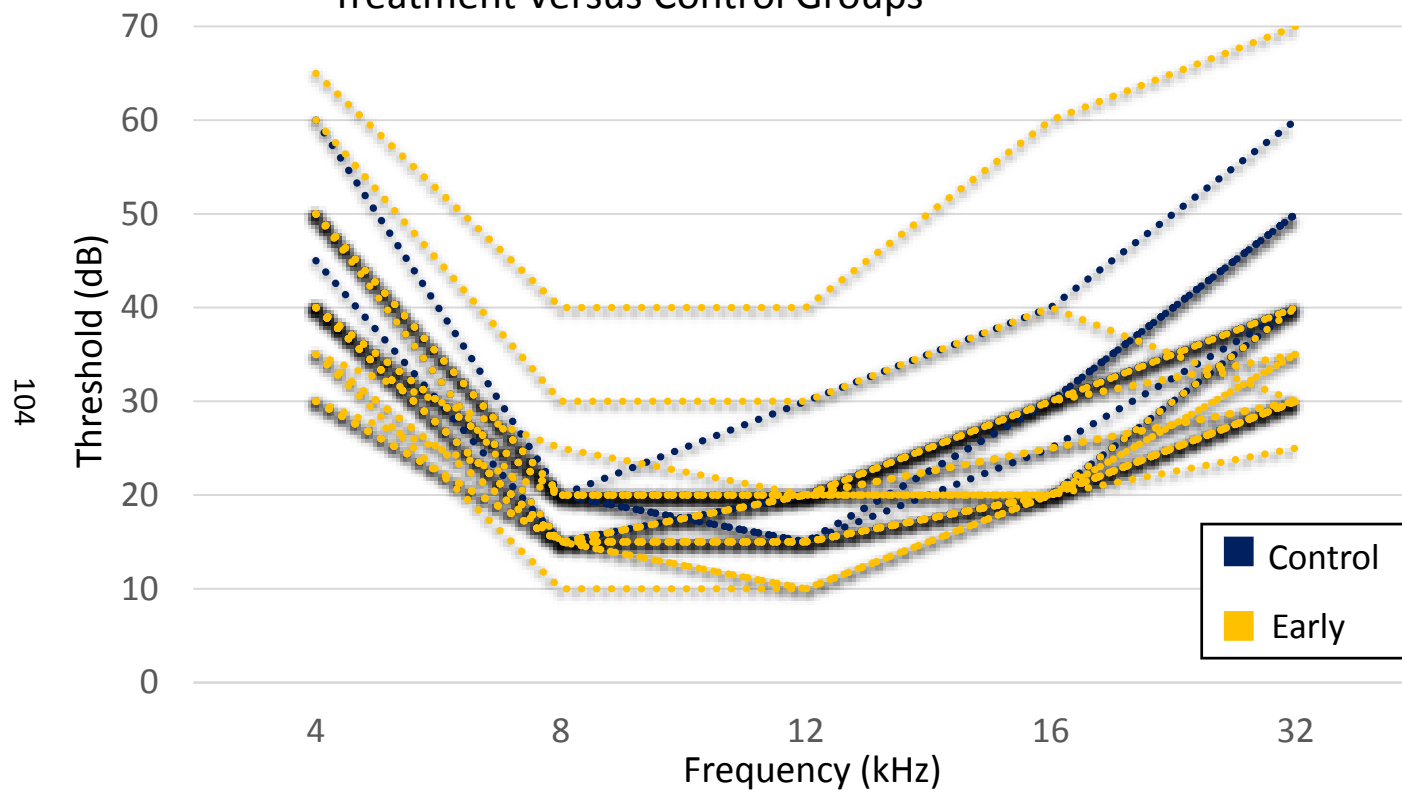
Figure 21. Specific Aim 2: 9 Month Individual ABR Data Early Antioxidant Treatment Versus Control Groups

105
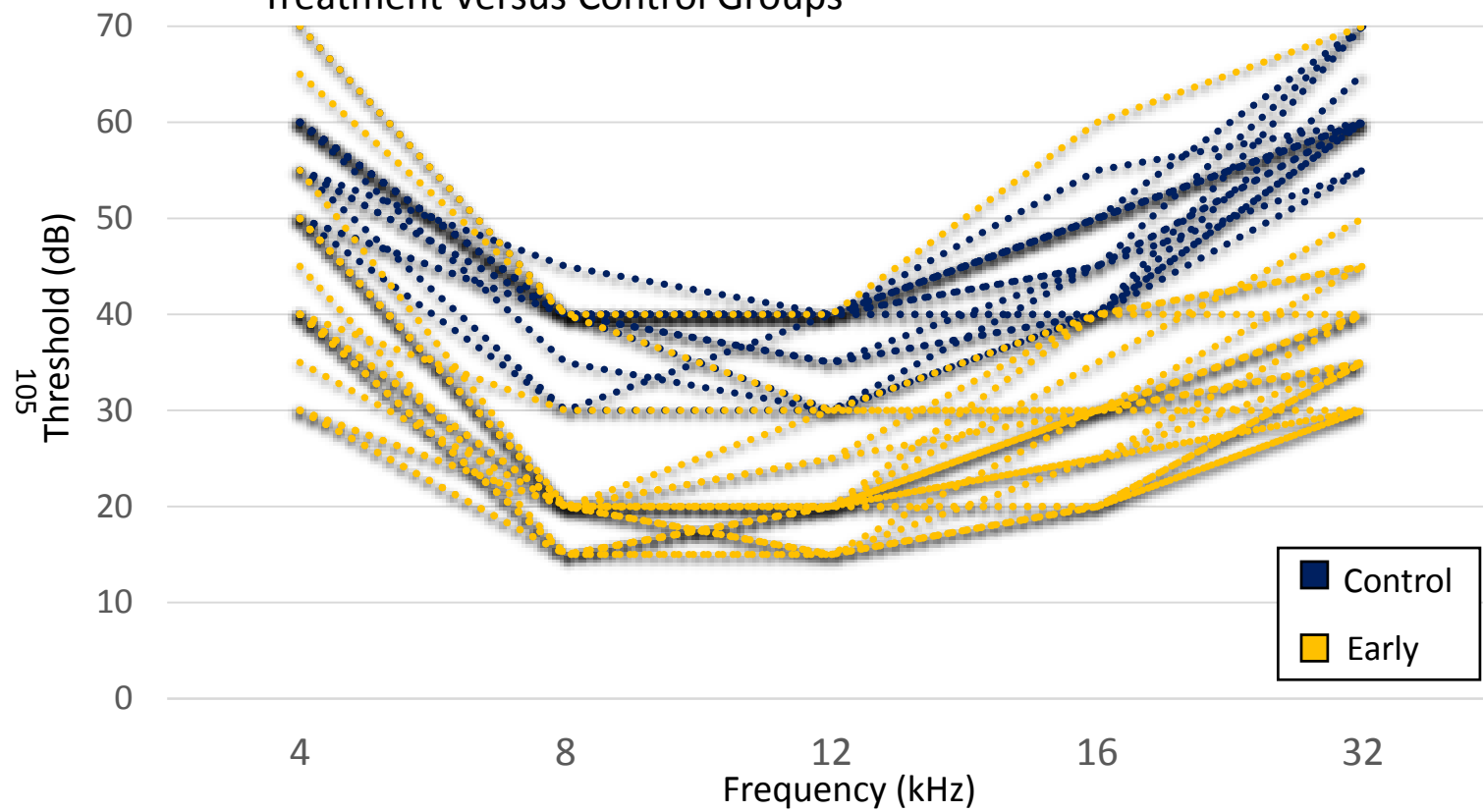
Figure 22. Specific Aim 2: 9 Month Mean ABR Thresholds Early Antioxidant Treatment Versus Control Groups

