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ENVIRONMENTAL AND GENETIC RISK FACTORS FOR TINNITUS

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Environmental and Genetic Risk Factors for Tinnitus

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To young researchers and science enthusiasts!

POPULAR SCIENCE SUMMARY OF THE THESIS

Tinnitus is a phantom auditory sensation, most often referred to as “ringing in the ears” with detrimental effect on quality of life. Between 4% and 37% of the global population has experienced tinnitus at some point in their life. For every 1 out of 10 individuals experiencing tinnitus, it becomes a severely impactful condition, affecting concentration, sleep, mood, and general quality of life. Despite its high prevalence and severe socio-economic burden, there is no successful treatment. The work presented in this thesis uses multiple scientific approaches to better understand the etiology of tinnitus, with the emphasis on the genetic landscape in order to gain insight into its molecular origins. First, we identify important gaps in knowledge on environmental risk factors associated with tinnitus. Second, we show using genetic epidemiology methods that severe tinnitus runs in families, which changes the current narrative that tinnitus would be generated purely due to environmental factors. Third, as tinnitus is commonly linked to hearing loss, we used a genome-wide biostatistical approach to reveal the genetic architecture of hearing loss, that will be further essential in distinguishing the two conditions. Fourth, we investigated the whole genome in relation to tinnitus to map correlated genomic regions and consequently, specific genes associated with tinnitus. Finally, we used a high-throughput sequencing of protein coding regions of the genome to identify disease-causing mutations impacting severe tinnitus. The work presented in this thesis provides insights from multiple aspects into the origins of tinnitus and will serve as a backbone to understanding the pathophysiology of the disorder.

LIST OF SCIENTIFIC PAPERS

I. Biswas R*, Genitsaridi E*, **TRPCHEVSKA N***, Lugo A, Akeroyd MA, Cederroth CR, Schlee W, Gallus S, Hall DA; *Low evidence for tinnitus risk factors: A systematic review and meta-analysis* – Manuscript

II. **TRPCHEVSKA N**, Bulla J, Hellberg PM, Edvall KN, Lazar A, Mehraei G, Uhlen I, Schlee W, Canlon B, Gallus S, Lopez-Escamez JA, Cederroth RC; *Sex-Dependant Aggregation of Tinnitus in Swedish Families;* Journal of Clinical Medicine, 2020,9(12):3812

III. Cederroth CR, PirouziFard MN, **TRPCHEVSKA N**, Idrizbegovic E, Canlon C, Sundquist J, Sundquist K, Zöller B; *Association of Genetic vs Environmental Factors in Swedish Adoptees with Clinically Significant Tinnitus;* JAMA Otolaryngol Head Neck Surg. 2019 Mar 1;145(3):222-229

IV. **TRPCHEVSKA N**, Scarcella D, Edvall NK, Lazar A, Uhlén I, Canlon B, Cederroth CR; *Lack of involvement of COMT Val158Met polymorphism in tinnitus severity* – Manuscript

V. **TRPCHEVSKA N***, Freidin MB*, Broer L*, Oosterloo BC, Yao S, Zhou Y, Vona B, Bishop C, Bizaki-Vallaskangas A, Canlon B, Castellana F, Chasman DI, Cherny S, Christensen K, Concas MP, Correa A, Elkon R, Estonian Biobank Research Team, Mengel-From J, Gao Y, Giersch ABS, Girotto G, Gudjonsson A, Gudnason V, Heard-Costa NL, Hertzano R, Hjelmborg JvB, Hjerling-Leffler J, Hoffman HJ, Kaprio J, Kettunen K, Krebs K, Kähler AK, Lallemend F, Launer JL, Lee IM, Leonard H, Li CM, Lowenheim H, Magnusson PKE, Meurs Jvan, Milani L, Morton CC, Mäkitie A, Nalls MA, Giovanni Nardone G, Nygaard M, Palviainen T, Pratt S, Quaranta N, Rämö J, Saarentaus E, Sardone R, Satizabal Barrera CL, Schweinfurth JM, Seshadri S, Shiroma E, Shulman E, Simonsick E, Spankovich E, Tropitzsch A, Lauschke VM, Sullivan PF, Goedegebure A, Cederroth CR, Williams FMK, Nagtegaal AP; *Genome-wide association meta-analysis identifies 48 risk variants and highlights the role of the stria vascularis in hearing loss;* Am J Hum Genet. 2022 Jun 2;109(6):1077-1091. doi: 10.1016/j.ajhg.2022.04.010. Epub 2022 May 16.

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CONTENTS

LIST OF ABBREVIATIONS

EP	Endocochlear Potential
SV	Stria Vascularis
OHC	Outer hair cells
IHC	Inner hair cells
CN	Cochlear nucleus
AVCN	Anterior ventral cochlear nucleus
PVCN	Posterior ventral cochlear nucleus
DCN	Dorsal cochlear nucleus
SOC	Superior olivary complex
IC	Inferior culliculus
LL	Lateral lemniscus
MGN	Medial geniculate nucleus
WHO	World Health Organization
NIHL	Noise induced hearing loss
ARHL	Age-related hearing loss
AEP	Auditory evoked potential
ABR	Auditory brainstem response
AN	Auditory nerve
THI	Tinnitus Handicap Inventory
TFI	Tinnitus Functional Index
CBT	Cognitive behavioral therapy
CNS	Central nervous system
ANF	Auditory nerve fibers
SFR	Spontaneous firing rates
VNS	Vagus nerve stimulation
GABA	Gamma aminobutyric acid
MEG	Magnetic encephalography
EEG	Electro-encephalography
fMRI	Functional Magnetic resonance imaging
TDC	Thalamocortical dysrhythmia

OR	Odds ratio
TMD	Temporomandibular joint disorder
GWAS	Genome-wide association analysis
SNP	Single nucleotide polymorphism
UKBB	United Kingdom Biobank
MVP	Million Veteran Program
MR	Mendelian Randomization
WES	Whole exome sequencing
RR	Risk ratio
HR	Hazard ratio
PR	Prevalence ratio
λs	Recurrence risk (lambda)
CI	Confidence interval
STR	Swedish Twin Registry
SPHC	Stockholm Public Health Cohort
SLOHS	Swedish Longitudinal Occupational Survey of Health
ICD	International classification of diseases
MZ	Monozygotic
DZ	Dizygotic
LD	Linkage disequilibrium
STOP	Swedish tinnitus outreach project
ESIT_SQ	European school of interdisciplinary tinnitus research screening questionnaire
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
COJO	Conditional and joint association analysis
eQTL	Expression quantitative trait loci
DEG	Differentially expressed genes
GSMR	Generalized Summary-data-based Mendelian Randomization
SVN	Single nucleotide variants
CADD	Combined annotation dependent depletion

LDSC	Linkage disequilibrium score regression analysis
LSV	Large structural variants
CNV	Copy number variants
LoF	Loss-of-function

1 INTRODUCTION

1.1 GENERAL OVERVIEW OF AUDITORY NEUROANATOMY AND PATHOPHYSIOLOGY

Hearing is one of the 5 senses that are essential for evolution across species. Perception of sounds is fundamental tool for communication and survival [1,2]. Mammals exhibit a large variation in their sound perception, from bats using ultrasonic frequencies to navigate in their environment, to elephant communities using infrasonic sounds for communication. Sound perception and processing play a vital role for the development of the modern human, as it is crucial for language development, communication, and interaction, framing their experience in the environment. The human auditory system is a complex neuroanatomical structure with the purpose of processing auditory signals by perceiving information from the environment and processing them into signals that construct a visceral perception of the space [3].The processing of sounds starts within the peripheral auditory system with sensory input from both ears and progresses to more complex processing in the central auditory system with numerous interactions with various regions of the nervous system.
The auditory system in mammals consists of 3 main parts:
1) the outer and middle ear that conducts the sound waves from the environment to the inner ear,
2) the cochlea, part of the inner ear containing the sensory hair cells responsible for transducing mechanical movements to neural signals,
3) the auditory nerve and the ascending pathways, including the cochlear nucleus, superior olivary complex, lateral lemniscus, inferior colliculus, medial geniculate nucleus and the auditory cortex.

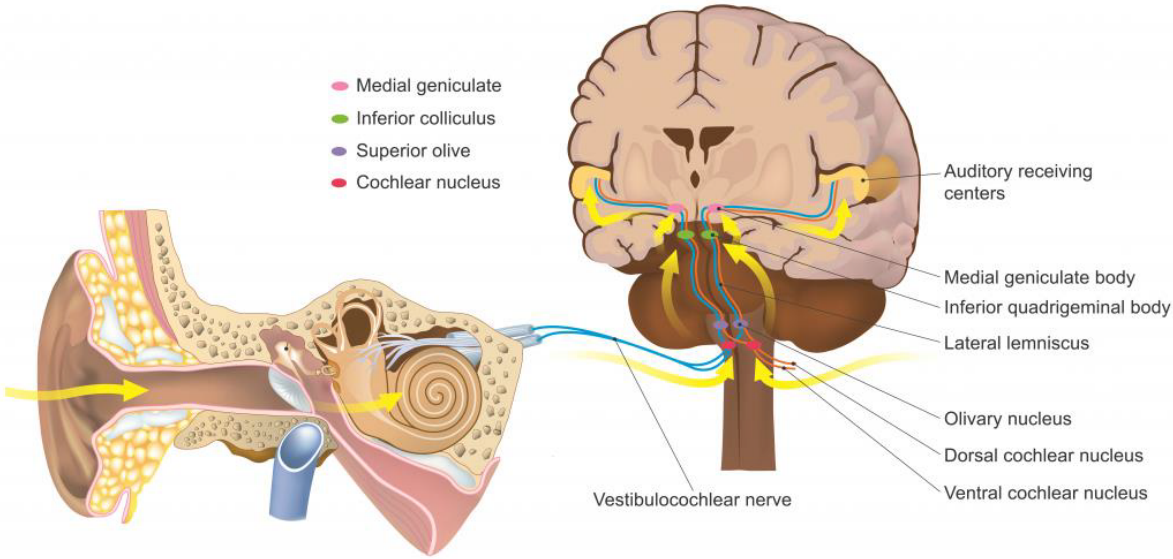
The outer ear is the visible, external cartilaginous part of the ear called pinna. Its function is to collect sound waves and make them propagate through the ear canal, causing vibration to the tympanic membrane, which is the innermost part of the outer ear. The middle ear contains the three small bones called ossicles (malleus, incus and stapes), each playing a role in the transmission of the sound waves into the cochlea. The cochlea represents a major part of the inner ear and forms a tubular structure with 3 fluid-filled compartments:

1) *Scala Vestibuli* - where the sound waves vibrations enter in the inner ear and it is filled with perilymph, a high Na+ charged fluid.
2) *Scala Media* - filled with endolymph, a fluid high in K+, which borders the basilar membrane, along which is located the organ of Corti containing one row of inner hair cells and three rows of outer hair cells, and the Reissner's membrane.
3) *Scala Tympani* – connected to the *scala vestibuli* vie the helicotrema at the apical part of the cochlea, and also filled with perilymph.

The endolymph has a high potassium concentration and a high endocochlear potential (EP) of 80 mV that is maintained by the stria vascularis (SV), is essential for transduction of sound by the sensory hair cells. The interior of SV is isolated with marginal and basal cells,

involved in endolymph homeostasis [4,5]. Within the stria vascularis are the marginal cells that regulate the K+ concentration through the enzymatic activity of Na+K+ ATPase [6]. The combined action of the high K+ concentration and the DC potential cause the driving force for a potential change in the hair cells. Atrophy of the SV has been proposed as a potential origin of sensorineural hearing loss, however, specific cell types within the SV are yet to be profiled [7]. Transduction of sound begins at the cilia of the hair cells where sound waves are transmitted via the perilymph causing a displacement in the basilar membrane and the tectorial membrane on which the outer hair cells (OHC) are anchored. This movement results a deflection of OHC stereocilia that modulates the prestin-mediated electro-motility and amplifies the basilar membrane vibration forcing the stereocilia of the inner hair cells (IHC) to become deflected, leading to depolarization of the cell and subsequent generation of nerve impulses that are then relayed to the afferent neuron [8]. When the stereocilia hair cells are deflected by sound stimulation, transduction channels open and K+ enters the apical part of the hair cell. Depolarization opens voltage-gated calcium channels and an influx of calcium into the cell leads to neurotransmitter release. The sensitivity to sound frequency of hair cells and nerve fibers depends on their position along the basilar membrane of the cochlea. The cochlea has a tonotopic organization, such that, high frequency sounds stimulate the base of the cochlea and the low frequency sounds stimulate the apex. The OHCs act as amplifiers of the mechanical movement of the basilar membrane helping to increase the overall sensitivity of the cochlea to sound stimulation [9]. The cochlea is innervated by the auditory nerve, a branch of the VIII cranial vestibulocochlear nerve (**Figure 1**).

Figure 1. Illustration of the auditory pathway.
https://teachmeanatomy.info/neuroanatomy/pathways/auditory-pathway/



The afferent fibers have their soma in the spiral ganglion of the cochlea and 95% of their dendrites innervate IHCs and the remaining 5% innervate OHCs. Further, the axons of the afferent neural fibers terminate in 3 divisions of the cochlear nucleus (CN) within the brainstem: the anterior ventral cochlear nucleus (AVCN), the posterior ventral nucleus (PVCN) and the dorsal nucleus (DCN). Fibers from the cochlear nucleus project to the

ipsilateral and contralateral Superior Olivary Complex (SOC) and from there they terminate in the Inferior Colliculus (IC) in the midbrain, via the lateral lemniscus (LL). This crossing of the fibers results in a similar amount of auditory input in both sides of the cerebral cortiex, which is clinically relevant in the case of unilateral lesions only, where there may be lack of any clinical manifestation. Furthermore, the afferent fibers from the IC project to the Medial Geniculate Nucleus (MGN) which is part of the thalamic auditory nucleus and continuing toward the primary auditory cortex in the temporal lobe of the brain. The complexity of the auditory pathway is confirmed by the description of other fibers from the cochlear nuclei that proceed in several different diffuse pathways towards the primary and secondary auditory cortex as well as, related auditory fields in the brain [10]. Noteworthy, the tonotopic organization is maintained throughout the auditory system.

