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First Department of Otorhinolaryngology and Head and Neck Surgery, Faculty of Health Sciences - School of Medicine, National and Kapodistrian University of Athens

Διδακτορική διατριβή

PhD Thesis

Απώλεια ακοής λόγω έκθεσης σε μουσική: Ανάπτυξη παραδείγματος προσωρινής πτώσης του ουδού της ακοής και ο ρόλος της πρεστίνης ως βιοδείκτης.

Music Induced Hearing Loss: Development of a temporary threshold shift paradigm and role of prestin as a biomarker.

Ελευθερία Ηλιάδου, Ωτορινολαρυγγολόγος

Eleftheria Iliadou MD (Otolaryngology), FEBORL-HNS, MSc

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Eleftheria Iliadou, Otolaryngologist / Ηλιάδου Ελευθερία, Ωτορινολαρυγγολόγος

Supervisory committee / Τριμελής συμβουλευτική Επιτροπή:

1. Thanos Bibas, Professor in Otolaryngology, School of Medicine, NKUA / Αθανάσιος (Θάνος) Μπίμπας, Καθηγητή ΩΡΛ, Ιατρική Σχολή, ΕΚΠΑ

2. Konstantinos Pastiadis, Professor in School of Music Studies, AUTH, and Visiting Professor, School of Medicine, NKUA/ Κωνσταντίνος Παστιάδης, Καθηγητής τμήματος Μουσικών Σπουδών, ΑΠΘ, και Επισκέπτης Καθηγητής, Ιατρική Σχολή, ΕΚΠΑ

3. Christopher J. Plack, Professor, Division of Psychology, Communication and Human Neuroscience, University of Manchester, Manchester, UK & Department of Psychology, Lancaster University, Lancaster, UK

Μέλη εξεταστικής επιτροπής:

4. Aristidis Iliopoulos, Professor of Biology, Lab of Biology, School of Mediccine, NKUA / Αριστείδης Ηλιόπουλος, Καθηγητής Βιολογίας, Εργαστήριο Βιολογίας, Ιατρική Σχολή, ΕΚΠΑ

5. Christos Consoulas, Professor of Physiology, School of Medicine, NKUA / Χρήστος Κόνσουλας, Καθηγητής Φυσιολογίας, Ιατρική Σχολή, ΕΚΠΑ

6. Colleen Le Prell, Emilie and Phil Schepps Professor of Hearing Science, University of Texas, Dallas, US

7. Thanos Tzounopoulos, Assistant Professor, Department of Neurobiology and Center of the Neural Basis of Cognition, University of Pittsburgh and Carnegie Mellon University, Pennsylvania, US

Πρόεδρος Ιατρικής Σχολής: Νικόλαος Φ. Αρκαδόπουλος

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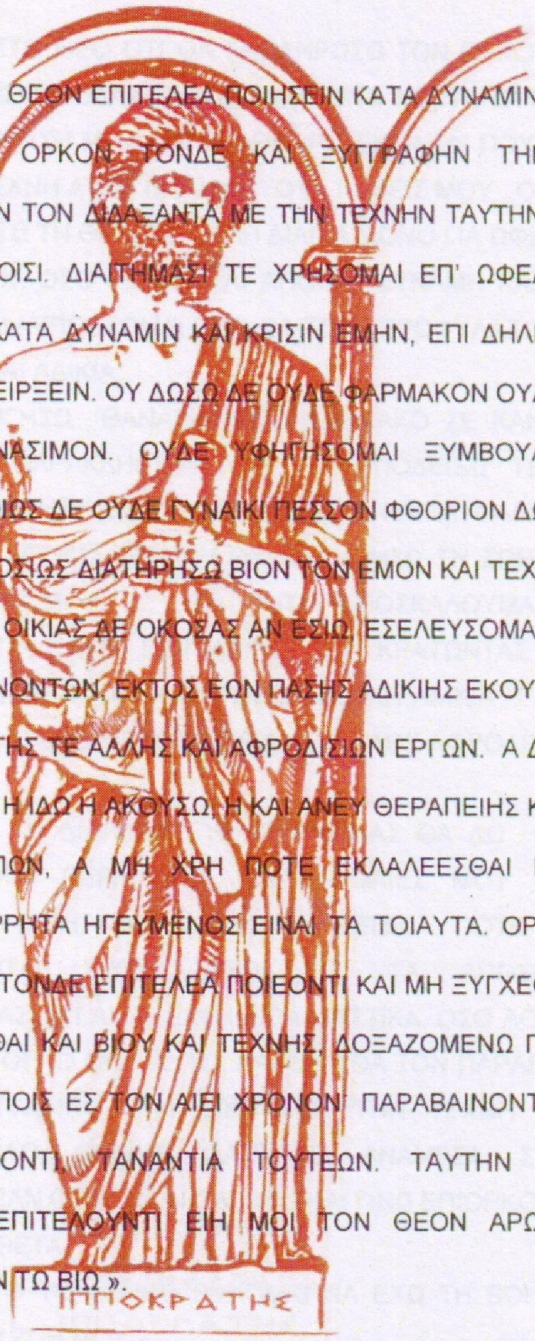
To the curious minds,
the knowledge seekers,
the science enthusiasts.

And to my mom, Daphne.