106
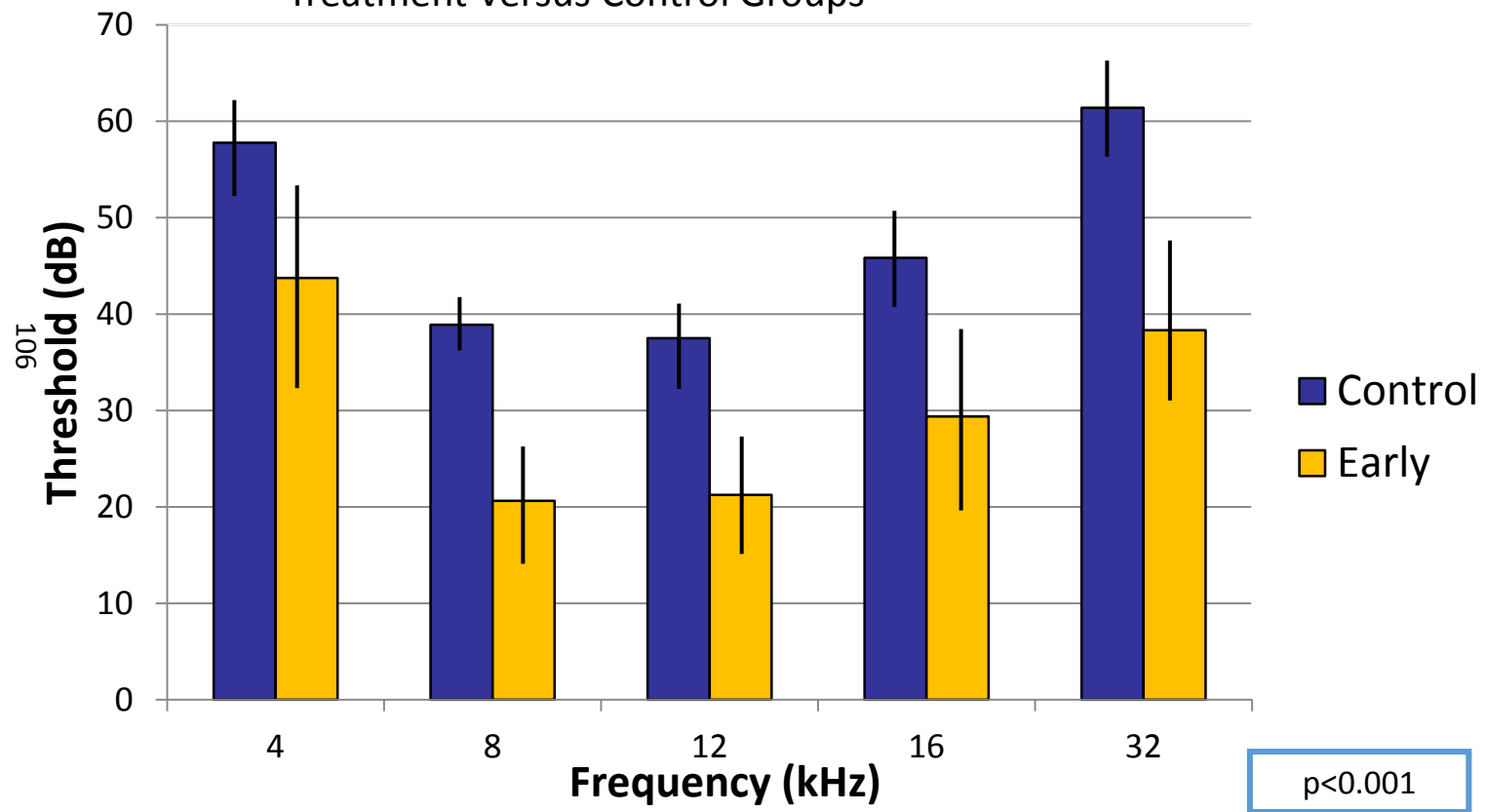
Figure 23. Specific Aim 2: 12 Month Individual ABR Data Early Antioxidant Treatment Versus Control Groups
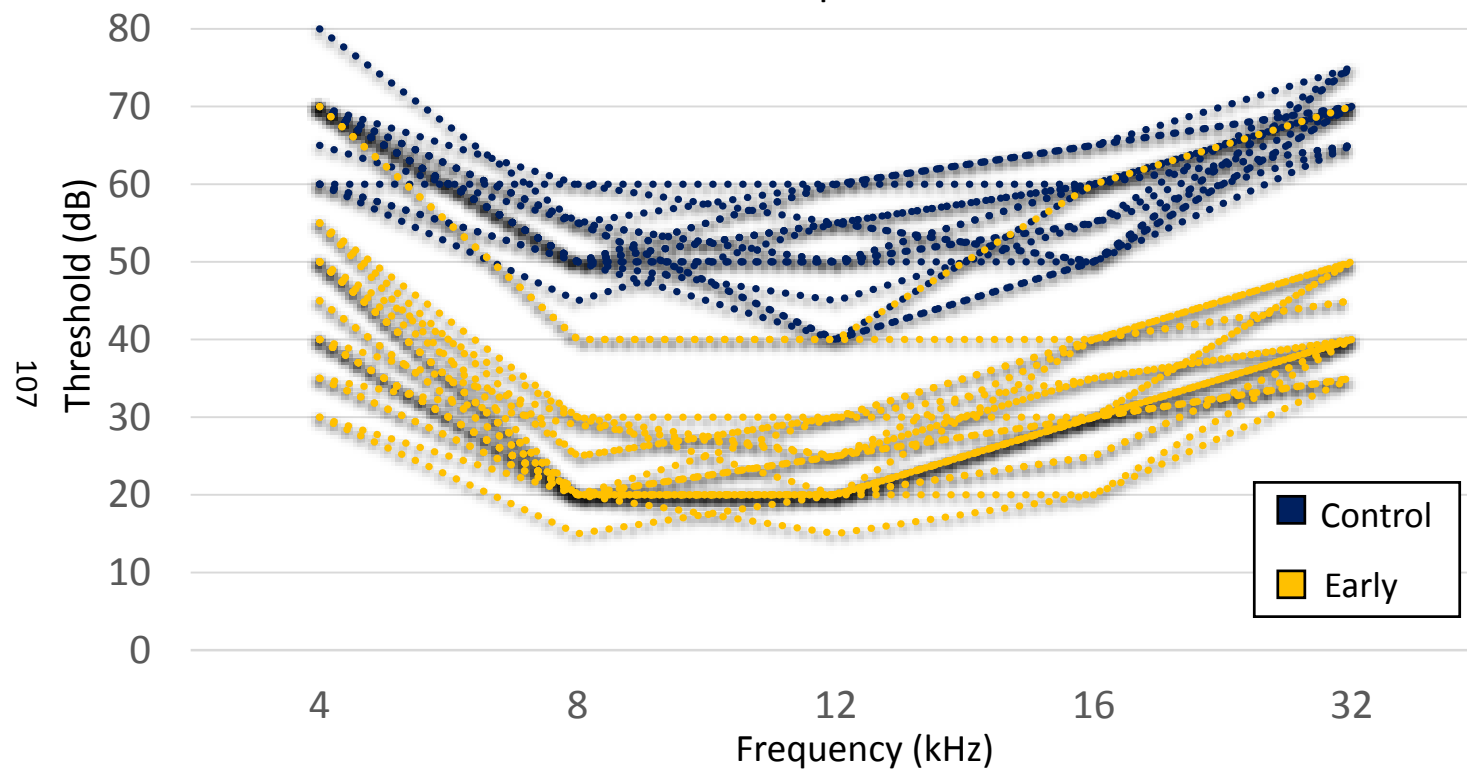
Figure 24. Specific Aim 2: 12 Month Mean ABR Thresholds Early Antioxidant Treatment Versus Control Groups
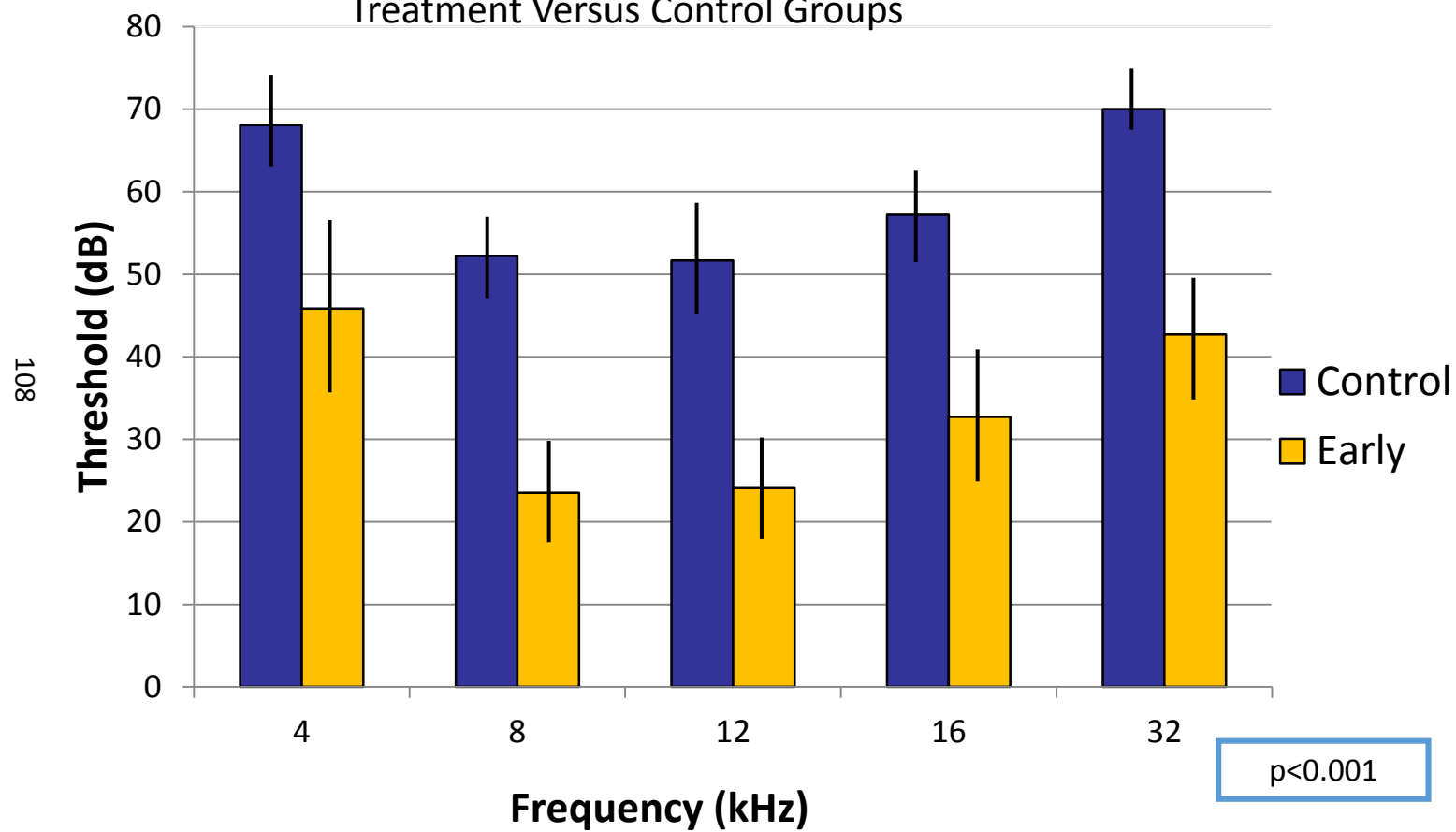
Figure 25. Specific Aim 2: 12 Month Mean ABR Threshold Shifts Early Antioxidant Treatment Versus Control Groups
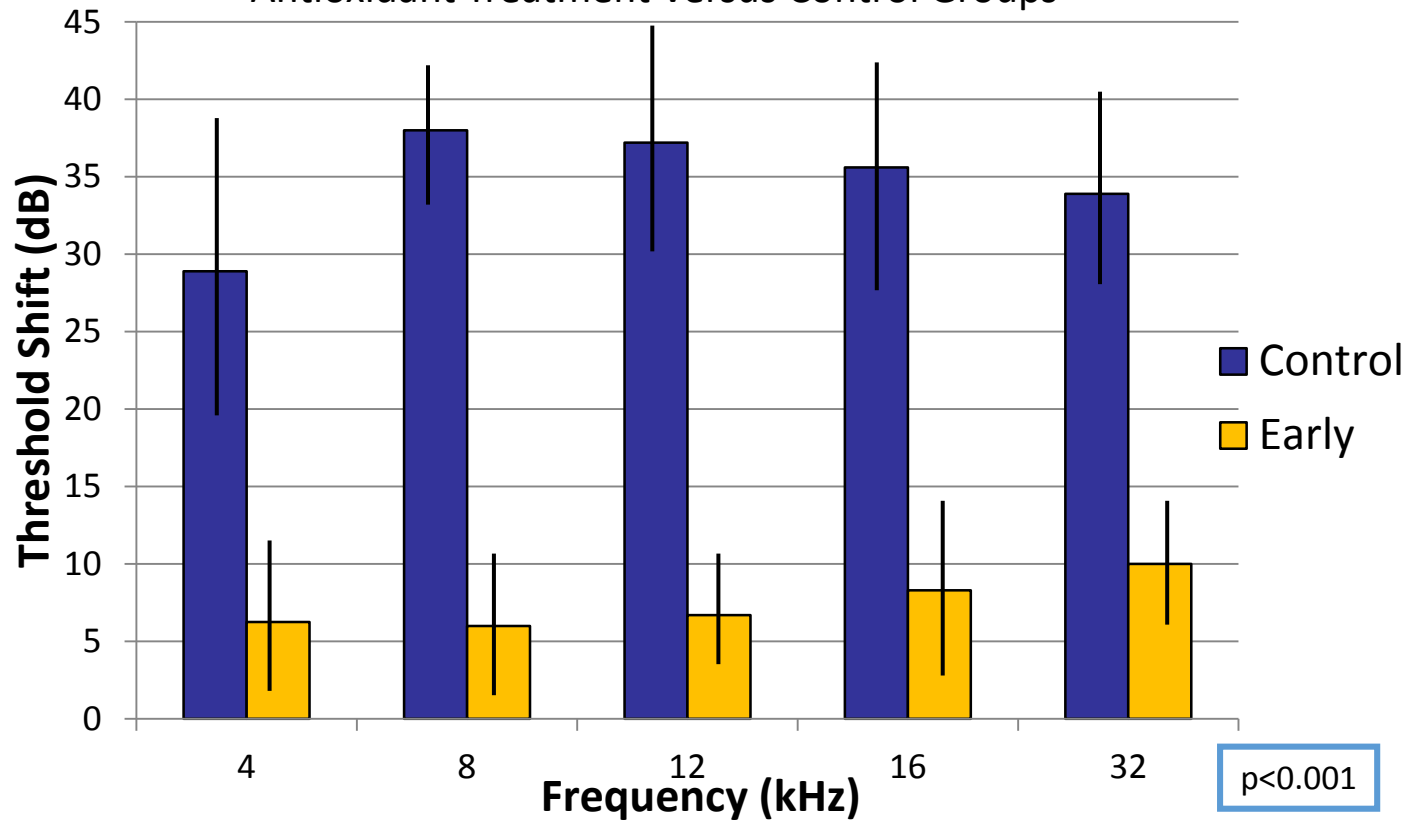
Figure 26. Specific Aim 2: Individual Pure Tone Averages Early Antioxidant Treatment Versus Control Groups