1.2 HEARING LOSS AND TINNITUS

1.2.1 Prevalence and social impact

Hearing loss refers to partial or complete inability to hear sounds and accounts for major burden on the global population [11]. Currently, 1.5 billion people (20.3%) globally live with some form of hearing loss, of which 403 million have moderate to complete hearing loss. Furthermore, hearing loss has been ranked 4th leading cause of years lived with disability [12]. The substantial, global socio-economic burden calls for urgent establishment of public health policies for prevention and treatment solution for this global health challenge [13]. The number of people living with disabling hearing loss has increased by 79.1% in the last 30 years, even though according to World Health Organization (WHO), 50% of hearing loss disorders are preventable [11,14]. Most of the causes leading to hearing loss, such as infectious diseases and noise exposure, are preventable if effective strategies of early detection and screening are integrated within the primary health care system [15]. Occupational noise exposure accounts for proportion ranging from 7-21% of diagnosed hearing loss in adults [16]. Furthermore, 30% of the working population in Sweden report hearing loss, emphasizing that cost-effective preventative regulations such as appropriate protective equipment and earplugs should be implemented [17]. Noise-induced hearing loss (NIHL) can not only result in temporary or permanent injury, affecting the hair cell, but also the auditory nerve fibers, known as cochlear synaptopathy [10,18].

The prevalence of hearing loss is increasing proportionally with every 10 years of age. Age-related hearing loss (ARHL) accounts for 96.2% of all hearing loss reports among adult population, which is also reflected in the poor ability to understand speech [11,19,20]. Age-related degenerative processes within the cochlea induced by cellular aging, ototoxic drugs, and cumulated noise exposure are responsible for ARHL [21,22]. Untreated hearing loss leads to a meaningful impact on psychological and physical health, causing to social isolation stress, mental fatigue, cognitive decline, depression and ultimately, a substantially decreased quality of life [23–28].

A related auditory condition with equally significant burden is tinnitus. Subjective tinnitus is a condition defined as a phantom auditory sensation - a conscious perception of a sound that

has no corresponding external source. Individuals can perceive tinnitus as a buzzing, ringing, or hissing sound in one ear, both ears or inside the head [29]. Some environmental risk factors, such as noise exposure, as well as otologic, neurological, and psychological comorbidities have been associated with tinnitus, affecting its generation and perception [30]. Recent studies have reported a broad range of tinnitus prevalence varying between 4.1% to 37.2% in the global population [31]. The prevalence of tinnitus among men varies between 10% and 49% and for women - 6% to 30% [32]. The prevalence of bothersome tinnitus ranges between 3% and 30.9% across the population [33]. Besides being an economic and social burden, chronic tinnitus has also been strongly associated with psychological distress, affecting concentration, attention, and other cognitive performances [34,35]. The level of distress depends on the severity of tinnitus perception, varying from a perception of tinnitus that does not affect every-day life to an incapacitating burden for individuals with catastrophic tinnitus perception [35]. Similarly to hearing loss, prevalence differs among different age groups as well, with a high proportion of 36% among people aged 86 and above, and 16% in people aged 50 and younger, respectively [33]. Tinnitus prevalence is also dependent on accompanying conditions, such as hearing loss, anxiety, vertigo, or head-and-neck problems [33,36]. There is a large body of research recognizing the relationship between bothersome tinnitus and several psychiatric disorders, most notably anxiety and depression, although a clear direction of the relationship has not been established [36–38]. Being highly prevalent in the population, severe tinnitus has a significant effect on life quality, leading to increased sick leave and disability pension [39]. A 10-year cumulative incidence of tinnitus of 12,7% has been reported in the USA and 5-year cumulative incidence of 18% in Australia suggesting the prevalence of tinnitus will double in the next ten years [40–43]. These estimates are projected to increase with further clarification in the phenotype of tinnitus subtypes [44]. The increased demand for treatment supports the global populational significance of tinnitus and consequentially increases health-care costs for society. Despite the clear need for an effective cure, tinnitus remains an untreated and neglected condition [13,44].

1.2.2 Assessment of hearing loss and tinnitus

Assessment of auditory function is done by basic audiometric test battery that evaluate pure-tone thresholds by air and bone conduction, as well as speech recognition tests in quiet and noisy environment. Clinical guidelines recommend that hearing is measured for each ear with pure-tone audiogram, by presenting pure-tone signals at frequency intervals of 250-8000 Hz [45], although hearing in humans reaches 20kHz. Individuals are regarded with disabling hearing impairment when their audiometric values correspond to > 40dB hearing level. Based on the average auditory threshold shifts, levels of hearing impairment can be assessed [46]. General auditory function can be assessed by measuring Auditory Evoked Potentials (AEP) that records the neural activity evoked by stimuli. The earliest neural activity is the auditory brainstem response (ABR), which output consists of 5 waves corresponding to the activity of the auditory nerve (AN), CN, SOC, LL, and IC. Assessment of ABRs has been also used in attempt to detect tinnitus however evidence to support this are scarce.
Conversely, there are currently no objective measures that can be used to clinically diagnose

or quantify tinnitus therefore, personal reporting using questionnaires, although not globally standardized is still the main form of assessment [47]. Due to the subjective definition of tinnitus, there is high variability in reporting. Tinnitus sensation has been described in both ears (bilateral) or one ear only (unilateral). In less than 10% of cases, tinnitus is characterized as pulsatile or rhythmical based on either vascular or somatic changes [48]. Tinnitus can be perceived in synchrony with the heartbeat, suggesting a vascular involvement in its generation [30]. Concerning the duration of tinnitus experience, it can be either acute, lasting less than three months, which is most commonly reported after an intense noise exposure such as blast injury, or chronic, lasting more than three months and developing over time. Tinnitus can appear intermittently, or a person can experience it constantly. Indeed, with increased occurrence of the occasional perception of tinnitus, the risk of getting constant tinnitus is increased [49]. Since constant tinnitus differs from occasional, with greater wave V latencies of the ABRs, it has been proposed that the neuronal maladaptive plasticity occurring in the IC freezes, with diminished ability to remit [49]. Variations in tinnitus severity and loudness are reported as well, ranging from catastrophically bothersome to rarely noticeable or unimpactful [50]. The variability in the reported prevalence is considered to be due to unclear diagnostic criteria, lack of agreed-upon definition of tinnitus, and the phrasing of the question used to define the presence of the condition [51,52]. There are variations in tinnitus prevalence due to the large number of instruments used for tinnitus assessment [14] as well as, different levels of tinnitus awareness and self-reported severity in specific geographic areas [32,51,52]. Many questionnaires, such as the Tinnitus Questionnaire, the Tinnitus Handicap Inventory (THI) [53], and the Tinnitus Handicap Questionnaire (THQ), are used to assess tinnitus severity; however, there is no evidence of their clinical interpretation [54,55]. Recently, the Tinnitus Functional Index (TFI) questionnaire for assessing severity, has been proposed as a reliable tool for distinguishing different patients, as well as varying levels of tinnitus perception [56]. The severity of tinnitus perception could be used as a homogenizing property and classification tool of tinnitus subtypes. The TFI is based on the question asking, “How much of a problem is your tinnitus?”, with the following response alternatives: 1)"not a problem", 2) "a small problem", 3)"a moderate problem", 4)"a big problem", and 5)"a very big problem." It includes eight subscales encompassing the global tinnitus perception, reflecting on 1) Tinnitus intrusiveness, 2) Sense of control, 3) Cognition, 4) Sleep, 5) Auditory, 6) Relaxation, 7) Quality of life, and 8) Emotional distress. Responses are then summed up to a score of 100. Higher scores indicate a greater effect of tinnitus on every item, giving an overview of the overall impact of tinnitus experience [54].

1.2.3 Management and treatment of hearing loss and tinnitus

Despite the high global prevalence and increased knowledge of the pathophysiology of hearing impairment there is no available treatment for hearing restoration and tinnitus perception [44,57,58]. Hearing aids are first line of treatment for hearing loss and are intended to compensate for impaired hearing. Well-fitted hearing aids are reported to improve cognitive performance and quality of life in individuals with both hearing loss and tinnitus [59–61]. However, only 14.2% of the population in need use hearing aids [62]. Cochlear implants can be

used to partially restore hearing by electrically stimulating the auditory nerve, improving speech perception, vocalization and cognitive performance [63]. Moreover, tinnitus perception has been mitigated in patients with cochlear implant, as well [64,65]. Increasing number of novel drug therapies for hearing loss are ongoing as active clinical trials addressing apoptosis pathways and oxidative stress of IHC [66,67]. In comparison, methods to treat tinnitus, such as medication and antidepressants, acupuncture, and neuromodulation, have failed to show beneficial clinical outcomes [57,68]. In attempts to treat acute tinnitus, pharmacological drug therapies have been used, such as lidocaine, vasodilators, and antiviral agents, showing only short-term improvements of tinnitus perception in a subset of patients [44,69,70]. Antidepressant medications and selective serotonin reuptake inhibitors have been used to treat chronic tinnitus in randomized trials, showing conflicting results [71–73]. Clinical trials investigating pharmacological treatment for tinnitus are ongoing however, with mixed success. In a phase 2 trial using trans-tympanic delivery of AM-101, an NMDA receptor antagonist, a reduction of tinnitus loudness was observed, which led to a double-blinded, randomized, placebo-controlled phase 3 trial. This trial has been interrupted due to a lack of efficacy. Similarly, AUT00063 - an agonist against a voltage-gated potassium channel, Kv3 - failed in a phase-2 trial [70]. A current tool used for managing the tinnitus experience is Cognitive Behavioral Therapy (CBT), which has been recommended in recent European Guidelines [37]. Indeed, CBT has been shown to be beneficial in managing the fear of tinnitus experience; however, psychological mechanisms of these improvements are not well understood [74].

Despite the high clinical demand and increasing research outputs, a definitive cure for tinnitus is still lacking. There are several limitations in the development process of drug discoveries. The lack of robust and translationally valid animal models, inappropriate outcome measures that are not objective, and the heterogeneity of tinnitus leading to the inclusion of non-responsive patients being the most important ones. Furthermore, outcome measures of tinnitus are not standardized, making the comparison between studies conflicting and impossible to meta-analyze. Reliable biomarkers, robust analysis of outcome measures, and the identification of tinnitus phenotypes, are considered as necessary steps towards a successful cure [44]. The current literature implies that there are gaps in knowledge of the biological mechanisms underlying tinnitus and it highlights the need for investigating new ways of addressing these hurdles. Ultimately, basic research is still needed to elucidate the pathophysiology of tinnitus and improve the understanding of its heterogeneity [44,69].

1.3 UNDERLYING MECHANISMS OF TINNITUS GENERATION

The phantom sound of tinnitus was previously considered to be an inner-ear or a cochlear problem, but there is accumulating evidence suggesting that the auditory and non-auditory regions of the Central Nervous System (CNS) are involved in its perception and severity, more specifically the primary auditory cortex and associated cortical structures [75–78]. However, the evidence of the primary pathology in tinnitus generation and perception is inconsistent among studies, where several approaches and theories have been proposed [79,80].

1.3.1 Cochlear synaptopathy

Since tinnitus is perceived as "ringing in the ears", it has been proposed that cochlear pathologies could be the cause of it. In most cases, tinnitus is accompanied by some level of hearing loss, suggesting a shared pathology between the two conditions [77]. Cochlear damage from noise exposure, aging, and ototoxic drugs can negatively impact, not only the hair cells, but also the synapses between the IHC and auditory nerve fibers (ANFs), a process called cochlear synaptopathy [10]. Cochlear synaptopathy is characterized in animals by a decrease in IHC-afferent neurons synaptic contacts that lead to a decrease in wave I amplitude of the ABRs in absence of hearing loss. In humans, cochlear synaptopathy cannot be detected through the standard pure-tone audiometry up to 8kHz. However, wave I amplitude has been shown to be lower in subjects with normal hearing up to 16kHz, that display speech-in-noise deficits [81–83]. In studies where animals were subjected to prolonged noise exposure, it has been shown that a temporary shift occurs in their hearing threshold, which then return to normal levels, but permanent damage to the afferent ANFs remains [18]. This vulnerability and swelling of the synapses of the ANFs has been shown to occur during aging as well, and is considered to contribute to the disruption of peripheral sensory input and may cause tinnitus perception in the ascending neurological structures [84–86].

1.3.2 Maladaptive plasticity

Tinnitus has been related to irregular neural activity in the auditory system. More specifically, increased burst-firing activity in different levels of the auditory system has been proposed as a general mechanism of tinnitus perception in humans, after using tinnitus-inducing agents [87,88]. Increased spontaneous firing rates (SFR) related to tinnitus have been reported along the auditory pathway, and have been associated with tinnitus after noise trauma [76,78,89]. Hyperactivity in the cochlear nucleus and increased neural synchrony compromise neural plasticity, causing a so-called maladaptive plasticity of the central nervous system [90,91]. These spontaneous firing rates and neural bursting have been reported in the fusiform cells, which are the primary output neurons from the DCN suggesting that tinnitus could be generated there, contributing to changes in the higher level auditory circuits [76,92]. These findings drive the notion that the perception of tinnitus can be explained by a chain of events, starting with reduced peripheral sensory input that triggers a compensatory imbalance of excitation and inhibition by affecting glutamate production. This, in turn, increases spontaneous firing rates and results in the reorganization of the cortical map (Eggermont and Roberts, 2012; Sedley, 2019). Whether reorganization of the cortical map is a tinnitus phenomenon, or more due to hearing loss is still a debate. Engineer et al., shows that vagus nerve stimulation (VNS) paired with tones can diminish tinnitus in rats, and reorganize the cortical plasticity to the original arrangement [95,96] – but many believe this is not a tinnitus correlate.

