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"Study of the Molecular Mechanisms of Age-Related Hearing Loss and the Impact of Hearing Aids on Cognitive Decline in Aging"

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*"**Non scholae sed vitae discimus**"*

Seneca, Epistulae Morales ad Lucilium c. 65 AD

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ABSTRACT

Hearing loss represents one of the most prevalent sensory impairments worldwide and is a major determinant of reduced quality of life in older adults. Among its various forms, age-related hearing loss (ARHL) is particularly clinically relevant due to its strong association with social isolation, cognitive decline, and neurodegeneration. Despite its clinical relevance, the molecular mechanisms underlying ARHL remain only partially understood.

This thesis explores oxidative and inflammatory processes contributing to cochlear and neural degeneration using complementary experimental models of ototoxicity (cisplatin and styrene exposure) and physiological aging in C57BL/6 mice, together with a clinical study assessing cognitive and emotional outcomes in elderly patients following hearing aid rehabilitation.

In experimental rat model, cisplatin administration caused dose-dependent auditory threshold elevation, degeneration of spiral ganglion neurons (SGNs) and afferent fibers, and reduced activation of the TrkB/AKT neurotrophic pathway, accompanied by increased $p75^{NTR}$ and cleaved caspase-3 expression. Styrene exposure induced marked oxidative stress and NLRP3 inflammasome activation, leading to elevated IL-1β and NF-κB signaling. Both the antioxidant rosmarinic acid and the anti-inflammatory anakinra attenuated cochlear injury, with anakinra providing earlier and stronger functional protection. In C57BL/6 mice, progressive high-frequency hearing loss at six months was associated with SGN loss and increased cochlear oxidative and inflammatory markers, indicating convergence of redox and immune mechanisms during auditory aging.

A clinical study on elderly new hearing-aid users (aged 60–75 years) showed modest improvements in cognitive performance (MMSE, MoCA) and reduced anxiety (HADS-A) after three months, suggesting short-term cognitive and emotional benefits of hearing rehabilitation.

Collectively, these results identify oxidative stress and inflammatory signaling as

convergent drivers of cochlear degeneration. Interventions that restore redox homeostasis and suppress inflammasome activation, combined with auditory rehabilitation, may provide a multifaceted approach to counteracting age-related hearing decline and its neurological consequences.

INTRODUCTION

1. Mechanisms of cochlear damage induced by ototoxic agents

The inner ear hearing organ, the cochlea, is a specialized neurosensory apparatus buried deep within the temporal bone. The cochlea is essential to our ability to hear. Cochlear function is mediated by a complex cellular architecture that composes the organ of Corti, located within the *scala media.* Two distinct types of sensory cells can be identified within the organ of Corti: inner and outer hair cells (IHCs, OHCs). The hair cells (HCs) form synapses at their basal region that connect with the axons of the spiral ganglion neurons (SGNs) of the auditory nerve, whose cell bodies are located in the spiral ganglion within the modiolus. The proper functioning of these sensory cells depends on the maintenance of the endocochlear potential of the endolymph fluid within the scala media, which is maintained by the *stria vascularis (Ashmore, 2008; Hudspeth, 2014)* (Figure 1).

The auditory sensory epithelium is particularly vulnerable to damage that results in cell death due to the lack of regenerative capacity of the HCs (Wang et al., 2025). "Sensorineural Hearing Loss" (SNHL), the most common form of permanent hearing loss, refers to the impairment of the sensory or neuronal components of the auditory system. Crucially, SNHL is often the result of damaged and degenerating cochlear HCs. Following the degeneration of the HCs, the SGNs also degenerate, ultimately resulting in neural death over a timeframe ranging from weeks to months following the death of HCs (Bohne and Harding, 2000; Zilberstein et al., 2012). Also, recent evidence shows that cochlear synaptopathy, the loss of inner hair cell synapses, may be widespread in ears with otherwise intact HC populations and normal audiograms, a condition referred to as "Hidden hearing loss" (Kujawa and Liberman, 2009; Schaette and McAlpine, 2011).

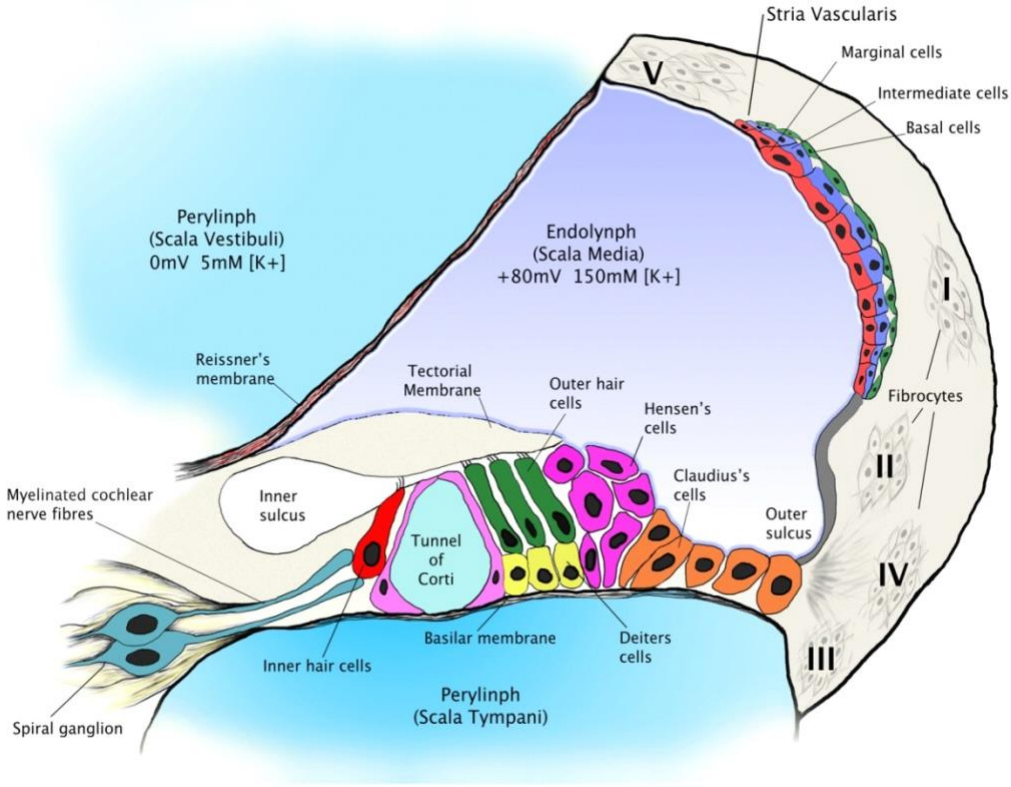

Figure 1. Graphical illustration of the cochlea. Representative cross-sectional illustration of the cochlea depicting the three distinct scalae (scala tympani, scala vestibuli, and scala media), the organ of Corti with inner hair cells (IHCs) and outer hair cells (OHCs), the *stria vascularis*, and the neurons of the spiral ganglion (Fetoni et al., 2011).

Our research group is committed to investigating the various causes of damage to the sensory cells and the neural components of the cochlea that lead to SNHL. The studies conducted in our laboratory aim to identify the molecular mechanisms involved, which may arise from exposure to environmental factors. Noise represents a serious occupational hazard and a significant environmental pollutant due to industrialization (Hong et al., 2013). Additionally, lifestyle choices such as the use of earphones to listen to loud music have increased the risk of SNHL specifically in young adults (Ishida et al., 2025; Khoza-Shangase and Mokhethi, 2025; Reijers et al., 2025). Early exposure to loud noise leads to noise-induced hearing loss (NIHL) associated with oxidative

stress and *stria vascularis* dysfunction (Fetoni et al., 2022b). These molecular alterations include increased production of reactive oxygen species (ROS), lipid peroxidation, dysregulation of endogenous antioxidant responses, and elevated expression of hypoxia-inducible factor 1- alpha (HIF-1α) and vascular endothelial growth factor C (VEGFC). These mechanisms appear to exacerbate the pathological condition compared with what is typically observed in ARHL (Fetoni et al., 2011; Kujawa and Liberman, 2006). The detrimental effects of noise exposure extend beyond the peripheral auditory system and involve cognitive processes. Early noise exposure has been shown to accelerate the presbycusis phenotype and to induce hippocampal-dependent alterations (Paciello et al., 2023).

SNHL can arise from exposure to certain pharmacological agents commonly used in clinical settings, such as aminoglycoside antibiotics and platinum-based chemotherapeutic drugs, as well as from various environmental and occupational factors, including exposure to heavy metals and organic solvents, such as styrene (Fetoni et al., 2021; Fetoni et al., 2016; Kros and Steyger, 2019). SNHL can be triggered by certain common drug compounds as a side-effect; these compounds can therefore be referred to as “ototoxic compounds”, or “ototoxic drugs”. Ototoxic drugs, such as cisplatin and gentamycin, have been found to damage the cell populations of the organ of Corti, leading to partial or complete loss of OHCs, sporadic loss of IHCs, and degeneration of SGNs (Anniko and Sobin, 1986; Fetoni et al., 2004), leading to hearing loss.

In previous studies, gentamycin and cisplatin were investigated for their ototoxic effects, given their extensive clinical use and their distinct yet partially overlapping mechanisms of toxicity (Fetoni et al., 2014; Fetoni et al., 2012; Fetoni et al., 2004). Both agents can induce cochlear damage through oxidative stress, inflammation, and programmed cell death, ultimately leading to irreversible loss of HCs and auditory neurons (Chung et al., 2008; Clerici et al., 1996; Garcia-Berrocal et al., 2007; Hirose et al., 1997; Kim et al., 2010; Murphy and Daniel, 2011; So et al., 2007). More recently,

attention has been directed toward elucidating the effects and molecular pathways underlying styrene-induced ototoxicity, with a particular focus on oxidative stress and inflammatory processes in the cochlea (Fetoni et al., 2021; Fetoni et al., 2016; Paciello et al., 2024).

Despite extensive research, effective therapies for hearing loss are still lacking. A deeper comprehension of the molecular and cellular mechanisms underlying cochlear vulnerability is therefore critical to guide future preventive and therapeutic strategies.

1.1. Cisplatin-induced cochlear damage

Cisplatin, or *cis*-diammine-dichloro-platinum (II), is a platinum-based chemotherapeutic agent widely used against various solid tumors, including cervical cancer, lung cancer, and ovarian and testicular cancers (Chattaraj et al., 2023; Liang et al., 2025; Volarevic et al., 2019). Cisplatin's efficacy is attributed to its ability to bind to the nitrogen at position 7 of the purine ring in DNA, thereby forming DNA-platinum cross-links that lead to the arrest of both transcriptional and replicative processes. In addition, cisplatin can also target mitochondria, inducing oxidative stress, uncoupling oxidative phosphorylation, and activating various signal pathways to kill tumor cells (Tchounwou et al., 2021). Despite its efficacy as an antitumoral drug, it frequently causes bilateral, progressive, and irreversible SNHL. A 2022 meta-analysis reported the prevalence of ototoxic hearing loss associated with cisplatin to be at 43% in patients undergoing chemotherapy and identified cisplatin-induced ototoxicity as a global health burden (Dillard et al., 2022).

Cisplatin preferentially accumulates in the OHCs, *stria vascularis*, and the SGNs through specific transporters and ion channels. Once inside the cells, cisplatin can persist for months and even years, contributing to both acute and delayed toxicity (Breglio et al., 2017).

Studies suggest the involvement of the organic cation transporter 2 (OCT2) and the copper transporter 1 (Ctr1) for the passage of cisplatin from the systemic circulation across the *stria vascularis* and into the endolymph (Ciarimboli et al., 2010; More et al., 2010). However, it cannot be excluded that cisplatin may reach the cochlea by permeating through the basolateral membrane (Hellberg et al., 2013; Hellberg et al., 2009). Within the *scala media*, cisplatin can damage HCs either through transporter-mediated uptake or through indirect mechanisms. Evidence suggests that the major influx of cisplatin into HCs occurs via the Ctr1 and copper pumps ATP7A and ATP7B, which are responsible for cisplatin sequestration and efflux in HCs (Ding et al., 2011).

Cisplatin-induced ototoxicity may also arise, at least in part, from the disruption of cochlear homeostasis, in addition to its direct toxic effects on HCs. The resulting imbalance can indirectly compromise HC survival. (Liu et al., 2016). Early signs of cisplatin-induced ototoxicity, resulting from cumulative chemotherapy exposure, include a progressive deterioration of hearing thresholds, as observed both in patients and in animal models (Dreschler et al., 1985; Pisani et al., 2025; Van Der Biest et al., 2025).

Cisplatin induces ototoxic damage through two main mechanisms. Its primary mechanism involves DNA binding, which halts replication and triggers cell death. In parallel, cisplatin can impair mitochondrial function, promoting the generation of reactive oxygen species (ROS), oxidative stress, and inflammation, processes that ultimately lead to apoptosis (Berndtsson et al., 2007; Gentilin et al., 2019). The cytotoxic mechanisms underlying cisplatin-induced damage in the organ of Corti are complex and not yet fully understood. Among the best-characterized pathways is the p53-dependent apoptotic cascade activated by DNA damage. When genomic insult occurs, the sensor kinase, Ataxia telangiectasia Mutated (ATM), is activated and initiates a signaling cascade that results in the activation of the tumor suppressor p53. Activated p53 upregulates the pro-apoptotic protein Bax (Bcl-2-associated X protein), which promotes mitochondrial cytochrome c release and subsequent caspase-3 activation, leading to programmed cell death (Wang et al., 2004).

Cisplatin induces the generation of reactive oxygen species (ROS) through several mechanisms. A key pathway involves the activation of nicotinamide adenine dinucleotide phosphate oxidase 3 (NOX3), an isoform highly expressed in the cochlea. NOX3 upregulation in supporting cells (SCs) and OHCs has been shown to play a central role in the onset of SNHL following cisplatin treatment (Banfi et al., 2004; Mohri et al., 2021). In addition, cisplatin promotes the overproduction of oxidases and depletes endogenous antioxidant defenses, further enhancing oxidative stress and contributing to its ototoxic effects (Pisani et al., 2025; Wang et al., 2023).

A large amount of evidence suggest that the cochlea can mount an inflammatory response as a result of ototoxic insults. Various transcriptional factors have been linked to cisplatin-mediated inflammation in the organ of Corti, spiral ligament and *stria vascularis*; particularly NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) is involved in pro-inflammatory release of cytokines including TNF-α (Tumor necrosis factor-alpha) , IL-1β (Interleukin-1 beta) , and IL-6 (Interleukin-6) (Chung et al., 2008; So et al., 2007). The JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway is a signaling pathway that plays a critical role in the inflammation processes in cisplatin-induced ototoxicity (Kaur et al., 2011). Under cellular stress, the transcriptional factor STAT1 becomes phosphorylated at tyrosine 701 (Tyr^{701}) and serine 727 (Ser^{727}), which promotes its translocation to the nucleus. Once in the nucleus, STAT1 drives transcription of genes involved in inflammation such as inducible nitric oxide (iNOS) and cyclooxygenase-2 (COX-2) (Fetoni et al., 2015; Rosati et al., 2019). Although the oxidative stress and inflammation-related pathways are implicated in cisplatin ototoxicity, these mechanisms are not exclusive to pharmacologically induced damage. Many of the same molecular pathways are also activated during age-related processes (Bielefeld, 2013; Skalleberg et al., 2020; Tan and Song, 2023; Yuan et al., 2024). Moreover, cisplatin-induced molecular pathways may exacerbate hearing loss in individuals with a genetic predisposition to auditory dysfunction (Lavinsky et al., 2015; Ninoyu and Friedman, 2024).

The mechanisms discussed above represent only a fraction of the complex signaling networks involved in cochlear cell injury, many of which remain poorly understood. Continued research is therefore essential not only to clarify how cisplatin triggers cochlear damage, but also to better understand the interplay between drug-induced, genetic, and age-related factors contributing to hearing loss. From a therapeutic perspective, the identification and development of pharmacological interventions capable of mitigating cisplatin-induced ototoxicity remain a major challenge and a key goal for future studies. At present, clinical interventions are largely limited to prosthetic

devices, such as hearing aids, which offer only partial benefit due to the irreversible structural damage to the cochlea. Recently, however, The U.S. Food and Drug Administration (FDA) approved the first pharmacological agent, sodium thiosulfate; unfortunately, its use is currently restricted to pediatric patients with non-metastatic solid tumors. Therefore, a deeper understanding of the molecular mechanisms underlying cisplatin-induced ototoxicity is essential to identify new therapeutic strategies applicable to broader patient populations (Fetoni et al., 2014; Tan and Song, 2023; Tan and Vlajkovic, 2023).

Specifically, the effect of cisplatin on the neural structures of the cochlea is poorly understood. For this reason, we investigated the effects of cisplatin on the neural structures (afferent neurites and SGNs) and the underlying molecular mechanisms of apoptosis in the cochlea under cisplatin exposure in a rat model. This study is presented in Chapter 1 of this thesis (pg. 34-45)

1.2. Styrene-induced cochlear damage

Hearing loss is one of the most common occupational diseases, and growing scientific evidence indicates that exposure to certain industrial solvents increases the risk of SNHL (Beaver and Schneider, 2023; Hormozi et al., 2017; Tihanyi et al., 2025; Vyskocil et al., 2012). Among these solvents, styrene, widely used in the production of plastics, fiberglass, and synthetic rubber (Papaleo et al., 2011), has been identified as a major ototoxic compound. During industrial processing at high temperatures, styrene vapors can be released and inhaled, entering systemic circulation and accumulating in various organs (Miller et al., 1994). Scientific studies have demonstrated the toxicity of styrene in various organs as well as its potential carcinogenicity (Brodkin et al., 2001; Volarevic et al., 2019). Its toxicity and possible carcinogenicity have been recognized by the International Agency for Research on Cancer (IARC) (Giampaoli et al., 2024). The inner ear is also a focus of the investigation into styrene's toxic effects, because hearing loss is observed in workers exposed to high levels of styrene. Numerous clinical studies have been conducted to investigate how styrene interacts synergistically with noise exposure in contributing to hearing loss, given that these two factors commonly coexist in occupational settings (Johnson et al., 2006; Morata et al., 2011; Muijser et al., 1988; Sisto et al., 2011; Sliwinska-Kowalska et al., 2003).

Occupational exposure to styrene is assessed by measuring its urinary metabolites. Mandelic acid (MA) and phenylglyoxylic acid (PGA) are the biological markers used to detect styrene uptake in an exposed worker (Morata et al., 2002; Morioka et al., 1999; Wigaeus et al., 1984). To evaluate the effects of styrene specifically on cochlear physiology and functionality, audiological tests, including pure tone audiometry (PTA) and distortion product otoacoustic emissions (DPOAEs), have been conducted on workers exposed to styrene (Johnson, 2007; Sisto et al., 2016). Findings reported in the literature indicate that workers exposed to styrene exhibit elevated auditory thresholds

in the high-frequency hearing range compared to control groups. Also, in many cases, workers present signs of insomnia and depressive symptoms (Johnson et al., 2006; Sliwinska-Kowalska et al., 2020; Werder et al., 2018; Zhu et al., 2025).

Research effort has also been directed toward understanding which biological processes within the inner ear are altered following styrene exposure and how these alterations contribute to hearing loss. Of specific interest is the possible involvement of ROS and the assessment of oxidative stress biomarkers in exposed workers. Interestingly, elevated oxidative stress biomarkers have been reported in association with DPOAE alterations, suggesting a link between cochlear dysfunction and oxidative stress in styrene-exposed workers (Sisto et al., 2016). In humans, the repair of oxidatively damaged DNA and RNA generates extracellular oxidized guanine derivatives, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) and 8-oxo-7,8-dihydroguanine (8-oxoGua), which are excreted in urine and serve as reliable indicators of oxidative damage. The levels of 8-oxodGuo and 8-oxoGua are not significantly influenced by diet or normal cell turnover, making them a promising biomarker of oxidative stress associated with chemical exposure, including styrene (Pigini et al., 2023; Pilger and Rudiger, 2006; Valavanidis et al., 2009).

Molecular biology studies in the auditory organ performed using animal models of styrene exposure have demonstrated that hearing loss is associated with the activation of apoptotic pathways, involving both caspase-9 and caspase-8, particularly in OHCs (Chen et al., 2007; Makitie et al., 2002). Subsequently, further studies have shown that styrene induces oxidative stress in the cochlea that leads to cell death. Detection of oxidative stress has been assessed in OHCs and SGNs (Fetoni et al., 2016). Further, a strong interplay between oxidative stress and inflammation has been observed (Fetoni et al., 2019). Experimental data show that styrene exposure triggers inflammatory responses in the cochlea (Fetoni et al., 2021). This study reported increased expression of inflammatory markers such as NF-κB, TNF-α, and IL-1β, along with a reduction in the anti-inflammatory cytokine IL-10. In the same study, synapse loss in the inner hair

cells (cochlear synaptopathy) and elevated hearing thresholds were observed following styrene exposure, indicating that inflammation contributes to cochlear dysfunction.