110
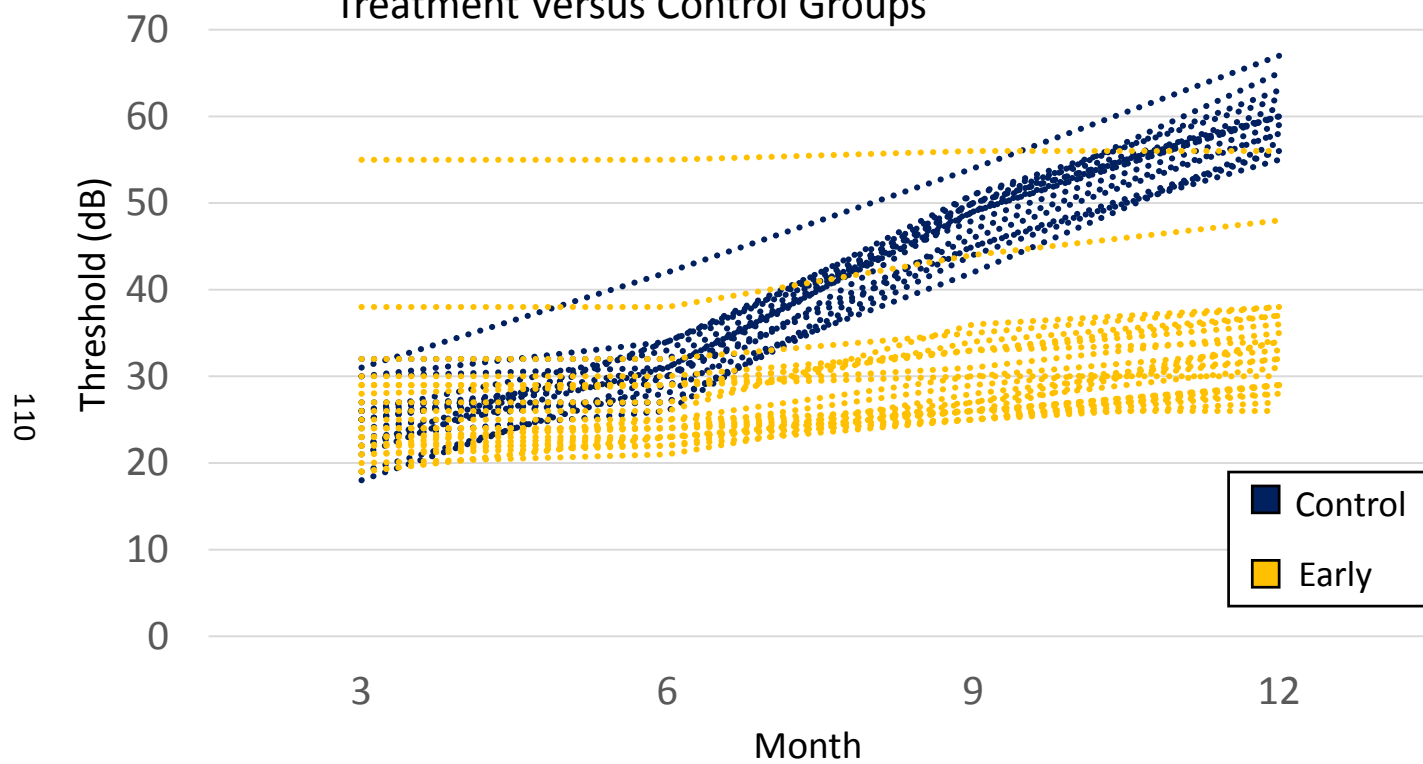
Figure 27. Specific Aim 3: 3 Month Baseline Individual ABR Data Delayed Versus Control Groups
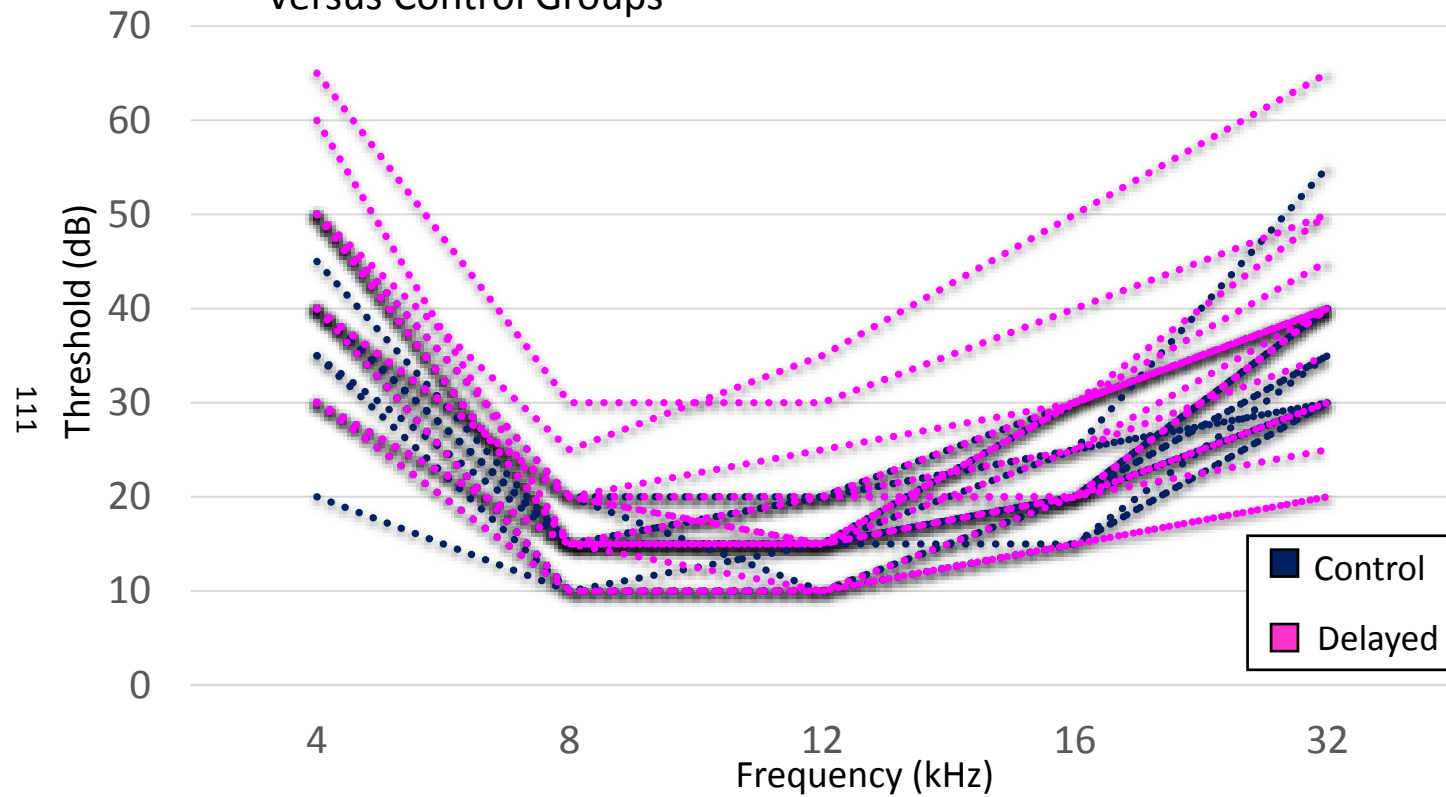
**Figure 28.** Specific Aim 3: 3 Month Baseline Mean ABR Thresholds Delayed Versus Control Groups
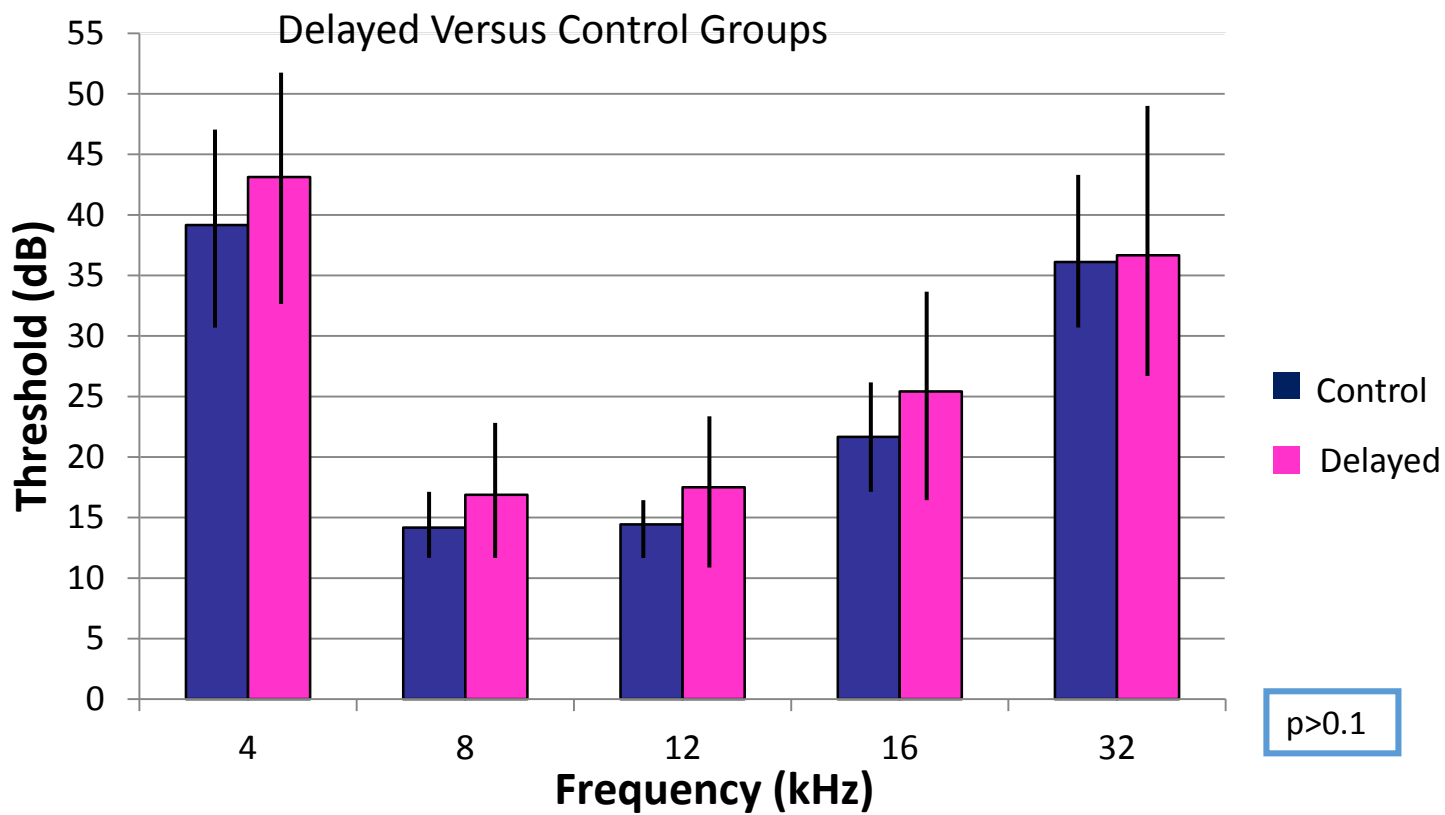
Figure 29. Specific Aim 3: 6 Month Individual ABR Data Delayed Versus Control Groups