1.3.3 Central gain

Central gain contains a temporal dynamic that is explained by the difference in reaction and adaptation of different brain structures along the auditory pathway, to the lack of peripheral sensory input [97,98]. This temporal profile of the increased gain suggests the possible

involvement of multiple neuronal mechanisms in the homeostatic plasticity occurring during tinnitus [98]. It has been shown, in mice, that the increased central gain and hypersynchrony, and SFR in the DCN can be partially explained by reduced activity of KCNQ2/3 and HCN channels mainly in the fusiform cells that control the excitability of neuronal and sensory cells [99,100]. Changes in neurotransmission can be explained by differences in gene expression of GABA subunits, modulating their activity [80]. Moreover, it has been proposed in animal studies, that both pathogenic plasticity in the DCN, and homeostatic plasticity emerging from inhibitory mechanisms, are implicated in tinnitus [96,101].

Homeostatic plasticity is explained as negative feedback aiming to stabilize neural activity by controlling excitatory and inhibitory mechanisms and synapses across the auditory pathway. These changes then translate into a tonotopic reorganization at the cortical level, showing altered representations of specific frequencies [80,102]. Homeostatic plasticity has been explained in the context of tinnitus generation in mice, pointing out the importance of down-regulatory mechanisms of the inhibitory synapses [103,104]. Tinnitus behavior in mice have been abolished with drugs that enhance inhibition, as opposed to drugs that reduce excitation [104]. Overall, these findings promote new prospects into tinnitus understanding by suggesting the importance of the descending auditory system. Whether these findings apply to humans is still debated, as many of the clinical trials that have been performed were of low sample size and with unclear tinnitus definitions [57].

1.4 TINNITUS AND NON-AUDITORY BRAIN STRUCTURES

1.4.1 Tinnitus and the limbic system

Studies have contributed to the identification of brain regions beyond the auditory pathway that are associated with tinnitus [77,80,105,106]. Changes in the connectivity in attentional circuits [106,107], emotional-distress regions [107–109] or temporofrontal attentional networks [77,106,109–111] have been observed previously in patients with tinnitus. As part of the limbic brain region, the amygdala is a complex functional structure that is highly sensitive to emotional processing, as well as sound interpretation, especially in auditory fear conditioning and stress responses, forming a negative emotional association to tinnitus perception [105]. Functional connectivity within the auditory network and amygdala, as well as the hippocampus, have also been shown in rats [112]. An fMRI study replicated these findings in tinnitus patients, identifying changes in effective connectivity in the amygdala and hippocampus having a strong influence on acoustic interpretation and perception [108]. The hippocampus gives a temporal dimension to sound perception, affecting learning and memory [113]. In guinea pigs, a remodeling of the hippocampal cholinergic input has been related to tinnitus behavior, validating the active functional connection between tinnitus perception and the limbic system [114,115]. Additionally, the limbic system and the parahippocampal region have been associated with tinnitus perception, chronicity, and severity [76,108,116]. The activation of the frontoparietal region has been associated with the functional connectivity with the auditory cortex in tinnitus cases by MEG, EEG and fMRI studies [77,80,111,117]. Brain regions beyond the classic auditory pathway, such as the ventromedial prefrontal cortex and the nucleus accumbens, are part of the

frontostriatal ‘gating’ system and have been identified as relevant components in tinnitus development and its cognitive perception. This gating system aims at distinguishing the relevant sensory stimulus and controlling the flow of sensory information through the descending pathways, which has been shown to be compromised in tinnitus patients [110].

The paralimbic associations with the ventromedial prefrontal cortex are related to long-term habituation of tinnitus, causing an anatomical reorganization of the auditory cortex, possibly leading to chronic tinnitus [114,118]. These findings indicate that long-range connectivity between auditory and cognitive-related brain regions is required for conscious tinnitus perception [80,118]. These findings have been conducted both in animals and humans using neurofunctional imaging and confirm the importance of limbic and auditory interactions in tinnitus pathophysiology and its perception. Similar mechanisms have been associated with other conditions like depressive disorders, chronic pain, hyperacusis or insomnia [110,112,113,119,120].

1.4.2 Thalamocortical dysrhythmia

The change in the common oscillatory pattern in the brain is called thalamocortical dysrhythmia (TCD), and it has been proposed as an underlying mechanism in tinnitus and other divergent neurological disorders [121]. It is explained as a specific brain wave’s oscillatory signature that is characterized by resting-state activity and cross-frequency coupling of low- and high-frequency oscillations [121–123]. Using machine learning, Vanneste et al. demonstrated an increased theta-beta and theta-gamma coupling for specific areas related to a specific disorder [124]. For tinnitus, there is an increased coupling at the auditory cortex. However, for pain and Parkinson’s - increased coupling is seen in the somatosensory and motor cortex, respectively, indicating a distinct TCD signature for tinnitus [124].

1.5 TINNITUS AND ENVIRONMENTAL FACTORS

1.5.1 Modifiable risk factors

In order to reduce the personal and economic burden of tinnitus upon society, adequate intervention strategies should be implemented, particularly preventions aimed towards modifiable risk factors associated with tinnitus. Tinnitus has been associated with many life-style related risk factors such as smoking, coffee intake, noise exposure, obesity and alcohol consumption [30,125–127]. However, the direction of the relationship cannot be established. Moreover, conflicting results have been reported regarding the effect of smoking on tinnitus generation. Some studies have shown that smoking increases the risk of tinnitus [32,128,129], while others have not been able to validate these findings [127,130]. Similarly, there are ambiguities regarding the effect of alcohol on tinnitus [32,128,129]. Recently published systematic review investigating modifiable risk factors for tinnitus, found increased risk for tinnitus in smokers and obese people, however, majority of the findings were based on cross-sectional data and a significant between-study heterogeneity [125].

1.5.2 Tinnitus Comorbidities

Auditory and non-auditory comorbidities have been associated with tinnitus based on observational and epidemiological studies [131]. Most notably, hearing loss has been most frequently related to tinnitus, reporting adjusted odds ratios (OR) between OR=2.31 and OR=5.15 [40,42,129,132–134]. This is consistent with reports showing an increased risk of tinnitus among professional musicians (Schink et al., 2014;Langguth et al., 2013; Nondahl et al., 2010;). Similarly, two studies have reported temporomandibular joint disorder (TMD) to increase the odds of having tinnitus [136,137]. Otosclerosis, neck and ear injury, dizziness, chronic kidney disease, and sleep disorders have shown a similar pattern of increased risk for tinnitus [40,134,138]. Psychiatric disorders have been commonly related with tinnitus and its psychological burden [139]. More specifically, depression, anxiety and negative coping mechanism have high prevalence in tinnitus subjects which is proportionally correlated with quality of life [119,140,141]. Additionally, cancer showed an increased risk of tinnitus, as well as drugs used in cancer treatments such as cisplatin, carboplatin, and ototoxic antibiotics, primarily aminoglycosides, which have an established ototoxicity [142–144]. Recently, hypothyroidism has been shown to have a strong relationship with tinnitus in adults [145]. Otitis media, hypertension, diabetes, BMI, and vascular incidents have shown no significant risk with tinnitus [128,129,134]. Similar negative relations were found between alcohol consumption and tinnitus [128]. No liability to tinnitus was reported with respect to drugs, such as aspirin, macrolides, and oral contraceptives [143,144,146]. Military service, cell phone use and socio-economic status have shown to pose insignificant risk to tinnitus [133,147,148]. Coffee intake was associated with decreased risk of tinnitus, as well as alcohol consumption of more than 140 grams per week [128,149]. Sex differences are observed, showing an increased risk of tinnitus in women [42,129,134], however this has not been consistently reported in the literature. Further high-quality longitudinal cohort studies and improved quality control of the analysis and assessment of bias is needed in order conclude the relationship between tinnitus and environmental risk factors.

1.6 TINNITUS AND GENETIC RISK FACTORS

1.6.1 Genetic epidemiology

In our quest to understand the molecular mechanisms involved in tinnitus generation or perception, a genetic investigation is needed. Despite the known association with environmental factors, not all subjects with tinnitus have reported some of the environmental risk factors, suggesting that individual genetic background is important to understand the tinnitus phenotype. Yet, studies aiming to map the genetic foundation of tinnitus are sparse [150].

In a genetic context, tinnitus was first mentioned as a secondary symptom of monogenic disorders for which many genes and inheritance patterns have been identified. Secondary tinnitus has been associated with autosomal dominant non-syndromic hearing loss and genes such as *ACTG1, CEACAM16, COCH, GJB2, GJB3*, have been identified [151–154]. Additionally,

tinnitus has been mentioned in other monogenic diseases and related genes, such as *GLA* gene in Fabry disease [155], *MNF2* gene in Hereditary motor and sensory neuropathy VI [156], *NF2* in Neurofibromatosis type 2 [157], *VHL* in von Hippel-Lindau syndrom [158], *CACNA1A* - Episodic ataxia type 2 [159].

1.6.2 Tinnitus as monogenic vs. complex trait

Monogenic disorders are a result of a single gene mutations that have a clear Mendelian pattern of inheritance with one dominant, two recessive alleles or X-chromosome linked transmission [160]. Despite the ability to identify genes in monogenic diseases causing tinnitus, their importance is of limited value for understanding the molecular mechanism of tinnitus, because tinnitus appears as a secondary symptom due to the significant pathologies of the monogenic disorders. In contrast, primary tinnitus has been contributed to additive genetic factors and multifactorial genetic etiology, defining tinnitus as a complex trait [161]. Complex traits, in general, are difficult to link to a specific genetic marker due to several of the following characteristics. Incomplete penetrance of some genes causes the genotype at a specific locus to affect the probability of the diseases, but not fully determine the outcome and manifestation [162]. Genetic heterogeneity means that mutations in several different genetic loci, or different mutations in the same locus can cause the same phenotype of the complex trait [163]. These conditions contribute to the challenges of studying complex traits such as tinnitus. In comparison, hearing loss and deafness have been contributed to Mendelian pattern of inheritance as syndromic hearing loss. However, studies show that syndromic hearing loss accounts for only 30% of hereditary hearing loss, indicating that hearing loss is largely a complex trait [164,165].

1.6.3 Aggregation studies

Familial aggregation studies are used in genetic epidemiology to estimate the recurrence risk of a trait within a family, which is the initial step to identify hereditary conditions [166]. The earliest familial aggregation study for tinnitus was conducted in 198 European families, showing a familial correlation between siblings of 0.16 and 1,7-fold increased likelihood of developing tinnitus with an affected sibling [167]. However, the authors speculated that these findings were due to increased awareness of tinnitus within the families.

1.6.4 Twin studies

Twin studies estimate disease concordance in monozygotic and dizygotic twins suggesting a role of genetic factors, assuming both sets of twins share the same parental environment [150]. Two studies have used a twin cohort to assess the heritability of tinnitus and provided insight into the genetic contribution [168,169]. Bogo et al. showed the genetic additive factor to be 40%, suggesting the mostly environmental foundation for tinnitus generation [168]. This study was based on questionnaire data asking about tinnitus annoyance, without further characterizations. Maas et al. conducted a pioneering observation into genetic contribution to tinnitus in twins, showing a heritability of bilateral tinnitus of 56%, and further stratification by sex increased this estimate to 68% in men, showing that the influence of the environment

is minimized when tinnitus subtypes are specified (Maas et al., 2017). These observations demonstrate the lack of clear Mendelian inheritance and possible polygenic involvement in tinnitus generation, confirming it as a complex trait. However, twin studies are unable to account for the effect of shared-environment and gene/environment interaction simultaneously, which can be assessed with adoption studies.

1.6.5 Linkage and association studies

Linkage analyses have been proven useful for Mendelian diseases such as cystic fibrosis [170]; however, this approach has been less successful in complex disorders with high genetic heterogeneity and multilocus effects like tinnitus. Case-control association testing has been used to identify the association of tinnitus with several genes involved in cardiovascular function (*ACE, ADD1*), serotonin transporter function (*SLC6A4*), neurotrophic factors (*BDNF*), dopamine receptor (*COMT*), and ion channel pathways (*KCNE1,KCNE3*) [150] (Table 1).

Gene	**Size (N)**	**Method**	**Gene function**	**Reference**
No associations				
ACE	89	PCR-RFLP	Angiotensin I converting enzyme	[171]*
BDNF	240	PCR-RFLP	Brain Derived neurotrophic factor	[172]
COMT	40	PCR	Catechol-O-methyltransferase	[173]
GDNF	52	PCR-RFLP	Glial cell derived neurotrophic factor	[174]*
KCNE1	201	Sanger	Potassium voltage-gated channel subfamily E regulatory subunit 1	[175]*
KCNE3	288	Sanger	Potassium voltage-gated channel subfamily E regulatory subunit 3	(Sand et al., 2011)*
HTR1A	88	Sanger	5-hydroxytrypthamine receptor 1A	[176] *
	Positive associations			
ADD1	89	PCR-RFLP	Adducin 1	[171]*
SLC12A2	128	SNP	Solute carrier family 12 member 2	[177]*
KCNE1(rs915539)	128	SNP	Potassium voltage-gated channel subfamily E regulatory subunit 1	[177]ap*

KCTD12	95	Sanger	Potassium channel tetramerization domain containing 12	[178]*
SLC6A4	54	PCR and VNTR	Solute carrier family 6 member 4	[179]*

*Studies that have not undergone multiple testing correction

ADD1 p.G460W genotype was found to be positively associated with tinnitus, showing increased frequency of *W* allele among tinnitus patients compared to controls [171]. *ADD1* polymorphism was previously related to hypertension and cardiovascular disease that are known to injure the stria vascularis in the cochlea, suggesting a potential pathophysiological mechanism for tinnitus [180,181]. Similarly, *KCNE1* and *SLC12A2* were found to be significantly associated with tinnitus in noise-exposed males with tinnitus [177]. However, when analyzing both males and females, no significant association was found for *KCNE1* (Sand et al., 2010). *KCNE1* and *SLC12A2* encode a potassium channel subunit that has previously been shown to be involved in auditory neural transmission, suggesting a possible involvement in tinnitus generation (Sand et al., 2010). Genes encoding receptors that increase inhibitory neurotransmission are of interest to analyze, in the attempt to describe the neuronal hyperactivity within the central auditory pathway in cases with chronic tinnitus [182]. *KCTD12* encodes a potassium channel protein that is associated with *GABAb2* receptor and has shown to be significantly associated with tinnitus, although the study was underpowered [178]. Similarly, a *5-HTTLPR* polymorphism in the gene *SLC6A4* that regulates serotonin neurotransmission was found to be significantly associated with an analog visual scale that measured tinnitus impact on the quality of life and discomfort level [179]. Many of the candidate-gene studies are limited by being underpowered, which can explain the lack of significant associations, or false-positive associations and many were not subjected to correction for multiple comparisons.