ΕΚ ΤΟΥ ΙΠΠΟΚΡΑΤΕΙΟΥ ΟΡΚΟΥ

ΚΕΙΜΕΝΟ

« ΟΜΝΥΜΙ ΤΟΝ ΘΕΟΝ ΕΠΙΤΕΛΕΑ ΠΟΙΗΣΕΙΝ ΚΑΤΑ ΔΥΝΑΜΙΝ ΚΑΙ ΚΡΙΣΙΝ ΕΜΗΝ ΟΡΚΟΝ ΤΟΝΔΕ ΚΑΙ ΞΥΓΓΡΑΦΗΝ ΤΗΝΔΕ. ΗΓΗΣΕΣΘΑΙ ΜΕΝ ΤΟΝ ΔΙΔΑΞΑΝΤΑ ΜΕ ΤΗΝ ΤΕΧΝΗΝ ΤΑΥΤΗΝ ΙΣΑ ΓΕΝΕΤΗΣΙΝ ΕΜΟΙΣΙ. ΔΙΑΙΤΗΜΑΣΙ ΤΕ ΧΡΗΣΟΜΑΙ ΕΠ' ΩΦΕΛΕΙΗ ΚΑΜΝΟΝΤΩΝ ΚΑΤΑ ΔΥΝΑΜΙΝ ΚΑΙ ΚΡΙΣΙΝ ΕΜΗΝ, ΕΠΙ ΔΗΛΗΣΕΙ ΔΕ ΚΑΙ ΑΔΙΚΙΗ ΕΙΡΞΕΙΝ. ΟΥ ΔΩΣΩ ΔΕ ΟΥΔΕ ΦΑΡΜΑΚΟΝ ΟΥΔΕΝΙ ΑΙΤΗΘΕΙΣ ΘΑΝΑΣΙΜΟΝ. ΟΥΔΕ ΥΦΗΓΗΣΟΜΑΙ ΞΥΜΒΟΥΛΙΗΝ ΤΟΙΗΝΔΕ. ΟΜΟΙΩΣ ΔΕ ΟΥΔΕ ΓΥΝΑΙΚΙ ΠΕΣΣΟΝ ΦΘΟΡΙΟΝ ΔΩΣΩ. ΑΓΝΩΣ ΔΕ ΚΑΙ ΟΣΙΩΣ ΔΙΑΤΗΡΗΣΩ ΒΙΟΝ ΤΟΝ ΕΜΟΝ ΚΑΙ ΤΕΧΝΗΝ ΤΗΝ ΕΜΗΝ. ΕΣ ΟΙΚΙΑΣ ΔΕ ΟΚΟΣΑΣ ΑΝ ΕΣΙΩ ΕΣΕΛΕΥΣΟΜΑΙ ΕΠ' ΩΦΕΛΕΙΗ ΚΑΜΝΟΝΤΩΝ, ΕΚΤΟΣ ΕΩΝ ΠΑΣΗΣ ΑΔΙΚΙΗΣ ΕΚΟΥΣΙΗΣ ΚΑΙ ΦΘΟΡΙΗΣ ΤΗΣ ΤΕ ΑΛΛΗΣ ΚΑΙ ΑΦΡΟΔΙΣΙΩΝ ΕΡΓΩΝ. Α Δ' ΑΝ ΕΝ ΘΕΡΑΠΕΙΗ, Η ΙΔΩ Η ΑΚΟΥΣΩ, Η ΚΑΙ ΑΝΕΥ ΘΕΡΑΠΕΙΗΣ ΚΑΤΑ ΒΙΟΝ ΑΝΘΡΩΠΩΝ, Α ΜΗ ΧΡΗ ΠΟΤΕ ΕΚΛΑΛΕΕΣΘΑΙ ΕΞΩ, ΣΙΓΗΣΟΜΑΙ, ΑΡΡΗΤΑ ΗΓΕΥΜΕΝΟΣ ΕΙΝΑΙ ΤΑ ΤΟΙΑΥΤΑ. ΟΡΚΟΝ ΜΕΝ ΟΥΝ ΜΟΙ ΤΟΝΔΕ ΕΠΙΤΕΛΕΑ ΠΟΙΕΟΝΤΙ ΚΑΙ ΜΗ ΞΥΓΧΕΟΝΤΙ ΕΙΗ ΕΠΑΥΡΑΣΘΑΙ ΚΑΙ ΒΙΟΥ ΚΑΙ ΤΕΧΝΗΣ, ΔΟΞΑΖΟΜΕΝΩ ΠΑΡΑ ΠΑΣΙΝ ΑΝΘΡΩΠΟΙΣ ΕΣ ΤΟΝ ΑΙΕΙ ΧΡΟΝΟΝ· ΠΑΡΑΒΑΙΝΟΝΤΙ ΔΕ ΚΑΙ ΕΠΙΟΡΚΕΟΝΤΙ ΤΑΝΑΝΤΙΑ ΤΟΥΤΕΩΝ. ΤΑΥΤΗΝ ΤΗΝ ΕΠΑΓΓΕΛΙΑΝ ΕΠΙΤΕΛΟΥΝΤΙ ΕΙΗ ΜΟΙ ΤΟΝ ΘΕΟΝ ΑΡΩΓΟΝ ΚΤΗΣΑΣΘΑΙ ΕΝ ΤΩ ΒΙΩ ».



Brief Resume

Eleftheria Iliadou is an Otolaryngologist currently working as Laryngology Fellow at the Royal National ENT Hospital (UCLH, London, UK) and as Clinical Research Associate at the UCL Ear Institute (London, UK), and at the Performing Arts Medicine Clinic of the National and Kapodistrian University of Athens (NKUA, Greece).

She received her medical degree in 2011 (Medical School of Aristotle University of Thessaloniki, Greece). In 2016, she completed her postgraduate studies on pediatric ENT in the University of Montpellier. In 2017, she obtained her MSc on Medical Research Methodology with Honours (Medical School of Aristotle University of Thessaloniki, Greece). She completed her thesis on audiometric data analysis (Multivariate Statistical Methods for Analyzing Audiometric Data and their Application to the Case of Idiopathic Sudden Sensorineural Hearing Loss) under the supervision of Professor Athanasios (Thanos) Bibas. In 2019, she obtained the post-graduate certificate on Audiology and Oto-neurology of the National and Kapodistrian University of Athens (NKUA, Greece). Since 2019, she has been working on her PhD project on the role of specific biomarkers in music-induced hearing loss.

In parallel, she has been working on the clinical coordination of the Horizon 2020 research project SMART BEAR (Smart Big Data Platform to Offer Evidence-based Personalised Support for Healthy and Independent Living at Home), and as clinical investigator in multiple RCTs and other research projects about voice, balance, and hearing disorders [UNITI (Unification of Treatments and Interentions for Tinnitus Patients), Holobalance (HOLOgrams for

personalized virtual coaching and motivation in an ageing population with BALANCE disorders (HOLOBALANCE) and REGAIN (REgeneration of inner ear hair cells with GAmma-secretase INhibitors to regain hearing in patients with sensorineural hearing loss)].

She is participating in the under- and post-graduate teaching activities of the NKUA Medical School, teaching Medical Research Methodology and Hearing and Voice disorders of performing artists and the general population. Since October 2022, she has been an expert member of the London Hampstead Research Ethics Committee.

Σύντομο βιογραφικό σημείωμα

Η **Ελευθερία Ηλιάδου** είναι ωτορινολαρυγγολόγος και εργάζεται σήμερα ως Λαρυγγολόγος στο Royal National ENT Hospital (UCLH, Λονδίνο, Ηνωμένο Βασίλειο) και ως κλινική ερευνήτρια στο Ear Institute UCL (Ηνωμένο Βασίλειο) και την Κλινική Ιατρικής των Παραστατικών Τεχνών της Α' Πανεπιστημιακής ΩΡΛ Κλινικής του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών (ΕΚΠΑ, Ελλάδα).

Πήρε το πτυχίο της Ιατρικής το 2011 (Ιατρική Σχολή του Αριστοτελείου Πανεπιστημίου Θεσσαλονίκης, Ελλάδα). Το 2016 ολοκλήρωσε τις μεταπτυχιακές της σπουδές στην παιδο-ΩΡΛ στο Πανεπιστήμιο του Μονπελιέ. Το 2017, απέκτησε το μεταπτυχιακό της δίπλωμα ειδίκευσης (MSc) στην Ιατρική Ερευνητική Μεθοδολογία με άριστα (Ιατρική Σχολή του Αριστοτελείου Πανεπιστημίου Θεσσαλονίκης, Ελλάδα). Ολοκλήρωσε τη διπλωματική της

εργασία με θέμα την ανάλυση ακοολογικών δεδομένων (Multivariate Statistical Methods for Analyzing Audiometric Data and their Application to the Case of Idiopathic Sudden Sensorineural Hearing Loss) υπό την επίβλεψη του καθηγητή Αθανάσιου (Θάνου) Μπίμπα. Το 2019, απέκτησε το μεταπτυχιακό πιστοποιητικό στην Ακοολογία και Ωτονευρολογία του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών (ΕΚΠΑ). Από το 2019, εργάζεται στο διδακτορικό της έργο σχετικά με το ρόλο συγκεκριμένων βιοδεικτών στην απώλεια ακοής που προκαλείται από τη μουσική.

Παράλληλα, εργάζεται στον κλινικό συντονισμό του ερευνητικού έργου SMART BEAR (Smart Big Data Platform to Offer Evidence-based Personalized Support for Healthy and Independent Living at Home) του Ορίζοντα 2020, καθώς και ως κλινική ερευνήτρια σε πολλαπλές κλινικές μελέτες και άλλα ερευνητικά έργα σχετικά με τις διαταραχές φωνής, ισορροπίας και ακοής [UNITI (Unification of Treatments and Interventions for Tinnitus Patients) , Holobalance (HOLOgrams for personalized virtual coaching and motivation in an ageing population with BALANCE disorders (HOLOBALANCE) and REGAIN (REgeneration of inner ear hair cells with GAmma-secretase INhibitors to regain hearing in patients with sensorineural hearing loss)].