Alongside peripheral damage in the auditory system, detrimental effects have also been reported in the central auditory system (Muly et al., 2004). Altered basal synaptic transmission and decreased dendritic spine density in the auditory cortex (ACx) have been observed, suggesting that the increase of ROS and inflammatory mediators may drive pathological alterations in both the peripheral auditory system and the central auditory system (Paciello et al., 2024).

In conclusion, these findings identify styrene as an occupational ototoxic agent that acts synergistically with noise to damage the auditory system. Oxidative stress and inflammation appear to be key mediators of this process, and combined antioxidant and anti-inflammatory approaches may represent promising therapeutic strategies for preventing styrene-induced hearing loss (Fetoni et al., 2008; Fetoni et al., 2016; Pisani et al., 2023).

However, the precise molecular interactions between oxidative and inflammatory pathways remain unclear, underscoring the need for further research to clarify these mechanisms and their relevance to other forms of SNHL, such as ARHL (Jiang et al., 2007; Shi et al., 2017).

For these purposes, we investigated whether the NLRP3 (NLR family pyrin domain containing 3) inflammasome mediates the interplay between oxidative stress and inflammation in styrene-induced cochlear damage, and we assessed the protective effects of an antioxidant (rosmarinic acid, RA) and an anti-inflammatory (anakinra, ANA) treatment. This study is presented in Chapter 2 (pg. 46-66).

2. Age-Related Hearing Loss: From Sensory Aging to Cognitive Decline

ARHL, also referred to as presbycusis, is a progressive, bilateral, and symmetrical sensorineural deficit influenced by both environmental and genetic contributors (Fischer et al., 2016). According to the most recent findings from the "Global Burden of Diseases, Injuries, and Risk Factors Study", among 371 evaluated diseases, ARHL ranks as the fourth leading cause of years lived with disability (Ferrari and Collaborators, 2024). In addition to its high prevalence, hearing loss has been linked to cognitive decline in the elderly (Loughrey et al., 2018; Readman et al., 2025; Thomson et al., 2017). Interestingly, this association is particularly evident in individuals with hearing loss who do not use hearing aids, as they exhibit higher rates of cognitive decline and a greater incidence of dementia compared with hearing aid users (Yeo et al., 2023). A relationship has also been observed between auditory impairment and dementia: for every 10 dB reduction in hearing ability, there is an increased risk of dementia, as observed in cognitive assessment studies showing an independent correlation between hearing loss and cognitive decline (Deal et al., 2017; Lin et al., 2011). According to *The Lancet Commission on Dementia Prevention, Intervention, and Care,* hearing loss is one of 14 modifiable risk factors for dementia across the lifespan. The population attributable fraction (PAF) for hearing loss alone accounts for approximately 7% of dementia cases, suggesting that timely identification and correction of hearing impairment could potentially prevent dementia in a significant portion of the population (Livingston et al., 2024). Moreover, data from the *World Health Organization* (WHO), indicate that unaddressed hearing loss carriers an annual global cost of almost US$ 1 trillion.

Understanding the relationship between hearing loss and dementia is crucial for public health, as it can guide preventive strategies and help reduce the global economic burden, with dementia alone costing an estimated US$ 1.3 trillion annually. The

association between hearing loss and cognitive decline has been established through longitudinal clinical studies (Deal et al., 2017; Fritze et al., 2016; Gallacher et al., 2012; Lin et al., 2011). These investigations, which assess hearing and cognitive function across diverse populations through repeated auditory evaluations and extended follow-up periods, suggest that elevated pure-tone audiometry (PTA) thresholds may be a risk factor for cognitive decline. Participants also underwent comprehensive cognitive testing across multiple domains, including frontal (reasoning, planning and executive control), temporal (memory and language), parietal (attention, numerical, and visuospatial abilities), and limbic (emotional regulation) functions (Gopinath et al., 2009; Lawrence et al., 2020). Moreover, hearing loss has been associated with a higher risk of social isolation, which can further accelerate cognitive decline and increase dementia susceptibility (Tomida et al., 2024; Yang and Jiang, 2024).

The pathophysiological relationship between ARHL and dementia remains incompletely understood; however, multiple explanatory models have been proposed. Recent review by Uchida and collaborators (Uchida et al., 2019) outlines four main hypotheses (the cognitive load, common cause, cascade and overdiagnosis hypotheses), describing how hearing loss may contribute to cognitive decline. Among these, the cascade hypothesis suggests that cochlear damage leads to cognitive deterioration, by negatively affecting brain structures through reduced sensory input. Human studies have shown that ARHL is associated with brain atrophy, especially in the auditory cortex (Lin et al., 2014). In older adults, the reduced auditory input alters the neural activation in response to speech. This sensory decline also affects brain regions involved in higher-level cognitive functions that are critical for speech comprehension, such as working memory and processing speed (Peelle et al., 2011).

Reduced auditory input decreases the activity of neural pathways, leading to their functional decline. This phenomenon reflects the principle of neuroplasticity, the capability of the brain to reorganize in response to environmental stimuli and learning. According to the “use it or lose it” model, limited engagement of auditory processing

can accelerate deterioration of listening and other auditory-related abilities, like speech comprehension, social awareness and spatial perception (Kleim and Jones, 2008). Consistent with this model, evidence from animal studies indicates that cochlear impairment results in synaptic and neurophysiological degradation in the brainstem and hippocampus (Hockley et al., 2023; Paciello et al., 2023; Park et al., 2018; Xie, 2016; Yuan et al., 2014).

Collectively, these findings indicate that peripheral hearing loss can contribute to central neurodegeneration and cognitive decline. Consequently, it is crucial to evaluate strategies to prevent neurodegeneration in patients already affected by hearing loss. However, hearing loss manifests heterogeneously in the population. ARHL is a multifactorial condition ranging from mild to severe, reflecting the cumulative effects of lifelong insults to the auditory system (Gates and Mills, 2005). The development of ARHL is influenced by multiple factors, including noise exposure, ototoxic agents, trauma, vascular and metabolic alterations, hormonal changes, diet, and immune activity, all superimposed on genetically driven aging processes (Bielefeld et al., 2010; He et al., 2019). Currently, there is no pharmacological treatment for ARHL, which, unlike conductive hearing loss, is irreversible. While hearing aids and cochlear implants can partially compensate for auditory deficits, they do not restore natural hearing; however, regular hearing aid use may help preserve cognitive function and improve long-term outcomes.

2.1. Age-related degenerative changes in the cochlea

In presbycusis, the cochlea degeneration involves the *stria vascularis*, HCs, primary afferent neurons, and the auditory nerve. Based on histological studies, Schuknecht categorized presbycusis into distinct types according to the predominant pattern of tissue degeneration (Schuknecht, 1994; Schuknecht and Gacek, 1993).

Sensory presbycusis is characterized by age-related degeneration of HCs, primarily affecting the basal region of the cochlea, which is responsible for high-frequency sound. Ultrastructural changes begin with stereocilia disorganization, followed by progressive OHC loss that advances from base to apex, while IHC loss occurs later and to a lesser extent. This degeneration is accompanied by loss of supporting cells, and gradual degeneration of cochlear dendrites and SGNs.

Neural presbycusis is characterized by a progressive loss of SGNs through life, beginning early and advancing with age. Despite an intact sensory epithelium, this neuronal loss leads to reduced speech discrimination, while pure-tone hearing thresholds remain relatively stable. The degeneration is diffuse along the cochlea, with only slightly greater severity at the basal turn.

Strial presbycusis refers to age-related hearing loss primarily caused by atrophy of the *stria vascularis*, a degenerative process that may have a genetic component, as it often affects multiple close family members. Clinically, it is characterized by a flat or mildly sloping audiometric configuration with preserved speech discrimination, indicating intact auditory processing within the residual sensitivity range. Histopathological findings show degeneration of the *stria vascularis*, particularly in the middle and apical turns of the cochlea. The marginal cells are the most severely affected and, in severe cases, only a single basal cell layer persists. Functionally, loss of strial cells disrupts endolymph homeostasis and the energy metabolism essential for cochlear HC function.

Indeterminate presbycusis, as later described by Schuknecht, is characterized by a flat or abrupt high-frequency hearing loss without a consistent, well-described

histopathological correlate. It is hypothesized that these cases result from functional impairment of cochlear cells, such as metabolic or biochemical dysfunction, rather than from cellular loss, making them undetectable with conventional light microscopy.

These different types of presbycusis can occur independently, but they frequently overlap in elderly individuals. When combined, their additive effects produce complex audiometric profiles reflecting multiple underlying pathologies. Although the precise etiology of presbycusis remains unclear, current evidence indicates that it arises from an interplay of factors, including intrinsic aging of the auditory system, chronic noise exposure, systemic diseases and ototoxic treatments, as well as genetic susceptibility that increases individual vulnerability.

Clinical and population-based studies have revealed consistent epidemiological patterns in presbycusis. Family members often exhibit similarly elevated hearing thresholds and parallel age-related declines, supporting a genetic contribution to its inheritance (Christensen et al., 2001; Gates et al., 1999). Subsequent targeted studies have identified several gene mutations associated with age-related auditory decline. For example, the ion channel $K_V7.4$, encoded by *KCNQ4* and expressed in HCs, SGNs, in cochlear nuclei and inferior colliculus, is crucial for K^+ extrusion. The loss of function of $K_V7.4$ in HCs results in a chronic depolarization, causing chronic cellular stress (Beisel et al., 2005; Peixoto Pinheiro et al., 2021; Rias et al., 2025). Furthermore, the loss of $K_V3.1$ (*KCNC1*), expressed in SGNs, the brainstem, and the inferior colliculus (Zettel et al., 2007).contributes to age-related deficits in temporal auditory processing and discrimination (Cai and Sesti, 2009; Keithley, 2020; Macica et al., 2003). Recent advances in “genome-wide association studies” (GWAS), enabled by large-scale biobanks and international consortia, have identified additional genetic variants associated with ARHL. The integration of extensive phenotypic datasets has facilitated the discovery of multiple genes implicated in the molecular mechanisms of presbycusis (DeStefano et al., 2003; Wells et al., 2020).

Although no animal model fully replicates human ARHL, studies in experimental

models have greatly advanced our understanding of the molecular and cellular mechanisms underlying cochlear degeneration and age-related hearing loss. Due to their anatomical and physiological similarity to humans, short lifespan, and well-established genetic tools, mice are the preferred model for studying the cellular and genetic basis of ARHL (Ohlemiller, 2006; Ohlemiller, 2009). Among 80 inbred strains, 19 display early-onset hearing loss before 3 months of age, while 16 exhibit late-onset loss (Ohlemiller, 2019; Zheng et al., 1999). At least 10 inbred strains carry the *ahl* (age-related hearing loss) locus on chromosome 10, which contributes to ARHL and corresponds to the cadherin 23 (*Cdh23*) gene. Cadherin 23 is involved in the function of the HC mechanotransduction as one of the main components of the stereocilia tip links (Liu et al., 2012). The *ahl* allele corresponds to a single nucleotide substitution in *Cdh23*, where adenine replaces guanine at position 753 ($Cdh23^{753G->A}$) (Zheng et al., 2005). Mouse strains homozygous for $Cdh23^{753G->A}$ exhibit progressive HC degeneration and accelerated hearing loss in aging. One of the most widely used strains is the C57BL/6, which is homozygous for $Cdh23^{753G->A}$ and shows progressive hearing loss with notable HC pathology and hearing threshold elevation by 6 months of age (Ikaheimo et al., 2022; Jeng et al., 2021; Noben-Trauth et al., 2003). The C57BL/6 mouse exhibits a cochlear degeneration pattern resembling the major categories of ARHL described by Schuknecht; however, the $Cdh23^{753G->A}$ mutation contributes substantially to its auditory phenotype, preventing it from being a pure ARHL model. Nevertheless, it is widely regarded as an accelerated model of ARHL under appropriate experimental conditions. In summary, although animal models have provided substantial insight into the pathogenesis of cochlear degeneration in presbycusis, none fully reproduces the complexity of human ARHL. Ongoing advances in genetic tools such as CRISPR-Cas9 are opening new avenues for exploring molecular and cellular mechanisms of ARHL in the cochlea.

Regarding the molecular pathways involved in presbycusis, prior studies have highlighted the central role of mitochondria in aging as the primary source of ROS

(Kujoth et al., 2005; Someya et al., 2009). Environmental factors such as noise exposure, ototoxic agents, and age-related reductions in cochlear blood flow exacerbate oxidative stress and ROS production, leading to oxidative stress and mutations in mitochondrial DNA (mtDNA) (Tan and Song, 2023). While the exact molecular mechanism leading from oxidative stress to hearing loss is not fully understood, models have been suggested. For instance, ROS-induced mtDNA mutations may establish a vicious cycle (the "mitochondrial clock theory"), whereby accumulated mtDNA damage amplifies ROS generation, triggering apoptotic cascades and cell death (Liu and Yan, 2007; Pickles, 2004).

In addition to ROS, inflammation and inflammation-related molecular pathways are putatively a major component of presbycusis. Inflammation is an essential immune defense process triggered by pathogens, cellular debris, or other stressors. Once activated, involved cells release cytokines that promote macrophage recruitment, vasodilation, and increased vascular permeability to eliminate the source of damage. However, excessive or prolonged inflammation may result in tissue injury (Mittal et al., 2014). In the cochlea, macrophages derived from bone marrow are mobilized following various insults, including noise trauma, exposure to ototoxic substances, and aging (Hirose et al., 2017). The inner ear can attract immune cells and express cytokines, demonstrating an active local immune capacity (Fujioka et al., 2006; Fujioka et al., 2014). Indeed, gene expression related to immune signaling increases within hours of acoustic injury (Tan et al., 2016; Yang et al., 2016), with cytokines such as TNF-α, IL-6, and NF-κB among the key molecules produced.

The NF-κB pathway is a key regulator of inflammatory signaling, oxidative stress responses, and cell survival. It consists of a family of transcription factors (RelA, RelB, c-Rel, NF-κB1, and NF-κB2) that can form various homo- and heterodimers, each with distinct transcriptional effects (May and Ghosh, 1998). Under resting conditions, NF-κB dimers are retained in the cytoplasm by inhibitory IκB proteins. Activation occurs through phosphorylation and ubiquitination of IκB, leading to its degradation and the

rapid nuclear translocation of NF-κB, which enables gene transcription (Senftleben et al., 2001). The activation pattern of NF-κB is highly cell-specific; not every stimulus activates the pathway in all cell types (Pahl, 1999). ROS and NF-κB signaling pathways have been found to have multiple ways to interact, linking oxidative stress to immune regulation (Morgan and Liu, 2011).

While inflammation typically supports tissue defense and repair, in the cochlea, its effects can be either beneficial or damaging (Adams et al., 2009; Fujioka et al., 2006; Tahera et al., 2006; Tan et al., 2016). For instance, cytokine production in the lateral wall and immune participation from the endolymphatic sac contribute to the cochlear inflammatory environment (Harris and Ryan, 1995). After injury, macrophages and monocytes infiltrate regions such as the spiral ligament, spiral ganglion, and scala tympani (Fredelius and Rask-Andersen, 1990; Hirose et al., 2005; Okano et al., 2008; Tornabene et al., 2006). Following acoustic trauma, fibrocytes in the cochlear lateral wall produce proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 (Fujioka et al., 2006), which recruit macrophages to the damaged areas (Wakabayashi et al., 2010). These macrophages may assist in debris clearance from dying HCs (Hirose et al., 2017) and potentially support IHC synapse regeneration (Kaur et al., 2019). How inflammation contributes to ARHL is poorly understood. Therefore, studying the immune and inflammatory activity within the cochlea remains an important aspect of understanding ARHL.

In this thesis, we used C57BL/6 mice to investigate the contribution of oxidative stress and inflammation to cochlear degeneration. By examining these processes as mice age, we aimed to capture and describe the events of cochlear degeneration and identify a potential therapeutic window for interventions targeting oxidative and inflammatory pathways. This study is presented in Chapter 3 (pg. 67-78).

3.1. The Use of Hearing Aids and Their Impact on Cognitive Decline

According to the *cascade hypothesis* of ARHL proposed by Uchida and collaborators (Uchida et al., 2019) introduced above, auditory deprivation from cochlear damage may trigger neurobiological changes that contribute to cognitive decline. Older adults with untreated moderate to profound hearing loss face higher risks of communication difficulties, social withdrawal, depression, reduced quality of life, and cognitive impairment (Fortunato et al., 2016; Uchida et al., 2019), which can often be mitigated through hearing aid use.

Hearing loss also impacts social functioning, notably increasing the risk of loneliness (Bowl and Dawson, 2019; Shukla et al., 2021). Loneliness, a subjective sense of social disconnection distinct from social isolation (Courtin and Knapp, 2017) has been linked to depression (Erzen and Cikrikci, 2018; Lawrence et al., 2020; Pronk et al., 2013), and to adverse physical conditions such as cardiovascular disease, stroke, and increased mortality (Tomida et al., 2023; Tomida et al., 2024). Wagner et al. found slightly higher loneliness scores among hearing aid users, but no significant differences between users and non-users when adjusted for hearing level. However, loneliness remained higher among those with poorer hearing regardless of hearing aid use (Wagner et al., 2025). Furthermore, loneliness is associated with reduced performance across cognitive domains such as memory, verbal fluency, and processing speed, and with accelerated cognitive decline over time (Guarnera et al., 2023).

By improving auditory input and verbal communication, hearing aids can enhance mood, promote social engagement, and support participation in cognitively stimulating activities (Amieva et al., 2015). At present, hearing aids remain the primary clinical strategy for managing hearing loss.

Technically, hearing aids capture sound signals through a microphone; since this process reduces the signal amplitude, a pre-amplifier is placed at the initial stage to boost the input and convert it into a sinusoidal waveform. This waveform is then

processed and transformed first into an electrical and subsequently into a digital signal, allowing it to enter the signal processing unit (CPU). Within the CPU, the signal is digitally modified according to the programmed parameters. At the output, a digital-to-analog converter (DAC) transforms the signal back into an electrical form, which is then transmitted to the amplifier to increase its voltage and drive the receiver, where it is reconverted into sound.

Hearing aid devices can be divided into three main categories: BTE (Behind-The-Ear), ITE (In-The-Ear), and RITE (Receiver-In-The-Ear). BTE aids, the earliest type, includes a microphone that captures the sound, which is processed electronically and delivered to the loudspeaker. The sound travels through a rigid curved tube and then through a thin plastic tube to a custom-made earmold. This system introduces a delay of approximately 13–14 milliseconds, a factor that must be considered during fitting, as it may complicate use in unilateral hearing loss or frequency-specific hearing loss cases. ITE hearing aids are custom-made systems fitted entirely inside a shell built from an impression of the external auditory canal. ITE hearing aids are more efficient in amplifying high frequencies than low or mid frequencies. A potential issue with ITE is the Larsen effect (feedback), which can create a high-pitched whistle due to sound emitted from the speaker and being re-captured by the microphone, leading to a feedback loop. RITE devices also include a microphone, battery, and electronic circuitry, but the receiver is separated and positioned directly inside the external auditory canal, connected via a thin electrical wire. This configuration allows for smaller and more discreet designs and eliminates the temporal delay observed in BTEs. However, RITE hearing aids are less effective at reproducing low-frequency sounds and are therefore best suited for patients with preserved low-frequency hearing. Newer ITE designs, such as the “Lyric” (Phonak) or the “ampli-mini” (Amplifon), represent a new category of extended-wear hearing aids offering near-invisible placement and a more natural acoustic experience. Other design innovations include adaptive, user-tailored technologies, for example the “Intent” model (Oticon), that optimize sound

processing based on the listener's environment.

Commonly reported reasons for not acquiring hearing aids include the perception that they are unnecessary, excessive cost, inconvenience, and negative experiences reported by other users. Similar barriers have been identified in previous investigations (Fischer et al., 2011; McCormack and Fortnum, 2013).

Cognitive impairment can be assessed using purpose-built questionnaires. The Mini–Mental State Examination (MMSE) is a 30-point questionnaire widely used in clinical and research settings to screen for dementia and assess the severity and progression of cognitive impairment (Folstein et al., 1975). Administering the MMSE takes approximately 5–10 minutes and is conducted in a quiet, comfortable environment free from distractions. The examiner should provide clear, paced instructions to ensure the participant fully understands each task. Another cognitive test relevant for the work in this thesis is the Montreal Cognitive Assessment (MoCA) (Nasreddine et al., 2005).