1.6.6 Genome-wide association studies

The feasibility of using large-scale genomic data has accelerated the research of tinnitus with regards to mapping its genetic landscape. Genome-wide association studies (GWAS) have a significant role in the improved understanding of the genetic architecture and the mechanism of many common and complex diseases, for instance, type 1 and type 2 diabetes [183,184], prostate cancer [185], inflammatory bowel disease [186], major depressive syndrome [187] and breast cancer [188]. The first tinnitus GWAS was performed in an adult population of a homogeneous ethnical background using 167 tinnitus cases and 794 controls [189]. None of the SNPs reached genome-wide significance. However, a subsequent gene-set enrichment analysis asking whether any biological pathway is significantly associated with the results of the GWAS revealed 7 metabolic pathways. Most notably, pathways involved *NRF2*-mediated oxidative stress response, endoplasmic reticulum stress response, and serotonin reception mediated

signaling pathways [189]. Identified pathways have been previously associated with hearing loss, as well as tinnitus [71,154,190]. Authors describe several limitations of the study, such as limited statistical power due to sample size, lack of characterization of the tinnitus phenotype, a cross-sectional study design of the sample set that was not initially optimized to observe the genetic contributors to tinnitus, but hearing loss [189]. No follow-up analysis was done to account for the overlapping heritability between tinnitus and hearing loss; therefore, results from the gene-set enrichment analysis could be potentially due to a hearing loss phenotype. Recent large GWAS study on tinnitus using UK Biobank (UKBB) as a discovery data, identified 6 significant loci, 3 of which were replicated in the Million Veteran Program cohort (MVP) [191]. Mendelian Randomization (MR) showed that tinnitus is associated with hearing loss in bidirectional way, suggesting that genomic distinction between tinnitus and hearing loss is still a challenge [191].

1.6.7 Whole exome sequencing

Whole exome sequencing (WES) is a "bottom up" approach in genomic research of tinnitus. It focuses on isolated protein coding areas of the genome which are expected to have causal implication on proteins by disturbing their function, that can potentially explain the pathology of diseases such as tinnitus [192]. However, only one study has used this approach to investigate tinnitus, so far [193]. Amanat et al., have used extreme phenotype of tinnitus and analyzed rare variants associated with tinnitus, providing a framework for investigating rare as well as common variants. Similar method (whole genome sequencing) has been used for hearing loss, identifying more than 300 novel genes [194], demonstrating the importance of growing genomic research and the opportunity for replication and further insights [195,196].

2 RESEARCH AIMS

The overall goal of this doctoral thesis is to elucidate potential mechanisms involved in tinnitus generation by identifying environmental risk factors and quantifying their effect over tinnitus, as well as map out the genetic landscape of tinnitus.

2.1 AIM I

- Identifying environmental risk factors for tinnitus.

The first aim is to identify environmental risk factors and their contribution to tinnitus, as well as establish the gaps in knowledge. This will in turn promote potential ways of improving the understanding of risk factors and their influence over tinnitus that will lead to improved prevention and limit the incidence of tinnitus.

2.2 AIM II

- Distinguish the environmental from the genetic risk factors by identifying genetic associations with tinnitus.

A second aim is to distinguish the environmental from the genetic risk factors for tinnitus, by mapping its genetic architecture. This will launch the research towards the identification of genetic markers and stratify tinnitus patients based on their genetic risk.

2.3 AIM III

- Understanding the genetic signature of tinnitus, individually and in the context of hearing loss.

The third aim will drive the use of large-scale genomic data and high through-put technologies in order to generate insights into molecular mechanism and pathways involved in tinnitus pathophysiology. Dissecting the genetic foundation of tinnitus will uncover novel biological insights that will facilitate clinical advances by identifying potential drug targets, as well as improve therapeutic optimization, diagnostic and prognosis of tinnitus.

3 MATERIALS AND METHODS

3.1 OBSERVATIONAL EPIDEMIOLOGY

The most common approach to assessing risk in humans is using epidemiological observational studies, as opposed to interventional studies in animals. Observational studies are used to estimate the relative risk of an environmental factor (exposure) over the disease or trait of interest (outcome) [197]. Observational studies consist of cross-sectional, case-control, and cohort studies, forming most of the epidemiological literature associated with tinnitus.

• Cross-sectional studies are analytical studies (involving a comparison group) used to quantify the relationship between the exposure and the outcome. However, the information regarding exposure and outcome are collected at the same time point, making it impossible to measure temporality and therefore, infer a causal relationship. Thus, this study design is ineffective when we want to understand the potential mechanism by which the disease of interest and the risk factors interact.

• In case-control studies, affected and unaffected subjects are identified and subsequently compared by the distribution of the risk exposure in both groups. Case-control studies are usually used to assess the risk factors related to an outcome; however, it is not always clear if the exposure occurred before or after the outcome. Hence, temporality is compromised, and we can only determine whether a population with the outcome is more or less likely to have the exposure.

• In cohort studies, a distinct group of disease-free individuals (exposed and unexposed), are followed over a period of time. Subsequently, the occurrence of the outcome is compared between the exposed and unexposed group, assessing temporality. Therefore, cohort studies can effectively infer causality.

In order to measure the effect of the exposure on the outcome, measures of associations are needed. Relative risk (RR), odds ratios (OR), prevalence ratio (PR) and hazard ratios (HR) are used to quantify the relationship between exposure and disease among two groups. RR compares the risk of health event in population groups that are exposed and unexposed to a specific risk factor [198,199]. RR are standard measure of association in cohort studies [200]. Similarly, HR is estimating the number of exposed subjects with the outcome divided by person-years of observation in one group, divided by unexposed subjects with the outcome in another group, called a rate ratio. One assumption for HR is that the rate is constant over time [201]. In contrast, OR refer to the odds that an outcome will occur given a specific exposure, compared to the odds of the outcome occurring in absence of the exposure and are commonly used in case-control studies [202]. However, there is a common misuse of the interpretation of the OR as “risk”, that usually stems from the selection of the controls [203]. If controls are selected persons from a base-population at risk in the beginning of follow up, then corresponding estimate is RR compared to controls selected from a population at risk at the end of follow up, which corresponds to OR [204,205]. Similar misuse is reported between the use

of OR and PR in cross-sectional studies, where mathematical calculations are the same, but the study design differs where PR is used only when prevalent cases are included [198,206].

In tinnitus research, the majority of the literature consists of cross-sectional studies demonstrating a substantial evidence-based gap in the knowledge [207]. For example, a recent systematic review and meta-analysis showed that smokers have a significantly increased risk of tinnitus. However, the relevance of this association is negligible since most of the data was comprised of cross-sectional studies that are unable to establish causality [208].

One of the aims of this doctoral thesis is to elucidate the environmental risk factors for tinnitus, by conducting a systematic review and meta-analysis of the literature (**STUDY I**) and provide the most updated and comprehensive evidence of the risk factors association with tinnitus. We identified a search string for MEDLINE using Medical Subject hearings and text related to tinnitus, exposure, and prevalence. We established comprehensive eligibility criteria regarding study design, where we excluded case reports, book chapters, letter to editor, dissertations, and thesis. Additionally, we excluded animal studies, and human subject and population-based tinnitus surveys were included. As part of this work, we have identified only 25 cohort and case-control studies that can effectively estimate the causal relationship between tinnitus and environmental risk factors, reporting measures of association. Meta-analysis was performed only for studies that used an appropriate reference group and reported compatible statistical measures that were optimized for pooling.

3.2 GENETIC EPIDEMIOLOGY

3.2.1 Aggregation studies

Genetic epidemiology offers approaches aimed at genetic dissecting of complex traits such as tinnitus. The first step toward characterizing the genetic background to complex traits is to investigate its aggregation within the family, despite their non-Mendelian pattern of inheritance [166,209]. The general approach is to determine whether having a relative with tinnitus increases one's risk of developing tinnitus. This is assessed by a recurrence risk ratio or sibling recurrence risk (λs) and is defined as the risk of siblings of probands (the sample individual) to a specific condition, relative to the population prevalence [210].

In **STUDY II**, we employed this approach in order to investigate the familial clustering of different subtypes of tinnitus [211]. To estimate the familial aggregation, we asked 2457 tinnitus subjects how many siblings they had with tinnitus. The prevalence in the general population of bilateral and unilateral tinnitus was calculated using data from the Swedish Twin Registry (STR; N=67,615) [212]. Prevalence of severe tinnitus was found using data from one wave of the Stockholm Public Health Cohort (SPHC, N=72,295) [213] and one wave of the Swedish Longitudinal Occupational Survey of Health (SLOSH) (N=19,992) [214]. LifeGene data set was used to estimate the prevalence of constant tinnitus (N=26,696) [215]. Clinically ascertained tinnitus individuals in the SPHC were identified through record-linkage with the National Patient registry

(ICD10:H93.1). We identified 608 families reporting tinnitus in at least one first-degree relative. λs is calculated as

$$\lambda = \frac{Kr}{K}$$

where *Kr* is the prevalence of tinnitus among siblings in the family, and *K* is populational prevalence

$$Kr = \frac{N.of\ affected\ relative\ of\ affected\ proband}{total\ N.of\ relative\ of\ affected\ proband}$$

resulting in λ scores. A score greater than 1 indicated that the sibling has an increased risk to be affected by tinnitus, if their sibling is affected [216]. We estimated λs scores for bilateral, unilateral, constant, and severe tinnitus. Further, we implemented a percentile bootstrap approach to provide an accurate estimate and significance level for the calculated lambda score. A limitation in familial aggregation studies is that it does not take into consideration the underlying biology for complex traits; therefore, the evidence of familial aggregation should not be taken as a statement of direct genetic effect [166].

3.2.2 Twin and Adoption studies

Twin studies are important for establishing a genetic component to complex traits [209]. Twin studies are particularly useful due to their genetic similarities and shared environment allowing for a comparison among monozygotic (MZ) or dizygotic (DZ) twin pairs and estimate heritability of complex traits [217]. Heritability (h^2) is estimated as the proportion of total variance that is accounted for by the genetic components:

$$h^2 = \frac{V_A}{V_P}$$

Where V_A = genetic variance with additive and dominant components and V_P is the total phenotype variance [217]. Twin studies have been previously used to estimate tinnitus heritability establishing a genetic component to tinnitus generation [168,169]. However, twin study design cannot account for gene/environment interaction due to the shared environment. Adoption studies can overcome this limitation due to their ability to separate genetic from environmental influence on a person's development [218].

In **STUDY III** we conducted an adoption study to investigate clinically significant tinnitus and its association with genetic factors [219]. We used a case-control study design with Swedish nationwide registry data to identify adoptees and their biological and adoptive parents and use their clinical assessment of tinnitus. We used multivariate logistic regression to calculate OR for tinnitus in adoptees with at least one affected biological parent compared to unaffected biological parents. In this model, we used adoptees' birth year, sex, educational attainment, and county of birth as covariates. Secondary outcome was an OR based on comparison between at least one affected adoptive parent and unaffected parents. Logistic regression is a standard method to estimate the association of independent variable and a binary (dependent)

outcome, resulting in estimation of probability of an outcome given the value of the predictor, or independent variable [220,221].

3.3 GENETIC ASSOCIATION ANALYSIS

Linkage and association analyses are two types of statistical methods used to identify the genetic basis of complex disroders. Linkage analysis relies on the idea that haplotype inheritance among generations without recombination, causes whole regions of the genome to be transmitted within families [210,222]. Association studies use case-control association testing based on a comparison of the allele frequencies between affected and unaffected subjects in the population. Significantly higher frequency of a given allele among the affected subjects compared to the unaffected is considered as evidence for disease susceptibility [222,223]. A positive association of a genetic variant with the disease of interest means that either the genetic variant is directly causative, or it is in linkage disequilibrium with a locus that directly affects the expression of the phenotype [224]. Linkage disequilibrium arises when there is an increased frequency of haplotype combinations of alleles at physically linked loci that have been inherited together across generations in the population. Since linkage disequilibrium (LD) is dependent on the population's history, an allele can be positively associated with the trait of interest, either in an isolated population or a very large, mixed population [222]. A potential issue with association studies in large, mixed population studies is ethnic stratification that can lead to false-positive association simply due to a higher frequency of the allele [223]. Most association studies, aiming to map the genetic landscape for tinnitus, employ a candidate-gene approach looking at the variation associated with the disease within a number of pre-defined genes [223]. Identifying patterns of LD in association studies and their use for localizing regions of the genome tagged by intercorrelated single nucleotide polymorphisms (SNPs) have been used to investigate specific regions of the genome and the evaluation of candidate genes associated with a complex disease [222].

In **STUDY IV** we conducted a case-control genetic association analysis evaluating the involvement of *Val*^{158}*Met* polymorphism of *COMT* in tinnitus generation and perception. We recruited a total of 1449 participants from the Swedish Tinnitus Outreach Project (STOP) and the Karolinska Hospital. Tinnitus cases and controls were identified by set of questionnaires - Intro_3 question in STOP questionnaire - "Do you have tinnitus?" as well as, the ESIT-SQ question A17 - "Over the past year, have you had tinnitus in your head or in one or both ears that lasts for more than five minutes at a time?", answering "Yes", and "No", respectively. We used both questionnaires to identify so-called "super cases " and "super controls" in order to create a consistent phenotype.