113
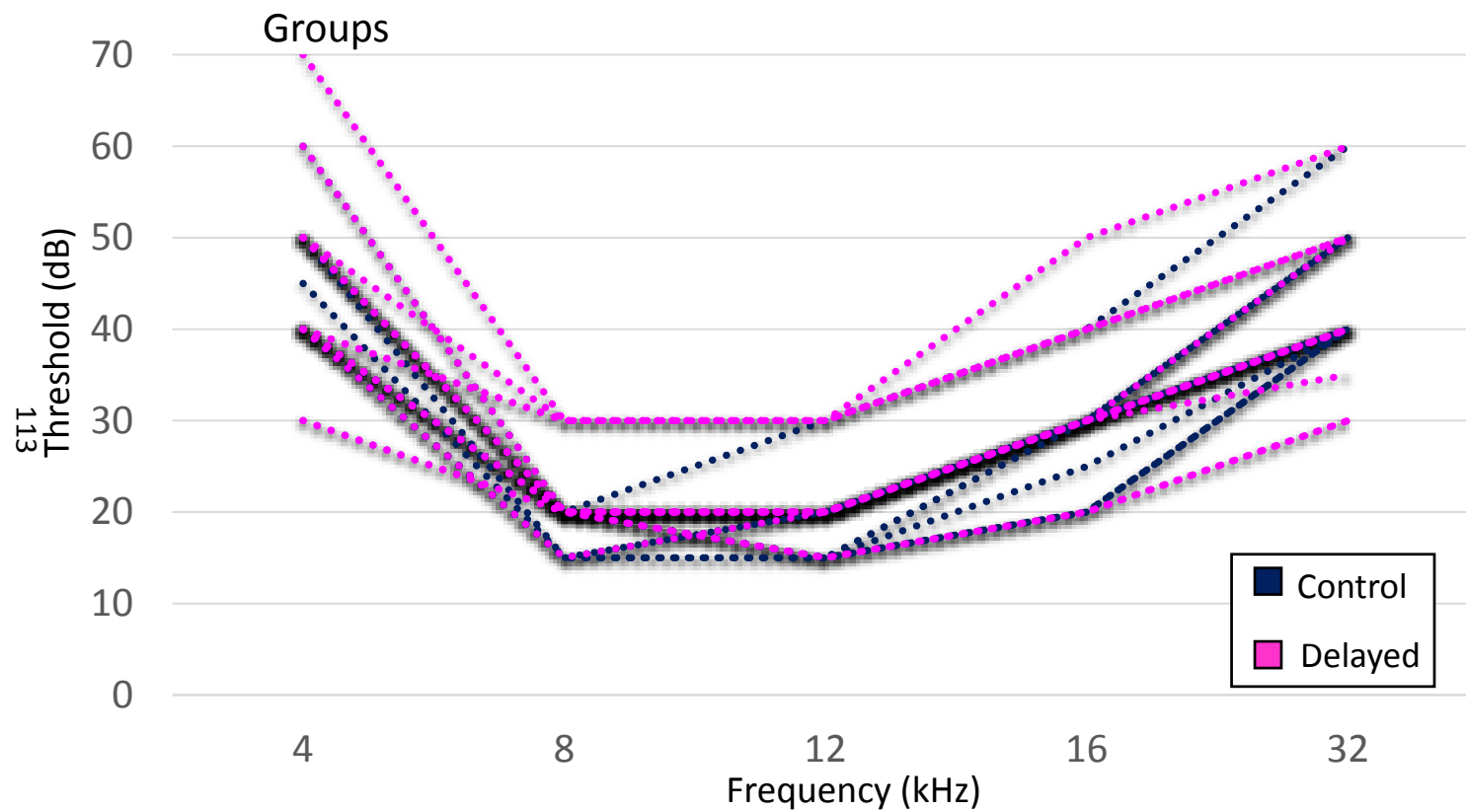
Figure 30. Specific Aim 3: 6 Month Mean ABR Thresholds Delayed Antioxidant Treatment Versus Control Groups

114
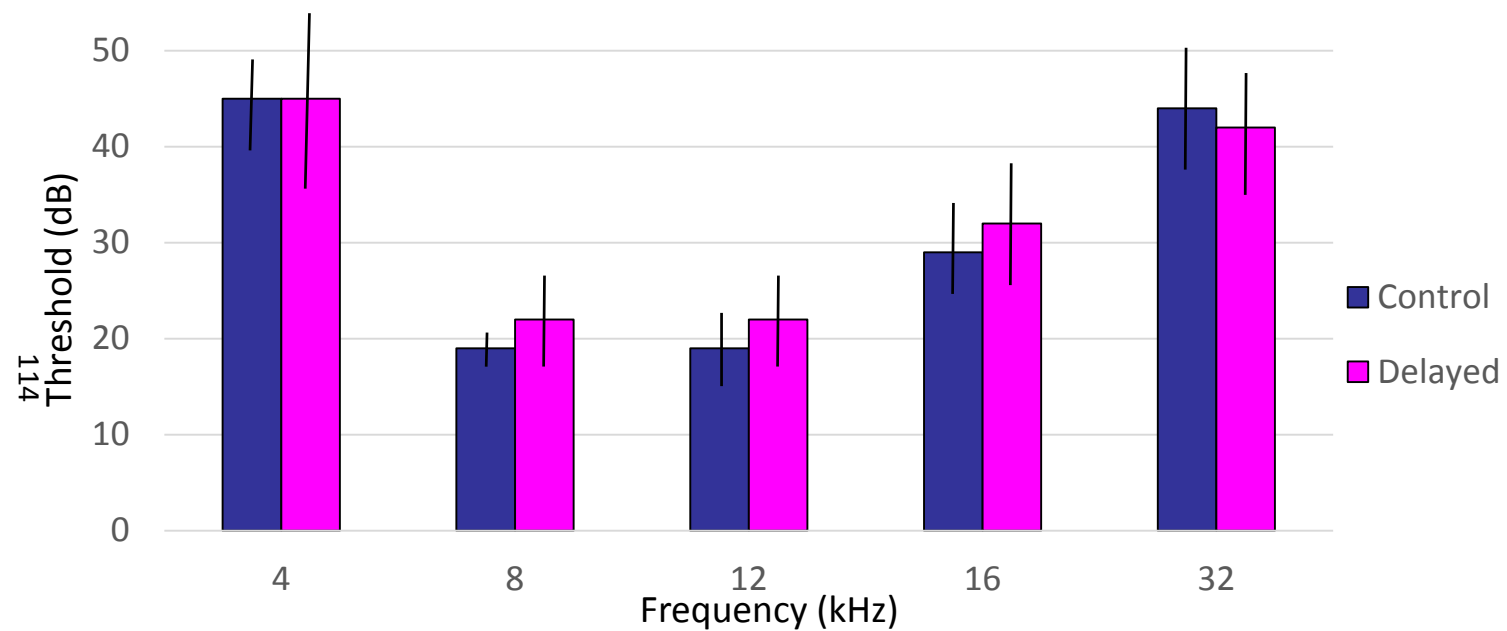
Figure 31. Specific Aim 3: 9 Month Individual ABR Data Delayed Versus Control Groups