Επιπλέον, συμμετέχει στις προπτυχιακές και μεταπτυχιακές διδακτικές δραστηριότητες της Α’ Πανεπιστημιακής ΩΡΛ Κλινικής του ΕΚΠΑ, διδάσκοντας μεθοδολογία ιατρικής έρευνας και διαταραχές ακοής και φωνής καλλιτεχνών και του γενικού πληθυσμού. Από τον Οκτώβριο του 2022 είναι εμπειρογνώμονας μέλος της Επιτροπής Ηθικής και Δεοντολογίας Έρευνας του Hampstead του Λονδίνου.

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Moreover, I would like to thank Professor Konstantinos Pastiadis for his help in designing and developing the experimental setting of this project, including the processing of the audio material, microphone and headphones calibrations, and sound level measurements. His analytical way of thinking, and his insightful feedback all these years not only enhanced the quality of the thesis but has also inspired me to always remain curious about how sound and hearing (and the whole world) works. He always believed in me, even in moments when I doubted myself.

I would also wish to express my gratitude to Professor Chris Plack for the time and effort he dedicated to guiding me through the ups and downs of this academic journey. His expertise and support have been instrumental in shaping this research. His passion for science and music has been contagious.

His constructive criticism, and meticulous editing of my manuscripts, always challenged me to think critically, express my ideas more clearly, and improve my writing skills. I am confident that the knowledge I have gained will serve me well in my future endeavors.

Moreover, I wish to acknowledge the support of the many who helped with the completion of this study:

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Finally, I would like to thank my family, whose love and support has always been my pillar.

My dear parents, Daphne and Dimitris, who taught me the value of being humble and kind, working hard without complaining, and not losing faith. Thank you for always believing in me, supporting me in pursuing my own dreams, and just wanting me to be happy. I do my best.

My sister, Martha, who is fed up with me always studying, but has accepted me for who I am. Thank you for all our conspiracies and inside jokes, for always laughing the bad times out, and for still being my “big sister”.

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Executive Summary

This is the dissertation of the PhD project entitled “Music Induced Hearing Loss: Development of a temporary threshold shift paradigm and role of prestin as a biomarker.” It consists of:

1. A general part (Chapter I, titled “Introduction – General considerations”)
2. A specific part (Chapters II-VII)

This PhD project aimed to determine the significance of a newly proposed biomarker, blood prestin, in noise-induced hearing loss (NIHL). Prestin is the 5th member of an 11-member membrane anion-transporter superfamily (solute carrier family 26 or SLC26). In the cochlea, prestin plays a crucial role in the electromotility of outer hair cells. It was recently shown that free serum prestin can be detected in the blood circulation of rodents and humans by means of Enzyme-linked Immunosorbent Assay (ELISA). This permeation of prestin to blood circulation is hypothesized to occur either directly through the blood-labyrinthine barrier or via engulfment by phagosomes.

The principal motivation for conducting this study was the determination of the role of blood prestin in NIHL and is based on previous findings that supported that blood prestin levels may be associated with the dynamic changes that occur in the cochlea after exposure to hazardous levels of noise. Blood prestin levels have shown variation after a single loud exposure, in rodents; in Parham et al. (2019), serum prestin levels increased immediately after 2 hours exposure to high-level noise and then decreased to near or below baseline at 14 days. In addition to these acute changes, in a recent human study by Parker et al.

(2022), blood prestin levels of adults with normal hearing also showed weak negative correlation with average daily noise exposure levels.

To investigate changes in blood prestin levels in temporary NIHL in humans, it was decided to measure blood prestin levels in adults with normal hearing before and after exposure to high levels of music.

Prior to this, it was essential to:

1. Map the current knowledge about blood prestin in animals and humans, and standardise the process of collecting blood samples and measuring prestin by means of ELISA, as well as assessing their test-retest and intra-rater reliability.

Given the limited existing knowledge on prestin blood levels in humans, a scoping review of relevant literature and an observational study on adults with normal hearing were initially conducted. This phase of the project aimed to identify regular blood prestin levels in individuals with normal hearing and their relationships with factors like age, sex, and chronic noise exposure. We showed that serum prestin levels did not differ significantly across time of day, and that our test-retest reliability between duplicates of the same sample was excellent. Neither lifetime noise exposure, age, nor sex correlated with serum prestin levels.

2. Develop a music exposure paradigm to safely induce Temporary Threshold Shift (TTS).

After a relevant scoping review on experimental studies on NIHL, for practical and ethical reasons, it was decided to focus on temporary rather than permanent hearing loss. A time-efficient music exposure paradigm, that was shown to induce substantial TTS and distortion product otoacoustic emission (DPOAE) amplitude shifts, without evidence of permanent hearing consequences, was developed. Seventeen participants with normal hearing were included in this phase of the study. All but two participants presented clinically significant TTS or decrease in their DP amplitude in at least one frequency. No participant showed any permanent threshold shift.

Once our methods were standardized and the reliability and safety of our paradigm were deemed satisfactory, according to the results of the abovementioned pilot study, I proceeded to assess blood prestin level in individuals with normal hearing before and after their exposure to our music paradigm. Fourteen adults with normal hearing were included in this stage. All participants presented TTS or a decrease in DP amplitude in at least one frequency after music exposure. Mean serum prestin level progressively increased following music exposure, reaching a maximum at 2 h and returned to pre-exposure level by 1 week. Our findings show that blood prestin level may change after exposure to high levels of music and suggest that the role of serum prestin level as a proxy marker for temporary cochlear dysfunction after exposure to hazardous levels of noise should be further explored.

Περίληψη

Η παρούσα διδακτορική διατριβή με τίτλο "Απώλεια ακοής που προκαλείται από τη μουσική: και ο ρόλος της πρεστίνης ως βιοδείκτης" αποτελείται από :

1. ένα γενικό μέρος (Κεφάλαιο I, με τίτλο "introduction – General considerations")
2. ένα ειδικό μέρος (Κεφάλαια II-VII)

Η παρούσα διδακτορική μελέτη είχε ως στόχο να διερευνήσει την αξία της πρεστίνης αίματος ως βιοδείκτης στην προσωρινή απώλεια ακοής που προκαλείται από θόρυβο. Η πρεστίνη είναι το 5ο μέλος μιας οικογένειας διαμεμβρανικών μεταφορέων ανιόντων (solute carrier family 26 ή SLC26). Στον κοχλία, η πρεστίνη συμμετέχει την ηλεκτροκινητικότητα των έξω τριχωτών κυττάρων. Πρόσφατα αποδείχθηκε ότι ελεύθερη πρεστίνη μπορεί να ανιχνευθεί στην κυκλοφορία του αίματος τρωκτικών και ανθρώπων με τη βοήθεια της Enzyme-linked Immunosorbent Assay (ELISA). Αυτή η διέλευση της πρεστίνης στην κυκλοφορία του αίματος φαίνεται να συμβαίνει είτε απευθείας μέσω του αίματος-λαβυρινθικού φραγμού είτε μέσω της απορρόφησης από φαγοσώματα.