- **Genotyping**

DNA was extracted using Oragene OG-500 DNA extraction protocol (DNA Genotek Inc. ON, Canada). After final purification, the DNA was stored in -80°C freezer for preservation

following standard guidelines (Szczepek et al., 2019). After evaluating DNA yield and quality using Nanodrop, we diluted the DNA with DNase-free water to a required concentration of 5 -20ng/µl. Then we performed 349-well plate TaqMan SNP genotyping with TaqMan Drug Metabolism Genotyping Assay (Thermofisher Scientific, cat no: 4362691), according to protocol. We used TaqMan Assay due to its low genotyping error and comprehensive results. TaqMan SNP genotyping assay requires forward and reverse PCR primers to discriminate 5' nuclease allele and two TaqMan probes that are labelled with fluorescent dyes, increasing the fluorescent signal with each PCR cycle. We used Applied Biosystems® ViiA™ 7 Real-Time PCR instrument (Life Technologies, Inc.; Foster City, CA), to detect the signals. The Sequence Detection System (SDS) Software uses the fluorescent signals during the plate read to create allelic discrimination plot detecting values in each cell and to indicate which allele is present in each sample.

- **Statistical Analysis**

According to the principles of genetic association studies, we hypothesized that increased frequency of the SNP allele in cases compared to controls, indicates that the presence of the SNP allele may increase the risk of tinnitus [225]. We used ANOVA in order to account for type 1 error probability (false positive) due to multiple comparison. The basic principle of ANOVA is to test for mean differences among groups of population by examining the amount of variation within each group, compared to the variance between groups. There are 3 basic assumptions in ANOVA testing; 1) each sample is drawn from a normally distributed population; 2) these populations have the same variance; 3) the groups are independent, and all other factors are controlled for [226]. Based on general genetic modeling for single SNP with alleles *AA, AB* and *BB*, we get a 2 x 3 contingency table. We used a logistic regression model with 0/1 outcome in cases and controls for the variable with 3 genotype levels *Val/Val, Val/Met, Met/Met*, with *Val/Val* set as a reference group. This additive model accounts for the effect of each allele by assuming increased risk by a factor for each allele. Alternatively, we analyzed a dominant model, by pooling suspected risk alleles (*Val/Met + Met/Met*), under the hypothesis based on existing literature, that carrying *Met* allele increases the risk of tinnitus, creating a 2 x 2 contingency table. Logistic regression method provides ORs for tinnitus, comparing the odds of tinnitus based on the genotype. For computing and analysis, we used R package "SNPassoc" (Rstudio, v1.4.7 2009-2021, PBC).

3.4 GENOME-WIDE ASSOCIATION STUDIES

Another approach using association studies with complex traits are Genome-wide association studies (GWAS). GWAS are hypothesis-free, case-control analyses used to explore the association between common genetic variants across the whole genome and a phenotype of interest [227]. These studies use the non-random inheritance of genetic variants that are in LD to estimate the association between hundreds of thousands up to millions of genetic polymorphisms and a trait of interest [228]. GWASs typically identify common risk variants with small effect sizes that can be concentrated in a specific genomic region or spread across different regions. GWA studies are population-based and can leverage the advanced

genotyping methods to study the relationship between genetic variation and a phenotype. The overall methodology of GWAS utilizes a large sample-size cohort of cases and controls based on clearly defined phenotype. Each individual is genotyped at a defined set of SNPs on chip arrays or sequencing strategies across the whole genome. After primary quality control, such as genotype calling and exclusion of duplicate SNPs and individuals, principal components are calculated to demonstrate clustering of individuals on genetic basis. In order to maximize genomic information, untyped variants are imputed using haplotype phasing and reference population [229]. Finally, genetic association analysis is performed and all SNPs are then independently evaluated for their association with the phenotype of interest and summary statistics are generated [230].

From a statistical point of view, GWAS are assumed to have a non-bias case-control selection from a population-based cohort [227]. The study population in a GWAS should be matched for ethnical background to avoid population stratification and cryptic relatedness, which would inflate type 1 error and generate false associations [227]. Given the comprehensive genetic information generated in a GWAS, population stratification can and should be corrected for in the association analysis using the genotyping data. Basic interpretation of a GWAS analysis is that one or more tag SNPs reside on a haplotype LD block with functional variants that have a biological effect on the phenotype. The identified SNPs are subjected to a stringent genome-wide significant threshold of p-value $5x10^{-8}$ after Bonferroni correction for multiple testing to reduce the likelihood of false-positive associations [231]. Independent replication and follow-up analysis, such as conditional analysis to identify independent signals in one locus and fine-mapping of the genomic region are required to identify which SNPs are causally associated with the disease. The sample size of the GWAS directly influences the statistical power of association of the analysis, and it is a key principle in the study design. Meta-analysis of GWAS data is an obvious solution to overcome the restrictions of sample size and can obtain in silico replication, and explore potential sources of heterogeneity, assuming standardization of the phenotype characteristics and exposures among individual cohorts [232]. Genome-wide association studies remain a robust approach for future tinnitus research, assuming the presence of a large sample size that will account for some considerations of the study design. A large meta-analysis is the most feasible option for initial discovery and validation efforts that can detect novel signals and elucidate the biomedical relevance of tinnitus [232]. In our aim to understand the genetic background of tinnitus in the context of hearing loss, we first performed a GWAS meta-analysis on subjects with age-related hearing loss and then for tinnitus (**STUDY V** and **STUDY VI,** respectively).

In **STUDY V** [233] and **STUDY VI**, we leveraged large-scale GWAS summary statistics from 17 population-based cohorts and conducted a meta-analysis. Samples based on questionnaire data for hearing loss and tinnitus were selected from the Swedish Twin Registry (STR) and genotypes were generated with SNP&SEQ platform in Uppsala University using Illumina Infinitum assay. We obtained raw genotype samples from STR and performed standardized quality control where we excluded with more than 10% missingness and discrepancies in observed sex and relatedness using PLINK 2.0 [234] and R studio. Post-QC genotype files were

prepared for phasing with SHAPEIT [235] and EAGLE [236], where we statistically estimate haplotypes from genotypes and proceed to imputation. We used IMPUTE2 [237] software to infer unobserved genotypes based on Haplotype Reference Consortium panel (HRC1.1) [229]. Imputation significantly increases the statistical power by evaluating the association of a tagged SNPs with reference SNPs that are not directly genotyped. After imputation and filtering SNPs with low imputation accuracy (INFO > 0.1), we conducted association analysis using BOLT_LMM algorithm [238]. BOLT_LMM computes association statistics between phenotype and genotype using linear-mixed model that avoids confounding by adjusting for principal components and genetic relatedness matrix (GRM). The linear mixed model accounts for relatedness by modeling the covariance between phenotype and genotype as a random, polygenic effect, while controlling for false positive associations [238]. After generating association summary statistics for STR we combined summary statistics from 16 other cohorts. We then performed standardized quality control using EasyQC software, where we excluded monomorphic SNPs, accounted for SNP missingness <0.05, excluded duplicate SNPs and SNPs with imputation score < 0.5. We meta-analyzed all summary-statistics using METAL software, by conducting inverse-variance weighted fixed-effect model, by computing β-coefficients and their standard errors [239]. We generated Manhattan plot with genome wide-significant SNPs and Quantile-Quantile plot used for visually examining the population stratification. The QQ-plots plots the observed test statistics against the values that would be obtained from a theoretical distribution and computing test-statistics to account for population stratification and other confounders.

- **LD Score Regression**

To distinguish between inflation of test statistics from true polygenicity (many small genetic effects) and bias, such as population stratification and cryptic relatedness, we used LD score regression [240]. LD score regression intercept quantifies the contribution of each factor by estimating the relationship between test statistics and linkage disequilibrium and for traits with polygenic architecture, intercept is close to 1 [240]. Additionally, we used LD Score regression to estimate genetic correlation between hearing loss and range of disorders, such as tinnitus, and to evaluate the extent of shared genetic background.

- **Conditional and joint association analysis (COJO)**

After obtaining results from meta-analysis, we used COJO to reveal possible secondary association SNPs for one genome-wide significant locus using GCTA software. COJO adopts a stepwise selection procedure to select SNPs based on conditional p-values and then estimate the joint effect of all selected SNPs after optimization, estimating LD from a reference samples from the UKBB [241].

- **Gene-set and pathway anaysis**

After identifying independent loci, we used MAGMA [242] to map genes to the identified SNPs. MAGMA gene analysis is based on multiple regression model for better statistical

performance. Gene-set analysis provides simultaneous analysis of multiple genes, identifying more genes and gene sets associated with hearing loss or tinnitus while correcting for type 1 error. This analysis provides additional insight into molecular and functional mechanisms based on the genetic component [242].

- **Expression analysis**

We obtained gene expression data in human tissue from Genotype Tissue Expression (GTX) project, v8 [243], aiming to characterize variation in gene expression levels across human tissues. Normalized expression was used to obtain differentially expressed gene sets (DEG) for each tissue, performing two-sided t-test per gene per tissue. Further, we used expression quantitative trait loci (eQTLs) to identify genetic variants that could explain the variation in gene expression levels for phenotype [244]. Using generalizes summary statistics Mendelian randomization (GSMR) we set to test the causal association between tinnitus and hearing loss [245]. Mendelian randomization is an analysis that uses genetic variants as instrumental variables independent of confounding factors, to infer credible causal associations [246,247]. GSMR is leveraging power from multiple genetic variants accounting for LD between them, based on summary GWAS data. Particular attention should be given when interpreting results from MR, especially distinguishing the signals of causality from pleiotropy (a single locus can directly affect multiple phenotypes) [248]. For that purpose, HEIDI-outlier score is used to detect genetic instruments that have pleiotropic effect on both risk factors [245].

- **Molecular insights**

In order to understand the biological relevance of our hearing loss GWAS findings and identified genomic loci, we sought expression data from already published mouse cochlea, profiling 15 different cell types [249,250]. We calculated the expression specificity by first aggregating the count per gene per cell type and filter out genes that are not expressed in any cell type or are not orthologous between mouse and human [251]. After normalizing the expression to 1 transcript per million per cell type, gene expression specificity was calculated dividing normalized expression in the cell type and sum of the expression in all cell types. Specificity ranged from 0 to 1, where a higher value indicates that the gene is more specific to the corresponding cell type, compared to it expression across all cell types. We then estimated SNP-heritability enrichment in hearing loss gene-level associations. By identifying cell-types specific genes that are enriched in our hearing loss genes, we gained an insight into functionality of each cell type in hearing loss.

Due to lower statistical power and fewer identified association and mapped genes in the tinnitus GWAS, we were unable to perform this analysis regarding tinnitus molecular pathways.

3.5 BURDEN ANALYSIS

GWAS analyses identify common variants that are associated with common diseases across the genome. However, the majority of identified GWAS variants are located in non-coding region of the genome [228], indicating that rare variants might also contribute to the polygenic complex model of tinnitus. Whole exome sequencing is a technique that targets only the protein-coding (exome) part of the genome [192]. WES studies are used to discover rare variants with large effect that are associated with a phenotype, and direct causal effect is easier to infer [252]. WES has been previously used for analyzing pathogenic variants for hearing loss [253–256], however, there is only one study using WES to understand the effect of rare variants on tinnitus [193]. Amanat et al., used subjects with extreme tinnitus phenotype in order to have a more homogenous population with strong genetic effect.

In **STUDY VII** we aimed to investigate the association of rare single nucleotide variants (SNV) and copy number variants (CNV) with severe tinnitus. Participants were selected from the STOP project based on questionnaire data. Severe tinnitus cases were identified by chronic and constant tinnitus for more than 6 months, and THI score > 58. Controls were obtained as healthy subjects from the SweGen project. Genomic libraries were prepared with Illumina TruSeq PCR_free DNA kits and were sequenced by NovaSeq6000 platform. After variant calling and annotation, we filtered out variants with MAF <0.01 and low CADD score, keeping likely pathogenic variants in the database. We performed a gene-burden analysis (GBA) to explore rare variants associated with our cohorts. Gene-burden testing is used to compare individuals carrying the rare variants in cases and controls [257]. A tabulation of the count of cases and controls generates a 2 x 2 contingency table and for each gene, ORs and 95% confidence intervals are computed. *P*-values are calculated and corrected for multiple testing. Burden analysis can improve the interpretation of missense and loss-of-function variants and identify candidate genes for complex disorders.

3.6 ETHICAL CONSIDERATIONS

In this research project, we discuss both epidemiological and genetic analyses where ethical considerations should be discussed. Ethical permits have been obtained for all our studies. Studies used in GWAS meta-analysis confirmed the presence of ethical permits and informed consent for all participants.