The above cognitive tests may leave out the possibility that a treatment, e.g., hearing aids usage, may alter the emotional state of the patients that may influence test results of the cognitive tests. This missing information may be acquired using another type of questionnaire. The Hospital Anxiety and Depression Scale (HADS) is a 14-item questionnaire designed to assess emotional distress in patients with chronic medical conditions. The HADS evaluates the presence and severity of anxiety and depressive symptoms in medical patients. The HADS is brief and easy to administer, typically requiring about 5 minutes. It is well suited for screening and monitoring emotional distress over time but is not a diagnostic tool for specific psychiatric disorders.

In the last study presented in this thesis, we utilise MMSE, MoCA, and HADS to assess the level of cognitive impairment in clinical patients fitted with hearing aids (Amplifon). The tests are repeated 3 and 6 months after starting the use of the hearing aids to investigate if any change in cognitive impairment occurs following hearing aid use. This study is presented in Chapter 4 (pg. 79-89).

RESEARCH AIMS

ARHL is the most common sensory impairment in the elderly and represents a major public health concern worldwide. Characterized by a progressive, bilateral, and symmetrical decline in auditory sensitivity, ARHL primarily affects high-frequency sounds and speech discrimination, profoundly impacting communication and quality of life. Unlike other chronic conditions, ARHL remains largely underdiagnosed and undertreated, despite its strong association with social isolation, depression, reduced independence, and cognitive decline.

Epidemiological data underscore the magnitude of this problem: more than one in four individuals over the age of 60 experiences disabling hearing loss, and the World Health Organization estimates that by 2050, approximately 2.1 billion people aged 60 years and older will be affected. This rising prevalence, driven by global demographic aging, highlights the urgent need for effective preventive and therapeutic strategies.

Beyond its direct impact on auditory perception, ARHL has increasingly been recognized as a modifiable risk factor for dementia. Accumulating evidence links auditory deprivation to accelerated brain atrophy, reduced cognitive stimulation, and altered neural network function. The biological mechanisms underlying ARHL involve complex interactions between genetic predisposition, oxidative stress, chronic inflammation, vascular compromise, and cumulative environmental insults such as noise exposure or ototoxic drugs. Nevertheless, the precise molecular and cellular pathways remain incompletely understood, limiting the development of targeted interventions.

Understanding the mechanisms underlying inner ear damage is a fundamental step toward the experimental and clinical development of innovative therapeutic strategies for ARHL. In this context, our research group has recently contributed to clarifying the causes of cochlear injury induced by exogenous factors, thereby opening new perspectives for prevention and treatment.

Within the framework of this doctoral project, the research activities were primarily

aimed at investigating the molecular pathways involved in SNHL triggered by exogenous agents, to delineate the mechanisms of cochlear vulnerability. Subsequently, the focus was extended to age-related processes to elucidate the cellular and molecular events that drive auditory decline during aging.

In brief, over the course of the doctoral program, the research activities focused on three main objectives:

1. To investigate the molecular mechanisms underlying cisplatin- and styrene-induced ototoxicity.
2. To explore the molecular pathways contributing to cochlear degeneration in an *in vivo* model of physiological aging (C57BL/6 mice);
3. To evaluate the relationship between hearing loss, cognitive performance and mental distress over time in a cohort of elderly individuals with hearing impairment undergoing their first experience with hearing aids.

Chapter 1 (Pisani et al., 2024) focuses on elucidating the molecular mechanisms of cisplatin-induced ototoxicity, with a particular emphasis on neural damage. The findings revealed that cisplatin administration induces a significant increase in auditory thresholds, reduces the survival of SGNs, and causes a loss of afferent fibers. These functional and structural alterations were linked to an imbalance in TrkB/p75 signaling pathways and the subsequent activation of pro-apoptotic cascades, ultimately driving neuronal degeneration. These results not only clarified the neural basis of cisplatin-induced hearing loss but also provided a solid foundation for expanding investigations to other exogenous agents capable of compromising auditory function.

Chapter 2 (Hassler, Mohamed-Hizam et al., 2025) addresses another exogenous factor of considerable occupational relevance: styrene. Using an established experimental model of styrene-induced ototoxicity, we demonstrated that oxidative stress and inflammatory responses are central mechanisms mediating cochlear damage caused by this volatile compound. Building on these mechanistic insights, two targeted

molecules were tested: rosmarinic acid, a potent antioxidant compound, and Anakinra, an IL-1 receptor antagonist with anti-inflammatory properties. Both compounds were shown to be effective in reducing styrene-induced hearing loss. These results, which hold clear translational significance, open promising perspectives for the prevention of occupational hearing loss and the protection of auditory health in individuals exposed to chemically hazardous environments.

In Chapter 3 (Pisani, Hassler et al., under revision) we investigated the role of oxidative stress and inflammation in the progression of ARHL by examining the temporal dynamics of cochlear degeneration throughout the lifespan of C57BL/6 mice. This mouse strain, which exhibits a well-characterized and predictable pattern of ARHL, provided an ideal model for dissecting the sequence of molecular and cellular events underlying auditory decline. The results obtained highlighted the complex interplay of oxidative damage and chronic inflammatory processes in cochlear aging, thus contributing to a deeper understanding of the mechanisms driving ARHL.

Finally, **Chapter 4** focuses on the clinical dimension of hearing loss, specifically its relationship with cognitive impairment. Here, our study evaluated a cohort of elderly individuals with hearing loss who were undergoing their first experience with hearing aids (defined as "New Users"). By investigating MMSE and MoCA scores before and after a 6-month rehabilitation period, we aimed to determine whether auditory rehabilitation through hearing-aid use could improve cognitive outcomes. In addition, we investigated HADS mental state test scores to determine how hearing-aid use may influence states of anxiety or depression. This approach provided valuable evidence on the potential benefits of hearing aid use, not only for auditory perception but also for the preservation of cognitive function in aging populations.

Taken together, these studies addressed complementary aspects of hearing loss, ranging from the elucidation of molecular mechanisms underlying exogenous and age-related

cochlear damage to the evaluation of clinical implications for cognitive health. The resulting findings, published in internationally recognized journals, provide a solid foundation for future translational research aimed at developing preventive and therapeutic strategies for hearing loss.

CHAPTER 1.

Unveiling neural impairment and the underlying molecular mechanisms of cisplatin-induced cochlear damage

1.Introduction

Cisplatin is a platinum-based chemotherapeutic agent widely used against solid tumors, including ovarian, lung, head and neck, and breast cancers (Boulikas and Vougiouka, 2004; Shah and Dizon, 2009). However, its clinical use is often limited by severe dose-dependent side effects, particularly ototoxicity, which remains one of the most significant complications of cisplatin therapy (Fetoni et al., 2015; Fetoni et al., 2022a; Rybak et al., 2007) Cisplatin-induced ototoxicity is characterized by progressive, bilateral SNHL often accompanied by tinnitus (Bokemeyer et al., 1998; Wei and Yuan, 2019). High-frequency hearing loss affects approximately 22% –75% of adult patients receiving cisplatin chemotherapy, potentially extending to lower frequencies with cumulative exposure (Rybak et al., 2009; Rybak et al., 2007).

Clinical and experimental evidence indicates that cisplatin-induced cochlear injury primarily targets OHCs, particularly in the basal and middle cochlear turns (Schacht et al., 2012; Sha et al., 2001; Tabuchi et al., 2011). The underlying mechanisms are multifactorial (Casares et al., 2012), with oxidative stress playing a central role through excessive generation of reactive oxygen and nitrogen species (ROS and RNS) (Fetoni et al., 2022a; Ramkumar et al., 2021). This redox imbalance triggers lipid peroxidation, inflammatory responses, and activation of the p53 signaling cascade, culminating in apoptotic cell death (Abi-Hachem et al., 2010; Casares et al., 2012). Furthermore, ROS-induced HC death is thought to contribute to secondary degeneration of SGNs (Zhai et al., 2011). Nevertheless, the molecular mechanisms underlying cisplatin-related neural injury remain incompletely understood.

To date, treatment options for SNHL have been largely limited to hearing aids and cochlear implants (CIs). CIs, primarily indicated for individuals with severe to profound hearing loss, yield variable outcomes depending on the survival and functional integrity of residual SGNs (Seyyedi et al., 2014). No current clinical therapies can rescue or regenerate degenerating SGNs. Therefore, elucidating the molecular pathways involved in cisplatin-induced cochlear and neural injury is essential for identifying potential therapeutic targets. In this study, we investigated the effects of cisplatin on cochlear neural structures and analyzed related molecular pathways, focusing on TrkB, p75, and phosphorylated AKT expression in SGNs to identify key mediators of cisplatin-induced neurotoxicity.

2. Materials and methods

2.1. Experimental animal model

We used two-month-old male Wistar rats weighing 200-250 g, obtained from the laboratories of the Catholic University. The animals were paired in cages and provided *ad libitum* access to food (4RF21, Mucedola, Settimo Milanese, Milan, Italy) and water. The housing environment was maintained at a temperature of 22-23 °C, with humidity levels at 60±5%, and a 12-hour light/dark cycle was strictly upheld. Stringent measures were taken to minimize animal stress and to reduce their numbers, aligning with the guidelines outlined in the European Community Council Directive of 24th November 1986 (86/609/EEC). All experimental procedures were carried out in accordance with the Laboratory of Animal Care and Use Committee of the Catholic University, School of Medicine of Rome, and received approval from the Italian Department of Health (Ministero della Salute, Prot. 1F295.111.EXT.77). The experiments involved a total of 30 animals, which were randomly assigned to two separate experimental groups: 1) the control group (Ctrl, N=15); 2) the cisplatin-

treated group (CIS, N=15). A cohort of 15 additional rats was used to perform the dose-response curve of cisplatin (8 and 12 mg/kg).

2.2. Cisplatin administration

Cisplatin (cis-diamino-platinum(II) dichloride) (Cat. No. P4394, Sigma-Aldrich, St. Louis, MO, USA) was freshly prepared by dissolving it in sterile saline solution at a concentration of 1 mg/ml. This solution was protected from light and heated for approximately 20 minutes to promote dissolution of the cisplatin. For drug administration, animals were deeply anesthetized with ketamine (87.5 mg/kg) and xylazine (12.5 mg/kg), and a single intraperitoneal dose of cisplatin (12 mg/kg) was delivered using an infusion pump (Axon Instruments, Foster City, CA, USA) at a controlled rate of 8 mL/h over ~30 min. Following treatment, rats received daily subcutaneous injections of 15 mL sterile saline to maintain hydration and reduce systemic toxicity associated with cisplatin exposure.

2.3. Auditory brainstem responses (ABRs)

Auditory function was evaluated by measuring ABRs bilaterally in all subjects. Measurements were obtained at low (6 kHz), mid (12, 16, 20 kHz), and high (24, 32 kHz) frequencies before treatment and three days post-administration. Animals were lightly anesthetized (ketamine 35 mg/kg, medetomidine 0.25 mg/kg) and placed in an anechoic chamber. Following a previously described method (Fetoni et al., 2022b; Paciello et al., 2021b), stainless steel electrodes were inserted subcutaneously in the right mastoid (active), vertex (reference), and left mastoid (ground). ABR recordings and auditory stimulus generation were conducted using a PC-controlled TDT 3 system from Tucker Davis Technologies (Alachua, FL, USA), which employs real-time digital signal processing. Monophonic presentation of pure tone bursts from 6 to 32 kHz (with

a rise/fall time of 1 ms, a total duration of 10 ms and a repetition rate of 20/s) was used. The responses were filtered in the range of 0.3-3 kHz, digitized and averaged over 512 discrete samples for each combination of frequency levels. Hearing threshold was defined as the lowest stimulus intensity eliciting a reproducible waveform.

2.4. Morphological analysis: H&E staining

Cochleae from five rats were collected three days after the onset of cisplatin treatment. The cochleae were fixed using a 4% paraformaldehyde solution in PBS. The fixation process was carried out at 4 °C. Following fixation, the cochleae underwent a decalcification process that spanned 15 days, using a 10% EDTA solution. The specimens were then immersed in a 30% sucrose solution for a period of 48 hours. The cochleae were embedded in optimal cutting temperature (OCT). Slices with a thickness of 12 μm were obtained through cryosectioning, using a Cryostat CM 1950 from SLEE. To assess ganglion neuronal cell damage, these sections were stained with hematoxylin and eosin (H&E). The staining procedure involved a 5-minute incubation in hematoxylin, followed by a 2- to 3-minute incubation in eosin. The stained samples were then mounted using Entellan® (Cat. No. 107960, Merck, Darmstadt, Germany). For further analysis, NIH ImageJ 1.43u (Image Processing and Analysis in Java) was employed. Neurons that displayed a distinct round nucleus and uniform cytoplasm were considered for cell counting. This counting was carried out within a specified area measuring 100x100 μm. Cell viability was determined by calculating the percentage of surviving cells relative to the control group.

2.5. Immunofluorescence analysis

To evaluate the effects of cisplatin on afferent fibers, immunofluorescence staining was performed using an antibody against NF200 (N41412, Sigma-Aldrich, St. Louis, MO, USA), a well-established neurofilament marker. Three days after treatment onset, cochleae were rapidly dissected from five animals per group and fixed in 4% paraformaldehyde in PBS (pH 7.4) at 4 °C. Samples were then decalcified for 15 days in 10% EDTA with daily solution changes, followed by cryoprotection in 30% sucrose for 48 h. Cochleae were embedded in OCT compound, sectioned at 12 μm thickness using a SLEE cryostat, and processed for subsequent immunostaining.

2.6. Western blot analysis

To investigate the molecular mechanisms underlying cisplatin-induced neurotoxicity in cochlear tissues, Western blot analyses were performed. Three days after cisplatin exposure, cochleae were dissected on ice, stored at −80 °C, and subsequently processed. Protein lysates (70 μg) were loaded onto 4-15% Tris-glycine polyacrylamide gels for electrophoretic separation. The proteins were then transferred onto nitrocellulose membranes at 100 V for 2 hours at 4 °C in transfer buffer containing 25 mM Tris (Cat. No. T4661, Sigma-Aldrich), 192 mM glycine (Cat. No. G8898, Sigma-Aldrich), 0.1% sodium dodecyl sulfate (SDS; Cat. No. L3771, Sigma-Aldrich), and 20% methanol (Cat. No. 322415, Sigma-Aldrich). Subsequently, membranes were incubated for one hour in a blocking buffer consisting of 5% skim milk (Cat. No. #1706404, Bio-Rad Laboratories) in TBST (Tris Buffered Saline, Cat. No. T5912, Sigma Aldrich and 0.1% Tween 20, Cat. No. P1379, Sigma-Aldrich), and then incubated overnight at 4°C with primary antibodies: Caspase 3 cleaved (Cat. No. AB3623, Millipore, Burlington, MA, USA), Caspase 3 (sc-727272, Santa Cruz Biotechnology, Dallas, TX, USA), p75 (ab52987, Abcam, Cambridge, UK), pTrkB

(Cat. No. 4619, Cell Signaling) and TrkB (ab18987, Abcam) pAKT (Cat. No. 9271, Cell Signaling) and AKT (Cat. No. 4691, Cell Signaling). After three 10-minute washes in TBST, the membranes were incubated for one hour at room temperature with HRP-conjugated mouse or rabbit secondary antibodies (1:2500; Cell Signaling, Danvers, MA, USA). To ensure equal protein loading between lanes, we re-probed the membranes with an anti-GAPDH antibody (1:10,000; Cat. No. ab8245, Abcam). Finally, protein expression levels were assessed and documented using UVItec Cambridge Alliance. All experiments were performed in triplicate for reproducibility.

2.7. Statistical analysis

Results are expressed as means and standard error of the mean (SEM), and statistical differences were evaluated using analysis of variance (ANOVA). Post-hoc comparisons were assessed with Tukey's Test (Statistica, Statsoft, Tulsa, OK, USA). A *P*-value <0.05 was considered significant.

3. Results

3.1. Cisplatin-induced hearing loss

To evaluate the impact of cisplatin exposure on hearing loss, auditory function was assessed by ABR recordings before and after 3 days (D3) of cisplatin injection (Figure 1). Results of the dose–response curve show that the best ototoxic effect of cisplatin is obtained with a dosage of 12 mg/kg (Figure 1A). Indeed, animals treated with cisplatin at a dosage of 12 mg/kg and analyzed 3 days after the onset of treatment showed a significant increase in the auditory threshold of 10-15 dB in the low frequencies and of about 20 dB in mid-high frequency regions, approximately 15-20 dB at the mid-high frequency region (Figure 1C). Notably, no significant differences in ABRs were

observed between rats treated with cisplatin at a dosage of 8 mg/kg and Ctrl animals (Figure 1B). Collectively, these results indicate that cisplatin-induced hearing loss is dose-dependent, with a dosage of 12 mg/kg causing the most significant ototoxic effect. Thus, based on these findings, immunofluorescence and Western Blot analyses were performed on animals treated with 12 mg/kg of cisplatin and analyzed after 3 days from the onset of treatment.

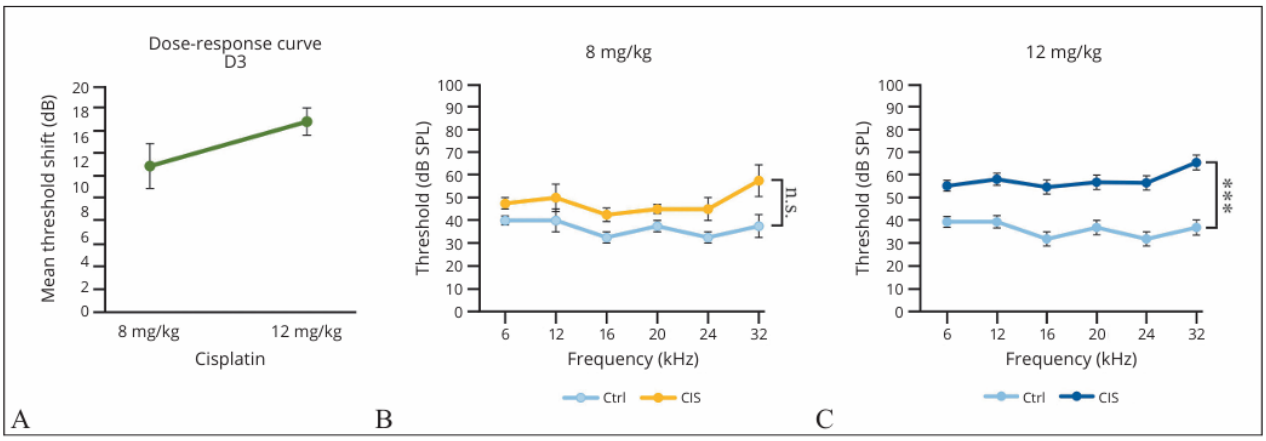

Figure 1. Hearing loss induced by cisplatin treatment. A: Graph (means±SEM) showing dose-response curve (mean ABR threshold shift values across all tested frequencies) for cisplatin at different doses (8 mg/kg; 12 mg/kg) after 3 days (D3) from the onset of treatment; B, C: Mean auditory threshold shift in cisplatin-treated animals measured at D3. Asterisks indicate significant differences between groups. ***P<0.001.

3.2. Cisplatin treatment induces SGN death

To analyze the ototoxic effect of cisplatin on SGN viability, we performed H&E staining on cochlear cryosections (Figure 2A, B). In the Ctrl group, the Rosenthal's canal was densely packed with SGNs (Figure 2A). After 3 days from the onset of cisplatin treatment, a marked reduction of SGNs was observed in middle and basal cochlear turns (about 40% of SGN loss), as shown by histograms representing the percentage of surviving neurons (Figure 2C). At the molecular level, we examined by

Western Blot analysis the expression of cleaved-caspase 3, which is considered a well-established marker for apoptotic cells (Sabirzhanov et al., 2012). Our findings reveal a significant increase in cleaved-caspase 3 expression following cisplatin treatment (Figure 2E). These findings collectively suggest that cisplatin has a detrimental impact on SGN viability, potentially through the induction of apoptotic cell death.