Οι ερευνητικές υποθέσεις στην παρούσα μελέτη βασίστηκαν σε προηγούμενα βιβλιογραφικά ευρήματα που υποστηρίζουν ότι τα επίπεδα πρεστίνης στο αίμα μπορεί να σχετίζονται με τις δυναμικές αλλαγές που συμβαίνουν στον κοχλία μετά από βραχεία ή χρόνια έκθεση σε δυνατό θόρυβο. Τα επίπεδα της πρεστίνης στο αίμα έχουν παρουσιάσει διακυμάνσεις μετά από μία και μόνο έκθεση σε δυνατό θόρυβο, σε τρωκτικά: στους Parham et al. (2019) τα επίπεδα

της πρεστίνης στον ορό αυξήθηκαν αμέσως μετά από έκθεση 2 ωρών σε δυνατό θόρυβο και στη συνέχεια μειώθηκαν κοντά ή κάτω από τη βασική τιμή σε 14 ημέρες. Επιπρόσθετα, σε μια πρόσφατη μελέτη σε ανθρώπους από τους Parker et al. (2022), τα επίπεδα της πρεστίνης στο αίμα ενηλίκων με φυσιολογική ακοή έδειξαν ασθενή αρνητική συσχέτιση με τα μέσα ημερήσια επίπεδα έκθεσης τους σε θόρυβο.

Για να διερευνηθούν οι αλλαγές των επιπέδων πρεστίνης στο αίμα στην περίπτωση απώλειας ακοής από δυνατό θόρυβο στον άνθρωπο, αποφασίσαμε να μετρήσουμε τα επίπεδα πρεστίνης στο αίμα σε ενήλικες με φυσιολογική ακοή πριν και μετά την έκθεση σε δυνατή μουσική.

Για την επίτευξη των στόχων της μελέτης, έπρεπε:

1. Να χαρτογραφηθούν οι τρέχουσες γνώσεις σχετικά με την πρεστίνη του αίματος σε ζώα και ανθρώπους και να τυποποιηθεί η διαδικασία συλλογής δειγμάτων αίματος και μέτρησης της πρεστίνης μέσω ELISA, καθώς και να αξιολογηθεί η αξιοπιστία τους

Δεδομένης της περιορισμένης υπάρχουσας γνώσης σχετικά με τα επίπεδα της πρεστίνης στο αίμα στον άνθρωπο, έπρεπε πρώτα να διεξαχθεί μια ανασκόπηση της σχετικής βιβλιογραφίας και, στη συνέχεια, μια μελέτη παρατήρησης σε ενήλικες με φυσιολογική ακοή. Αυτή η φάση αποσκοπούσε στον εντοπισμό με αξιοπιστία της πρεστίνης στο αίμα σε άτομα με φυσιολογική ακοή με τη βοήθεια της ELISA, καθώς και την αξιολόγηση της συσχέτισης των επιπέδων της πρεστίνης ορού με παράγοντες όπως η ηλικία, το φύλο και η χρόνια έκθεση σε θόρυβο. Στη φάση αυτή, σε ένα δείγμα 56 υγιών ατόμων, τα

επίπεδα πρεστίνης στον ορό δεν άλλαζαν σημαντικά κατά την διάρκεια της ημέρας ή της νύχτας, και ότι η αξιοπιστία της μεθόδου μας για την μέτρηση της πρεστίνης στο ίδιο δείγμα αίματος ήταν εξαιρετική. Τα επίπεδα της πρεστίνης στο αίμα δεν έδειξαν συσχέτιση με την χρόνια έκθεση σε θόρυβο, την ηλικία, ή το φύλο των συμμετεχόντων.

2. Να αναπτυχθεί ένα πειραματικό παράδειγμα έκθεσης σε μουσική για την ασφαλή πρόκληση προσωρινής απώλειας ακοής.

Μετά από μια σχετική ανασκόπηση της βιβλιογραφίας ήδη δημοσιευμένων πειραματικών μελετών για την βαρηκοΐα από θόρυβο, για πρακτικούς και δεοντολογικούς λόγους, επιλέχθηκε η επικέντρωση στην προσωρινή και όχι στη μόνιμη απώλεια ακοής από θόρυβο. Έτσι, αναπτύχθηκε ένα πειραματικό παράδειγμα έκθεσης σε δυνατή μουσική σε συνθήκες εργαστηρίου, το οποίο δοκιμάστηκε σε 17 συμμετέχοντες με φυσιολογική ακοή. Όλοι οι συμμετέχοντες πλην δύο παρουσίασαν κλινικά σημαντική αύξηση των ουδών ακοής τους ή μείωση του πλάτους των ωτοακουστικών εκπομπών τους σε τουλάχιστον μία συχνότητα. Κανένας συμμετέχων δεν παρουσίασε μόνιμη απώλεια ακοής.

Αφού, λοιπόν, η διαδικασία των μετρήσεων τυποποιήθηκε και η αξιοπιστία και η ασφάλεια τους κρίθηκαν ικανοποιητικές, ακολούθησε η μέτρηση των επιπέδων πρεστίνης στο αίμα σε άτομα με φυσιολογική ακοή πριν και μετά την έκθεσή τους στο μουσικό μας πειραματικό παράδειγμα. Δεκατέσσερις ενήλικες με φυσιολογική ακοή συμπεριλήφθηκαν σε αυτό το στάδιο της μελέτης. Όλοι οι συμμετέχοντες παρουσίασαν παροδική αύξηση των ουδών του τονικού

ακοογράμματος τους ή μείωση του πλάτους των DPOAE σε τουλάχιστον μία συχνότητα μετά την έκθεση στη μουσική. Τα μέσα επίπεδα πρεστίνης στον ορό αυξήθηκαν προοδευτικά μετά την έκθεση στη μουσική, φθάνοντας στην μέγιστη τιμής τους στις 2 ώρες. Επέστρεψαν στα επίπεδα που είχαν πριν από την έκθεση σε 1 εβδομάδα μετά από αυτήν. Τα αποτελέσματά αυτά δείχνουν ότι τα επίπεδα πρεστίνης στο αίμα μπορεί να μεταβληθούν μετά από έκθεση σε δυνατή μουσική και υποδεικνύουν ότι ο ρόλος των επιπέδων πρεστίνης στον ορό αίματος ως βιοδείκτης των αλλαγών που γίνονται στον κοχλία μετά την έκθεση σε δυνατή μουσική θα πρέπει να διερευνηθεί περαιτέρω.

Abbreviations

DPOAE: Distortion product otoacoustic emissions

EHF-PTA: Extended high-frequency pure tone audiometry

ISSHL: Idiopathic sensorineural hearing loss

MIHL: Music-induced hearing loss

NIHL: Noise-induced hearing loss

NIOSH: National Institute of Occupational Safety and Health

OAE: Otoacoustic emissions

OHC: outer hair cell

PTA: Pure tone audiometry

PTS: Permanent threshold shift

SPL: Sound pressure level

SNHL: Sensorineural hearing loss

TEOAE: Transient evoked otoacoustic emissions

TTS: Temporary threshold shift

WHO: World Health Organization

Definitions

dBA: Decibels of sound pressure level adjusted using the A-weighting filter; a level frequently used to measure occupational and environmental noise exposures.

dB HL: Decibels of hearing level; a level used to measure audiometric hearing threshold levels referenced to the average human ear. “dB HL” is a relative measure, and the actual sound pressure level (in decibels) can vary depending on the specific calibration and equipment used in a given test or measurement.

ER: Exchange Rate is the change in average noise level (in dB) that corresponds to a doubling or halving of allowable exposure time. As an example, a 5 dB exchange rate permits a doubling or halving of exposure duration for every 5 dB increase or decrease in average noise level, respectively, e.g., 8 hours of noise exposure permitted at 90 dBA, 4 hours at 95 dBA, 2 hours at 100 dBA, etc.

LAVG: Average noise level (dBA) measured using an exchange rate other than 3 dB (often 5 dB).

LEQ: Equivalent continuous average noise level (dBA) measured using a 3 dB exchange rate.