Epidemiological studies investigate the distribution of the predictors for health-related events in a study population. Epidemiological research is examining human exposure to different environmental factors and compute their association with an health outcome [258]. The goals of epidemiological studies are to generalize the understanding of risk factors and their effect on distribution of health or disease in the population, and asses the consistency of epidemiologic data and ethological hypotheses by providing foundations for preventative procedures that will promote health in the population [259]. Ethical issues arising in epidemiological studies are related to potential risks, privacy, consent and conflict of interest [260]. Potential societal benefits should be balanced with the risk for individuals in epidemiological studies, which

requires respect of individual rights, privacy, and confidentiality, while applying scientific approaches for restoring public health. These risks are especially important to minimize in studies of children, elderly people and marginalized populations [260]. Epidemiologists are obligated to distribute newly acquired information from epidemiological studies that should be relevant to every populational group defined factors such as sex, race, ethnicity, socioeconomic factors [261]. Additional ethical issues can arise from failing to disclose conflict of interest that can affect public trust in epidemiological data and health research. It has been reported that financial interest can influence researchers' commitment to a scientific approach, especially in clinical trials that damages public confidence in epidemiology [262]. Therefore, ethical committees are obligated to inspect any and all potential harmful interactions, censorship or interference in the process of conducting epidemiological research. One way to protect individuals involved in epidemiological studies is informed consent [263]. Informed consent is based on information and voluntariness [264]. Voluntariness refers to the decision of the participant to be involved in the study without influence or coercion. Informedness on the other hand, requires the participant to have sufficient information to enable them to freely make a decision whether to refuse or consent to participate. In the last decades with the progress of digitalization and data generation, most research requires multidisciplinary collaboration between different specialists such as clinicians, epidemiologists, geneticists, microbiologists and bioinformaticians. This add a level of complexity of a research project making it difficult for everyone involved to be fully educated in all possible consequences [265], creating so called "information gap" leading to more restrictive version of consent criteria than it is needed in order to protect the interest and safety of the participants [266,267]. A written informed consent form is the "gold standard" for biomedical research, allowing the participant to exhibit autonomy and self-governance and competence [265]. Privacy and confidentiality are basic concerns for genetic and epidemiological research which is addressed by creating sophisticated coding and anonymization methods [268]. Potential ethical concerns in genetic studies can arise from the fact that genetic data can provide information, not just on the individual, but their relatives and related population [269]. In particular, GWAS pose some ethical challenges, mostly due to the international collaborative nature of the projects and data generated by consortiums and biobank. Moreover, GWAS are considered hypothesis-generating studies rather than hypothesis-testing, with complex statistical and scientific approaches that are difficult to comprehend, generating very large amount of data that can be re-analyzed for different purposes, making it difficult for ethical committee to reach a consensus [270,271]. International involvement in GWAS has increased complexity of standards for data sharing and protection. This has led to establishing a stringent de-identification methods and strict consent for the use of DNA for future research by the General Data Protection Regulation, within Europe [272]. In addition, there is an agreement within the research community that genetic results that are medically relevant and would have not been diagnosed otherwise should be disclosed to that individual [273]. Arguably, one of the current ethical challenges for GWAS and general genetic research is diversity and generalizability of results for the global population. The lack of inclusivity in GWAS is failing to generate knowledge that can promote health regardless of

race and geographical region [274]. Only 20% of GWAS studies have been investigating common disorders in population of non-European ancestry, showing underrepresentation of Asian, African and indigenous population [274,275]. Ethical guidelines and standards for genetic studies are continuously improving with the goal of securing safety for inevitably fast progression of genomic data analysis and machine learning approaches that change the perspectives of human health.

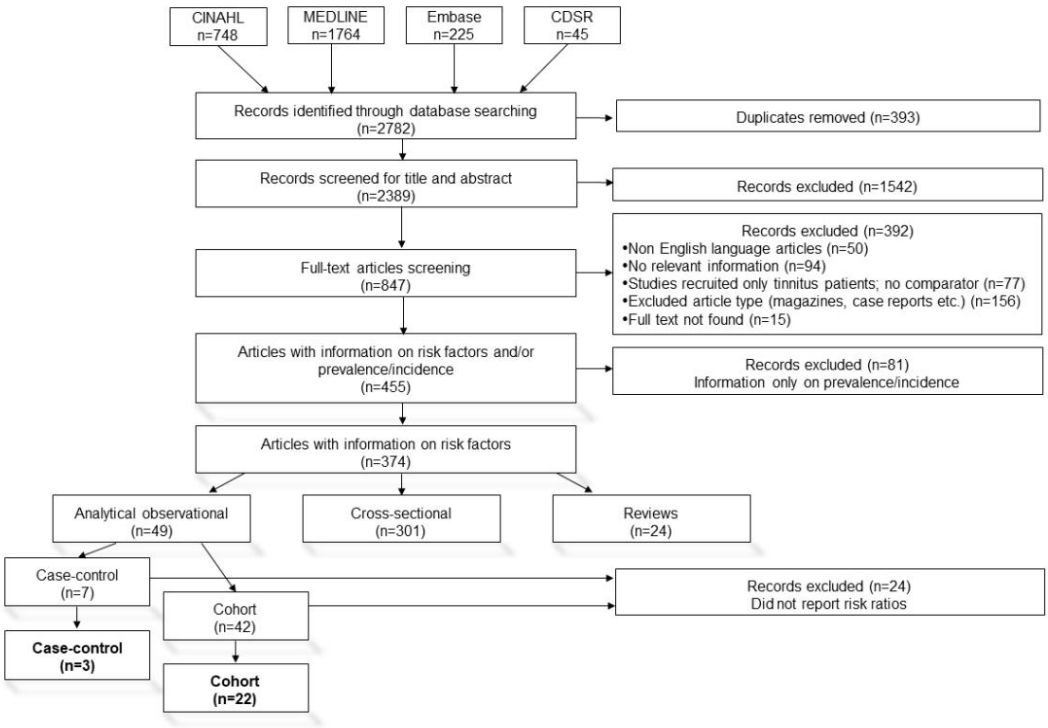
4 RESULTS

4.1 **STUDY I -** LOW EVIDENCE FOR TINNITUS RISK FACTORS: A SYSTEMATIC REVIEW AND META-ANALYSIS - MANUSCRIPT

In order to provide the most updated and comprehensive overview of environmental risk factors associated with tinnitus, we are conducting a systematic review and meta-analysis. The primary aim of this systematic review is to identify all publications that report information on the relationship between tinnitus and various environmental risk factors. A secondary aim is to quantify the association between any potential risk factor and tinnitus and identify tinnitus prevalence in specific populations.

We identified 374 studies that passed the primary eligibility criteria, 13% (49) of which were analytical observational studies. Only 25 studies met the quality threshold with reported measures of association, 22 of which were cohort studies (**Figure 2**.)

Figure 2. Schematic presentation of identified studies



Of the cohort studies, 10 were prospective, evaluated hearing-related information from participants that have been followed up after number of years. In contrast, 12 studies reported risk factors based on retrospectively collected symptom. We identified a large heterogeneity among studies based on their study design, analysis, and adjustment for covariates. All studies reported risk factors that can be divided into 6 main categories: hearing related; lifestyle risk factors, socio-demographic; comorbidities; treatments and therapy; other. High-quality studies and studies with appropriate study design were used in the meta-analysis.

Tinnitus was moderately associated with unspecified hearing loss (RR, 1.94; 95% CI, 1.41-2.67), occupational noise exposure (RR, (1.63; 95% CI, 1.61-1.65) and otitis media (RR, 1.63; 95% CI, 1.61-1.65). Increased risk of tinnitus was associated with sensorineural hearing loss ((RR, 3.68; 95% CI, 2.93-7.04;), and platinum therapy (RR, 324 2.81; 95% CI). Leisure noise exposure was not associated with tinnitus (RR, 1.36; 95% CI, 0.70-2.62). We analyzed studies reporting lifestyle factors impacting tinnitus, such as computer use, nutrition, physical activity, alcohol use, coffee and tobacco consumption and drug addiction. We found that only high alcohol consumption was associated with tinnitus, decreasing the risk of tinnitus (RR, 0.94; 95% CI, 0.91-0.96). Conversely, low alcohol consumption (RR, 1.00; 95% CI, 0.85-1.19) and smoking (RR, 1.15; 95% CI, 0.81-1.62) were not associated with tinnitus. We did not find any of the socio-demographic risk factors to be associated with tinnitus. Out of all comorbidities, depression (RR, 1.31; 95% CI, 1.28- 1.34) and temporo-mandibular joint disorder (RR, 2.06; 95% CI, 1.30-3.27) showed increased risk of tinnitus. Association with diabetes indicated a preventative effect on tinnitus development (RR, 0.85; 95% CI, 0.82-0.88). There was no evidence of association between tinnitus and the following comorbidities: heart failure, hypertension, body mass index, stroke, rheumatoid arthritis, migraine, head injury, and whiplash. This study shows a very limited knowledge on tinnitus related risk factors. The lack of prospective cohort studies and high-quality data acquisition and analysis results in a gap in knowledge and inability to infer causal relationship between tinnitus and environmental risk factors.

4.2 **STUDY II -** SEX-DEPENDENT AGGREGATION OF TINNITUS IN SWEDISH FAMILIES

The second aim of this thesis is to distinguish the environmental and genetic risk factors for tinnitus. Therefore, we performed a familial aggregation study to assess the recurrence risk of specific forms of tinnitus within the family, namely bilateral, unilateral, constant, and severe. Prevalence was higher in males for all tinnitus subtypes. The highest λs we found for severe tinnitus 7.27 (95% CI (5.56–9.07). λs for bilateral tinnitus λs_{Bil} = 1.79 (95% CI (1.55–2.04)) was similar to the one for unilateral tinnitus λs_{Unil} = 1.99 (95% CI (1.45–2.56)). Constant tinnitus showed slightly higher λs = 2.29(95% CI (2.01–2.58)). Following a sex-stratified analysis we found that λs for severe tinnitus was consistently higher in women (λs = 10.25 (7.14–13.61)). compared to male λs = 5.03 (3.22–7.01). Overall, higher lambda scores were observed among women, compared to man suggesting a greater genetic susceptibility in particular to severe and constant tinnitus.

4.3 **STUDY III** – ASSOCIATION GENETIC VS. ENVIRONEMNTAL FACTORS IN SWEDISH ADOPTEES WITH CLINICALLY SIGNIFICANT TINNITUS

Adoption studies are a powerful tool for evaluating the interaction between genetic and environmental risk factors, accounting for shared family environment. We conducted an adoption study to answer whether clinically significant tinnitus is associated with genetic risk factors, using data from adopted subjects (N=11,060) and their biological (N=19,015) and adoptive parents (N=17,025) from the Swedish nationwide registry. We employed a case-control study design using ICD codes for tinnitus cases. The OR for tinnitus of adoptees with and affected adoptive parent was not significant 1.00 (95% CI, 0.43-2.32), indicating that

shared family environment with adoptive parent does not influence tinnitus among adoptees. In contrast, biological parent with tinnitus increased the odds of tinnitus in adoptees by a factor of 2.22 (95% CI, 1.03-4.81). These findings suggest that genetic factors are associated with clinically significant tinnitus.

4.4 **STUDY IV –** LACK OF INVOLVEMENT OF *COMT* VAL^{158}MET POLYMORPHISM IN TINNITUS SEVERITY **-** MANUSCIPT

We performed genetic association analysis in a case-control settings to test for the implication of *COMT* variant on constant and severe tinnitus. Using additive genetic model, we did not find and association between *COMT* genotypes and constant tinnitus. (OR = 0.88; 95% CI: 0.62-1.24; $p = 0.108$). Similarly, no association was identified when we stratified by sex. The dominant model, combining all – *Met* variants did not show any evidence of association with constant tinnitus. We then stratified our study population based on THI score >58 for tinnitus severity. Here, the additive model showed no effect of genotype among people with severe or negligible tinnitus (OR = 0.89; 95% CI: 0.55-1.41; p = 0.029). In the sex stratified analysis, men with *Met/Met* genotype showed decreased odds of severe tinnitus, when compared to those with negligible tinnitus controls (OR = 0.13; 95% CI: 0.02-0.56; $p = 0.009$). The dominant model showed male specific trend (OR = 0.25; 95% CI: 0.09-0.64; $p = 0.007$). These findings suggest a possible preventive effect of *COMT* polymorphism on tinnitus only in males.

4.5 **STUDY V -** GENOME-WIDE ASSOCIATION META-ANALYSIS IDENTIFIES 48 RISK VARIANTS AND HIGHLIGHTS THE ROLE OF THE STRIA VASCULARIS IN HEARING LOSS

GWAS studies are an established approach to outline the genetic blueprint of complex traits and common diseases. Leveraging 17 independent cohorts and a total of 723,266 individuals of European descent we conducted a GWAS meta-analysis for age related hearing loss. Cases were defined by wither clinical diagnosis of tinnitus (2 studies, FinnGen and Estonian Genome Center) or self-reported hearing loss. We identified 48 significant and independent loci ($p < 5x10^{-8}$). LDSC intercept was 1.0039 (0.0095) indicating inflation of test statistics due to polygenicity, rather than confounding. We estimated SNP-heritability on the liability scale to vary between 0.033 (0.002) and 0.061(0.003) based on populational prevalence. Out of 48 independent loci, 10 were novel associations with LD < 0.6. Prioritized genes were examined for their relationship with hearing loss in human or in mice and 17 loci were in or near genes with known effect on hearing loss. Pathway analysis showed enrichment in sensory perception of mechanical stimulus, sensory perception of sound and negative regulation of actin filament polymerization. Out of 48, 8 lead NSPs encoded missense mutations. Further in-silico analysis identified 2 SNPs to be highly deleterious and to likely disrupting gene function. We analyzed our identified loci of hearing loss and their association with other traits and diseases. We found a positive correlation of hearing loss with depression, obesity, insomnia, neuroticism alcohol dependance, attention deficit hyperactivity disorder and smoking. Using mouse cochlear and brain cell specific expression profiles, we identified the involvement of spindle and root cells and basal cells of the stria vascularis in hearing loss. In basal cells, 10 genes were associated with our GWAS significant SNPs. In spindle and root cells, *EYA4* and *HOMER2* were identified in sensory perception of sound and mechanical stimulus pathway, respectively. These finding suggest that common

variants in genes expressed in cells of the stria vascularis have a significant impact on hearing function.