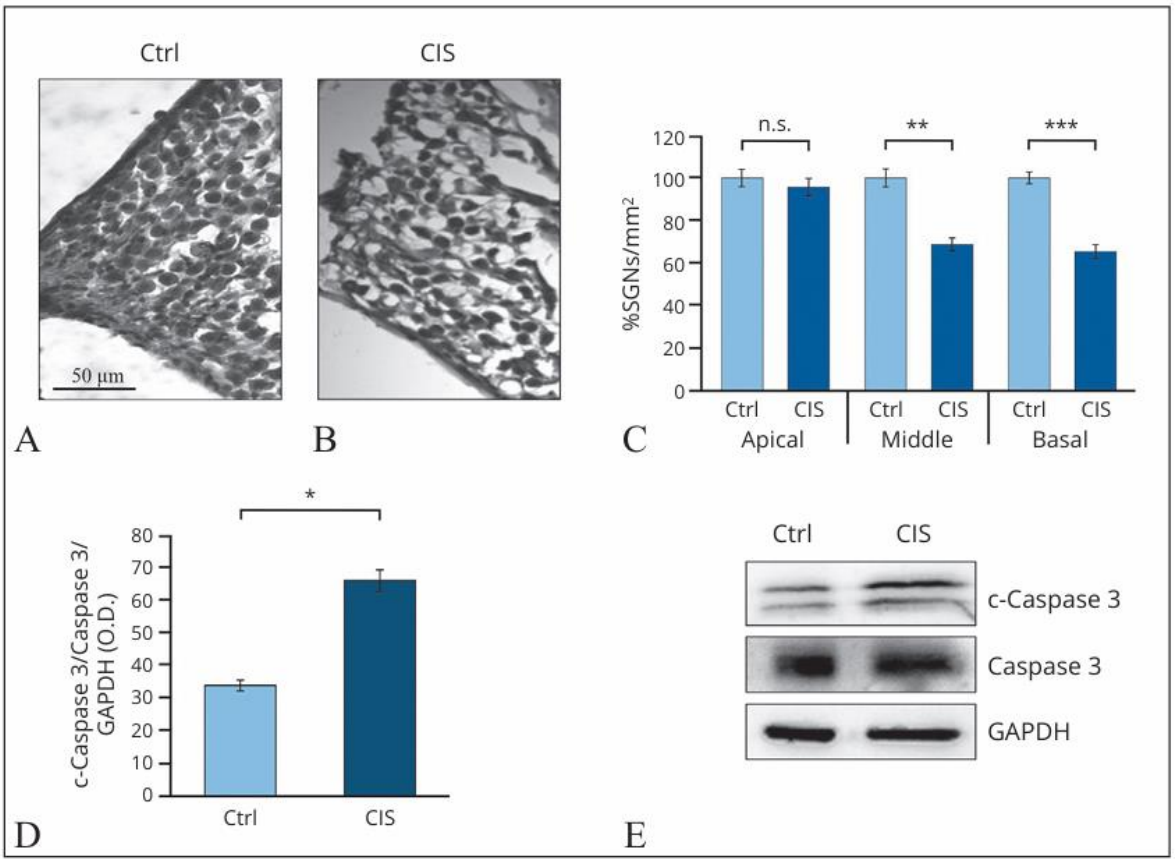

Figure 2. Cisplatin treatment induces neuronal death by the activation of the pro-apoptotic pathway. A, B: representative H&E images of cochlear cryosections showing SGNs of Ctrl (A) and cisplatin (B) groups; C: histograms (means±SEM) show SGN viability; D: representative Western blot image showing an increase of caspase 3-cleaved expression in cisplatin-treated cochleae compared to control samples; E: Bar graphs showing results of densitometric analyses for protein expression normalized to GAPDH. Data are expressed as mean±SEM. Asterisks indicate significant differences between groups. *P<0.05; **P<0.01; ***P<0.001.

3.3. Cisplatin induces a severe decrease of cochlear primary afferent fibers

To investigate the impact of cisplatin on the cochlear neural compartment, immunofluorescence analysis for NF200 was performed. A marked reduction in primary afferent fibers was observed in cisplatin-treated animals compared to controls (Figure 3A–B). High-magnification images further revealed a pronounced disorganization and loss of fiber integrity in the cochleae of cisplatin-exposed rats (Figure 3E–F) relative to controls (Figure 3C–D). These findings indicate that cisplatin induces a substantial degeneration of cochlear primary afferent fibers, highlighting its detrimental effect on the neural compartment.

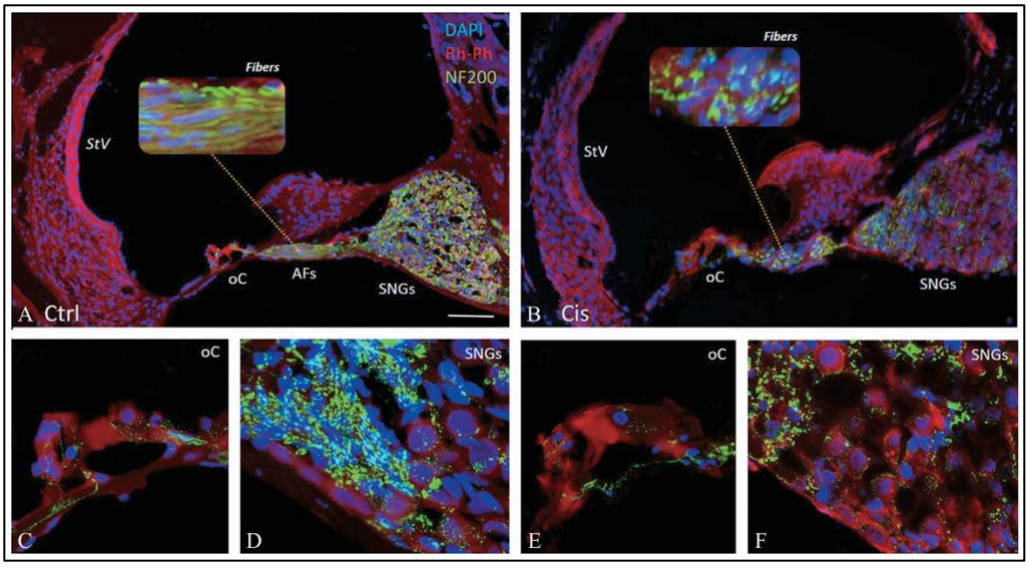

Figure 3. Cisplatin induces a severe decrease of cochlear primary afferent fibers. Cochlear cryosections stained with NF200 (green fluorescence), Rh-Ph (red fluorescence), and DAPI (blue fluorescence). In the Ctrl group (A), we observed normal afferent neural fibers organization, as shown in the high magnification images representing the organ of Corti (C) and SGNs (D). In the cisplatin-treated animals (B) we observed a decrease of primary afferent fibers (as shown by the decrease of neurofilament signal density-NF200); E, F: high magnification images showing a

severe impairment in the fiber organization in cisplatin-treated animals. Scale bar: 25 µm. StV: *stria vascularis*; Oc: organ of Corti; SGNs: spiral ganglion neurons; AFs: afferent fibers.

3.4. Molecular mechanisms underlying cisplatin-induced cochlear damage

To elucidate the molecular mechanisms underlying cisplatin-induced neural damage in the cochlea, Western blot analyses were performed focusing on the TrkB signaling pathway, which is critically involved in neuronal survival (Ohira and Hayashi,2009; Pisani et al., 2023b). Our results revealed a marked decrease in phosphorylated TrkB (pTrkB) levels following cisplatin exposure (Figure 4A). This reduction in TrkB activation was accompanied by a significant decrease in phosphorylated AKT ($pAKT^{S473}$), a key kinase mediating cell survival downstream of neurotrophic and stress-related pathways. Conversely, cisplatin treatment induced a significant upregulation of p75 expression, consistent with the activation of apoptotic signaling (Bamji et al., 1998).

Together, these findings suggest that cisplatin disrupts TrkB-dependent survival signaling while promoting p75-mediated apoptosis, providing a molecular basis for the observed degeneration of spiral ganglion neurons and loss of primary afferent fibers.

4. Discussion

The results of this study provide novel insights into the impact of cisplatin on auditory function and the associated molecular mechanisms underlying cochlear neurotoxicity. Using complementary experimental approaches, we demonstrated that:

1) Cisplatin-induced hearing loss is dose-dependent, with 12 mg/kg producing the most pronounced ototoxic effect;

2) Cisplatin negatively affects spiral SGN viability by activating apoptotic cell death;
3) Cisplatin causes a substantial reduction in cochlear primary afferent fibers, reflecting its detrimental effect on cochlear neural compartments;
4) Cisplatin disrupts TrkB signaling and promotes apoptosis in cochlear neural cells, which may account for the observed SGN loss and fiber degeneration.

It is well established that cisplatin accumulation within the cochlea triggers HC degeneration (Baker et al., 2015; Fetoni et al., 2022a) leading to SRHL. This effect is largely driven by cisplatin-induced oxidative stress, which enhances the production of ROS and RNS (Fetoni et al., 2022a; Wang et al., 2023).

These reactive species act as major mediators of cellular injury, promoting lipid peroxidation, mitochondrial dysfunction, and activation of apoptosis (Gentilin et al., 2019). In particular, mitochondrial ROS release triggers cytochrome c release and activation of caspase-9 and caspase-3, leading to irreversible cell death (Cheng et al., 2005).

Here, we show that increased oxidative stress also damages SGNs, resulting in apoptotic neuronal death. Consistently, we observed a significant increase in caspase-3 activation and a decrease in primary afferent fiber density and integrity following cisplatin administration.

TrkB signaling plays a pivotal role in neuronal survival, neurotransmission, and differentiation (Huang and Reichardt, 2001; Minichiello, 2009; Patapoutian and Reichardt, 2001; Schimmang et al., 2003). Activation of TrkB engages multiple intracellular cascades, including the PI3K/Akt pathway, which maintains neuronal survival by preserving mitochondrial integrity and inhibiting pro-apoptotic signaling (Dai et al., 2017; Wu et al., 2019). Activated Akt counteracts the effects of Bcl-2 family members (Bad, Bax) and suppresses Fas ligand-mediated apoptosis via FOXO phosphorylation (Zhang et al., 2011).

Our results revealed a marked reduction in TrkB levels after cisplatin exposure, accompanied by a significant reduction in Akt phosphorylation, a key kinase regulating

cell survival downstream of growth factors, oncogenes, and cellular stress. Conversely, we observed a significant increase in p75 expression following cisplatin treatment, indicating activation of apoptotic pathways.

These findings suggest that cisplatin impairs cochlear neural cells by disrupting TrkB signaling and promoting apoptosis, providing a molecular explanation for the observed SGN loss and degeneration of primary afferent fibers. All experiments were conducted 3 days post-cisplatin administration to assess early activation of cell death pathways. Notably, these experimental results align with emerging clinical evidence indicating that cisplatin induces auditory neuropathy spectrum disorder-like hearing loss, characterized by SNHL that predominantly affects speech perception and comprehension. Lesions of SGNs and their fibers often result in speech distortion, commonly associated with disabling tinnitus.

CHAPTER 2.

Targeting NLRP3 inflammasome activation in styrene-induced ototoxicity: comparative efficacy of rosmarinic acid and anakinra in mitigating oxidative and inflammatory damage

1. Introduction

The ototoxicity of organic solvents is well documented and represents a major concern in occupational medicine (Fuente and McPherson, 2006; Pigini et al., 2023; Pleban et al., 2017; Sisto et al., 2020). Styrene, an industrial organic solvent, is widely used in the production of plastics and synthetic rubber, and exposure to high levels of styrene has been associated with auditory damage and hearing loss, particularly in occupational settings (Chen et al., 2007; Gagnaire and Langlais, 2005; Lataye et al., 2003; Sisto et al., 2016) .

Accumulating evidence suggests that styrene exposure elevates mitochondrial ROS, with the consequent rise of lipid peroxidation and cell death (Fetoni et al., 2021; Fetoni et al., 2016; Paciello et al., 2024). We have previously shown that styrene increases ROS production in the cochlea and that antioxidant therapy with Q-ter, the soluble form of Coenzyme Q10, significantly attenuates styrene-induced hearing loss (Fetoni et al., 2016). It is also known that a strong relationship exists between ROS and inflammation. A close link between oxidative stress and inflammation has been well established. ROS generation promotes the expression of pro-inflammatory cytokines, such as TNFα, thereby amplifying cochlear injury (Tan et al., 2016; Tornabene et al., 2006; Wakabayashi et al., 2010). Elevated ROS production and inflammation have been reported to occur in SNHL caused by noise exposure, ototoxicity (Fetoni et al., 2014; Fetoni et al., 2016; Paciello et al., 2021b), and aging (Bielefeld et al., 2010; Fujimoto and Yamasoba, 2014; Huang and Tang, 2010; Ohlemiller, 2006).

The ability of ROS to drive inflammation involves the nuclear factor kappa B (NF-κB) signaling pathway, which induces cytokine production (Yamamoto et al., 2009). The relationship between oxidative stress and NF-κB is bi-directional. NF-κB-dependent genes have been shown to impact the levels of ROS, but, in turn, ROS production can also influence NF-κB activity (Blaser et al., 2016; Collins et al., 2012; Gloire et al., 2006; Morgan and Liu, 2011; Yamamoto et al., 2009). The existence of an interplay between ROS and inflammation in styrene-induced auditory system damage has also been demonstrated, both in the cochlea and in the auditory cortex (Fetoni et al., 2021; Paciello et al., 2024).

The interplay between ROS and inflammation may be driven by the inflammasome complex. Inflammasomes are molecular complexes functioning as sentinels of innate immunity by detecting pathogen-associated molecular patterns (PAMPs) or endogenous danger-associated molecular patterns (DAMPs) (Martinon et al., 2002). Recent evidence suggests that the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is involved in the interplay between ROS and inflammation (Abderrazak et al., 2015). NLRP3 activation and oxidative stress have been reported to act in a positive feedback loop, in which ROS accumulation participates in the initiation of inflammasome signaling (Ziehr and MacDonald, 2024). NLRP3 inflammatory cells, including macrophages and neutrophils, in turn can elevate ROS production (Mittal et al., 2014). When activated, NLRP3 initiates the maturation of proinflammatory cytokines, such as interleukin-1β (IL-1β), to promote innate immune defenses (Pennisi et al., 2017).

Here, we hypothesized and investigated if the NLRP3 inflammasome could be a molecular candidate in mediating the interplay between ROS and inflammation in styrene-induced cochlear damage. Furthermore, to better understand the different impacts of oxidative stress and inflammation in ototoxicity, we also verified the efficacy of both antioxidant (rosmarinic acid - RA) and anti-inflammatory (anakinra - ANA) protective treatments against hearing loss.

2. Materials and methods

2.1. Animals

For this study, a total of 66 male adult Wistar rats of 2 months of age were randomly assigned to the following groups: (1) normal hearing control animals ("Ctrl" group; n = 13); (2) animals exposed to styrene administered by gavage ("Styrene" group; n = 13); (3) animals exposed to styrene and treated with Rosmarinic Acid ("Styrene +RA" group; n = 13); (4) animals treated with styrene and anakinra ("Styrene +ANA group; n = 13); (5) Sham group receiving vehicle oral gavage ("Ctrl-Sham" group; n = 7); (6) Sham group exposed to styrene and receiving vehicle intraperitoneal injection ("Styrene-Sham" group; n = 7). Animals were housed two per cage in a controlled environment with a temperature of 22-23 °C, humidity of 60 ± 5%, and a 12 hours light/dark cycle, with free access to food (*Mucedola* 4RF21, Italy) and water. To minimize animal suffering and numbers, we adhered to the guidelines set by the European Community Council Directive of November 24, 1986 (86/609/EEC) and followed the procedures approved by the Laboratory of Animal Care and Use Committee of the Catholic University, School of Medicine of Rome, and the Italian Department of Health (*Ministero della Salute*, n° 1F295.195). A diagram detailing the experimental plan is shown in Figure 1.

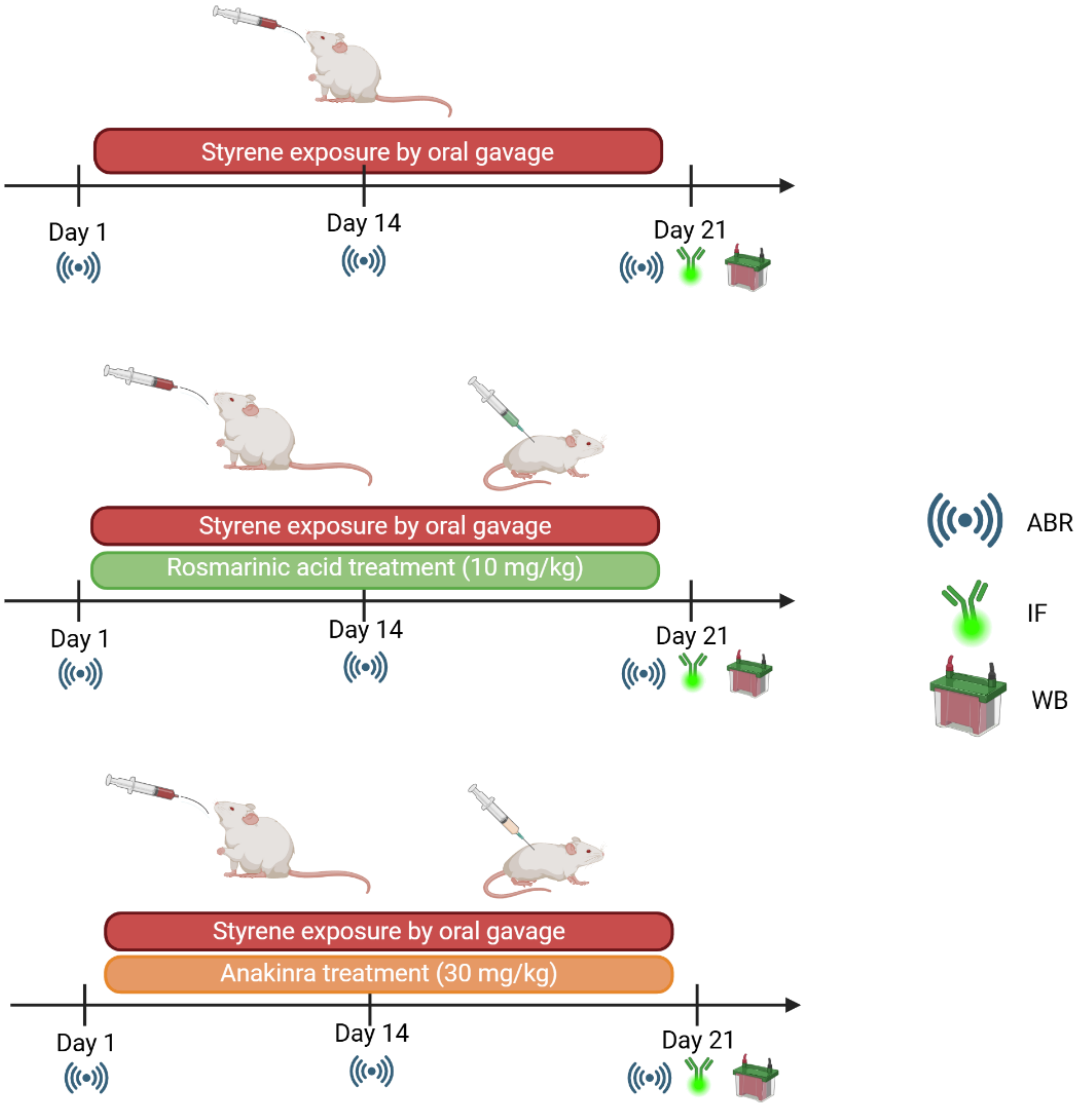

Figure 1. Schematic Representation Of The Experimental Plan. Styrene was administered daily (400 ml/kg) via oral gavage for 3 weeks (from day 1 to day 21), 5 days per week, in adult Wistar rats. At the end of the treatment period (day 21) , the animals were sacrificed, and cochlear tissues were collected for experimental analyses. Parallel to styrene exposure, two groups of animals received treatments: one with rosmarinic acid (RA, 10 mg/kg) and the other with anakinra (ANA, 30 mg/kg). ABR: auditory brainstem responses; IF: immunofluorescence; WB: western blot.
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2.2. Pharmacological treatments

To induce ototoxicity, styrene (styrene > 99%, Sigma Corporation, product id: S4972) was dissolved in olive oil (0,4 ml/kg body weight) and administered by gavage at a dosage of 400 mg/kg per day, 5 days a week for 3 consecutive weeks, a dosage regimen we previously found to provide an ototoxic effect (Fetoni et al., 2021; Paciello et al., 2024). To test the effectiveness of an anti-inflammatory or antioxidant treatment against styrene-induced ototoxicity, a cohort of animals was treated with an intraperitoneal (i.p.) injection of RA (96%, cod. Number 536954, Sigma-Aldrich, Italy; 10 mg/kg) or ANA (Kineret, Swedish Orphan Biovitrum AB; 30 mg/kg) 1 hour before styrene oral gavage. The dosage was chosen on the basis of previous experiments documenting ANA and RA protective effects against hearing loss (Fetoni et al., 2015; Paciello et al., 2021b).