Chapter I

Introduction – General considerations

1.1. Hearing loss

According to the estimation of the World Health Organization (WHO), one out of five people worldwide live with hearing loss (Haile et al., 2021). An estimated 430 million have hearing loss of moderate (more than 35 decibels) or higher severity (loss of more than 50 decibels) in the better hearing ear (McDaid et al., 2021). In the Eastern Mediterranean region, 78 million people present some degree of hearing impairment (Orji et al., 2020). By 2050, 10% of the human population will require hearing rehabilitation (Cieza et al., 2020; World Health Organization, 2021). Most of them will not have access to interventions (World Health Organization, 2021). The annual cost for Europe is estimated to range between 555 and 675 billion euros depending on hearing aid ownership (Shield, 2018).

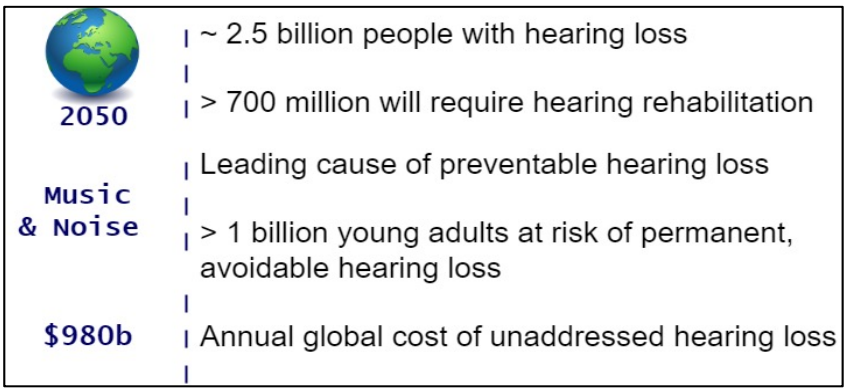


Figure 1.1 Hearing loss in numbers.

Apart from the financial impact, the personal and societal burden are also significant. According to the Global Burden of Disease study, it is one of the

leading causes of living years with disability (Haile et al., 2021). Multiple studies imply association of hearing impairment with psychological and physical diseases, such as cognitive disorders and dementia (Lin, Ferrucci, et al., 2011; Lin et al., 2023; Livingston et al., 2017)), anxiety and depression (Dawes et al., 2015), accidents (Li et al., 2013) and higher mortality rate (Holwerda et al., 2012). Moreover, hearing loss has been identified as one of the modifiable risk factors for dementia (Livingston et al., 2017). People suffering from hearing loss have fewer career opportunities, are less productive and tend to retire sooner than normal hearing people (Saunders et al., 2018). Children and adolescents face difficulties in developing their cognitive abilities, and completing their education can be extremely challenging (Stevens et al., 2013). On the other hand, adults with hearing impairment tend to isolate themselves by reducing any participation in social events (Saunders et al., 2018). The lack of communication with their environment lowers significantly their quality of life (Holwerda et al., 2012).

1.2. Noise induced hearing loss

The leading cause of preventable hearing damage is overexposure to noise (Deafness and Hearing Loss, n.d.; Le et al., 2017). Hearing loss caused by noise exposure is defined as noise-induced hearing loss (NIHL) (Le et al., 2017). NIHL is the second most common self-reported occupational illness or injury, despite decades of relevant studies, workplace interventions, and regulations in national and European level (National Institute for Occupational Safety and Health. Division of Biomedical and Behavioral Science, 1998; Śliwińska-Kowalska & Zaborowski, 2017a; World Health Organization, 2021;

World Health Organization, 2022). It is also the second most common cause of sensorineural hearing loss after presbycusis (Śliwińska-Kowalska & Zaborowski, 2017a). Urbanization and industrialization of modern society causes inevitably an increase of environmental noise levels (Silva et al., 2020). People are exposed to hazardous noise during their everyday life, their occupation (occupational noise) and their leisure time (recreational noise) (Ma et al., 2022; Welch & Fremaux, 2017). Along with the concerns about NIHL in the general population, overexposure to hazardous levels of music may also lead to varied degree of hearing loss (Thom et al., 2018). Over 1 billion young adults may be at risk of permanent, avoidable music-induced hearing loss (MIHL) due to unsafe listening practices (World Health Organization., 2021). Worldwide, 16% of disabling hearing loss in adults is attributed to occupational noise, while all ages seem to be affected by high-level music and noise exposure as well (Katbamna & Flamme, 2008; Śliwińska-Kowalska & Zaborowski, 2017a). There is also evidence that other hearing disorders (e.g., TTS, tinnitus, and hyperacusis) may be associated with music exposure (Di Stadio et al., 2018a; Thom et al., 2018). As pleasant as recreation might be, it can also lead to exposure to high-level sounds (noise and music), which can frequently be underestimated by people, society and stakeholders (Silva et al., 2020).

Our current understanding of the pathophysiological mechanisms of NIHL is limited and mostly based in preclinical studies (Nordmann et al., 2020). For many decades, noise-induced cochlear trauma and related mechanisms have been studied in animal models (Ryan et al., 2016). In animals, NIHL presents

as a spectrum comprising three primary types of harm (Hertzano et al., 2020). Temporary threshold shift (TTS) is the transient loss of hearing sensitivity that recovers in few days following noise exposure (Ryan et al., 2016). TTS seems to be a mechanical process that involves pro-inflammatory, pro-apoptotic and metabolic processes that affect peripheral and central structures of the auditory pathway, including cochlear hair cells, dentrites, stereocilia, connections between the tectorial membrane and outer hair cell stereocilia, reticular lamina, basilar membrane, inner and outer hair cells, cochlear synapses, supporting cochlear cells, cochlear endothelium and fibrocytes (Ryan et al., 2016). Next, cochlear synaptopathy is the loss of neural connections between inner hair cells and afferent cochlear neurons (Kujawa & Liberman, 2009; Liberman & Kujawa, 2017). Finally, sensitivity losses that do not recover are labeled as noise-induced permanent threshold shift (NIPTS or PTS). Mechanisms such as generation of reactive oxygen species (ROS) have been associated with these pathways (Lin, Furman, et al., 2011). A particular case of NIHL is the acute acoustic trauma, which is caused by exposure to extremely loud noise. It may be unilateral or bilateral (Rosenhall et al., 2023). Although an exact noise exposure threshold is not known, acoustic trauma may occur after either impulse noise (e.g., gunfire) or brief continuous noise. A recent literature review about acoustic trauma due to loud continuous noise showed that diffuse-field related levels above 120 dBA for 10s or more, or above 130 dBA for 2–3s, can lead to acoustic trauma, although these levels are less intense than the ones tolerated by participants in TTS studies (Berger & Dobie, 2019). Pathophysiological mechanisms that have been associated comprise noise-induced cochlear microcirculation and lateral wall pathologies (Shin et al.,

2019). Acoustic trauma may be entirely or partially reversible, or in some cases irreversible (Singh et al., 2022). In contrast to TTS, it requires early management with a course of systemic +/- intratympanic steroids (Singh et al., 2022).

1.3. Diagnosis of noise-induced hearing loss

The gold standard of assessing cochlear pathology, that is histological preparation and microscopy, has limited applicability in human, due to obvious ethical constrictions and requirement of pre-mortem noise-exposure history and hearing assessment (Kujawa & Liberman, 2009). Many results of preclinical studies cannot be confirmed and translated in humans (Dobie & Humes, 2017; Ryan et al., 2016). Moreover, human studies are largely limited to use of behavioural and far-field electrophysiological tests. Behavioural tests, such as pure tone audiometry (PTA), demand participation and interaction of the subject with the researcher, having an impact on their accuracy and reliability, and being affected by the subject's cognition or alertness (Heinrich et al., 2015). Moreover, PTA has poor sensitivity to inner or outer hair cell (OHC) loss, and thus to early diagnosis of NIHL (Clark et al., 1987). On the other hand, electrophysiological and other objective testing, such as auditory brainstem response or otoacoustic emissions (OAEs), require specialised equipment and personnel, and their clinical and research value in monitoring cochlear dysfunction is not entirely understood (Le Prell et al., 2012).