4.6 **STUDY VI –** GENOME-WIDE ASSOCIATION META-ANALYSIS ON TINNIITUS INDICATED LOW INDEPENDENCE FROM HEARING LOSS - MANUSCRIPT

Similar to **STUDY V**, we conducted a GWAS on tinnitus in order to investigates its genetic landscape. We curated a summary statistic for 16 independent cohorts comprising 56,467 tinnitus cases and 475,859 controls. GWAS meta-analysis revealed 2 significant loci. LDSC intercept was 0.9729 (0.0074) indicating polygenicity and lack population stratification. We estimated SNP-heritability on the liability scale to be 0.069 (0.0038) given the prevalence ration (10%). Two genomic loci were identified as significant, with independent signals rs2263514 on chromosome 5 and rs4350491 on chromosome 14. We prioritized genes and used MAGAM for a gene-set analysis to identify molecular pathways associated with tinnitus. Pathways involved in oxidative stress repair and neuron death were identified but none reached statistical significance (Bonferroni corrected p-value <0.05). Only one SNP rs158921 is annotated as exonic with CADD core of 21.2 suggesting deleteriousness of the SNP. The gene *NDUFAF2* was the gene mapped closest to rs158921 and rs2263514, that is involved in mitochondrial respiratory chain complex I assembly [276]. *TMX1* was the gene mapped closest to rs4350491 and is involved in endoplasmic reticulum oxidative stress response, implicated in Meniere's Disease and tinnitus [277]. We found significant positive genetic correlation with tinnitus and associated disorders such as back pain, depression, headache, occupational noise exposure and anxiety. No somatic tissue was significantly associated with tinnitus in the GTEx v8 dataset. We applied GSMR to test for potentially causal association between hearing loss and tinnitus and we found 34 SNPs as genetic instruments. However, HEIDI p-value was 0.285, indicating that the genetic instruments were not independent, and pleiotropy cannot be excluded when we interpret the results. The findings from the GWAS meta-analysis suggest the codependent nature of tinnitus and hearing loss

4.7 **STUDY VII –** COMPREHENSIVE ANALYSIS OF CODING AND NON-CODING RARE VARIANTS IN THE GENOMES OF SWEDISH PATIENTS WITH SEVERE TINNITUS - MANUSCRIPT

We aimed to explore the effect of rare variants on Swedish patients with severe tinnitus by analyzing single nucleotide variants (SNV), large structural variants (LSV), and copy number variants (CNV) using Whole Exome Sequencing (WES). Using Gene burden analysis we found 8 genes with burden of Loss-of-Function variants (LoF) and 4 of them were mutation-intolerant genes (*KIAA1109, FAM135A, TUT4, DNAH7*). Missense SNVs were found in 3 genes: *CACNA1E*, *DHX37* and *NAP1L3.* We found rare variants in genes that have been associated with tinnitus such as *ANK2, AKAP9* and *TSC2.* Additionally, we found 4630 large structural variants (LSV), 37 of which were classified as likely pathogenic, according to

AnnotSV. We found ultra-rare variants overlapping the mutation-intolerant gene *NAV2* and *TMEM132D.* We analyzed copy number variants (CNV) that were categorized as likely pathogenic, however, non were in overlapping candidate genes that show enrichment of SNV We used Allen Brain Atlas of adult mouse brain to investigate the candidate gene expression and found that the cortex, hippocampal region, cerebellum, and olfactory bulbs showed expression of *NAV2* and *CACNA1E*. The special distribution of the *CACNA1E*. gene was comparable with the expression profile in human brain. These findings implicate *CACNA1E*, *NAV2,* and *TMEM132D* in severe tinnitus.

5 DISCUSSION

This thesis uses several methods to infer insights into the environmental and genetic background of tinnitus, as well as understanding tinnitus in the context of hearing loss. We highlight several points of discussion that became apparent in this thesis.

5.1 GAPS IN KNOWLEDGE

In our systematic review we reviewed the literature on environmental risk factors for tinnitus. However, only 6% of all studies passed the quality control and reported quantified measures of association based on case-control and cohort studies. As previously shown, we confirm the role of hearing loss increasing the risk of tinnitus. Our findings show causal link between TMJ and tinnitus as previously suggested [207,278]. The most striking finding from performing the systematic review was the paucity of high quality analytical observational studies, which prevented us from deducing cause and effect relationship between exposures and tinnitus. Furthermore, our systematic review, highlighted the numerous gaps in knowledge such as the lack of evidence for associations between tinnitus and the role of nutrition, physical activity, and social environment. Similarly, the relationships between tinnitus and cardiovascular disorders [207], anxiety [279], and otosclerosis [42], are based on cross-sectional studies or an insufficient number of case-control studies where a clear direction of causation was impossible to infer.

5.2 DISCREPANCIES IN TINNITUS DEFINITION

Tinnitus definition is another concern that was highlighted in our **STUDY I**. Our findings revealed that only 13 out of 25 studies relied on ICD codes for tinnitus by using healthcare or health insurance databases, while the remaining ones used questionnaires for self-reported tinnitus assessment. However, the discrepancies in tinnitus assessment are obvious when comparing medically defined tinnitus vs. self-reported outcomes. For instance, ICD 10 codes for tinnitus specify only left ear, right ear, bilateral or unspecified tinnitus, and most often as a secondary symptom to an otologic disorder, whereas tinnitus questionnaires use a number of questions to identify tinnitus, mostly related to the tinnitus experience being occasional, constant or severe. Recently, it has been shown that the more often occasional tinnitus occurs, the higher the odds are for tinnitus to become constant, and once constant, tinnitus correlates with delayed ABR Wave V latencies [49]. These findings suggest a tinnitus subtype that is not considered in clinical practice. Indeed, it is possible for individuals to get tagged with tinnitus ICD code H93.1 despite of tinnitus being mild or non-clinically relevant, for instance as after a noise exposure - an acute but transient tinnitus, or for having hearing loss as a primary complaint and accompanied with tinnitus as a symptom (a secondary condition). The proportion of individuals with occasional or constant tinnitus, acute or chronic, visiting a specialty clinic for tinnitus as a primary complaint, to our knowledge, is still unknown.

Moreover, Lugo et al., established a relationship between self-reported severe tinnitus and suicide attempts, but not among subjects with clinical diagnosis of tinnitus [280]. In this context, the tinnitus diagnosis preceded the self-report data on suicide attempts, which could indicate that individuals that have been exposed to medical attention were less susceptible to suicidal attempt, than those that had not seen a doctor for the purpose of tinnitus. In clinical settings, tinnitus is usually assessed in relation to a primary disorder such as hearing loss. This suggests that the relationship between tinnitus severity and suicide attempts could have been underestimated if only ICD codes were considered [280]. The lack of standardization regarding tinnitus assessment in clinical practice, increases the risk of misclassification of cases and controls, compromises the quality of tinnitus research, and jeopardizes the meta-analysis in our **STUDY I**, by introducing high variability in the measured outcomes. In addition to scarcity of available data for meta-analysis, a significant heterogeneity in tinnitus definition, selection of statistical models and adjustment factors contribute to lack of clear causal inference between tinnitus and the investigated environmental risk factors.

5.3 TINNITUS SUBTYPES AND GENDER BIAS

Important limitation in epidemiological tinnitus research is the lack of studies addressing different tinnitus subtypes and association with sex. The association between tinnitus and sex has been investigated in the past with conflicting results [47] and recent reports suggested greater burden of severe tinnitus in women than in men [280,281]. However, a recent global systematic review suggested that no sex bias existed for either any or severe tinnitus [31]. In **STUDY II** we show that women with mean age 51.05 (4.06) with severe tinnitus have 10 time the risk of also having a sibling with tinnitus ($\lambda s = 10.25$; 95% CI (7.14-13.61)), indicating that severe tinnitus is more genetically influenced in women than in men [211]. A limitation in this study is that we were not able to control for hearing loss or chronic ear diseases that can potentially influence tinnitus generation. Importantly, we did not have information regarding the tinnitus subtype reported in siblings and if it is different from the one reported in the proband. However, the prevalence of self-reported severe tinnitus (2.55% (2.45–2.65)), was similar to the one of clinically diagnosed (2.77% (2.65–2.89)), suggesting that reporting severe tinnitus could be considered as a proxy for clinically diagnosed. A greater prevalence of stress and anxiety has been reported in women with constant and severe tinnitus, the basis of which could also be potentially explained by genetics (Schlee et al., 2017). These findings will require replication in other populations.

5.4 GENETIC AND GENOMICS OF TINNITUS

In recent years, the emerging of the genomic era has accelerated the genetic investigation of tinnitus, aiming to determine its molecular signatures. Early studies were not able to show a strong evidence of familial effect in self-reported tinnitus for more than 5 minutes [167]. With increased sample size and better characterization of tinnitus, considering laterality and constant and severe tinnitus, we were able to identify strong familial aggregation of tinnitus

[211]. Maas et al., showed high heritability for bilateral tinnitus in men, suggesting a significant portion of tinnitus to be due to genetics [169]. Furthermore, in **STUDY III** we used longitudinal adoption data to address the bias due to shared environment that influences the results of twin studies and identified an association between tinnitus in adoptees and their biological parents, but not the adoptive parents (Cederroth et al., 2019). Although most adoptees were diagnosed with tinnitus in adulthood when familial environmental effect are weakened, we still consider that the transmission of tinnitus from biological parent to offspring to be associated with genetic factors. Despite the evidence for genetic background for tinnitus, attempts for determining molecular signatures have been scarce. Early candidate genes studies were unable to report significant findings due to lack of replication and underpowered design [150].

A meta-analysis showed association of rs4680 variant in Catechol-O-methyltransferase (*COMT*) gene with stress and anxiety, while also providing evidence differences in association based on sex [282]. *COMT* inactivates dopamine, epinephrine and norepinephrine which can influence estrogen response elements and estradiol production regulating cognitive function in women [283]. In our **STUDY IV** we investigated the relationship between tinnitus severity and *COMT Val*^{158}*Met* polymorphism showing a protective effect in men with *Met/Met* genotype (OR= 0.25, CI (0.09-0.64)). Our null association within women could be due to lack of adjusting for menstrual phase and hormonal birth control use in our logistic regression model. Some studies show a differences between man and women in responsiveness to treatments for tinnitus [284–286]. The bias that we observe in **STUDY IV** suggests an influence of sex in the pathophysiological mechanisms leading to constant and severe tinnitus, therefore sex and severity should be considered as key elements for refining tinnitus definition.

Genome-wide association studies can identify susceptibility variants that can give insight into novel biological mechanisms and processes. The first GWAS of tinnitus did not show any significant associations due to small sample size [189]. A significant increase in sample size using UKBB discovery data and MVP as a replication cohort, a tinnitus GWAS revealed the polygenic profile of tinnitus identifying 6 significant genomic loci [191]. Several variants were identified in genes *COL11A1, MSRA* and *ZNF318* that have been related to various forms of hearing loss in human and mice [187,287,288]. Moreover, Clifford et al., found a high correlation between tinnitus and hearing loss, showing bidirectional relationship, suggesting a common molecular pathway. Similarly, tinnitus was highly correlated (rg = 0.6, SE 0.056, p = 1.40E−26) with hearing difficulty in a GWAS assessing hearing difficulty in 250,000 UKBB individuals [289]. In **STUDY V**, we generated a GWAS on hearing loss using a sample size of 723,266 individuals mapping 48 genomic loci associated with hearing loss [233]. We confirmed variants previously reported in association with hearing loss and identified additional 10 novel genomic loci.

A strong limitation to genetic studies of hearing loss is the lack of cochlear tissue in the GTEx database that restricts deeper expression analysis and hinders novel findings for hearing disorders. Despite the possible limitation of using mice expression data to infer genetic and molecular mechanisms of hearing loss in humans, we have found that 16 out of

18 genes associated with hearing loss have been reported in mice, supporting the translational reliability of our findings. Further studies are needed to investigate the mechanisms involving the sensory cells and the stria vascularis with appropriate molecular techniques and prior to cell death. Moreover, we showed a genetic correlation between hearing loss and depression, insomnia, and smoking. As expected, we found that tinnitus was highly correlated with hearing loss (UKBB samples, rg = 0.6294, SE = 0.0564, p = 4.21E-29). In **STUDY VI** we aimed to distinguish the genetic signature of tinnitus and hearing loss, where we performed a GWAS meta-analysis on 56,467 tinnitus cases and 475,859 control individuals. Despite the large sample size, we identified only 2 genomic loci, which were insufficient for fully informative analysis. We performed a GSMR to test for causal associations between tinnitus and hearing loss with the identified SNPs as genomic instruments, showing hearing loss to have a promotive effect on tinnitus (beta= 0.384 (0.033), p-value = 2.13e-31). However, pleiotropy cannot be excluded from the interpretation of the results, suggesting that the identified SNPs are not independent and have a distinct effect on both hearing loss and tinnitus. Noise around tinnitus diagnosis was a significant limitation to this study, where we used self-report of "any tinnitus" in combination with ICD codes for tinnitus, showing a large heterogeneity that could not be compensated by the large sample size. The combination of heterogeneous phenotypes has been shown to dilute genetic signals, suggesting that genetic differences between tinnitus subtypes should be taken into consideration in future GWAS studies [290]. The risk of misclassification is evident and despite the large sample size we were not able to replicate the association with tinnitus found by Clifford et al., [191]. A major difference in both studies is the tinnitus definition and their statistical approach, where they used an ordinal definition based on frequency of tinnitus reported in the UKBB, as opposed to case/control model usually used in association studies. Furthermore, their replication cohort (MVP) comprises individuals with deep audiologic phenotyping, long-term noise exposure, blast injuries and other comorbities making them more susceptible to auditory disorders. Indeed, we reached out to incorporate their cohort in our analysis, but access was not granted.