115
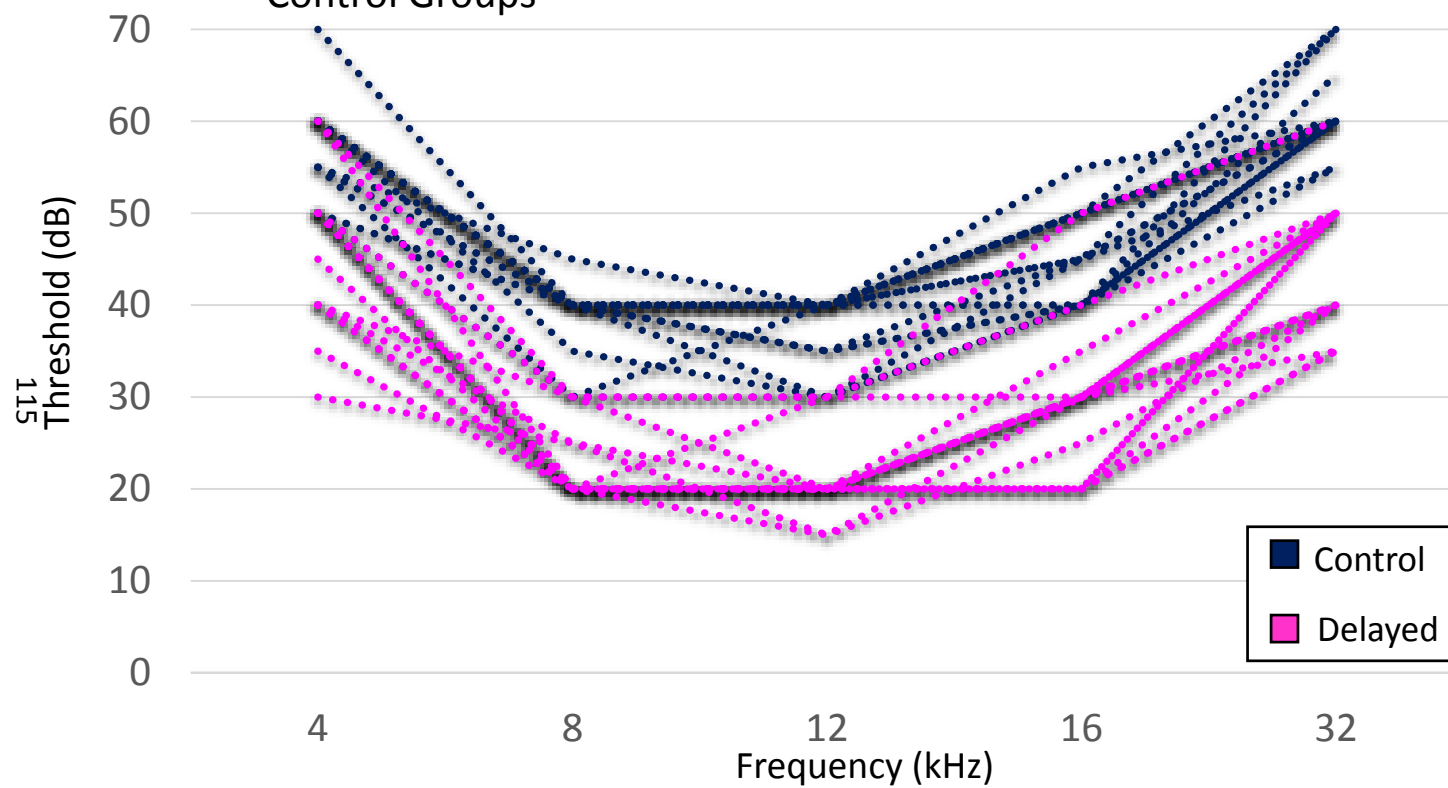
Figure 32. Specific Aim 3: 9 Month Mean ABR Thresholds Delayed Versus Control Groups
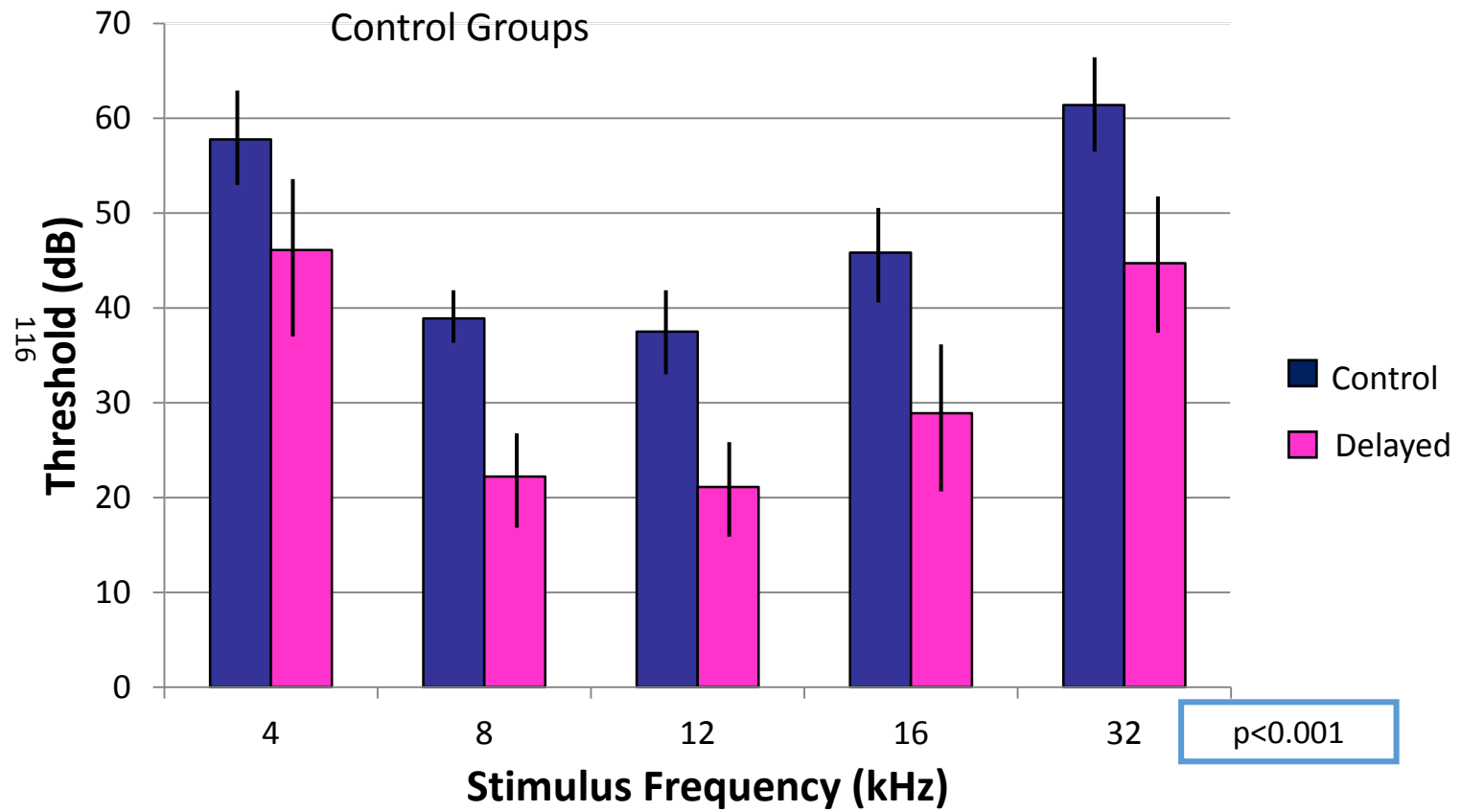
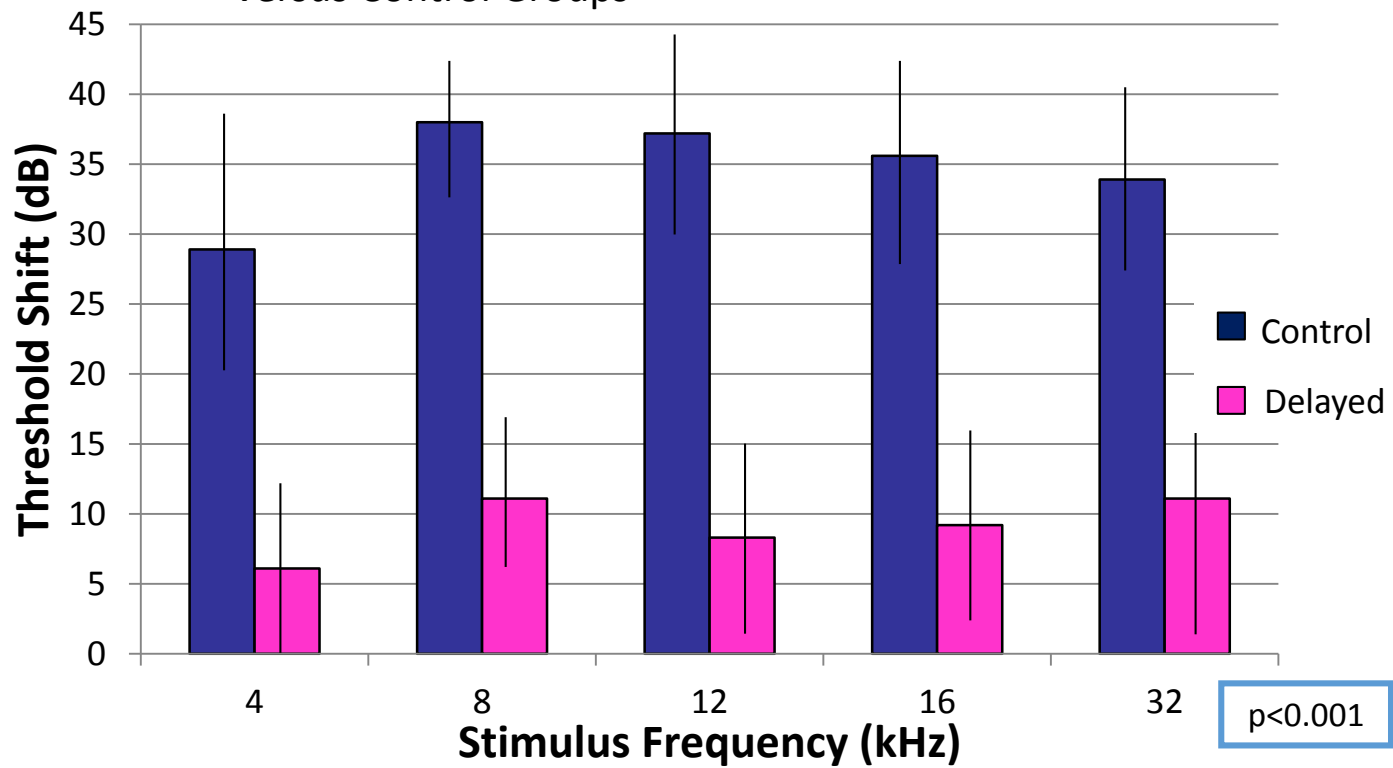**Figure 33.** Specific Aim 3: 12 Month Individual ABR Data Delayed Versus Control Groups

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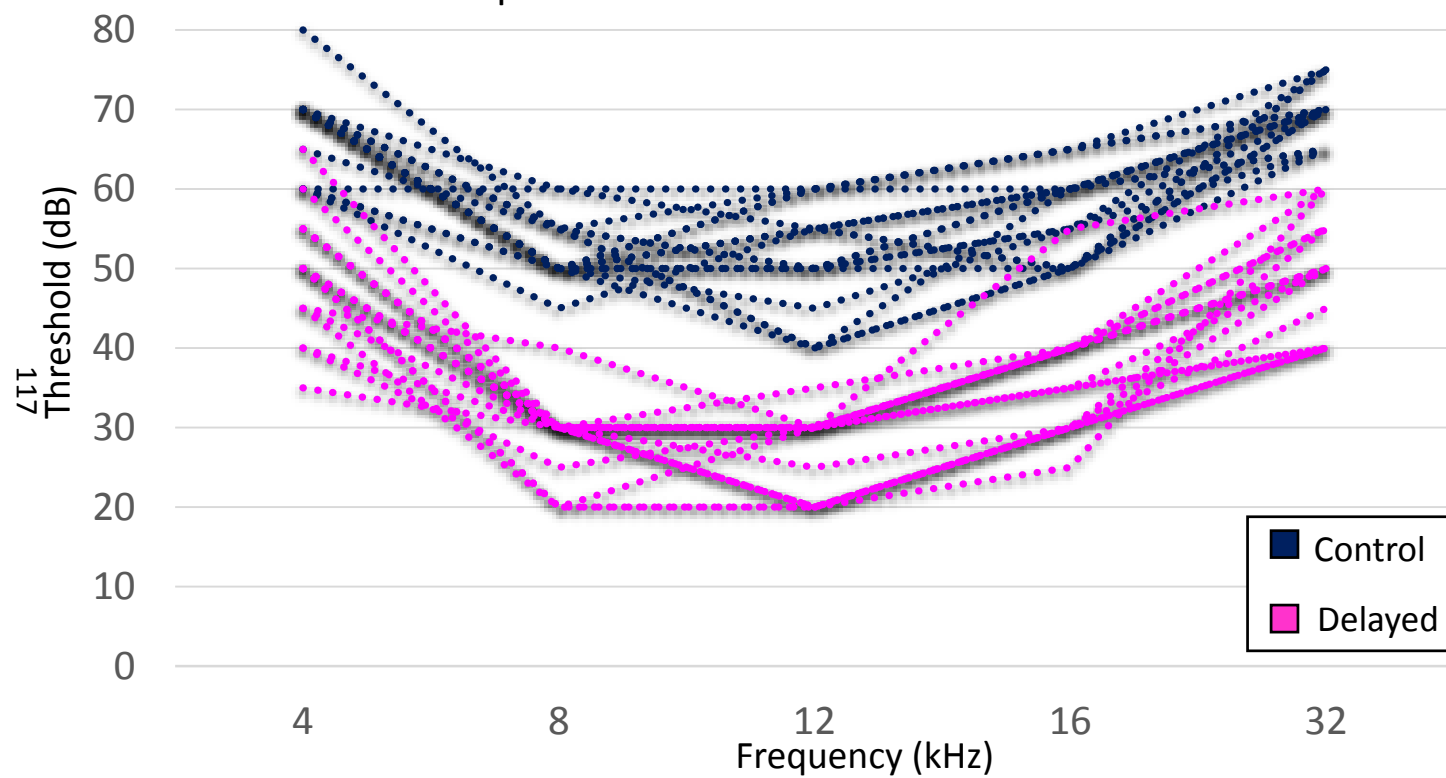
Figure 34. Specific Aim 3: 12 Month Mean ABR Thresholds Delayed Versus Control Groups