The knowledge gap in current hearing research on noise exposure is large.

Current hearing tests such as DPOAEs may be useful in evaluating the dynamic changes that happen in the cochlea during or after exposure to noise that may lead to temporary or permanent hearing loss. Nevertheless, they show high variability, depending on a high number of factors, such as individual variability, probe fitting and distance from tympanic membrane, f1:f2 ratio etc. Although factors such as genetics, middle ear power transfer, outer ear resonance, melanin levels, or sex are known to play some role in one's vulnerability to noise, there is no prognostic model for susceptibility to noise exposure. Research, development and validation of simple, inexpensive tests that could detect with sufficient accuracy and sensitivity the presence and time course of cochlear damage before it is symptomatic or permanent are of high priority. In the long term, the identification of factors or markers that associate with one's susceptibility in noise could also make part of a predictive model that could serve as risk calculator.

1.4. Aims of the PhD project

The main aim in this PhD project was to observe and investigate aspects of the relevance and validity of a newly proposed biomarker in NIHL. This new biomarker is prestin, the 5th member of an 11-member membrane transporter superfamily (solute carrier family 26 or SLC26), which encodes anion transporters and related proteins (Liberman et al., 2002). Prestin was until recently thought to be found exclusively in the cochlea (Parham, 2015). However, it was recently detected in the myocardium as well (Zhang et al., 2021). In the cochlea it is situated in the lateral wall of OHCs and is responsible for their electromotility (Liberman et al., 2002). Free prestin can be also

identified in the blood by means of Enzyme-linked Immunosorbent Assay (ELISA) (Dogan et al., 2018; Hana & Bawi, 2018; Parham et al., 2019; Sun et al., 2019). This is a rapid immunochemical test that involves an antibody (in the present case, prestin-specific) that can detect the presence of a ligand (prestin) in a liquid sample (blood). The procedure allows the quantification of peripheral prestin in the blood. Passage of cochlear prestin in the circulation has been hypothesized to happen directly through the blood-labyrinthine barrier or via engulfment by phagosomes. Although there is no published literature nor any pre- or clinical research paradigms on the release of cardiac prestin in the systemic circulation, it could be hypothesized that this might be the case following cardiac insult as in a myocardial ischaemia or infarction.

The objectives of the projective were:

1. To thoroughly review existing studies pertaining to the measurement of blood prestin level of animals and humans, identifying constraints, methodological pitfalls, or gaps in current knowledge.

Since knowledge on prestin blood level is limited, it was decided to start by exploring fundamental aspects in previously published scientific papers. I conducted a scoping review of the literature entitled “Blood Prestin Levels in Normal Hearing and in Sensorineural Hearing Loss” [(Iliadou et al., 2021), Chapter II],

2. To determine if blood prestin level differ in the plasma or the serum, if they are affected by the time of the day, by age or sex, and if their measurement in our experimental setting had acceptable reliability.

In order to test for changes in the blood prestin level in NIHL, I needed to first standardize our prestin measurement procedures and test for our inter- and intra-rater/subject variability. Hence, I conducted an observational, repeated measures study entitled “Blood Prestin Levels in Adults with Normal Hearing” (Chapter III). Based on the methodological pitfalls and the knowledge gaps identified during the review, I proceeded with the design of the experimental setting and conditions, the identification of regular blood prestin level in adults with normal hearing and their relationship with time of day, age, sex and chronic noise exposure. I also assessed their test-retest reliability. Once the procedure of collecting the blood samples and measuring prestin in the blood was standardized, and since the test-retest variability was proven satisfactory, and the effects of sex, age and time of day were identified, I was able to proceed with the measurement of prestin level in MIHL.

3. To develop a general understanding of how to create a safe lab-based music (> 80 dB A) exposure paradigm by documenting and exploring methodological and ethical aspects of experimental studies that describe noise and/or music exposure as an intervention.

To select the safest and more reliable methods of using music in an experimental setting, a scoping review of the literature was conducted to provide insights on previously used experimental paradigms.

4. To develop and validate a safe and reliable music exposure paradigm that could be used in studies requiring temporary cochlear dysfunction.

After reviewing the relevant literature, a music exposure paradigm that safely and reliably created TTS was created and validated in a sample of 17 adults with normal hearing (Chapter V).

5. To assess blood prestin level in adults who have been exposed to high-levels of music, and to investigate their correlation with other indicators of transient hearing impairment, such as elevation of PTA thresholds or reduction in DP amplitude.

Before committing to a large-scale study requiring time and resources, I used the newly developed music exposure paradigm in order to assess the changes of blood prestin in temporary hearing loss in a small-scale pilot study (Chapter VI).

Chapter II

Blood Prestin Levels in Normal Hearing and in Sensorineural Hearing Loss: A Scoping Review

Abstract

Objective: Recently it has been hypothesized that blood prestin level may reflect cochlear damage and thus serve as an easily measurable, early sensorineural hearing loss biomarker. This is a scoping review aiming to identify and critically appraise current evidence on prestin blood level and their temporal variation in rodents and humans with normal hearing and with sensorineural hearing loss.

Methods: This study was designed and held according to PRISMA Extension for Scoping Reviews (PRISMA-ScR) guidelines. With no limitation with regards to study type, animal and human studies focusing on prestin blood level in normal hearing and in sensorineural hearing loss were sought in major databases such as Medline, Central. Scopus, PROSPERO and Clinicaltrials.gov. Results have been then hand-searched. A data charting form was developed including the parameters of interest.

Results: Seven studies focusing on measuring prestin blood level by means of ELISA in rodents and human subjects with normal hearing and noise-induced, drug-induced, or idiopathic sudden hearing loss were found eligible and were included in the analysis. According to these proof-of-concept studies, prestin can be detected in the circulation of subjects with no hearing loss, however

normal ranges remain unclear. After cochlear damage, blood prestin level seem to initially rise and then return to near or below baseline. The degree of their change relates with subjects' degree of hearing loss, damaged cochlear region, and recovery.

Conclusions: Prestin blood level and their temporal variation seem to correlate with cochlear damage, however methodological weaknesses, such as small sample size, lack of detailed phenotyping, insufficient exclusion of confounding factors and short follow-up do not allow for robust conclusions. To conclude, current findings support the value of studying blood prestin level in normal hearing and hearing loss and highlight a need for larger scale longitudinal research.

2.1. INTRODUCTION

Biomarkers, or biological markers, are defined as patients' characteristics that can be measured objectively, accurately and reproducibly ("Biomarkers and Surrogate Endpoints," 2001). They serve as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Strimbu & Tavel, 2010). In the case of sensorineural hearing loss (SNHL), which accounts for most of the hearing loss (HL) cases, they could identify early hearing impairment, potentially before it becomes measurable by standard audiometric procedures.

Although OAE amplitude decrease and extended high-frequency pure tone threshold elevation seem to correlate with early signs of NIHL, their use in

clinical practice and research is limited by their high variability. To date, no biomarker has been developed or validated in the case of SNHL. However, since the pathogenesis of many SNHL types occurs in a specific cell type in the inner ear, the OHCs, these cells are suggested as a good target of future research and precision medicine (Eggermont, 2017, 2019; Kujawa & Liberman, 2015; Matsuoka et al., 2019). This type of cell is a main and early target of aging, various ototoxic substances and overexposure to noise or acoustic trauma (Kujawa & Liberman, 2015; Ryan et al., 2016). OHC loss or dysfunction may, in addition, have a pathogenetic role in idiopathic SNHL (ISSHL) (Sun et al., 2019). Consequently, discovery of an OHC-specific biomarker and the assessment of the conditions under which it could help in the diagnosis and management of SNHL is of great priority.