Another limitation in our study is the use of partially overlapping samples and the small number of significant loci in the GSMR analysis that might impact the findings. In general, GWAS shortcomings include the challenges around the interpretation of the results, including identifying the functional mechanisms behind each of the associated risk loci, which can be difficult, time-consuming, and costly. Not every gene in the associated region is sufficiently annotated to evaluate its disease relevance, and functional variants might reside in non-coding regions of the genome, and most of the identified variants have small effects on the trait, but GWAS provide an unbiased means to discover enriched biological pathways and offer translation of the findings in clinical care (McCarthy et al., 2008). Higher penetrance, variants with a lower frequency, and non-coding variants can be accounted for with high-throughput sequencing of the genome and complement the GWAS results (Uffelmann et al., 2021). The use of WGS or WES that can increase the genomic resolution for complex disorders and build on GWAS findings and this approach has been effective in investigating schizophrenia

[291]. Identifying rare variants with larger effect size associated with tinnitus, can complement our current GWAS findings and identify potential molecular mechanisms implicated in severe tinnitus. An extreme phenotype strategy can improve the power of genetic studies by showing burden of rare variants in extreme cases causing a large effect on the disease risk [292,293]. The first study considering extreme tinnitus phenotype among patients with Meniere's disease reported a burden of rare missense variants in 24 synaptic genes identified by WES [294]. In **STUDY VII**, we identified a burden of rare LSV within *NAV2*, *TMEM132D* and *CACNA1E.* We were also able to replicate the involvement of missense variants in *ANK2,* previously reported by Amanat et al., [294]. *ANK2* has a role in organization of the cytoskeleton by linking spectrin-actin proteins, mostly expressed in the postcentral gyrus of the parietal lobe of the brain, known as primary somatosensory cortex [295]. *NAV2* and *TMEM132D* have been involved in neuronal development, morphogenesis and differentiation [296,297]. *TMEM132D* is expressed in the frontal cortex and has been implicated in emotional processing of depression and panic [298]. *CACNA1E* on the other hand, encodes subunit of proteins in calcium high-voltage activated channels, causing firing patterns modulations and over-excitability of the neurons - a shared mechanism associated with tinnitus and epilepsy [99,299]. Overall, the findings indicate that there is a greater neurological role is implicated in severe tinnitus, rather than purely audiological. We show that there is a significant burden of missense variants and LSV, supporting *NAV2*, *TMEM132D* and *CACNA1E* as candidate genes for severe tinnitus and genetic signatures independent of hearing loss. Additionally, the extreme phenotype approach for performing burden analysis can improve the tinnitus subtyping and further studies are needed to replicate our findings as well as to verify the molecular mechanisms involved.

6 CONCLUSIONS

In this thesis work, we aimed to investigate tinnitus in several contexts in order to better understand its pathophysiology and functional mechanisms, by examining environmental risk factors, genetic considerations and genomic insights associated with tinnitus and we have drawn several conclusions.

- Hearing loss, occupational noise exposure, otitis media, temporomandibular joint disorder, ototoxic platinum exposure and diabetes are the most reliable risk factors related to tinnitus, based on our systematic review.
- Epidemiological studies investigating the relationship between environmental risk factors and tinnitus are of considerably low quality. The lack of analytical observational studies with high quality study design and estimation of measures of association are highlighting a large gap in knowledge which hinders the progress of tinnitus research.
- Absence of objective diagnostic measures and the lack of consensus regarding tinnitus assessment contribute to high heterogeneity in tinnitus definition, which significantly increases the risk of misclassification in analytical studies.
- Tinnitus has a moderate heritability but improving on the identification of tinnitus subtypes can increase the consistency of tinnitus cases. Specifically, we show genetic evidence of independent signatures severe tinnitus.
- We show familial aggregation of severe tinnitus, with highest recurrence risk in siblings of women with severe tinnitus (λs=10.25 (7.14–13.61)), compared to men, underlining the importance of sex consideration for future studies.
- We distinguish the environmental from the genetic effect on clinically significant tinnitus in Swedish adoptees, showing increased odds (OR=2.22 (95%CI, 1.03-4.81)) of adoptees having tinnitus if their biological parents were diagnosed with tinnitus, but not the adoptive ones. These findings show that genetic factors are implicated with familial clustering of tinnitus with no effect of shared environment.
- *COMT Val^{158}Met* polymorphism is not associated with constant tinnitus, but *Met/Met* allele has a protective effect in males with tinnitus (OR = 0.25; 95% CI: 0.09-0.64; p = 0.007), confirming a gender bias in tinnitus subtypes.
- We identified 48 genomic loci associated with hearing loss in a GWAS meta-analysis, 10 of which were novel associations, suggesting an important role of the lateral wall of the cochlea, the stria vascularis and the outer sulcus in hearing loss.
- A GWAS meta-analysis of tinnitus reveals only 2 associated genomic loci despite the large sample size (N=523,326). A summary-based mendelian randomization analysis identifies a causal association between hearing loss and tinnitus, however, identified SNPs were highly dependent and with strong effect on both hearing loss and tinnitus. High pleiotropy indicates that based on common genetic variants, “any tinnitus” and hearing loss cannot be genetically distinguished.

- The use of WES and extreme phenotype strategy, focusing only on severe tinnitus cases reveals a burden of missense and LSV in *CACNA1E*, *NAV2*, and *TMEM132D* as putative new candidate genes for severe tinnitus.

7 POINTS OF PERSPECTIVE

Tinnitus research has expanded significantly in the last years with increased efforts and initiatives for multidisciplinary collaborations, where academics, clinicians, industry partners and patient organizations are contributing to common projects aiming to improve the fundamental knowledge and understanding of tinnitus [300]. Additionally, the continuous growth of genetic and genomic methodologies and technologies has improved the feasibility to understand the genetic signature of tinnitus [196]. However, despite the accelerated tinnitus research, there is no effective cure, and it still lags behind other neuropsychiatric disciplines. One of the reasons for the slow progress of tinnitus research is the lack of consensus within the field regarding tinnitus definition, objective measures, and treatment outcomes [301]. In this thesis work we have identified some of the challenges in tinnitus research, leveraging epidemiological and genetic data.

7.1 IMPROVED CLINICAL PHENOTYPING OF TINNITUS

We have shown evidence that the latest ICD11 definition of tinnitus https://icd.who.int/ct11/icd11_mms/en/release – "having ringing in the ears in the absence of corresponding external stimuli" is not optimal and produces high heterogeneity in observational epidemiology, as well as in genetic analyses. Future perspectives should incorporate update of the definition of tinnitus, as we show that temporality, sex, and overall burden are important components of identifying tinnitus patients. Therefore, there needs to be a distinction between acute tinnitus that can be a symptom of an associated disorder (hearing loss, Meniere's diseases or vestibular schwannoma), and constant or severe tinnitus, which should be recognized as a primary disorder [302]. Our data suggest that there is evidence to distinguish constant tinnitus from occasional, and that severe tinnitus is more genetically influenced [49,211,219,294]. This information points towards the importance of multidisciplinary approach to identifying objective tinnitus measurements, where audiometric assessment, electrophysiological imaging and genetic testing should be used to refine the profiling of tinnitus patients. This concept of tinnitus assessment will increase the accuracy by identifying homogeneous populations that will ultimately improve the quality of analytical studies and produce more robust and comparable results. Several international initiatives have undertaken the challenge to generate large tinnitus specific data to facilitate the progress of tinnitus research , namely Tinnitus Research Initiative (TRI) dataset [303], STOP, European School of Interdisciplinary Tinnitus research (ESIT) [300], and TIN-ACT. Recently, the importance of accessing gender bias in tinnitus research has been highlighted [211,280,304,305] and initiatives to further investigate this aspect has been undertaken by "Tinnitus Genetic and Environmental Risks" project (TIGER). If the results from these initiatives are implemented clinically, it will advance the recognition of tinnitus as an otologic and neurological disorder and fast-track future research. Moreover, changing the perspective of tinnitus definition and including standardized multidisciplinary assessment in clinical practice, will drastically improve upon current phenotyping strategies [306]. Improved phenotyping of tinnitus patients will help to

address the existent gap in knowledge in observational and epidemiological studies, as well as to avoid current misclassification in much needed genetic studies [301,307].

7.2 ADVANCEMENT IN GENETIC DESIGN FOR TINNITUS REFINEMENT

As we have demonstrated, genetic factors contribute to tinnitus development, paying special attention to severe tinnitus. Further advancement in genetic studies in tinnitus is paramount for the discovery of tinnitus biomarkers that will give an insight into tinnitus pathophysiology, molecular mechanisms, and genetic signature. A big challenge in genetic studies remains the lack of well-defined tinnitus phenotype and refining of patient selection for a homogeneous population [196]. Candidate gene studies have shown limited success for defining tinnitus, mostly due to the polygenic nature of tinnitus, where individual genetic variants show small effect on complex disorders such as tinnitus. Conversely, GWAS have made an advantage in understanding the genetic architecture of tinnitus by pinpointing variants in specific genomic loci that are then used to investigate their biological relevance. Already generated GWAS data can be used for advancement in clinical settings and personalized medicine, by mapping out the loci that can be used as therapeutic targets, markers to stratify clinical population based on genetic risk and promote predictive diagnostic tools [227]. Despite the large number of genetic variants identified in GWAS, relatively few risk loci have been comprehensively studied. Most of the risk loci in GWAS are located in non-coding genomic regions with small effect size and low penetrance, which complicates post-GWAS investigation. However, novel epigenomic methodologies and genome engineering tools are used to understand the functional relevance of GWAS findings [308]. Efforts of compiling large scale GWAS investigating tinnitus and hearing loss are progressing, and several identified loci have been replicated [191,233,289]. Future studies with more homogeneous phenotype and large sample size that include non-European populations will be needed. Considering the genetic and clinical overlap of tinnitus and hearing loss, more studies will be useful to assess the shared and non-shared genetic background between the two. Additional challenge in understanding GWAs findings is the prevalent pleiotropy in complex traits such as tinnitus, where identified variants have been associated with multiple complex traits [309,310]. Constant improvement of in silico methodologies has been used to prioritize set of GWAS signals that are likely to be causally associated with the phenotype in specific loci. Fine-mapping is becoming the standard practice in GWAS where a set of variants based on the linkage disequilibrium patterns and association signals are defined as credible variants [311]. Fine-mapping allows further dissection of the locus and prioritizing likely affected gene which can be seen as the most important finding in interpretation of GWAS loci. One way of achieving that is identifying genes correlated with our variants of interest that are associated with a molecular quantitative trait locus (mQTL) with specific molecular phenotype. GTEx is an accessible and available catalog of expression QTLs of 49 human tissues [243]. However, cochlear tissue is not one of them, which is a severe limitation for comprehensive genetic investigation to audiological traits such as tinnitus and hearing loss. Future efforts should be made to incorporate detailed sections of the cochlear tissue in GTEx catalogue that will enable the integration of genetic and transcriptional expression data to be analyzed and

ultimately identify genes with expression association with tinnitus. Therefore, transcription wide association analysis (TWAS) will be complementary to tinnitus GWAS and identify significant eQTLs that impact gene expression that are not well explained by individual tagging of SNPs [312]. Leveraging eQTL mapping of causal genes can be used in the discovery of biological pathways and improve interpretation of GWAS findings that can elucidate some of the molecular mechanisms involved in tinnitus. Moreover, employing Mendelian Randomization (MR) approach to identify causal relationship between tinnitus and hearing loss using genetic variants as randomization instruments, can possibly identify SNPs to increase the robustness of the causal inference [313]. Similar efforts have been made by Clifford et al., where MR showed a bidirectional effect between tinnitus and hearing loss, failing to identify specific variant that contribute to the direction of causal relationship [191]. Future improvements in phenotyping might improve the outcome from this analysis. Moreover, refining the molecular understanding of variants in a gene of interest can improve interpretation of MR [313].

7.3 CLINICAL APPLICATION OF GENETIC OUTCOMES

An important possible application of GWAS data is the ability to predict disease risk and identify individuals with different levels of risk that can be further channeled for clinical intervention or preventative measures, by calculating polygenic risk scores (PRSs). PRSs are determined by calculating the weighted sum score of risk alleles based on the effect sizes identified in a GWAS of a target population [230]. PRS can be useful for stratified screening and identification of individuals with increased risk for a disease, which can optimize the effectiveness of screening and prevention programs, early detection of disease and treatment outcomes. However, PRSs prediction accuracy is low, which is the biggest limitation that prevents the clinical implementation of PRS. Secondly, PRSs generalizability across different population is limited, since most of the PRS are based on GWASs of population with European ancestry [314]. Future perspectives involve improvement of statistical and in silico methods to overcome challenges of PRS and their implementation in clinical practices to profile tinnitus patients. Another useful application of identified genomic regions and target genes implicated in GWAS is in drug discovery and repositioning [315,316]. For example, the use of a monoclonal antibody ustekinumab is used to neutralize a p40 subunit of interleukin-12 and 23, pathways that have been identified by GWAS as risk factors for psoriasis [317]. A similar approach can be used to identify drug targets for tinnitus and hearing loss. Moreover, GWAS can be used to understand drug mechanisms and variability in response, as well as drug repurposing. Future use of GWAS in pharmacogenomics will improve the investigation of genetic associations and cellular mechanisms involved in understanding variation in drug response and improved prognosis of drug therapies [318]. While GWAS methods give an insight into many genomic loci associated with tinnitus, they explain only a fraction of its heritability due to small and moderate effect of the genomic variants. Identifying rare variants in the genome that are associated with tinnitus can complement current understanding of gene expression and biological mechanisms. Such efforts are have recently emerged by using WES in population with severe tinnitus [294]. Future studies using WGS, and WES can identify

variants with different effect on implicated genes. Gene-set enrichment analyses can be used to discover specific cells and tissues that can be further validated in animal studies and demonstrate their relationship to tinnitus pathophysiology.

Despite the remaining challenges, there is an optimistic outlook on tinnitus research. Large consortiums and industry organizations have supported the interdisciplinary nature of tinnitus research and yielded significant results that led us one step closer to understanding tinnitus and offering possible preventative and strategies and treatment alternatives. Improving methods to standardize tinnitus assessment and definition, will significantly impact the progress in interpretation of epidemiological, clinical, and genetic outcome. Facilitating active global collaborations will inevitably lead to novel insights that will refine fundamental tinnitus knowledge that can be translated and routinely used in clinical settings, and hopefully, lead us to useful strategy to improve quality of life of affected individuals.

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