118
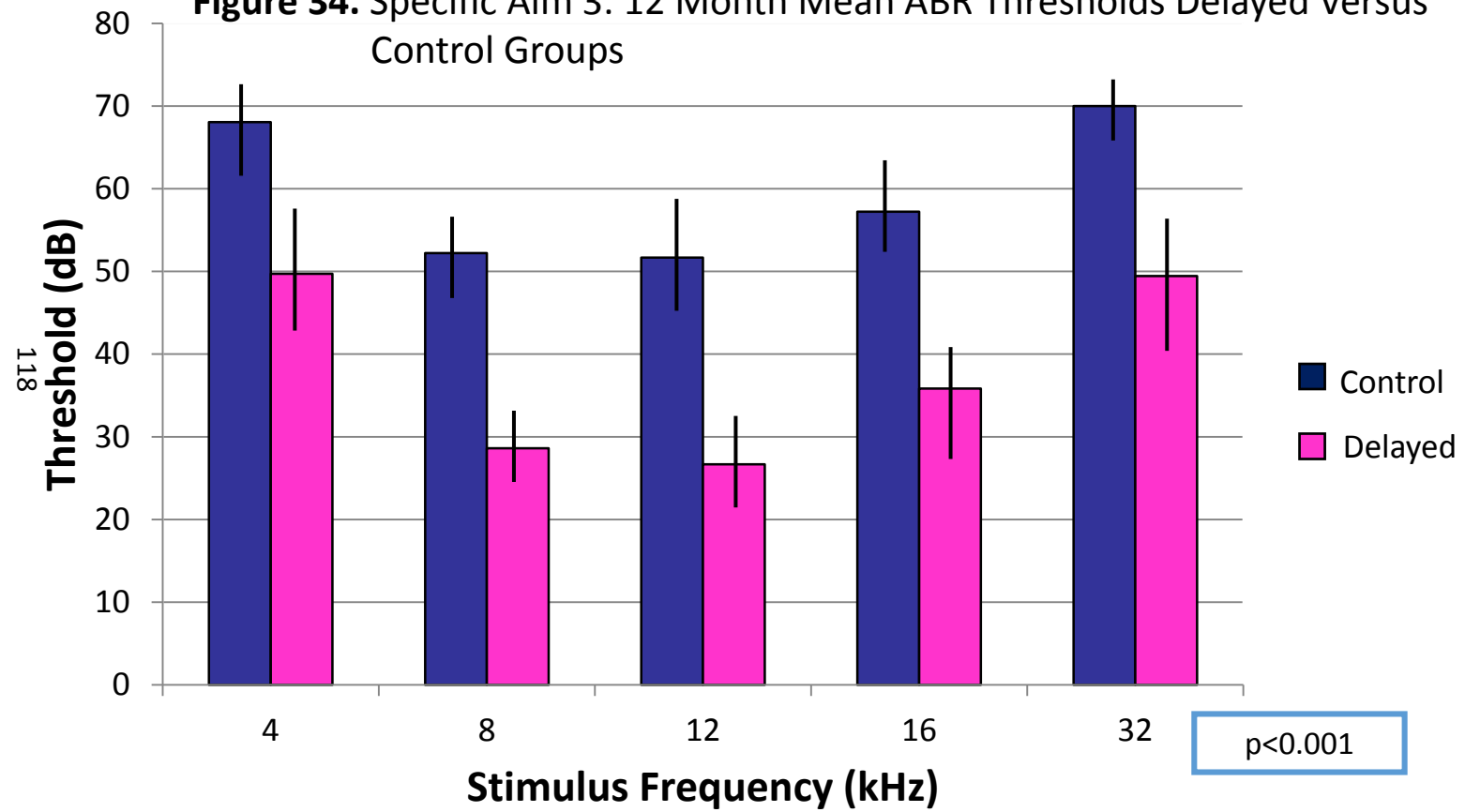
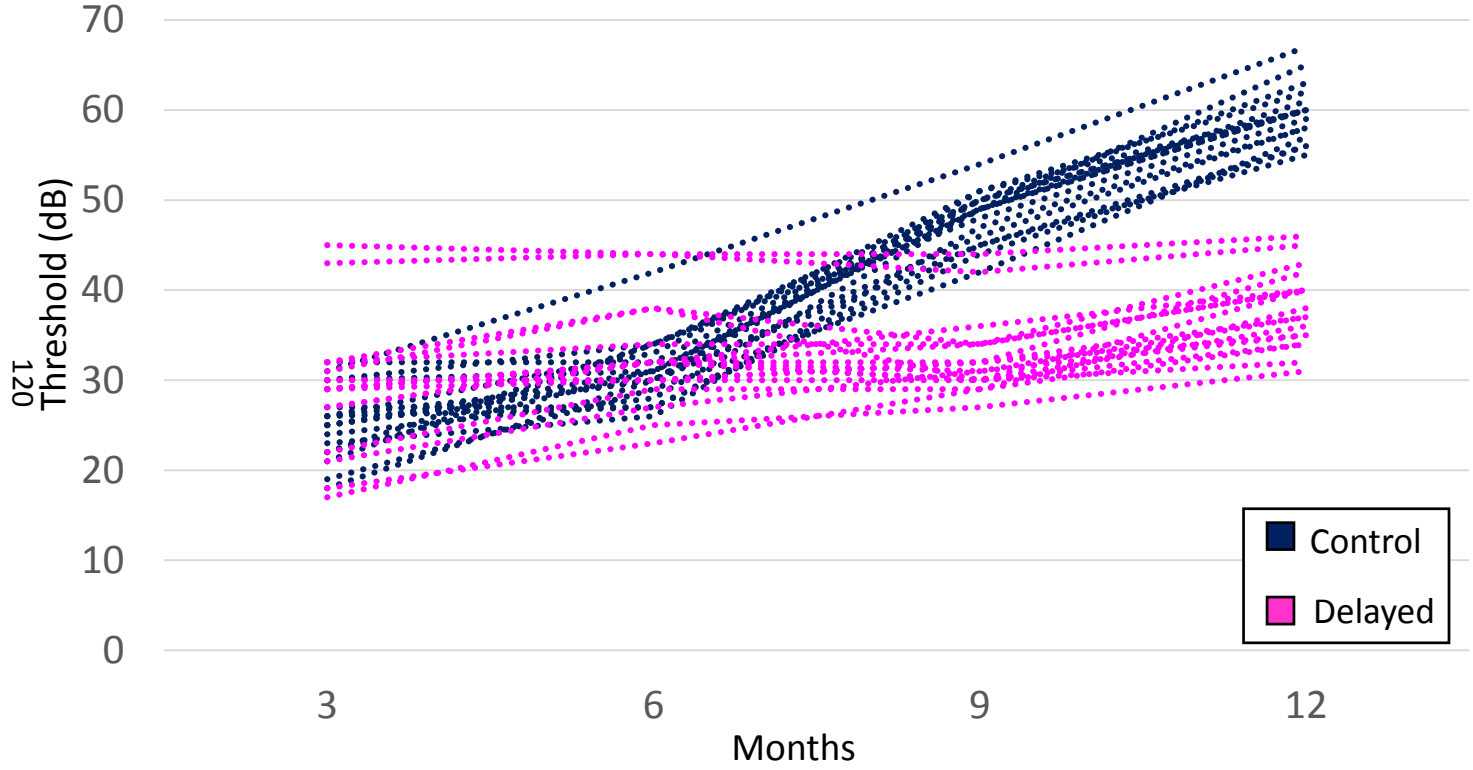**Figure 35.** Specific Aim 3: 12 Month Mean ABR Threshold Shifts Delayed Versus Control Groups

Figure 36. Specific Aim 3: Individual Pure Tone Averages Delayed Versus Control Groups

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ORIGINAL RESEARCH–OTOLOGY AND NEUROTOLOGY

A combination antioxidant therapy prevents age-related hearing loss in C57BL/6 mice

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

ABSTRACT

OBJECTIVE: Age-related hearing loss (ARHL) is characterized by gradual, progressive sensorineural hearing loss, which impairs communication, lending to clinical depression and social withdrawal. There are currently no effective treatments for ARHL. The purpose of this study is to evaluate the potential of a combination antioxidant therapy in preventing ARHL.

STUDY DESIGN: Randomized controlled trial.

SETTING: Animal study.

SUBJECTS AND METHODS: C57BL/6 mice, a recognized animal model of ARHL, were assigned to one of three groups: early treatment (n = 12), late treatment (n = 9), or control group (n = 9). Treatment groups of mice were fed with a combination agent comprising six antioxidant agents that target four sites within the oxidative pathway: L-cysteine-glutathione mixed disulfide, ribose-cysteine, NW-nitro-L-arginine methyl ester, vitamin B12, folate, and ascorbic acid. Auditory brainstem response (ABR) thresholds were recorded at baseline and every three months following initiation of treatment.

RESULTS: Threshold shifts from baseline were decreased in the treatment groups when compared to the control group at all tested frequencies ($P < 0.001$). The ABR threshold shift at 12 months of age for the control group was 34.7 dB with a 95% confidence interval (CI) of ±1.6. The mean threshold shifts for the early and late treatment groups were 7.5 dB (±0.87, 95% CI) and 9.2 dB (±1.6, 95% CI).

CONCLUSION: Combination antioxidant therapy effectively decreased threshold shifts on ABR within an animal model of ARHL. Combination antioxidant therapy, with further research and investigation, may provide a safe and cost-effective method of preventing presbycusis in the growing elderly population.

Age-related hearing loss (ARHL), or presbycusis, refers to a gradual progressive hearing loss that accompanies aging. ARHL is characterized by decreased hearing sensitivity, decreased speech recognition in noisy environments, slowed central processing of acoustic stimuli, and impaired sound localization. The hearing loss is typically described as downward-sloping high-frequency loss but may be associated with various types of auditory system dysfunctions that progress with aging.

Age-related hearing loss is one of the most common conditions affecting the elderly population. Approximately 35 percent of adults age 65 and older have been reported to have some degree of age-related hearing loss. It is projected that by 2025, approximately 24.5 million Americans will be affected.[1] Age-related hearing loss disables an individual's ability to communicate, thereby effectively jeopardizing their autonomy. Patients often experience associated depression and social withdrawal. Thus, age-related hearing loss presents a major public health concern.

Oxidative injury caused by free-radical damage is perhaps the most fundamental cause of age-related pathology in the biological aging of cells. Oxidative damage may be an important intrinsic factor in the pathogenesis of presbycusis. Increased concentrations of free radicals (reactive oxygen species and reactive nitrogen species [RNS]) are implicated as a mediator of oxidative stress and damage (Fig 1). Free radicals are known to produce mitochondrial genome deletions including the mtDNA[4977] deletion. Human temporal bone studies have revealed an increased incidence of such deletions in elderly individuals affected by presbycusis when compared to elderly individuals unaffected by the disease.[2] Folate and vitamin B12 function in DNA synthesis and repair, which may function in repair of mitochondrial genomic deletions. Human studies have revealed a correlation between vitamin B12 and folate deficiency with the presentation of presbycusis.[3] However, there have been reports within the literature that contradict this correlation.[4] Glutathione (GSH) is an important ubiquitous antioxidant found in the inner ear. Reduced levels of GSH were found in the inner ear of aged Fischer 344 rats when compared to young rats.[5] Inducible nitric oxide synthase (iNOS) is a crucial enzyme in the production of nitric oxide, a potent RNS. Our preliminary data revealed an increase in the expression of iNOS within the cochlea of aged Fischer 344 rats when compared to young rats.

Given the contribution of oxidative damage to the pathogenesis of presbycusis, antioxidants may prevent the onset or progression of disease. A number of antioxidants have
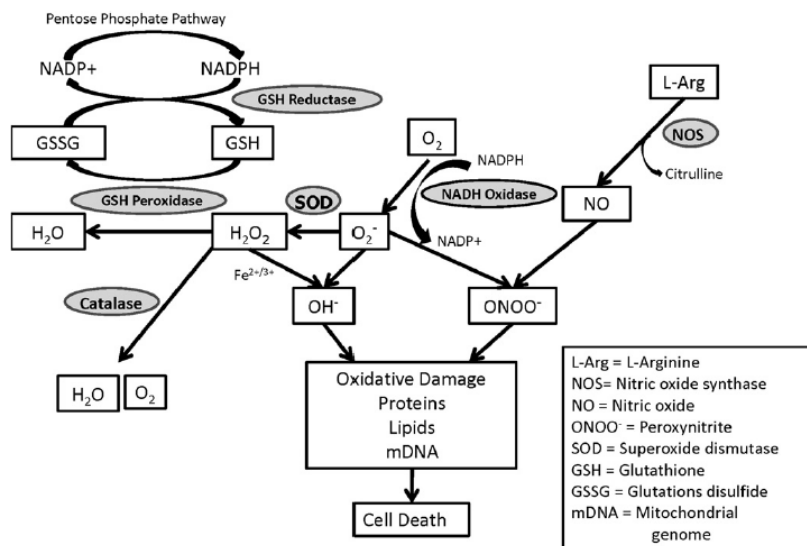
Figure 1 An abbreviated schematic of the oxidative pathway.

been investigated in the attenuation of presbycusis, including vitamin E, ascorbic acid, melatonin, folate, α-lipoic acid, L-carnitine, and rebamipide.[6-10] However, the associated results have been modest; but most previous studies targeted one or two sites within the oxidative pathway. We endeavored to create a combination antioxidant that targets multiple sites within the oxidative pathway. The objective of this study was to evaluate the potential of combination antioxidant therapy in the prevention of ARHL in an animal model as assessed by auditory brainstem response (ABR) testing. We hypothesized that ARHL may be halted or attenuated with the administration of a combination antioxidant agent.