Parallelly, animals of the “Ctrl-Sham” group underwent gavage procedure with olive oil (without styrene injection), and animals of the “Styrene-Sham" group received vehicle i.p. injection 1 hour before styrene administration. At the end of treatments (day 21), animals were sacrificed, and cochleae were collected and processed for immunofluorescence and western blot analyses. The schedule of experimental procedures is summarized in Figure. 1.

2.3. Auditory Brainstem Responses

Auditory thresholds were detected in all animals by recording ABRs, measured at different frequencies (6, 12, 16, 20, 24, and 32 kHz) before and after 7, 14, and 21 days of styrene treatment onset. Functional evaluations were performed under anesthesia (ketamine 35 mg/Kg + medetomidine 0.25mg/Kg, i.p.) in the anechoic room as described previously (Fetoni et al., 2014). The auditory threshold was defined as the lowest stimulus level able to produce a repeatable waveform-based onset. Results are shown as threshold shift values, referring to the difference between the auditory threshold after and before treatment.

2.4. Morphological analyses

To assess cochlear morphological alterations, HC survival was evaluated by using immunofluorescence analyses. Surface preparations of the organ of Corti were obtained as previously described (Pisani et al., 2025) from 3 cochleae from 3 animals/group, and samples were stained with ActinGreen 488 Ready Probes Reagent (Cat. No. R37110, Thermofisher Scientific). The samples were mounted with mounting medium (FluoSave, Cat. No. 345789, EMD Millipore Corp.), and a confocal microscope system (Nikon Ti-E, Confocal Head A1 MP, Tokyo, Japan) was used to capture images of the immune-labelled specimens using a 20× objective lens. To quantify OHCs, the epithelium was divided into successive 0.24-mm segments using a calibrated eyepiece reticle (approximately 3% of the total cochlear length). Counting began in the first intact segment distal to the apex (0.00–0.24 mm) and proceeded toward the hook region. Segments were subsequently grouped into apical (0–25%), middle (40–60%), and basal (70–100%) regions. Any segments showing mechanical damage from dissection were excluded from analysis.

An OHC was considered present if both an intact phalloidin-positive ring and its

associated stereociliary bundle were visible; it was deemed missing if these structures were absent or replaced by a supporting-cell scar. Counting was performed by an investigator blinded to the treatment group. The resulting averages were used for statistical analysis and are expressed as the percentage of surviving OHCs per cochlear turn.

2.5. Immunofluorescence analyses

To assess the oxidative-inflammatory damage in cochlear tissues, we performed immunofluorescence analysis to detect ROS amount and IL-1b expression in cochlear cryosections (3 cochleae from 3 animals/group).

Samples were collected and stored in 4% paraformaldehyde in PBS at +4 °C overnight. Next, after 10 days of incubation in EDTA (10%), tissues were transferred to sucrose (30%), included in OCT, and cut in longitudinal sections (12 μm thickness) with a cryostat (CM 1950; SLEE).

For oxidative stress analyses, cochlear slices were incubated with 1 M DHE (Cat. No. D23107, Thermo Fisher, Waltham, MA, USA) in PBS for 30 min at 37°C and then coverslipped with an antifade medium (FluorSave). To detect IL-1β, the slices were incubated with a blocking solution (1% BSA, 0.5% Triton X-100, and 10% Normal Goat Serum in PBS 0.1 M) and then were incubated with anti-IL-1b primary antibody (Cat. No. sc-7884, Santa Cruz Tech.) 1:100 in PBS overnight at + 4°C. Specimens were washed in PBS and incubated for 2 h at RT in labeled conjugated goat anti-rabbit antibody (Alexa Fluor 488 IgG, Cat. No. A32731, Invitrogen) 1:400 in 0.1 M PBS. Finally, DAPI solution (Thermo Fisher, 1:500 in 0.1 M PBS) was used to visualize cell nuclei. A confocal laser microscope (Nikon Ti-E, Confocal Head A1 MP, Tokyo, Japan) with a 20× objective lens was used to acquire images.

2.6. Western Blot and Dot Blot

Cochleae from at least 4 animals/group were removed, kept on ice, and stored at -80 °C. Cold RIPA buffer (Cat. No. R 0278, Sigma Aldrich) was used to extract proteins from cochlear tissues, as described in (Pisani et al., 2025). Protein lysates obtained were loaded (70 µg) onto Tris-glycine polyacrylamide gels for electrophoretic separation and were then transferred onto nitrocellulose membranes at 330 mA for 2 hr at 4 °C in a solution containing 25 mM Tris, 192 mM glycine and 20 % methanol. Membranes were incubated for 1 hr with 5% skim milk in TBST and then incubated overnight at 4 °C with the following primary antibodies: NF-κB (1:1000, Cat. No. #8242, Cell Signaling Tech.), NLRP3 (1:1000; Cat. No. MA5-32255, Invitrogen), and Il-1b (1:500; Cat. No. sc-7884, Santa Cruz Tech.). For dot blots, 5 µl (3 µg/µl) of protein lysates were spotted into a TBST nitrocellulose membrane and processed as previously described (Paciello et al., 2024) to detect 3-Nitrotyrosine (3-NT) (1:1000; Cat. No. 05-233, Sigma-Aldrich), GSH (1:1000; Cat. No. ab19534, Abcam) and 4-HNE (Cat. No. HNE11-S, Alpha Diagnostic International).

After three rinses of 10 min in TBST, membranes were incubated for 1 h at RT with mouse or rabbit secondary antibodies HRP-conjugated (1:5000; Cat. No. 70765, Cell Signaling Tech.). Equal protein loading was set by using anti-GAPDH (1:10.000; Cat. No. 9485, Abcam). Semi-quantitative analyses were performed by using UVItec Cambridge Alliance.

2.7. Statistical analysis

Sample sizes were chosen with adequate statistical power (0.8) according to the results of prior pilot data sets or studies. Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used for statistical comparisons. For multiple group comparisons, analysis of variance (one-way ANOVA, two-way ANOVA or Kruskal-Wallis test) was employed, followed by Tukey correction or Dunn’s multiple comparisons test to compare means.

Post hoc comparisons were run only if F achieved $P < 0.05$ for interactions and there was no significant variance in homogeneity. The level of significance was set at 0.05. Results are presented as mean ± SEM.

3. Results

3.1. ANA and RA administration counteracts styrene-induced cochlear damage

To evaluate the efficacy of ANA and RA treatments, ABRs were recorded in all experimental groups at 7, 14, and 21 days after the onset of styrene exposure. No significant differences in auditory thresholds were observed between Ctrl and Ctrl-Sham groups, or between Styrene and Styrene-Sham groups (data not shown). At day 7, styrene-treated animals exhibited a mild, non-significant threshold shift of approximately 10 dB, while similar values were observed in the Styrene+ANA and Styrene+RA groups (Figure 2A). After 14 days of exposure, auditory thresholds further increased, with styrene-treated rats showing a threshold shift of about 20–25 dB (Figure 2B). Consistent with day 7 results, RA treatment produced a slight but non-significant improvement compared to the Styrene group (two-way ANOVA, $p > 0.05$). In contrast, ANA treatment exerted an earlier and more pronounced protective effect, significantly attenuating threshold shifts, particularly at middle frequencies (12 kHz: $p = 0.0046$; 16 kHz: $p = 0.0022$; 20 kHz: $p = 0.0194$; two-way ANOVA, Tukey's post hoc test).

By day 21, styrene ototoxicity reached its maximum effect, with threshold shifts of approximately 30–40 dB (Figure 2C).

At this time point, both RA and ANA showed a similar protective effect, decreasing threshold values of about 15-20 dB (Figure 2C; two-way ANOVA, Tukey's post-hoc test, Styrene *vs* Styrene+ANA: 6 kHz $p = 0.0012$, 12 kHz $p = 0.0005$, 16 and 20 kHz $p < 0.0001$, 24 kHz $p = 0.0026$, 32 kHz $p = 0.0005$; Styrene *vs* Styrene+RA: 6 kHz $p = 0.037$, 12 kHz $p = 0.02$, 16 kHz $p = 0.0026$, 20 kHz $p = 0.0002$, 24 and 32 kHz $p = 0.02$). Collectively, our functional data indicate that the antioxidant treatment showed slower otoprotection, that was evident from 21 days after styrene insult, whereas the

anti-inflammatory treatment showed an earlier protective effect, already evident after 14 days from styrene exposure.

At the morphological level, histological analyses in the organ of Corti confirm functional data. At the end of treatment (day 21), styrene exposure caused high HC percentage affecting mainly OHCs, mainly in the middle cochlear region, compared to the control group (Figure 2D-E; Kruskal-Wallis test, Ctrl *vs* Styrene $p < 0.0001$). Both ANA and RA treatments showed a protective effect against OHC damage, with about 20-30 % of cell survival compared to Styrene specimens (Figure 2E-J Kruskal-Wallis test, Styrene *vs* Styrene+RA $p = 0.024$; Styrene *vs* Styrene+ANA $p = 0.026$).

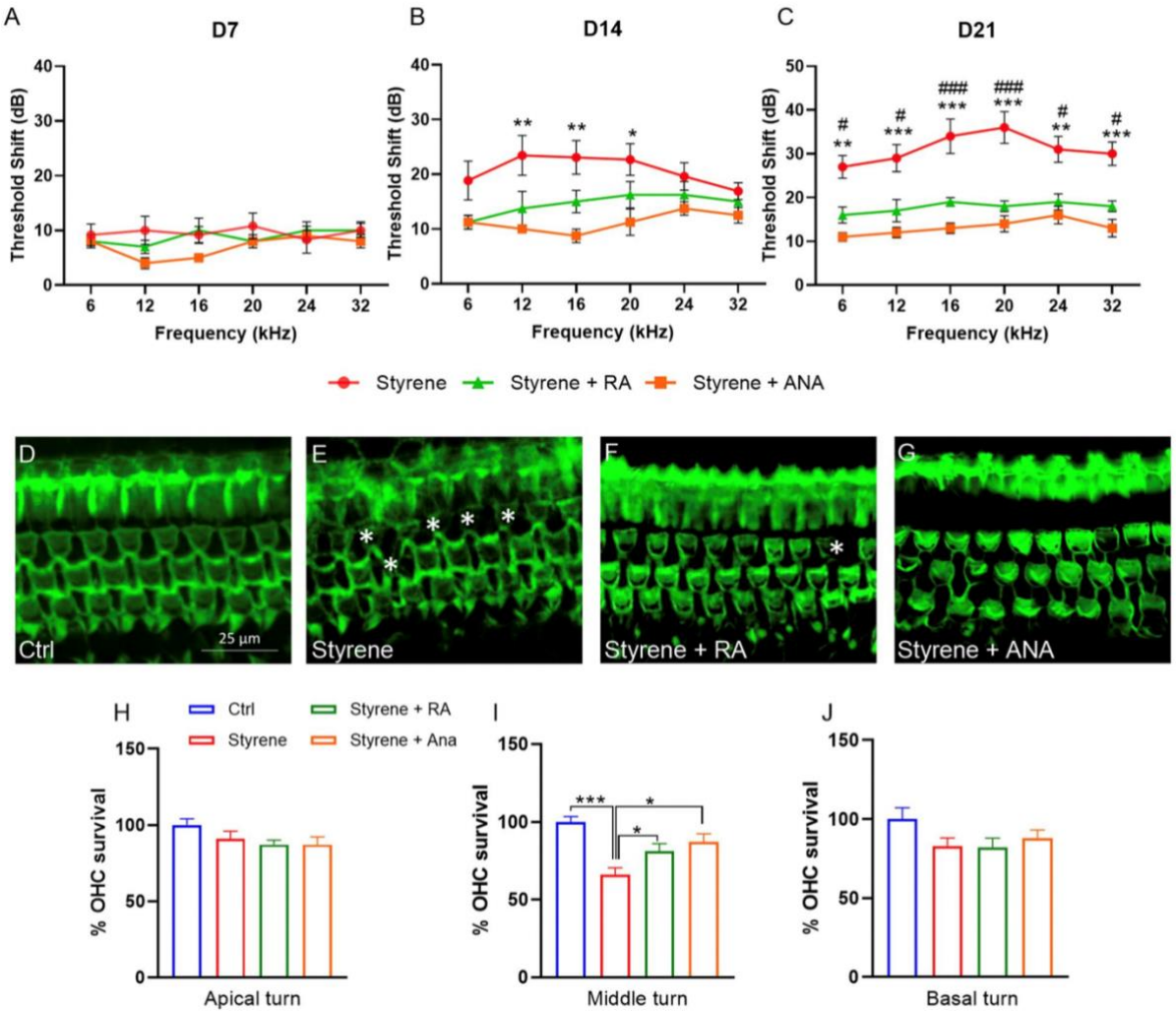


Figure 2. Styrene-induced cochlear damage. A-C: Graphs (mean +- SEM) showing auditory threshold shifts in animals treated with styrene (n = 13) and in animals co-treated with styrene and RA (Styrene + RA group; n = 13) or ANA (Styrene + ANA groups; n = 13). Functional evaluations were performed at 7 days (A), 14 days (B), and 21 days (C) from the onset of styrene treatment. Asterisks indicate significant differences between Styrene vs Styrene + ANA groups (*** $p < 0.001$; **$p < 0.01$). Hashtags indicate significant differences between Styrene vs Styrene + RA groups (### $p < 0.01$; #$p < 0.05$). D-G: Representative images of surface preparations of the organ of Corti. White asterisks mark missing cells, predominantly observed in the styrene-treated samples (E). Scale bar: 25 µm. H: Histograms (mean +- SEM) showing the percentage of OHC survival in all cochlear turns, normalized to control values. Asterisks denote significant differences among groups (** $p < 0.001$; **$p < 0.01$)

3.2. Efficacy of antioxidant and anti-inflammatory treatments against oxidative stress

Disruption of cochlear redox balance has been previously observed after styrene exposure (Chen et al., 2007; Fetoni et al., 2021). To assess the protective efficacy of RA and ANA treatments, we analyzed cochlear redox status by quantifying oxidative stress (ROS production and lipid peroxidation) and evaluating the endogenous antioxidant response through the levels of glutathionylated proteins. No significant differences were detected between Ctrl and Ctrl-Sham groups, or between Styrene and Styrene-Sham groups (data not shown). As expected, DHE staining revealed a marked increase in ROS production in cochlear cryosections of styrene-treated animals compared to controls, involving all cochlear substructures (Figure 3A–B). Both antioxidant (RA) and anti-inflammatory (ANA) treatments effectively reduced ROS accumulation, with ANA exerting a stronger protective effect, particularly in the organ of Corti and *stria vascularis* (Figure 3C–D).

Consistent with these findings, dot blot analysis revealed elevated levels of protein tyrosine nitration (3-NT), a marker of nitro-oxidative stress (Ischiropoulos and Beckman, 2003). NT levels were significantly higher in cochleae from styrene-treated animals compared to controls (Figure 3E–F; one-way ANOVA, $n = 5$; Ctrl vs. Styrene, $p < 0.0001$). Both RA and ANA significantly counteracted this effect (Styrene vs. Styrene+RA, $p < 0.0001$; Styrene vs. Styrene+ANA, $p = 0.0001$), with ANA restoring NT values to control levels (Ctrl vs. Styrene+ANA, $p > 0.05$). Lipid peroxidation, assessed by 4-hydroxynonenal (4-HNE) detection, was also significantly increased in cochlear lysates from styrene-exposed animals compared to controls (Figure 3G–H; one-way ANOVA, $n = 5$; Ctrl vs. Styrene, $p < 0.0001$). Both treatments significantly reduced 4-HNE levels (Styrene vs. Styrene+RA, $p = 0.0008$; Styrene vs. Styrene+ANA, $p < 0.0001$), with ANA again showing the strongest effect (Ctrl vs. Styrene+ANA, $p > 0.05$). To further investigate the endogenous antioxidant response,

we measured the levels of glutathionylated proteins, a marker reflecting the GSSG/GSH redox balance. A pronounced decrease in glutathionylated proteins was detected in styrene-treated cochleae compared to controls (Figure 3I–J; one-way ANOVA, $n = 5$; Ctrl vs. Styrene, $p < 0.0001$). Both RA and ANA treatments significantly increased glutathionylated protein levels following styrene exposure (Styrene vs. Styrene+RA, $p = 0.002$; Styrene vs. Styrene+ANA, $p < 0.0001$).

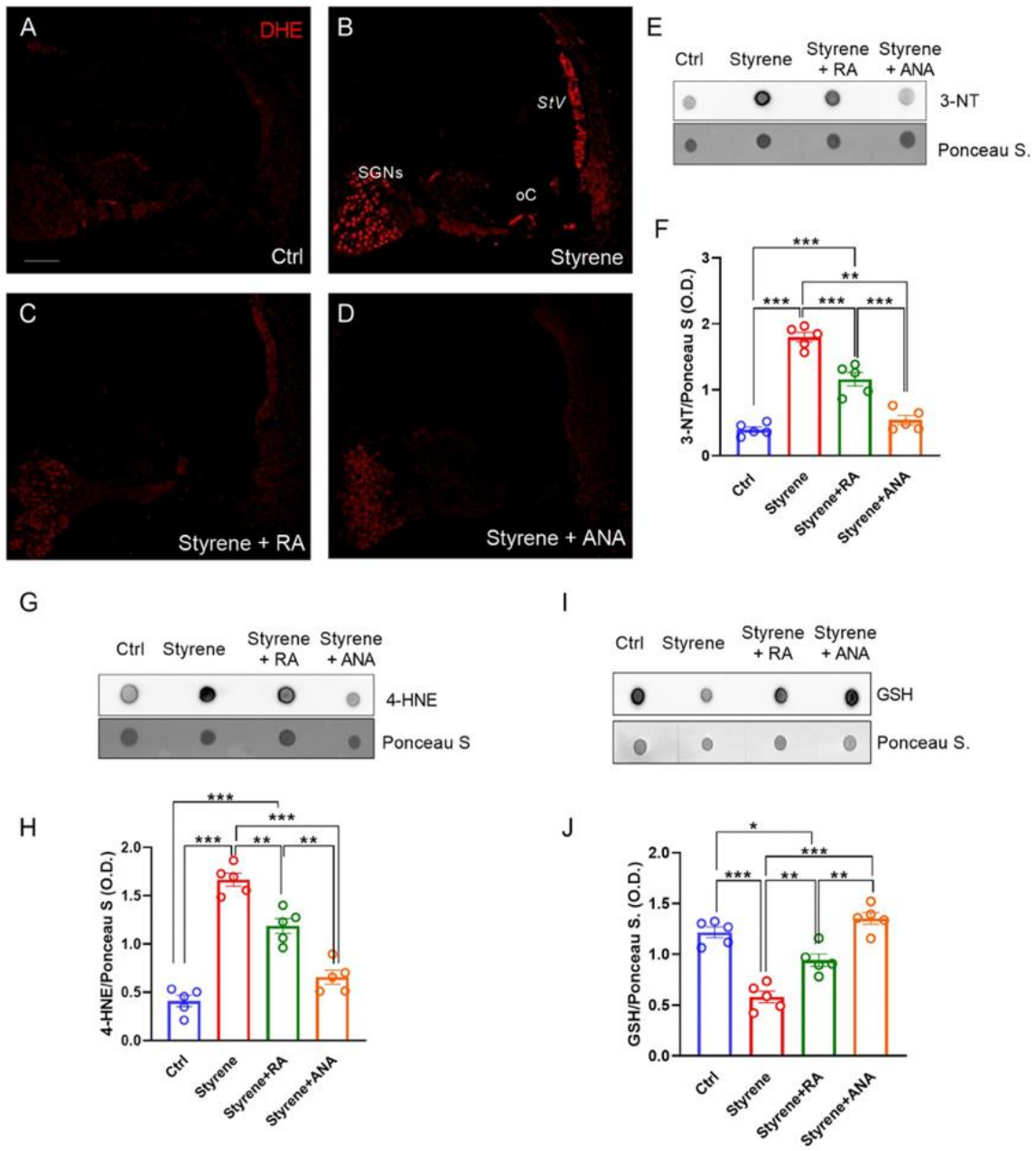


Figure 3. **Protective effects of RA and ANA against cochlear redox imbalance.** A-D: Representative images of cochlear cryosections (middle cochlear turn). Red fluorescence indicates ROS levels detected by the DHE assay. Scale bar: 50 µm. (E, G, I) Representative dot blots showing 3-nitrotyrosine (3-NT; marker of protein nitration) (E), 4-hydroxynonenal (4-HNE; marker of lipid peroxidation) (G), and glutathionylated proteins (GSH) (I) in all experimental groups. (F, H, J) Histograms (mean ± SEM) showing normalized optical density (O.D.) values with Ponceau S staining (n = 5 animals/group). Asterisks indicate statistically significant differences among groups (***p < 0.001; **p < 0.01; *p < 0.05).