Prestin is the 5th member of an 11-member membrane transporter super family (solute carrier family 26 or SLC26) which includes anion transporters and related proteins (He et al., 2014; Liberman et al., 2002). Prestin is situated in the lateral wall of OHCs and is responsible for their electromotility (Liberman et al., 2002; Parham, 2015). Prestin's exact role and regulation mechanisms have not been completely clarified (Matsunaga & Morimoto, 2016; Mazurek et al., 2007; Xia et al., 2013). Its deficiency is associated with moderate SNHL. In mice, prestin gene deletion can cause loss of OHC electromotility in vitro and 40-60 dB loss of cochlear sensitivity in vivo, while heterozygotes present a 6 dB elevation of hearing thresholds (Liberman et al., 2002). Moreover, in vitro OHC damage due to ototoxic substances and high intensity noise increases the expression rate of the responsible gene (Mazurek et al., 2007; Xia et al., 2013).

To date, there is only one observational study assessing the auditory results of prestin gene mutation in humans (two identical twins); its results also imply a sensorineural loss of about 40-60 dB (Matsunaga & Morimoto, 2016).

Apart from cochlear prestin, circulating prestin has also been observed in the blood of animals and humans with or without HL (Dogan et al., 2018; Parham, 2015; Tovi et al., 2018). Its presence in the blood has been detected by means of enzyme-linked immunosorbent assay (ELISA) and could be explained either by its small size (80 kDa), which may allow crossing of the blood-labyrinthine barrier, or by its engulfment by phagosomes after OHC apoptosis. ELISA is capable of detecting blood prestin even in small quantities, where less than 1% of OHCs are lost (long before audiological symptoms or abnormal audiometric outcomes appear) (Parham, 2015).

All the above arguments have led to the hypothesis that prestin blood level could reflect changes or damage in the cochlea, and more specifically in the OHCs, and thus serve as an easily measurable, early SNHL biomarker (Parham, 2015). Consequently, evaluating potential changes in prestin blood concentration in patients suffering from SNHL has attracted researchers' interest. A scoping review of all the available scientific evidence could help the design and execution of further research in this particular domain. To the best of our knowledge, no such review has been conducted to date.

2.2. MATERIALS AND METHODS

2.2.1. Objectives

This is a scoping review of current clinical and basic science literature on the measurement of prestin level in the blood of normal and hearing-impaired animals and human participants, aiming at systematically mapping the research conducted in this area. Identification of the limitations of previous works, methodological pitfalls, or gaps in current knowledge, are a prerequisite in order to understand under which conditions prestin blood level can have a meaningful interpretation.

The following research question was formulated: What is known from the literature about prestin blood level and its temporal variations in people and animals with or without SNHL?

2.2.2. Methods

The protocol of this study was drafted according to PRISMA Extension for Scoping Reviews (PRISMA-ScR) guidelines (Shamseer et al., 2015; Tricco et al., 2018).

2.2.3. Eligibility criteria

Population: humans and animals with or without SNHL

Intervention: measurement of prestin blood level

Comparator: not applicable

Outcome: prestin blood level in healthy controls and hearing impaired, temporal variation of prestin blood level, correlation of prestin blood level with HL

Inclusion Criteria: controlled experimental studies [controlled clinical trials (CCTs) and randomized controlled trials (RCTs)], observational studies [longitudinal and cross-sectional studies], reviews. Publication type and language: English, French, Spanish or German-language journal articles. Publication year: last 10 years. Particulars: There was no restriction in types of SNHL. Sudden HL, noise trauma, hereditary HL etc., were all included in the review. Both human and non-human studies have been included.

Exclusion Criteria: Studies in languages other than the aforementioned ones. No full text available.

2.2.4. Information sources

Major databases of Medline, Central and Scopus were searched for eligible studies by two reviewers independently. The grey literature was sought in PROSPERO, Clinicaltrials.gov, EU Clinical Trials Register and the lists of abstracts in major Audiology- and Otoneurology-related conferences of the past six years. The results were then hand-searched (Hopewell et al., 2002).

2.2.5. Search

The Medline search was conducted via Pubmed by using free text and MeSH terms. Predefined search strategies and selection criteria were used to evaluate the eligibility of studies. Final syntax follows:

(prestin) AND ((hearing loss) OR (hearing impairment) OR "Hearing Loss"[Mesh] OR "Hearing Loss, Sensorineural"[Mesh] OR "Hearing Loss,

Noise-Induced"[Mesh] OR "Hearing Loss, Sudden"[Mesh] OR "Deafness"[Mesh])

Adding a third search term such as “ELISA”, "Enzyme-Linked Immunosorbent Assay"[Mesh] or “antibodies” was finally rejected since a number of studies were omitted. The NOT Boolean Operator was tried in an effort to exclude conductive-HL-focused studies, but it was finally rejected since it did not change the number of results.

Search in the other aforementioned databases followed the same principles, using keywords and MeSH terms wherever available. Since this review is focusing only on studies measuring prestin in the blood and this has been introduced as a procedure only recently (Parham et al., 2014), our research was limited to the last ten years.

2.2.6. Selection of sources of evidence

All studies were screened, first by title and abstract and subsequently by full text in order to identify and exclude those that were irrelevant, duplicates, or in other than the approved language.

2.2.7. Data charting

A data charting form was developed including the parameters of interest for the particular study.

2.2.8. Data items

Data about article identification (author, journal, year of publication), article characteristics (e.g., country of origin, language, funding), population characteristics (human or other species, age, type of HL), prestin level measurement (methodology, setting, results) were extracted from the included studies. A comprehensive summary will be presented in Results (section 3.2).

2.2.9. Synthesis of results

We present the included studies and summarize the type of settings, populations and study designs, with emphasis on our predefined scientific queries (study's timeline, results in prestin blood level as measured in controls and hearing-impaired participants).

2.3. RESULTS

2.3.1. Selection of sources of evidence

Procedures followed for the selection of included studies are presented in Figure 2.1.

2.3.2. Characteristics of sources of evidence

Article identification information (author, year and journal of publication etc.) and comprehensive comments on the methodology and results of each one are included in **Table 2.1** and **Supplementary Material 2.1**.

Figure 2.1 Selection of sources of evidence. Seven studies fulfilled the predefined inclusion criteria.