Methods

Animal Model

C57BL/6 mice were chosen as the animal model for this study. These mice are an accepted model of age-related hearing loss. The hearing loss experienced by this strain has been well characterized and documented within the literature.[11] Onset of hearing loss occurs around five to six months of age with near-complete deafness by 18 months of age. The mice were housed within the RAR facilities at the University of Minnesota under conditions in accordance with the NIH guidelines for animal care, PHS Policy on Use of Laboratory Animals, and the Animal Welfare Act.

Combination Antioxidant Components

A total of six antioxidants were included within the combination therapy: vitamin B12, folate, ascorbic acid, NW-nitro-L-arginine methyl ester (L-NAME), L-cysteine-glutathione mixed disulfide (L-CySSG), and ribose-cysteine.

These agents together target four different sites within the oxidative pathway (Fig 2). Vitamin B12 and folate both target mitochondrial genomic deletions. Ascorbic acid is a free-radical scavenger that specifically targets reactive oxygen species. L-NAME is a nitric oxide synthase inhibitor, which was demonstrated to be protective against noise-induced hearing loss in an animal model and to decrease the production of nitric oxide within the perilymph.[12,13] L-CySSG and ribose-cysteine are both prodrugs for GSH. The antioxidant daily dosages administered were as follows: vitamin B12 0.08 mcg/g, folate 0.1 mcg/g, ascorbic acid 0.2 mg/g, L-NAME 0.25 mg/g, L-CySSG 0.2 mg/g, and ribose-cysteine 0.2 mg/g. The combination antioxidant agent was administered orally by incorporation into the mouse feed. Mice were randomized to one of three experimental groups: a control group (n = 9); an early treatment group, which received initiation of antioxidant therapy at three months of age (n = 9); and a late treatment group, which received initiation of antioxidant therapy at six months of age (n = 12). The sample size was determined based on a desired power of 0.8, a desired alpha of 0.05, and an estimated size effect delta of approximately 1.4 based on preliminary data.

Study Design

Prior to the initiation of all treatments, baseline ABRs were obtained from all of the mice. This was performed at three months of age. Figure 3 depicts a timeline of ABR analysis. Body temperature was actively maintained at 37°C by placing the animal on a thermal blanket. Subdermal needle electrodes were inserted in the standard vertex-positive, ipsilateral mastoid–negative montage and ground. ABR recordings, in response to calibrated acoustic signals, were
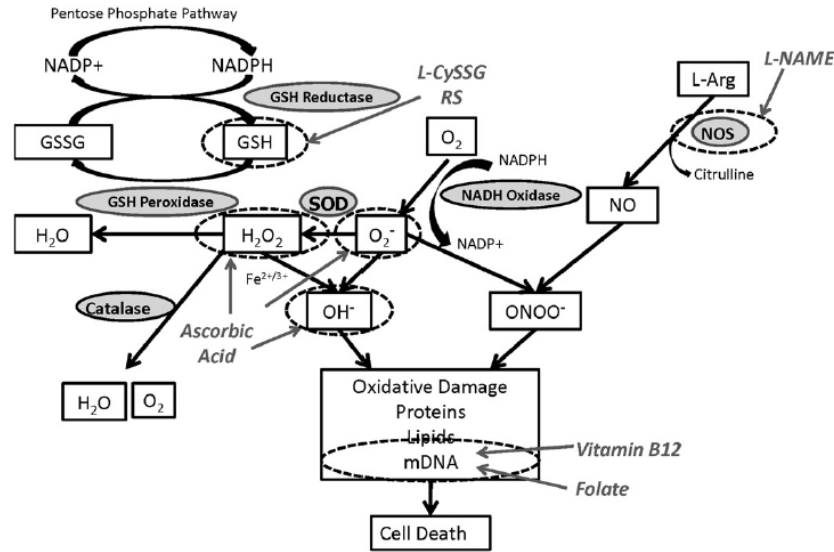
Figure 2   Specific sites of action of each component of the combination antioxidant within the oxidative pathway.

obtained in the mice to verify auditory sensitivity to tone bursts over a 4- to 32-kHz range. Total tone durations were 1 to 4 ms and consisted of Blackman rise/fall times and no plateau. The polarity of the signal was altered during a trial run to identify and reduce signal artifacts to minimize the acquisition of spurious signals. Five hundred twelve presentations were averaged for each intensity. The gain of the physiologic amplifier was set to 100,000× for ABRs. Thresholds were determined in 5-dB steps of decreasing stimulus intensity until waveforms lost reproducible morphology. ABRs were repeated every three months until the conclusion of the treatment period using this protocol. The final ABR will be obtained at the conclusion of the nine-month period. ABRs were compared by age and treatment groups for evidence of threshold shifts. Kruskal-Wallis analysis of variance (ANOVA) was performed to assess for statistically significant difference across groups. With $P <$ 0.05, post hoc Tukey method analysis was performed to confirm statistical significance.
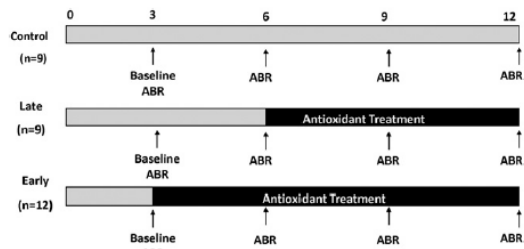
Figure 3   Timeline of auditory brainstem response testing performed within the study (age in months).

Results

Baseline ABRs were subjected to a Kruskal-Wallis ANOVA. Baseline ABR thresholds are presented in Figure 4. No statistically significant difference was found in the baseline ABR thresholds across experimental groups.

The mean values for six-month ABR threshold shifts were analyzed. At this time point, the early treatment group had received three months of antioxidant therapy. Both the control and late treatment groups had received no antioxidant therapy. The mean ABR threshold shifts for the control, early, and late treatment groups were found to be 5.6,
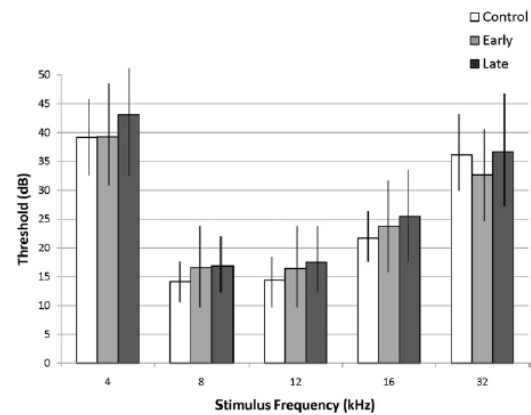
Figure 4   Baseline ABR thresholds for the experimental groups by frequency of stimulus. The *error bars* represent standard deviation. Kruskal-Wallis ANOVA of the mean thresholds across treatment groups revealed no significant difference ($P = 0.27$).

0.76, and 4.9 dB, respectively. Kruskal-Wallis ANOVA showed a significant difference between at least two of these values ($P \leq 0.004$). Post hoc Tukey method analyses were performed. This revealed a statistically significant difference in the threshold between the control versus early treatment groups as well as late versus early treatment groups. There was no statistically significant difference found between the control and late treatment groups.

The nine-month ABR data are presented in Figure 5. At this time point, the early treatment group had received six months of antioxidant therapy and the late treatment group had received three months of antioxidant therapy. The average threshold for the control group was found to be 14 to 23 dB greater than that of the early group ($P < 0.001$). Similarly, the threshold for the control group was found to be 11.7 to 17 dB greater than that of the late group ($P < 0.001$). There was no significant difference between thresholds for the early versus the late treatment groups at 4, 8, 12, and 16 kHz ($P > 0.05$). However, at 32 kHz, a statistically significant difference was noted in thresholds of the early treatment versus the late treatment groups ($P = 0.018$).

The ABR threshold shifts from baseline (3 months) to nine months were analyzed. The mean nine-month threshold shift for the control group was 23.2 dB with a 95% confidence interval (CI) of ± 1.6. The mean threshold shifts for the early treatment and late treatment groups were 4.3 dB (± 0.76, 95% CI) and 3.7 dB (± 1.3, 95% CI), respectively. A Kruskal-Wallis ANOVA with post hoc Tukey method revealed a statistically significant difference between threshold shifts for control versus early treatment groups and control versus late treatment groups ($P < 0.001$). There was no statistically significant difference between nine-month ABR threshold shift among the early treatment and the late treatment groups ($P > 0.05$).
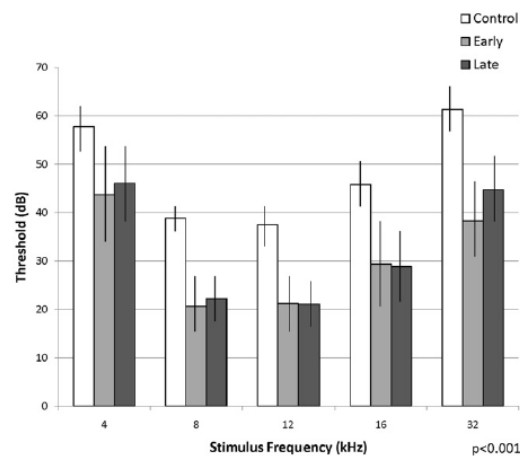
Figure 5 Auditory brainstem response thresholds for the experimental groups at nine months of age by frequency of stimulus. The *error bars* represent standard deviation.
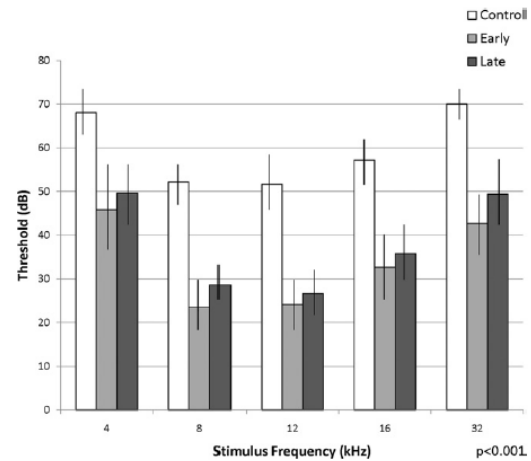
Figure 6 Auditory brainstem response thresholds for the experimental groups at 12 months of age. The *error bars* represent standard deviation.

The 12-month ABR threshold data are presented in Figure 6. At this time point, the early treatment group had received nine months of antioxidant therapy and the late treatment group had received six months of antioxidant therapy. The threshold for the control group was found to be 24.5 to 29 dB greater than that of the early treatment group ($P < 0.001$). Similarly, the threshold for the control group was found to be 18 to 24 dB greater than that of the late treatment group. The final mean threshold differences between the early and late treatment groups ranged from 2.5 to 7 dB. This difference was not found to be statistically significant at 4 kHz, 12 kHz, and 16 kHz ($P > 0.05$). However, at 8 kHz and 32 kHz, a statistically significant difference was noted in ABR thresholds of the early versus the late treatment groups (P value of 0.009 and 0.005, respectively).