3.3. Efficacy of antioxidant and anti-inflammatory treatments against cochlear inflammation

Once evaluating oxidative stress, we also analyzed inflammatory mechanisms induced by styrene and the ability effects of both RA and ANA to counteract inflammatory damage.

We first investigated NF-κB levels, a transcription factor responsible for transcriptional induction of pro-inflammatory cytokines and chemokines (Liu et al., 2017).Western blot data showed decreased NF-kB levels in animals treated with RA and ANA, compared to styrene (Figure 4A-B; Kruskal-Wallis test, n = 4; Styrene *vs* Styrene+RA p = 0.007; Styrene *vs* Styrene+ANA p = 0.02). Considering that NF-κB also has a role in regulating the activation of inflammasomes (Sutterwala et al., 2014). and that we previously documented that styrene can activate NLRP3 inflammasome in hippocampal tissues (unpublished results) we also evaluated the ability of RA and ANA in counteracting NLRP3 activation. Our western blot analyses showed elevated NLRP3 levels in styrene-treated samples compared to controls (Figure 4C-D Kruskal-Wallis test, n = 4; Ctrl *vs* Styrene p = 0.002). Interestingly, similarly to what was observed for NF-κB, NLRP3 levels were significantly reduced by RA and ANA administration (Figure 4C-D; Kruskal-Wallis test; Styrene *vs* Styrene+RA p = 0.04; Styrene *vs* Styrene+ANA p = 0.03).

In addition, we also evaluated IL-1β levels, by using both western blot (Figure 4E-F) and immunofluorescence (Figure 4G) analyses. IL-1 β was significantly higher in styrene-treated animals compared to controls, as documented by western blot (Figure 4E-F; Kruskal-Wallis test, n = 4; Ctrl *vs* Styrene p = 0.02) and immunofluorescence (Figure 4g1-g2), showing an increased cytokine expression in the SGNs, organ of Corti and stria vascularis. Treatment with RA showed a slight but significant effect in reducing IL-1b levels (Figure 4E-F; Kruskal-Wallis test, n = 4; Styrene *vs* Styrene+RA p = 0.04), with a marked fluorescence intensity still observed mainly in the stria

vascularis (Figure 4 g3). On the other hand, levels of IL-1β were found markedly lower in Styrene+ANA than in the Styrene group (Figure 4E-G; Kruskal-Wallis test, n = 4; Styrene *vs* Styrene+ANA p = 0.004). Notably, this result is consistent with the fact that ANA is the recombinant form of the naturally occurring IL-1 receptor antagonist.

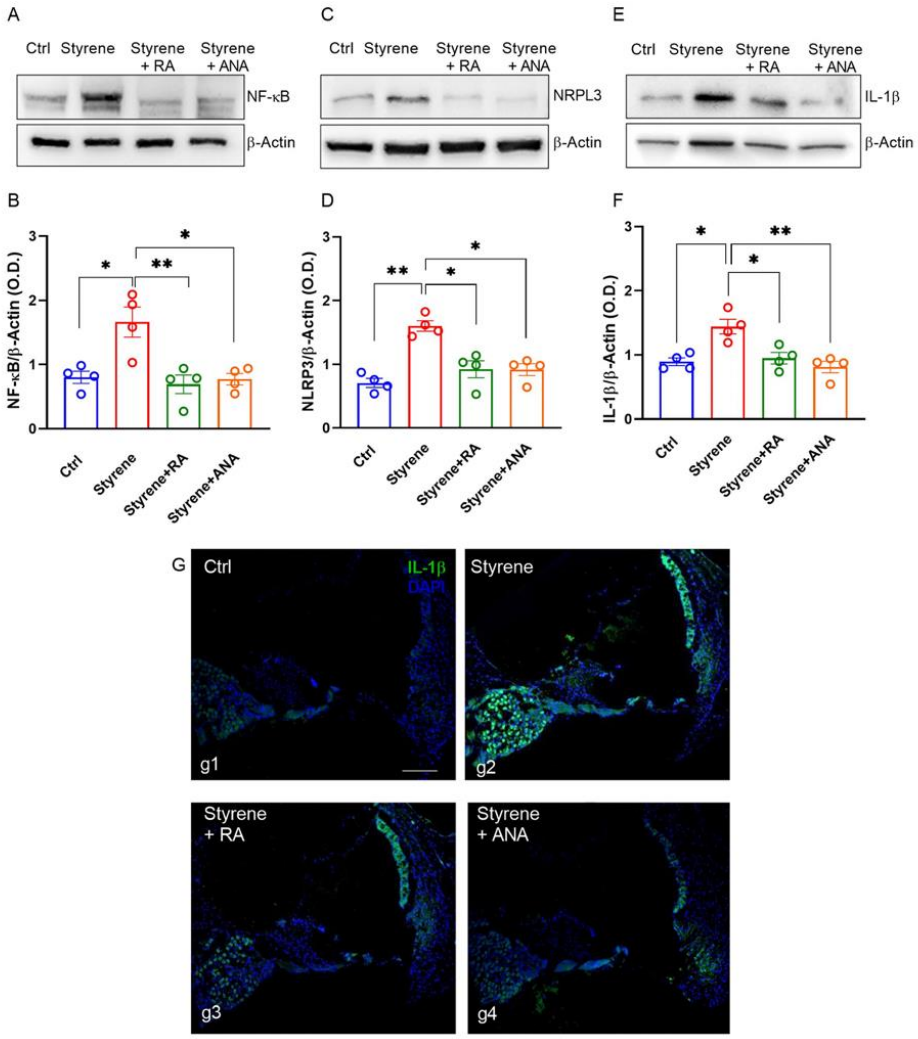


Figure 4. Cochlear inflammation and inflammasome activation. A,C,E: Representative western blot showing NF-kB (A), NLRP3 (C) and IL-1 β (E) levels in cochlear samples from all experimental groups. B, D, F, I: Histograms (mean +- SEM) showing optical density values (O.D.) normalized to β -actin (n = 4 animals/group). Asterisks indicate significant differences among groups (*** $p < 0.001$; **$p < 0.01$; *$p < 0.05$). G: Representative confocal images of cochlear cryosections stained with IL1- β (green fluorescence) and with DAPI (Blue fluorescence). Scale bar: 50 μm.

4. Discussion

In this study, we investigated the protective effects of two compounds, rosmarinic acid (RA), a phenolic antioxidant, and anakinra (ANA), an anti-inflammatory agent, against styrene-induced ototoxicity. Our results demonstrate that both treatments reduced NLRP3 inflammasome expression, indicating their capacity to modulate inflammatory signaling. However, our results demonstrate that ANA treatment exerts the strongest protective effect by counteracting the styrene-induced impairment of auditory function at an early stage. Blocking oxidative stress through an antioxidant treatment led to decreased inflammasome activation and reduced levels of the redox-sensitive transcription factor NF-κB and IL-1β. On the other hand, blocking inflammation was more effective, allowing a powerful effect in modulating pro-inflammatory cytokines. Several studies reported a key role of inflammatory factors in mediating cochlear damage induced by exogenous or endogenous factors, including ototoxic drugs, noise exposure, or immune challenges (Fujioka et al., 2006; Ihler et al., 2014; Kaur et al., 2011; Tan et al., 2016). We previously documented that styrene affects auditory function by causing oxidative stress and triggering the inflammatory response, with increased expression of the main inflammatory agents, including NF-κB, TNF-α, and IL-1β (Fetoni et al., 2021), and microglial activation (Paciello et al., 2024). Here, we studied the mechanism linking inflammation and ROS production by focusing on inflammasomes. The term ‘‘inflammasome’’ refers to the assembly of an intracellular multiprotein complex as a molecular platform that triggers an inflammatory response regulating the maturation and secretion of pro-inflammatory cytokines, such as IL-1β (Schroder and Tschopp, 2010). Among the different types of inflammasomes, NLRP3 is activated by a broad spectrum of stimuli, including pathogens, damaged or dead cells, and toxic compounds (McKee and Coll, 2020; Wei et al., 2021). Our data, for the first time, demonstrate an increase in NLRP3 levels in animals treated with styrene, suggesting a role of NLRP3 linking inflammation and oxidative stress in styrene-

induced cochlear damage.

To find a cause-and-effect link among increased inflammatory markers, NLRP3 activation, and increased oxidative stress, we compared the efficacy of antioxidant and anti-inflammatory treatments, thus evaluating the effect of scavenging ROS or counteracting inflammation on cochlear functions. As an anti-inflammatory drug, we chose ANA, a recombinant form of IL-1R we previously demonstrated to have a protective effect in a model of noise-induced hearing loss (Paciello et al., 2021b). Our data show that ANA has an early protective effect on styrene-induced hearing loss observed on day 14 from ototoxic exposure. Additionally, western blot and immunofluorescence analyses show a marked and significant decrease in IL-1β cochlear expression after ANA treatment, according to the well-known antagonistic effect of ANA on IL-1 receptor. The reduction of IL-1β probably contributed to reducing NF-κB levels, according to literature data showing that IL-1 activation facilitates NF-κB translocation to the nucleus, thus leading to the expression of downstream pro-inflammatory genes (Hayden and Ghosh, 2008). Moreover, several studies documented the efficacy of ANA treatment in decreasing both IL-1β and NF-κB in various disease models (Goncalves et al., 2015; Pignataro et al., 2022; Zhuang et al., 2016).

To target oxidative stress, we used RA, a phenolic compound, one of the major components of rosemary with remarkable biological effects, including antioxidant capacity (Tepe, 2008). We previously reported the otoprotective effect of RA treatment in a model of noise-induced hearing loss (Fetoni et al., 2018; Fetoni et al., 2015). Here, RA administration showed protective effects, although slower than ANA, against styrene damage on day 21 from the onset of treatment, attenuating hearing loss induced by styrene of about 25 dB. The NLRP3 decreased levels were observed both in RA and ANA-treated samples, indicating that blocking oxidative stress can result in blocking inflammasome activation induced by styrene, according to previous evidence showing the critical role of ROS production in triggering NLRP3 activation (Jin and Flavell,

2010; Zhong et al., 2013).

Namely, our data support the hypothesis of crosstalk between oxidative and inflammatory damage, in which NLRP3 inflammasome activation plays a key role. We hypothesize that styrene causes increased ROS production, leading to NLRP3 activity and inflammation. Treatment with RA can decrease oxidative stress, leading to reduced inflammation through diminished NLRP3 activity. On the other hand, ANA can act on two opposite sites: decreasing inflammation via the NLRP3 pathway and targeting IL-1β through the antagonizing effect on its receptor (Figure 5).

It should be noted that we employed a chronic model of styrene exposure to replicate conditions similar to those encountered in occupational settings and to propose a potential therapeutic strategy for preventing or mitigating the effects of repeated exposure to organic solvents in workers. Moreover, considering that the ototoxic effects of several volatile compounds, such as toluene, ethylbenzene, and xylene, share similar molecular mechanisms, involving disruption of cochlear redox balance (Fechter et al., 2007; Liu et al., 2024; Sliwinska-Kowalska et al., 2007) our findings may also be relevant to other ototoxic agents.

From a translational perspective, our results suggest that oral administration of the antioxidant RA, as well as the use of the anti-inflammatory drug ANA, both of which have been evaluated in several clinical studies (Noguchi-Shinohara et al., 2023; Osakabe et al., 2004; Park et al., 2023) could represent a promising therapeutic approach to counteract styrene-induced ototoxicity. Undoubtedly, it would also be of interest to evaluate the protective effects of both RA and ANA following acute exposure, such as to high doses of styrene, by thoroughly investigating their potential therapeutic benefits and clinical applications. In summary, our findings reveal that inflammasome activation plays a pivotal role in styrene-induced cochlear injury, linking oxidative stress to inflammation. Targeting these pathways through antioxidant and anti-inflammatory treatments effectively preserves auditory function, with ANA providing the earliest and most robust protection.

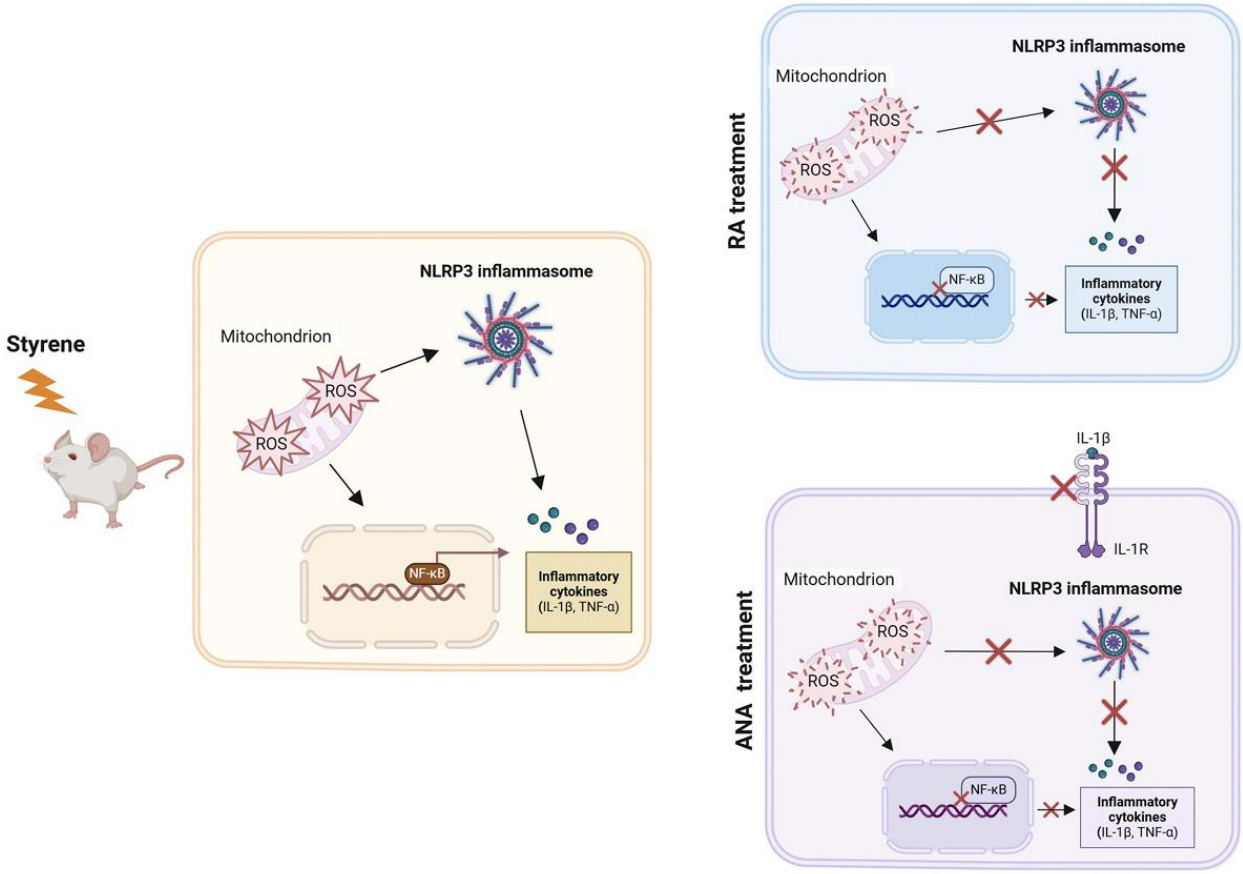


Figure 5. Molecular mechanisms of styrene cochlear damage and otoprotection. A: Styrene induces cochlear damage by increasing ROS production, which subsequently activates NLRP3 inflammasome and NF-κB transcription activity, leading to elevated pro-inflammatory cytokine production. B: The protective effect of RA reduces ROS production, thereby preventing NLRP3 activation and the upregulation of inflammatory markers. C: The molecular mechanisms of Anakinra acts on two fronts: it reduces oxidative stress and suppresses the inflammasome pathway while directly blocking IL-1β by antagonizing its receptor (IL-1R). Created with Biorender.

CHAPTER 3.

Oxidative Stress and Inflammation in Age-Related Hearing Loss: Temporal Dynamics in Cochlear Degeneration of C57BL/6 Mice

1. Introduction

ARHL is a progressive decline in auditory sensitivity associated with the degeneration of cochlear receptors. Its onset is multifactorial, involving genetic predisposition, environmental risk factors such as chronic noise exposure and ototoxic drugs, as well as comorbid conditions (Schuknecht, 1964; Willott et al., 1991). Clinically, ARHL typically begins in the high-frequency range, which is among the first to deteriorate with aging, and then extends to lower frequencies. Although widely studied, the molecular mechanisms driving presbycusis remain complex and not fully elucidated.

One major contributor to cochlear aging is the progressive accumulation of oxidative damage caused by reactive oxygen species (ROS) (Liguori et al., 2018). This process promotes mitochondrial dysfunction, reduced energy production, and tissue degeneration. Under physiological conditions, antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione S-transferase (GST), and glutathione peroxidase (GPX) maintain redox balance (Gutteridge and Halliwell, 1992). However, with aging, the imbalance between ROS production and antioxidant defenses leads to oxidative stress, which not only exacerbates cellular injury but also triggers inflammatory pathways. ROS activate nuclear factor kappa B (NF-κB) and other redox-sensitive transcription factors, enhancing the expression of pro-inflammatory cytokines including interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β) (Paciello et al., 2021a). This persistent low-grade inflammation, or "inflammaging," contributes to cellular senescence and tissue dysfunction (Zuo et al., 2019). Nevertheless, the precise timing and causal interplay

between oxidative stress and inflammation in the aging cochlea remain unclear.

C57BL/6 mice are a widely used model of ARHL, as they display a predictable progression of hearing loss that starts with high frequencies and gradually extends across the auditory spectrum (Fetoni et al., 2011; Fetoni et al., 2022b). These mice carry genetic mutations predisposing them to early-onset ARHL, making them particularly suitable for exploring the molecular mechanisms of presbycusis. Their phenotype shares several hallmarks with human ARHL, including synaptic dysfunction, hair cell loss, and impaired neural transmission.

In this study, we employed C57BL/6 mice to investigate the contribution of oxidative stress and inflammation to cochlear degeneration. By examining these processes across different ages, we aimed to define the kinetics of damage and identify a potential "therapeutic window" for interventions targeting oxidative and inflammatory pathways, to mitigate ARHL and preserve auditory function in aging populations.

2. Materials and methods

2.1. Animals

Male adult C57BL/6 mice (Charles River Laboratories, Lecco, Italy) were used. A total of 15 animals aged 3, 6, and 12 months (M) were studied. Mice were housed 3–5 per cage with ad libitum access to food (Mucedola 4RF21, Italy) and water, under controlled temperature (22–23 °C), constant humidity (60 ± 5%), and a 12-h light/dark cycle. All procedures were made to minimize animal suffering and to reduce their number, in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC). All procedures were performed in compliance with the Laboratory of Animal Care and Use Committee of the Catholic University, School of Medicine of Rome, and were approved by the Italian Department of Health (Ministero della Salute, Prot. 1F295.169.EXT.112).

2.2. Auditory Brainstem Responses (ABRs)

Auditory function was evaluated by ABR recordings at low (6 kHz), mid (12, 16, 20 kHz), and high (24, 32 kHz) frequencies at 3, 6, and 12 M. Mice were anesthetized (ketamine 35 mg/kg, medetomidine-domitor 0.25 mg/kg) and placed in an anechoic room. As described previously (Paciello et al., 2024; Pisani et al., 2025). tone bursts were presented monaurally in an open field via a horn tweeter (Tucker-Davis Technologies, TDT). ABRs were recorded using three subcutaneous stainless steel electrodes (active: posterior to the tested pinna; reference: vertex; ground: contralateral pinna). Auditory stimuli were generated and ABRs recorded using a PC-controlled Tucker-Davis Technologies (TDT) System 3 (Alachua, FL, United States) with real-time digital signal processing. Tone bursts of pure tones from 6 to 32 kHz (1 ms rise/fall time, 10 ms total duration, 20/s repetition rate) were presented monaurally. The responses were filtered, digitized, and averaged across 512 discrete samples at each frequency-level combination. Wave I and wave II amplitude–intensity (A–I) and latency–intensity (L–I) functions were analyzed to assess auditory nerve fiber integrity, following Fetoni et al. (Fetoni et al., 2013; Fetoni et al., 2021).

2.3. Morphological analysis: H&E staining

Histological changes in SGNs were assessed on cochlear cryosections (12 μm) obtained as previously described (Pisani et al., 2025). Cochleae were fixed in 4% paraformaldehyde (PBS, pH 7.5, 4 °C), decalcified for 15 days in 10% EDTA, cryoprotected in 30% sucrose for 48 h, embedded in OCT, and sectioned using a Cryostat CM1950 (SLEE). Sections were stained with hematoxylin and eosin (H&E; Abcam, ab245880) using a standard protocol (4-5 min hematoxylin, 45 s eosin) and mounted with Entellan® (Merck, Cat. No. 107960). The cross-sectional area of Rosenthal's canal in the middle turn was measured with NIH Image. Viable SGNs were

defined by a clear, round nucleus and homogeneous cytoplasm. SGN density (cells/mm²) was calculated within a 500 × 500 μm area using NIH ImageJ 1.43u. Counts were performed by two independent, blinded investigators. Neuronal survival was expressed as a percentage relative to 3 M controls.

2.4. Western blot and dot blot analyses

Cochlear tissues from 3 animals/groups were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail). Homogenates were sonicated (10 s on/20 s off, 3 cycles; Diagenode Bioruptor Standard) and centrifuged at 22,000×g and 4°C. Equal amounts of protein were boiled and resolved using SDS-PAGE, as previously described (Fetoni et al., 2021; Paciello et al., 2021a; Pisani et al., 2025). For dot blotting, 5 μl of lysate (5 μg/μl) was spotted onto TBST-prewetted nitrocellulose membranes (Paciello et al., 2021a; Pisani et al., 2025). Membranes were incubated overnight with primary antibodies against 3-nitrotyrosine (3-NT, 1:1000; Sigma-Aldrich, Cat. No. 05-233), 4-HNE (Alpha Diagnostic International, Cat. No. HNE11-S), and TNF-α (1:1000; Santa Cruz Tech., Cat. No. sc-52746), followed by HRP-conjugated secondary antibodies (1:5000; Cell Signaling Tech., Cat. No. 70765) for 1 h at RT. After TBST washes, signals were visualized using chemiluminescent substrates (Cyanagen, Bologna, Italy) and quantified with UVItec Cambridge Alliance. Equal protein loading was verified using GAPDH (SDS-PAGE) and Ponceau S (dot blot).

2.5. Statistical analysis

Sample size was determined by power analysis to achieve 80% power at $\alpha = 0.05$ based on pilot data and previous work. Data were analyzed blinded using Prism 8.0 (GraphPad Software, San Diego, CA, USA). One- or two-way ANOVA followed by

Tukey's post hoc test was applied when appropriate. Post hoc tests were performed only when ANOVA showed significant effects ($P < 0.05$) and variance homogeneity was verified. Results are presented as mean ± SEM, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Auditory dysfunction with advancing age

To evaluate the effects of aging on auditory function, ABRs were recorded in C57BL/6 mice at 3, 6, and 12 months (M) (Figure 1). Mean ABR thresholds (Figure 2A) showed a progressive hearing loss that initially affected high frequencies at 6 M and extended to all frequencies by 12 M. At 3 M, thresholds ranged between 30-40 dB across all frequencies. At 6 M, thresholds increased to ~ 50 dB at low/mid frequencies and ~ 60 dB at high frequencies, consistent with early presbycusis. By 12 M, thresholds exceeded 60 dB across the spectrum, confirming the progressive ARHL phenotype in this strain. To further characterize functional decline, we analyzed ABR waves I and II at 16 kHz. Wave I amplitude, reflecting inner hair cell–auditory nerve synaptic integrity, was significantly reduced in 6- and 12-month-old mice (Figure 2C), while latency increased with age (Figure 2D), suggesting cochlear synaptopathy and impaired neural conduction. Similarly, wave II amplitude declined significantly (Figure 2E), accompanied by increased latency (Figure 2F), indicating reduced afferent input and delayed signal processing in the cochlear nucleus.

Together, these changes demonstrate age-related neural dysfunction, characterized by reduced neural synchrony, impaired transmission efficiency, and progressive loss of functional auditory units.

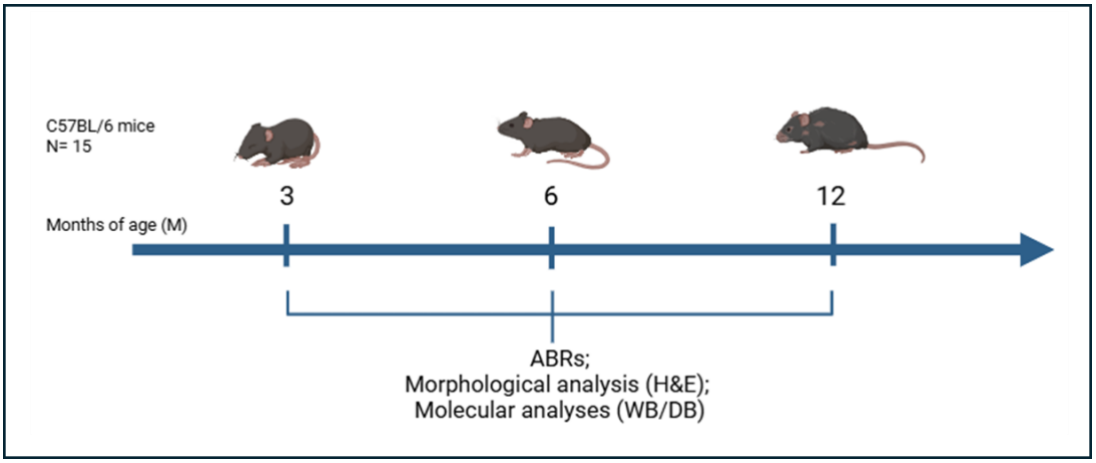


Figure 1. Experimental design and time schedule

Schematic representation of the experimental design. C57BL/6 mice of 3, 6, and 12 months (M) of age were used in this study. Auditory function was assessed during aging. Molecular analyses (WB/DB) were performed to analyze the modulation of oxidative stress and inflammation during aging. ABR: Auditory Brainstem Responses; H&E: Hematoxylin and Eosin; WB: Western Blot; DB: Dot Blot.

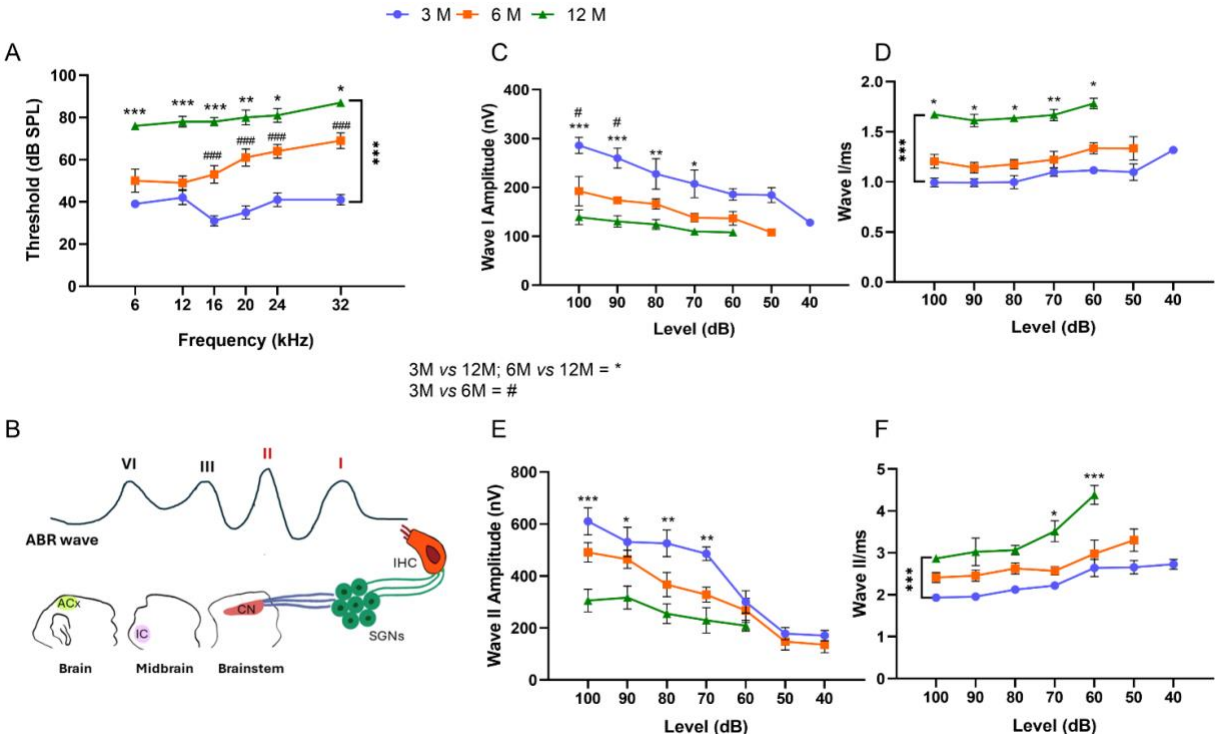


Figure 2. Age-related decline in auditory function in C57BL/6 mice

A: Graph shows ABR threshold values (means ± SEM) in C57BL/6 mice of 3, 6, and 12 months of age (M). A progressive worsening of auditory threshold was observed in mice during physiological aging. B: Schematic representation of ABR waves. **C, E**: Amplitude-intensity curves (mean ± SEM) showing a decreased amplitude of wave I (C) and wave II (E) in mice of 6 and 12 M at 16 kHz compared with mice of 3 M. **D, F**: L–I curve (mean ± SEM) of the P1 (D) and P2 (F) waveform (16 kHz) showing increased latency of waves during physiological aging. Asterisks indicate significant differences among groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) from two-way (A) and one-way ANOVA (B,C) with repeated measures. IHC: Inner Hair Cell; SGNs: Spiral Ganglion Neurons; CN: Cochlear Nucleus; IC: Inferior Colliculus; ACx: Auditory Cortex.

3.2. Progressive neuronal cell loss with advancing aging

To investigate the mechanisms underlying the ARHL phenotype, we assessed SGNs on cochlear cryosections stained with H&E. At 6M, we observed a moderate but significant reduction in SGN density (about 20%) compared to 3M mice (Figure 3A,B,D; one-way ANOVA, Tukey's *post-hoc* test, n = 3; p = 0.0128). At 12 M, a more substantial decline in neuronal survival was observed of about 80% compared to 3 M animals (Figure 3A,C,D; one-way Anova, Tukey's *post-hoc* test, n = 3; $p < 0.0001$). These results demonstrate a progressive degeneration of SGNs with age, with 12M representing a critical stage of marked neuronal loss.

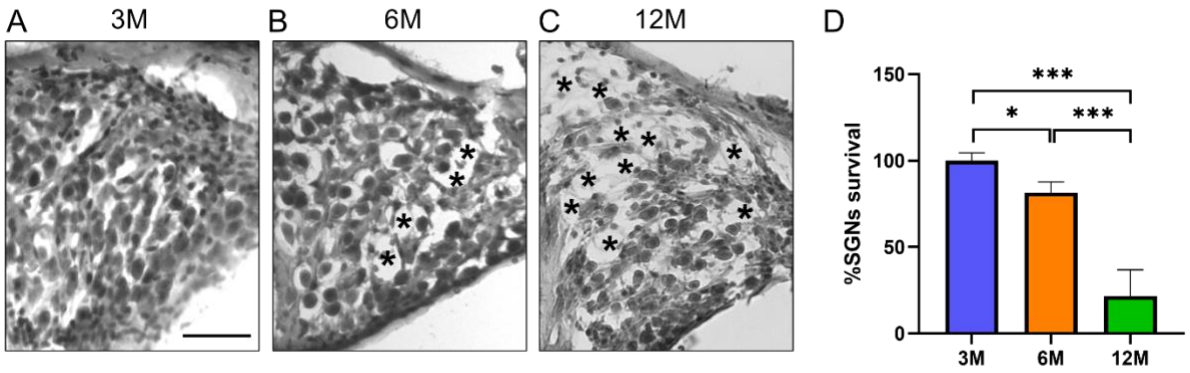


Figure 3. Age-related neuronal loss in C57BL/6 mice
A-C: Representative images of SGN cryosections at middle cochlear turn level. Black asterisks indicate a neuronal loss. D: Graph (means ± SEM) shows the percentage of SGN survival in C57BL/6 mice of 3, 6, and 12 months of age (M), normalized to mice of 3 months of age. A progressive worsening of SGN viability was observed in mice during physiological aging. Asterisks indicate significant differences among groups (*$p < 0.05$; ***$p < 0.001$) from one-way ANOVA. Scale bar: 50 µm.

3.3. Progressive cochlear oxidative damage and inflammation in age-related cochlear degeneration

Cochlear oxidative damage is a hallmark of age-related inner ear degeneration, yet its temporal dynamics remain unclear. Using dot blot analysis, we measured markers of oxidative stress and lipid peroxidation at different ages. A significant increase in 3-NT and in 4-HNE was detected at 6 and 12 months compared to 3M (Figure 4A,B,D,E; 3-NT: one-way Anova, Tukey's *post-hoc* test, n = 3; 3M *vs* 6M $p = 0.0347$; 3M *vs* 12M $p = 0.0013$; 6M *vs* 12M $p = 0.0381$; 4-HNE: one-way Anova, Tukey's *post-hoc* test, n =3; 3M *vs* 6M $p = 0.0099$; 3M *vs* 12M $p < 0.0001$; 6M *vs* 12M $p < 0.0001$).

Since inflammation often follows oxidative injury (Paciello et al., 2021a), we also examined TNF-α expression. A significant and progressive increase in TNF-α was observed at 6 and 12M compared to 3M (Figure 4C,F; one-way Anova, Tukey's post-hoc test, n = 3; $p < 0.0001$ for all comparisons).

These findings indicate that both oxidative stress and inflammation are evident in the cochlea of C57BL/6 mice starting from 6 M, when the early decline of auditory thresholds at high frequencies starts to be observed.

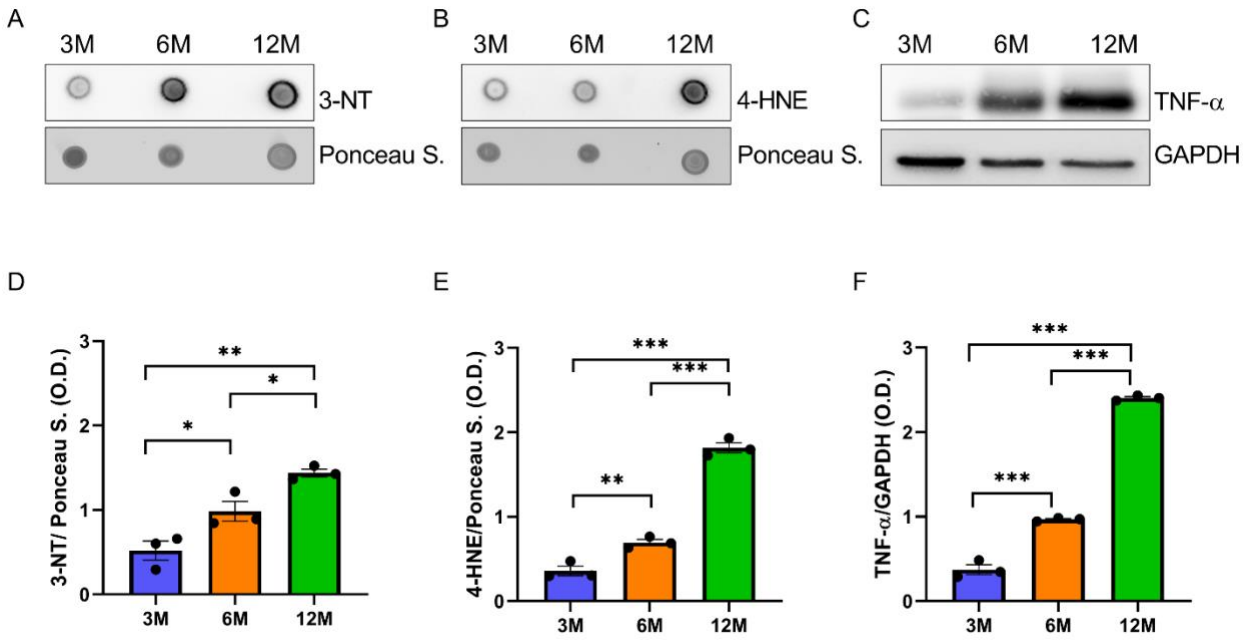


Figure 4. Increase of oxidative stress and inflammation in aging cochlea
A, B: Representative dot blots showing a progressive increase of protein tyrosine nitration (3-NT, n = 3 mice for each group) and lipid peroxidation (4-HNE, n = 3 animals for each group) in cochleae of mice during aging. Ponceau S staining confirmed equal protein loading. C: Western blot images showing elevated levels of TNF-a indicating an increase of inflammatory processes in cochleae of mice during physiological aging (n = 3 animals for each group). Asterisks indicate significant differences among groups (*p < 0.05; **p < 0.01; ***p < 0.001) from one-way ANOVA with repeated measures.

4. Discussion

This study investigated functional and molecular alterations associated with ARHL in C57BL/6 mice, focusing on the kinetics of oxidative and inflammatory damage. We found that aging is accompanied by a progressive decline in auditory function, as shown by elevated auditory thresholds, reduced neural transmission efficiency, SGN loss, and increased oxidative and inflammatory responses, all emerging at 6 M.

The progressive rise of ABR thresholds across frequencies indicated that hearing loss first affected the high-frequency range and later extended to lower frequencies, closely

resembling the clinical progression of presbycusis in humans (Wang and Puel, 2020). These findings reinforce the validity of C57BL/6 mice as a model of ARHL (Fetoni et al., 2011; Fetoni et al., 2022b). Genetic factors, such as the $Cdh23^{753A}$ (Ahl) mutation, which affects stereocilia integrity (Brown et al., 2008), together with metabolic alterations like aberrant cochlear glycogen accumulation compared to CBA mice (Willott et al., 1991), contribute to early-onset ARHL in this strain, reproducing the phenotypic features described by Schuknecht (Schuknecht, 1964; Schuknecht and Gacek, 1993).

Consistent with previous studies, ABR testing revealed elevated thresholds at high frequencies (20-32 kHz) from 6 M, progressing to profound hearing loss (>80 dB) at 12 M across all frequencies (Fetoni et al., 2011; Willott et al., 1993). Waveform analysis revealed reduced amplitudes and increased latencies of ABR waves I and II from 6 M onward, indicating synaptic degeneration, SGN dysfunction, and impaired central processing. This functional decline is consistent with reports of cochlear synaptopathy and ribbon synapse loss as key contributors to auditory nerve dysfunction in aging (Steenken et al., 2021). These electrophysiological changes also parallel clinical difficulties in speech discrimination in noisy environments, which rely on precise and synchronous auditory processing (Pronk et al., 2013). Histological analysis confirmed progressive SGN loss, most pronounced at 12 M, consistent with advanced neural degeneration. At the molecular level, elevated 3-NT and 4-HNE levels indicate nitro-oxidative stress and lipid peroxidation as major drivers of cochlear pathology from 6 M onward. Protein nitration can either reduce or enhance enzymatic activity, as shown for MnSOD, glutathione reductase, cytochrome c, and others (Cassina et al., 2000), thereby amplifying dysfunction within auditory tissues. In parallel, the rise in TNF-α highlights the contribution of inflammation, supporting the view that oxidative stress and inflammation act synergistically. TNF-α promotes ROS production, creating a vicious cycle that sustains cochlear degeneration (Li et al., 2023). The simultaneous onset of oxidative and inflammatory markers at 6 M suggests that these processes are

tightly linked and represent a pivotal turning point in ARHL. This convergence of cochlear stress, auditory nerve impairment, and central processing deficits identifies key pathogenetic mechanisms that could be targeted therapeutically.

Taken together, our findings suggest that 6 M represents a critical window for early intervention. Targeting oxidative stress and inflammation at this stage could be effective in preserving auditory function. Pharmacological modulation of these pathways holds promise for delaying or preventing cochlear degeneration and ARHL, but further studies are required to clarify the molecular crosstalk between oxidative and inflammatory signaling and to optimize therapeutic strategies and timing.

CHAPTER 4.

Results and observations on the rehabilitative effect of hearing aids on cognitive functions after 6 months of use in elderly patients.

1.Introduction

Speech comprehension difficulties associated with ARHL contribute to social isolation, a known risk factor for cognitive decline. ARHL may initiate a cascade in which hearing loss accelerates cognitive deterioration, while reduced social engagement further exacerbates both. Interventions such as hearing aid use may help interrupt this cycle. Previous studies have shown that hearing loss increases the risk of incident dementia and depressive symptoms (Conceicao Santos de Oliveira et al., 2023; Lin et al., 2011; Shukla et al., 2021). More recent evidence indicates that hearing loss interventions combining hearing aids and counselling can improve communicative function in elderly patients (Sanchez et al., 2024). The 2024 *Lancet* report on dementia identifies hearing loss as a modifiable risk factor and hearing aid use as a protective intervention (Livingston et al., 2024).