The ABR threshold shifts from baseline (3 months) to 12 months were analyzed (Fig 7). The ABR threshold shift at 12 months of age for the control group was 34.7 dB with a 95% confidence interval (CI) of ± 1.6. The mean threshold shifts for the early and late treatment groups were 7.5 dB (± 0.87, 95% CI) and 9.2 dB (± 1.6, 95% CI). The threshold shift for the control group was found to be 23 to 32 dB greater than that of the early treatment group ($P < 0.001$). Similarly, the threshold shift for the control group was found to be 23 to 29 dB greater than that of the late treatment group ($P < 0.001$). The final mean threshold shift differences between the early and the late treatment groups ranged from 0.2 to 5.1 dB. However, this difference was not found to be statistically significant at 4 kHz, 12 kHz, 16 kHz, and 32 kHz ($P > 0.05$). A statistically significant difference was revealed at 8 kHz alone ($P = 0.004$).
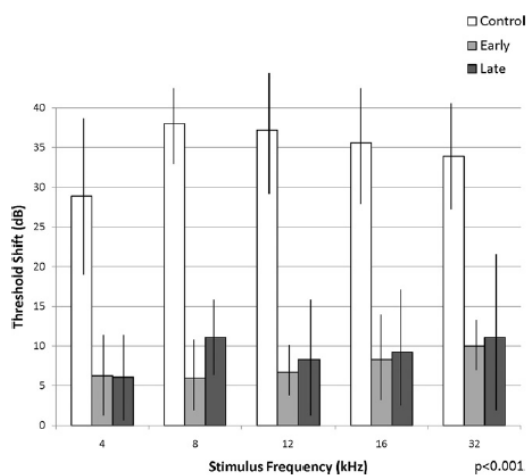
Figure 7   Auditory brainstem response threshold shifts at 12 months of age. Shown is the difference between the baseline ABR thresholds and the 12-month ABR thresholds for the experimental groups. The *error bars* represent standard deviation.

Discussion

Various antioxidant therapies for the prevention of age-related hearing loss have been tried, but with variable results. Most of these studies used an antioxidant that targets one or two sites within the oxidative pathway. The current study investigates a combination antioxidant agent composed of vitamin B12, folate, ascorbic acid, L-NAME, L-CySSG, and ribose-cysteine. Together these agents target four specific sites within the oxidative pathway: mitochondrial genomic deletions, nitric oxide synthase, reactive oxygen species, and glutathione.

Vitamin B12 and folate are both critical in DNA synthesis and repair. Level 2 evidence has revealed a correlation of both folate deficiency and vitamin B12 deficiency with age-related hearing loss.[3] However, there has been level 2 evidence refuting these findings.[4] There has also been level 1 evidence indicating that folate may prevent the progression of age-related hearing loss. A three-year randomized controlled trial of folate supplementation among 728 patients revealed a minimal but statistically significant reduction in the degree of age-related hearing loss in the treatment group as compared to the control.[8]

Ascorbic acid is a free-radical scavenger that acts to reduce reactive oxygen species. Level 1 evidence of ascorbic acid supplementation in Fischer 344 rats revealed an attenuation of age related hearing loss of 10 to 25 dB over the course of 22.5 months of treatment.[6] Similarly, clinical trials have revealed an approximate 5-dB attenuation of hearing loss at low frequencies with the administration of ascorbic acid and rebamipide over eight to 52 weeks.[14] With the addition of lipoid acid, the hearing result was improved at both low and high frequencies.[15]

L-NAME is a nitric oxide synthase (NOS) inhibitor. Within the cochlea, nitric oxide is produced by iNOS. NOS inhibitors, such as L-NAME, may slow or prevent the production of nitric oxide within the cochlea and its subsequent injury. L-NAME has been reported to block vestibular hair cell degeneration and has also been demonstrated to prevent the production of nitric oxide within the perilymph of guinea pigs.[13,16] Thus, L-NAME could potentially prevent the oxidative damage created by the presence of nitric oxide in the cochlea in ARHL.

GSH, an endogenous cellular antioxidant present in the cochlea, is a tripeptide consisting of L-cysteine (Cys), L-glutamic acid (Glu), and glycine (Gly). It is biosynthesized in two steps, catalyzed by the enzymes gamma-glutamyl-cysteine synthetase and glutathione synthetase. L-CySSG and ribose cysteine are both GSH prodrugs. L-CySSG is a mixed disulfide of Cys and GSH that has been found to be highly effective in protecting the liver from hepatotoxicity in a mouse model of oxidative stress induced by reactive oxygen species.[17] Ribose-cysteine is a Cys prodrug that in turn is critical in the biosynthesis of GSH. It has been demonstrated to reduce hepatic and renal toxicity induced by reactive oxygen species.[18,19] Ribose-cysteine has not previously been investigated in the management of ARHL.

This current study presents data from the administration of a combination agent composed of each of the aforementioned antioxidant agents. The rationale for creating a combination agent was to target multiple sites within the oxidative pathway to retard or prevent oxidative injury. The data demonstrate that the administration of this combination antioxidant agent did attenuate the presentation of ARHL within the animal model, C57BL/6 mice. The evidence of attenuation was present after as early as six months in the early treatment group as compared to the control group. However, on review of our most remote data point, it does not appear that there is a significant benefit in initiating antioxidant therapy before the onset of hearing loss. There was no significant difference in the hearing thresholds of the early and late treatment groups at the 12-month assessment point at most frequencies.

Administration of a combination antioxidant targeted at four sites within the oxidative pathway attenuated the presentation of age-related hearing loss in C57BL/6 mice. Further study is necessary to elucidate the optimal age of initiation, antioxidant combination, and dosing. However, these findings are promising for the possibility of using a combination drug therapy in the treatment and prevention of ARHL.

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Disclosures

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Appendix C

A variable amount of data is available regarding effective dose, tolerable upper intake level (UL), and toxicity for the antioxidants including within the combination antioxidant. The antioxidants which have been studied most extensively and have the greatest degree of available data include folate, vitamin B12 and vitamin C. In fact, these three antioxidant have established reference daily intake (RDI) or daily values (DV) for recommended intake (folate 400 mcg, vitamin C 60 mg, and vitamin B12 6 mcg) (Food and Drug Administration, 2015). However, the tolerable upper intake level for each of these antioxidants is significantly higher. Less extensive studies have been conducted to evaluate these characteristic for L-NAME, ribose cysteine and L-CySSG. A synopsis of the currently available data is presented within this appendix.

Vitamin C

According to the Institute of Medicine Dietary *Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids* (2000), the daily intake of vitamin C required to provide antioxidant protection is 90 mg/day for adult men and 75 mg/day for adult women based on intake necessary to maintain near-maximal neutrophil concentration with minimal urinary excretion of ascorbic acid. For smokers, the required daily intake of vitamin C is increased by 35 mg/day. Ascorbic acid or vitamin C functions as a potent antioxidant scavenger of reactive

oxygen species, but also functions as an antioxidant for some reactive nitrogen species, in collagen synthesis, as well as in synthesis and modulation of hormonal components of the nervous system (Institute of Medicine, 2000). The positive effect range for vitamin C reportedly ranges from 50 to 2,000 mg/day.

Reviews of high vitamin C intake indicates low toxicity (Johnston, 1999). The tolerable upper intake level (UL) for vitamin C in adults is set at 2 g per day with associated adverse effects of osmotic diarrhea and gastrointestinal disturbance noted above this level (Kallner et al., 1979). Typically, adverse effects begin to present with intakes of greater than 3 g/day. This is likely due to limitations of saturable intestinal absorption and renal tubular resorption; thus overload of ascorbic acid is unlikely in humans (Blanchard et al, 1997; Levine et al., 1996). The most common adverse effects noted with high doses of vitamin C are nausea, abdominal cramps, and diarrhea (Hoffer, 1971). However, additional potential adverse effects include increased oxalate excretion, renal stones, increased uric acid excretion, pro-oxidant effects, systemic conditioning ("rebound scurvy"), increased iron absorption leading to iron overload, reduced vitamin B12 and copper status, increased oxygen demand, and erosion of dental enamel (Herbert & Jacob, 1974; Hornig & Moser, 1981; McLaran et al., 1982; Rivers, 1987; Levine et al., 1996a; Wandzilak et al. 1994; Institute of Medicine, 2000).

Folate

According to the Institute of Medicine Dietary *Dietary Reference Intake for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline* (1998), the recommended daily allowance of folate is 400 mcg/day. Folate functions in deoxyribonucleic acid (DNA) synthesis (which depends on a folate coenzyme for pyrimidine nucleotide biosynthesis), purine synthesis, generation of formate in the formate pool, and amino acid interconversion. The bioavailability of folic acid is 85 to 100% for oral consumption (Gregory, 1997; Pfeiffer et al., 1997). The UL for folate is 1,000 mcg/day (Institute of Medicine, 1998). Various adverse effects are noted in association with high doses of folic acid including masking vitamin B12 deficiency and neuropathy in individuals with B12-deficiency (Chodos & Ross, 1951; Spies et al, 1948; Wagley, 1948; Institute of Medicine, 1998). Additionally, mental status changes, sleep disturbance, gastrointestinal effects have been reported (Hunter et al., 1970). However, studies using comparable higher doses failed to confirm the previously stated adverse effects (Gibberd et al., 1970; Hellstrom, 1971; Richens, 1971; Sheehy 1973; Suarez et al. 1947). In fact, extensive studies folate supplementation in females of reproductive age for prevention of neural tube defects have revealed folate to be safe at high doses in a cohort of 100 females over 7 to 10 years (Holmes-Siedle et al. 1992). These findings have been confirmed with two extensive large scale randomized controlled trials of folate supplementation in 4,753 and 5,502 women respectively with no adverse effect (Czeizel and Dudas, 1992; Czeizel et al.,1994).

Vitamin B12

According to the Institute of Medicine Dietary *Dietary Reference Intake for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline* (1998), the recommended daily allowance of vitamin B12 is 2.4 mcg/day. However, there is insufficient evidence to set a UL because there is no evidence that excess B12 intake causes adverse effect. "No adverse effects have been associated with excess B12 intake from food or supplements in healthy individuals" (Institute of Medicine, 1998). There is risk for patients with Leber optic atrophy. Vitamin B12 administration in patients with Leber optic atrophy increases the risk of irreversible neurologic damage from the optic atrophy due to a reduced ability to detoxify the cyanide intoxication (present in tobacco smoke, alcohol, and some plants) (Foulds 1968, Foulds 1969, Foulds 1970, Wilson and Matthews, 1966). Vitamin B12 is converted to one of two cobalamine coenzymes that are active in human metabolism: methylcobalamin and 5-deoxyadenosylcobalamin. Within the United States, Vitamin B12 refers to cyanocobalamin. The derivatives of vitamin B12 function as cofactor for methionine synthase which requires methylcobalamin and L-methylmalonyl-CoA mutase which also requires adenosylcobalamine.

L-NAME

The dosage range for L-NAME is reportedly 2 to 780 mg/kg (Wyse et al., 2001; Green et al., 1997). Most of the studies of L-NAME have been conductive using animal models. There is limited clinical data regarding its use. There is no

significant toxicity data available. L-NAME has been noted in animal models to attenuate noise induced hearing loss (Diao et al, 2007; Nagashima et al, 2010; Rhee, 2003).

Ribose-cysteine

There are minimal studies within the literature evaluating the toxicity of ribose-cysteine. Ribose-cysteine has been noted to be protective against acetaminophen induced hepatotoxicity and acetaminophen induced renal toxicity (Roberts et al., 1992; Lucas et al., 2000 Slitt et al., 2005). Ribose-cysteine has been purported to be effective in the inhibition of astrocytoma cell proliferation and effective in the treatment of colitis (Jurkowska et al., 2011; Oz et al., 2007). Ribose-cysteine has been reported to be effective within *in vitro* studies in the reduction of the prevalence of radiation-induced mutation and persistent chromosomal instability (Waldren et al., 2004). Within animal studies (swine), ribose-cysteine has been reported to reduce anastomotic leaks and death following rectosigmoid resection and radiation 3 weeks post-operatively (Rowe et al., 1993). In each of these studies no adverse effects or toxicities have been reported. A dosage ranging of approximately 140 to 3,000 mg/kg has been reported in the literature (Kader et al., 2014; Lucas et al., 2000).

L-CySSG

The literature regarding L-CySSG is scarce regarding toxicity. L-CySS G has been reported to be effective in the treatment of colitis (Oz et al., 2007).

Preliminary data reveals a trend toward attenuation of age related threshold

shifts in Fisher 344 rats. L-CySSG has also been found to be protective against

acetaminophen-induced hepatotoxicity (Berkeley et al., 2003).