Hearing loss is a major contributor to the overall disease burden in Europe, a problem expected to worsen with the continent's aging population (Bridget, 2019). Reported barriers to hearing aid use include perceived lack of need, discomfort, limited restoration of hearing, difficulty in handling and maintenance (especially among older adults), and social stigma. As a result, a large portion of the hearing-impaired individuals remain untreated, leading to significant economic and social costs. Greater knowledge and awareness of the potential benefit of hearing aids in the prevention of cognitive decline may improve hearing aid adoption in ARHL and thus contribute to overall public health.

How hearing aids affect elderly ARHL patients' cognition remains poorly understood,

especially in the short term (within six months of hearing aid use). The impact of the use of hearing aids on elderly cognitive function may be elucidated via the use of questionnaires that assess cognitive function. The present study aims to evaluate the relationship between hearing loss and the results of the cognitive tests Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA) in a sample of hearing-impaired elderly individuals experiencing hearing aid use for the first time (defined as new users). Additionally, the Hospital Anxiety and Depression Scale (HADS) is used to evaluate the mental state. The objective is to compare the scores obtained in these tests at three time points within a 6-month period of rehabilitation (0, 3, and 6 months) with hearing aids in order to analyze any potential improvement in cognitive function associated with hearing aid use in new users.

2.Materials and methods

2.1. Diagnostic of presbycusis: Pure-Tone Threshold Audiometry (PTA)

All the ARHL participants underwent audiometry at 0, 3, and 6 months from the first fitting of hearing aids. Pure tone audiometry was performed in a sound booth through headphones (frequency range 125 Hz–8000 Hz). Measurement was performed through air conduction. The procedure started with a clear audible stimulus; once a reliable response was obtained, the intensity was decreased in 10 dB steps until the tone was no longer perceived and then increased in 5 dB steps until the tone was heard again. The hearing threshold was defined as the lowest intensity level at which tones were perceived. The final PTA value was computed as the mean of the results from 500Hz, 1000Hz, 2000Hz, and 4000Hz thresholds.

Grade of hearing loss severity per participant was derived from the final PTA value. The classification schema goes as follows: Normal “0” (-10 to 15 dB HL), slight “1”

(16-25 dB HL), mild “2” (26-40 dB HL), moderate “3” (41-55 dB HL), moderately severe “4” (56-70 dB HL), severe “5” (71-90 dB HL), profound “6” (91+ dB HL) (Clark, 1981).

2.2. Questionnaires

The MMSE included 18 questions assessing key cognitive domains:

1. Temporal Orientation (5 points): Identify the current day, date, month, year, and season.
2. Spatial Orientation (5 points): Specify the location (e.g., hospital, city, province, country).
3. Registration (3 points): Repeat three words (e.g., *house, bread, cat*). Each correctly repeated word earns 1 point.
4. Attention and Calculation (5 points): Count backward from 100 by sevens (five subtractions).
5. Recall (3 points): Recall the three words from the registration task, in any order.
6. Naming (2 points): Name two common objects (e.g., a pencil and a watch).
7. Repetition (1 point): Repeat a short phrase (e.g., “no ifs, ands, or buts”).
8. Comprehension (3 points): Follow a three-step command, such as “Take this paper in your right hand, fold it in half, and place it under the table.”
9. Reading (1 point): Read and perform a written command (“Close your eyes”) without the examiner reading it aloud.
10. Writing (1 point): Write a complete, meaningful sentence with at least a subject and a predicate.
11. Drawing (1 point): Copy two intersecting pentagons. Both shapes must have five sides, five angles, and four intersections.

The maximum score is 30. A score of 24 or higher generally indicates normal cognitive function, while lower scores suggest impairment. Raw scores were adjusted for age

and education level to improve diagnostic accuracy.

- Mild: 19–23 points
- Moderate: 10–18 points
- Severe: ≤9 points

MoCA was utilised following MMSE. MoCA is a 30-point test designed to evaluate cognitive function across six domains:

1) Memory (5 points)
2) Executive function (4 points)
3) Attention, concentration, and working memory (5 points)
4) Language (5 points)
5) Orientation (6 points)

The MoCA incorporates several subtests to assess these domains:

- Trail Making Test (TMT-B): Connect numbers (1–5) and letters (A–E) in alternating order (e.g., 1–A–2–B). This evaluates visual attention and task switching.
- Cube Copying: Reproduce a three-dimensional cube from a two-dimensional outline to assess visuospatial planning and visuomotor coordination.
- Clock Drawing Test (CDT): Draw a clock showing a specific time with correctly positioned numbers and hands. This test measures planning, conceptualization, and symbolic reasoning and may be influenced by literacy and education level.
- Naming: Name three uncommon animals (e.g. lion, rhinoceros, camel). Difficulty in naming may suggest cognitive decline.
- Digit Span (Forward and Backward): Repeat sequences of numbers first in order, then in reverse. The backward task primarily assesses executive working memory.
- Letter A Tapping Test: Tap when hearing the letter “A” in a sequence of random letters. These measures sustained attention and concentration.

- Serial Sevens: Subtract 7 from 100 successively five times to assess attention and calculation ability.
- Sentence Repetition: Repeat two sentences read aloud by the examiner. Performance should be interpreted with consideration of educational background.
- Verbal Fluency (Letter F): Generate as many words as possible beginning with the letter “F” in one minute. This assesses phonemic verbal fluency.
- Abstraction (Wechsler Similarities Test): Identify the shared concept linking two objects to evaluate semantic knowledge and abstract reasoning.
- Delayed Recall: Recall five previously presented words after a short delay. Compared with the MMSE, the MoCA includes more words (5 vs. 3), fewer learning trials (2 vs. up to 6), and a longer delay (5 vs. 2 minutes), enhancing sensitivity to amnestic mild cognitive impairment (MCI).
- Orientation: Identify the current location (city, place) and time (date, month, year, day of the week).

The maximum score is 30, with higher scores reflecting better cognitive performance. For individuals with more than 12 years of education, 1 point was subtracted to adjust for educational bias. A score of 26 or higher is generally considered normal.

HADS was utilized following MoCA. In HADS, respondents indicate which of four response options best describes their emotional state over the past week. HADS is self-administered; participants are provided with the questionnaire and instructed to complete it independently, assigning the score they consider most appropriate for each item. Response order varies across items to reduce response bias.

- Subscale A (Anxiety): Consists of seven odd-numbered items assessing symptoms such as tension, nervousness, fear, worry, restlessness, and panic.
- Subscale D (Depression): Includes seven even-numbered items evaluating low mood, anhedonia, loss of interest in self-care, psychomotor slowing, and pessimism.

Each item is rated on a 4-point Likert scale (0–3). Subscale scores range from 0 to 21, with higher scores indicating greater symptom severity.

Cut-off scores:

- <8: No symptoms
- 8–10: Mild symptoms
- 11–15: Moderate symptoms
- >16: Severe symptoms, suggesting clinical anxiety or depression.

2.3. Hearing aids

The new users were fitted with the AMPLI-MINI E 6G LP-model of hearing aids (Amplifon). This model features 113 dB SPL peak output, 56 dB gain, 200 Hz – 8 kHz bandwidth, < 1 % total harmonic distortion, < 20 dB internal noise, and up to 30 hours of rechargeable use (≈ 24 h typical). Additional features included: Amplifon SmartFit 2.1 software for hearing experience optimization, Wireless Bluetooth connectivity, and a range of optional accessories (TV streamer, remote, phone clip, personal mics, and the Amplifon smartphone app). The device is classified as a Class 1 hearing aid under both MDS and LEA standards.

2.4. Statistical Analyses

Statistical significance in hearing loss severity, MMSE, MoCA, and HADS tests was assessed for paired time points (T0 vs. T3, T3 vs. T6, T0 vs. T6) using the sign test with significance level set at $p < 0.05$. All tests and data graphing were performed in MATLAB (v. 2025b, MathWorks Inc.).

3. Results

3.1. Degree of presbycusis in examined patients

The sample included 30 hearing-impaired participants, predominantly male, aged between 60 and 75 years. The education level in the group was heterogeneous: 1/30 (3.3%) had completed primary school, 4/30 (13.3%) lower secondary school, 14/30 (46.7%) upper secondary school, and 11/30 (36.7%) held at least a university degree. All participants had bilateral progressive SNHL. All participants were new users of hearing aids. Hearing loss severity was measured without the use of a hearing aid using PTA and classified into six categories, and the measurement was repeated at T0 (before fitting hearing aids), T3 (3 months following hearing aid use), and T6 (6 months following hearing aid use). Hearing loss severity ranged from mild to severe, and we found no difference in the grade of hearing loss severity between T0, T3, and T6 (Figure 1).

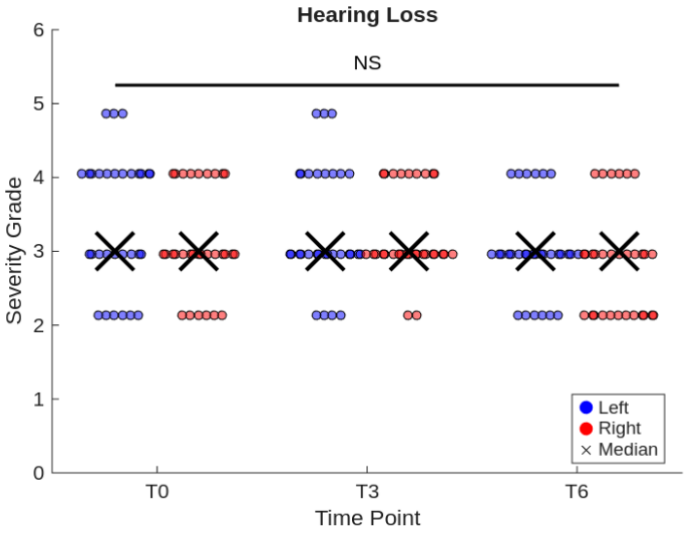


Figure 1. Hearing loss severity following hearing aid use. Severity was graded 0-6 (0 = normal, 1 = slight, 2 = mild, 3 = moderate, 4 = moderately severe, 5 = severe, 6 = profound) on y-axis. Time point is categorized on the x-axis. Results are grouped by ear (Left/Right). Median is denoted by X. NS = non-significant (Sign test).

3.2. MoCA test score

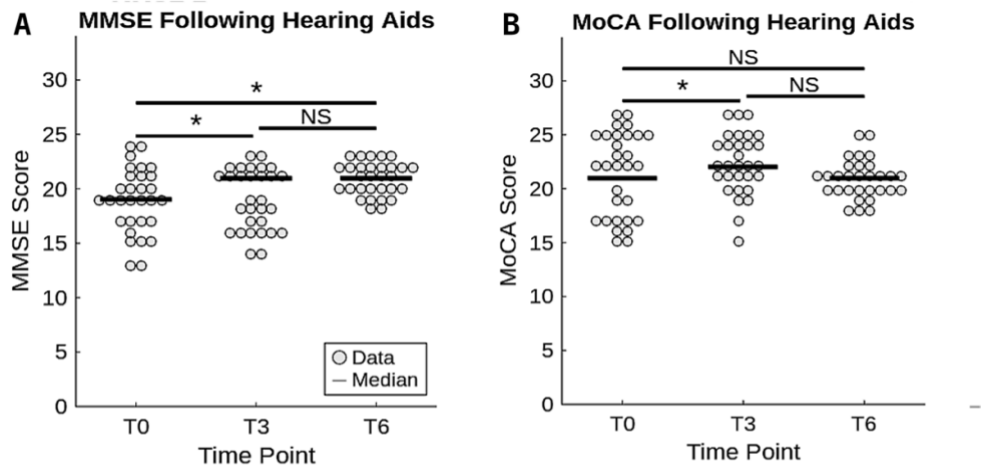
MoCA was measured at T0, T3, and T6. Median MoCA score was slightly (+1) higher at T3 compared to T0, with no detected statistically significant difference between T0 and T6 or T3 and T6 (Figure 2B).

3.3. MMSE test score

MMSE was measured at T0, T3, and T6. Median MMSE score was slightly (+2) higher at T3 and T6 compared to T0 and reached statistical significance using the sign test, with no detected statistically significant difference between T3 and T6 (Figure 2A).

3.4. HADS test score

HADS was measured at T0, T3, and T6. HADS-A (Anxiety subscale) and HADS-D (Depression subscale) test scores were analyzed separately. Median HADS-A score was slightly (-0.5) lower at T3 and T6 compared to T0, with no statistically significant difference between T3 and T6 (Figure 2C). Median HADS-D score showed no statistically significant difference between any time point (Figure 2D).



	Comparison	PValue	Significant	MedianDifference	Test statistic (S)	N
MMSE	'T0 vs T3'	1.9073e-06	true	-2	0	30
	'T3 vs T6'	0.1892	false	0	7	30
	'T0 vs T6'	0.0081	true	-2	7	30
MoCA	'T0 vs T3'	1.2207e-04	true	-1	0	30
	'T3 vs T6'	0.5235	false	1	9	30
	'T0 vs T6'	0.0872	false	0	9	30
HADS-A	'T0 vs T3'	7.6294e-05	true	0.5000	18	30
	'T3 vs T6'	0.2266	false	0	3	30
	'T0 vs T6'	1.4496e-04	true	0.5000	17	30
HADS-D	'T0 vs T3'	0.7539	false	0	6	30
	'T3 vs T6'	0.2188	false	0	5	30
	'T0 vs T6'	0.1797	false	0	7	30

Figure 2. Cognitive function and mental state tests following hearing aids use. (A) MMSE score before hearing aids (T0) and following hearing aids (3 months, T3; 6 months, T6). (B) MoCA score at T0, T3, T6. (C) HADS-A (Anxiety subscale) score. (D) HADS-D (Depression subscale) score. Table compiles the specific sign test results together. Test statistic S reports number of positive difference observations between time points.

4. Discussion

ARHL represents a major modifiable risk factor for the onset of dementia; therefore, its early diagnosis and treatment constitute a possible strategy to counteract the long-term consequences of cognitive decline in older adults. Here, we present a clinical study on 30 elderly participants (60-75 years old), newly fitted with hearing aids and assessed at three and six months after the start of use through questionnaire-based measurements of cognitive function. The main objective was to assess short-term cognitive and psychological responses to hearing rehabilitation. Results from the MMSE and MoCA cognitive tests indicated a slight improvement in cognitive function after three months of hearing aid use, with no further change at six months of age. At baseline, all participants displayed mild-to-moderate cognitive decline. The MoCA test, being particularly sensitive to early cognitive impairment, may be more suited to younger populations, whereas the MMSE remains the most widely used screening tool for older adults, effectively identifying global cognitive deficits and early dementia signs. Our results suggest that hearing rehabilitation gave a relatively quick improvement to cognitive performance, potentially contributing to slower cognitive decline. However, improved test scores may partly reflect better auditory perception of test instructions, especially tasks involving verbal repetition, rather than genuine cognitive improvement. Thus, lower baseline scores might have been influenced by hearing difficulties, potentially leading to an overestimation of cognitive impairment when hearing loss is untreated. Nevertheless, the slight post-intervention improvement in both MMSE and MoCA suggests that hearing aid use can facilitate performance in similar cognitive tasks and may enhance quality of life.

Regarding emotional state, the HADS mental state tests showed a small decline in the anxiety score (HADS-A) after three months of hearing aid use, with no significant change in depression scores (HADS-D). The observed decline in anxiety could be related to improved social interactions following better hearing, although this

mechanism was not specifically investigated.

In summary, this study provides preliminary evidence that hearing aids can modestly improve cognitive functioning and reduce anxiety in older adults with ARHL over the short term. Moreover, MMSE, MoCA, and HADS assessments proved to be practical, non-invasive tools for monitoring cognitive and emotional outcomes in hearing rehabilitation studies, even though they may not reveal the underlying mechanisms of improvement. Overall, hearing rehabilitation with high-quality hearing aids may enhance communication, social engagement, emotional well-being, and cognitive functioning, ultimately leading to a better quality of life for elderly individuals with ARHL.

GENERAL CONCLUSIONS

ARHL is among the most common chronic conditions in older adults and a major contributor to disability worldwide. Its pathology involves progressive high-frequency hearing loss, reduced speech discrimination, and eventual degeneration of cochlear HCs and SGNs. A central molecular mechanism implicated in ARHL is the interplay between oxidative stress and chronic low-grade inflammation that accumulates over the lifespan.

Here, we have studied the cellular and molecular basis of ARHL using experimental models that recapitulate some of the pathological processes typical of aging. By comparing damage caused by cisplatin, styrene, and normal aging in mice, the work sought to identify common pathways that may drive cochlear pathology in ARHL.

In Chapter 1, cisplatin administered to rats produced a dose-dependent increase in auditory thresholds together with marked loss of SGNs and depletion of primary afferent fibers. Molecular analysis showed reduced levels of phosphorylated TrkB and AKT and increased expression of the pro-apoptotic receptor $p75^{NTR}$ and cleaved caspase-3, indicating that loss of neurotrophic support and downstream PI3K/AKT survival signaling drives neural degeneration. Because cisplatin triggers many of the oxidative and inflammatory cascades that also characterize ARHL, these findings underscore the vulnerability of the neural cochlea to age-related injury and highlight the possibility of preserving SGNs by enhancing TrkB/AKT signaling during aging.

Chapter 2 examined styrene exposure, an industrial solvent known to induce cochlear oxidative stress, and demonstrated robust activation of the NLRP3 inflammasome with downstream elevation of IL-1β and NF-κB-dependent cytokines. Styrene also increased protein nitration and lipid peroxidation, confirming the central role of reactive oxygen species in triggering inflammatory cascades. Targeted interventions showed that the antioxidant rosmarinic acid attenuated ROS-mediated damage and reduced inflammasome activation, whereas the IL-1 receptor antagonist anakinra provided a rapid functional rescue. The results indicate that blocking IL-1 signaling

can interrupt the oxidative-inflammatory feedback loop that may also drive ARHL.

Chapter 3 followed the progression of ARHL in C57BL/6 mice, showing that auditory thresholds rise first at high frequencies at six months of age and become severe across the spectrum by twelve months, with corresponding declines in ABR wave amplitudes and significant loss of SGNs. Molecular profiling identified a simultaneous increase in oxidative markers such as 3-nitrotyrosine and 4-hydroxynonenal and in the pro-inflammatory cytokine TNF-α at the six-month time point. This timing aligns oxidative injury with the onset of chronic inflammation in the cochlea. The finding suggests that interventions deployed at this early stage could halt or slow subsequent cochlear neural degeneration.

Chapter 4 evaluated the rehabilitative effect of hearing aids in elderly patients newly fitted with amplification devices. The study observed modest but statistically significant improvements in global cognitive screening scores on the MMSE and a trend toward better performance on the MoCA after three months of use, effects that persisted but did not increase further at six months. Anxiety scores on the HADS-A also declined modestly, likely reflecting reduced listening effort and enhanced social engagement. These results indicate hearing aids may confer cognitive and emotional benefits, underscoring the value of hearing-aid use as a component of ARHL management.

Together, the studies show that cisplatin and styrene trigger common pathophysiology involving oxidative stress, inflammation, and elevated NF-κB signaling in the cochlea. The NLRP3 inflammasome appears to be a component of the molecular mechanism of styrene-induced ototoxicity that exhibits a similar pathological pattern in the cochlea as ARHL, suggesting that the NLRP3 inflammasome may be involved in the pathophysiology of ARHL. Early detection and intervention of oxidative stress and inflammation may prevent inflammasome activity and strengthen antioxidant defenses, which may ameliorate ARHL in the long term. The findings of the fourth study showed here also support hearing-aid use as an effective, non-pharmacological intervention

that can modestly improve cognitive function in the short term.

Future research should aim to validate NLRP3 inhibition and IL-1β blockade as viable pharmacological interventions to ARHL. Further preclinical studies should explore pharmacologic enhancement of TrkB/AKT signaling to protect SGNs during both acute ototoxic challenges and aging. The optimal timing, dosing, and delivery routes for antioxidant and anti-inflammatory agents should be defined to exploit the six-month therapeutic window identified in mice. Finally, longitudinal human studies that integrate auditory and cognitive assessments are needed to delineate how hearing rehabilitation translates into protection from cognitive decline. In all, the findings presented here suggest approaches that could postpone, mitigate, or prevent the disabling consequences of ARHL.

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