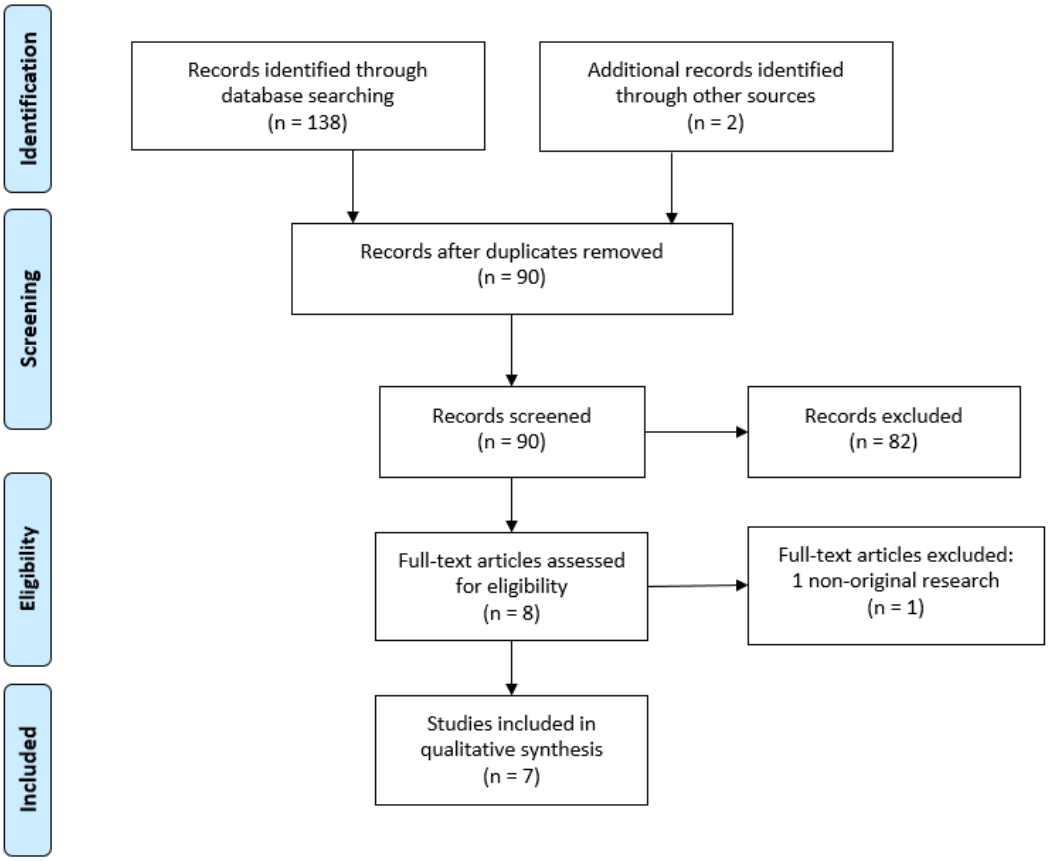


Table 2.1. Characteristics of included human studies. All prestin blood level measurements were conducted by means of ELISA.

Author, Year, Journal	Title	Population	Summary	Audiometric Assessment	Findings
Sun et al. *Ear, Nose and Throat Journal* 2019	A Preliminary Report on the Investigation of Prestin as a Biomarker for Idiopathic Sudden Sensorineural Hearing Loss.	Adults with unilateral ISSHL [n=14 (8 females), age = 57.9 (15.4), mean (SD)]	Measurement of prestin blood level in 14 patients hospitalized for ISSHL, before and after treatment with oral prednisolone 1 mg/kg for 5 days, IV Gingko biloba, intramuscular cobamamide (± batroxobin) and comparison with 24 adults with no history of hearing loss (age/sex-matched control group)	Pure tone audiometry - Arithmetic mean value of 0.5, 1, 2, and 4 kHz - Recovery according to Siegel's criteria (nominal)	- Prestin was detected in all participants (patients / controls) - Prestin level in ISSHL patients before treatment were significantly higher compared to control patients - Approximately half of the patients with ISSHL had higher prestin concentrations than the average level of the control group - Plasma prestin level before treatment in patients with ISSHL did not correlate with treatment outcomes
Hana and Bawi *Ibnosina Journal of Medicine and Biomedical Sciences* 2018	Prestin, otolin.1 Regulation, and Human 8.oxoG DNA Glycosylase 1 Gene Polymorphisms in Noise Induced Hearing Loss	Adults with occupational (NIHL). (n = 300, Age = 40.5±5.2, M:F = 93.2%)	Measurement of prestin blood level in 300 patients with NIHL in comparison with 200 workers (age, sex and occupational noise exposure-matched) with normal hearing	Pure tone audiometry - 0.5, 1, 2, 4, 6 kHz - Nominal according to degree of hearing loss	- Prestin was detected in all participants (patients / controls) - Significant positive correlations were detected between prestin level and the severity of NIHL, otolin-1 level, 8–OxoG, and Cys/Cys genotype

SD, standard deviation; ISSHL, idiopathic sensorineural hearing loss; NIHL, noise-induced hearing loss; IV, intravenous

2.3.3. Prestin blood level without hearing loss or noise trauma

Four out of seven included studies evaluated the level of prestin in the blood of human subjects or animals with no HL or exposure to noise or to ototoxic agents. In a recent clinical study by Sun et al. (2019), 24 people (13 females)

referred as of normal hearing capacity were age- and sex-matched to 14 (eight females) idiopathic sudden sensorineural hearing loss (ISSHL) patients. In this control group, which had no hearing loss history, prestin level ranged from 85.4 to 1628.25 pg/mL, with an average of 840.24 (± 496.22) pg/mL [mean (SD)]. Mean age was 57.9 years, however no further information on the control group's characteristics was available (**Table 2.1**).

A larger scale cross-sectional study by Hana and Bawi (2018), assessing prestin blood level in 300 workers with NIHL, also showed that prestin was present in the blood of 200 volunteers that served as controls [100.9 (± 16.7) pg/mL, mean (SD)] (**Table 2.2**). The mean age of the control group was estimated at 40.3 (± 3.9) years. Their occupational exposure to noise was measured using a sound level meter at their workplace and reported according to the duration of occupational noise exposure in months [18.2 (± 7.4) months, mean(SD)] and exposure level [87.0 (±7.6) dBA, mean (SD)] separately. Overall exposure was unclear since recreational exposure was not reported. The above information allowed the authors to match the control to the patient group by age, sex, and occupational exposure. The authors also asked patients and controls for a history of ototoxic drugs usage, hearing-related family history, and smoking, although they only controlled for smoking in the analysis.

Animal studies have also shown that prestin can be detected in controls' blood. Parham et al. (2019) observed that prestin level ranged from 125 to 245.7 pg/mL [177.9 (± 4.3) pg/mL, mean (SD)] in 46 male Wistar rats with no prior exposure to ototoxic noise or drugs. In another male Wistar rat model,

measuring prestin level after exposure to ototoxic factors (amikacin, cisplatin), Dogan et al. (2018) also showed that prestin could be detected in the blood of the control, ototoxic-drug-free, group [n=10, 377.0 (± 135.3) pg/ml, mean (SD)].

Table 2.2. Mean prestin concentration level in two human studies.

	Patients	**Controls**	**p-value**$^{+}$
Hana and Bawi, 2018	300 workers with NIHL	200 workers with normal hearing	
Prestin Level in pg/ml (SD), before treatment	169 (88.4)	100.9 (16.7)	0.04*
Prestin Level in pg/ml (SD), 1 month after treatment	114 (99.2)	-	0.04*
Sun et al., 2019	**14 patients with ISSHL**	**24 with normal hearing**	
Prestin Level in pg/ml (SD), before treatment	1955.98 (2501.48)	840.24 (496.22)	<0.01*
Prestin Level in pg/ml (SD), after treatment	1653.26 (1967.60)	-	0.06

SD, standard deviation; ISSHL, idiopathic sudden sensorineural hearing loss; NIHL, noise induced hearing loss.
* Significant at an alpha of 0.05
$^{+}$ p-value for comparison between patients and controls

2.3.4. Relation of blood prestin level to hearing loss

All included studies were focused on SNHL. Sun et al. (2019) included patients hospitalized with ISSHL. Hana and Bawi (2018) evaluated prestin blood level in patients with NIHL. Animal studies included rats and guinea pigs that were exposed to ototoxic substances, such as aminoglycosides and cisplatin (Dogan et al., 2018; Liba et al., 2017; Naples et al., 2018), and thus present SNHL due to ototoxicity; or to hazardous levels of noise, and thus present NIHL (Parham et al., 2019; Parham & Dyhrfjeld-Johnsen, 2016).

In the ISSHL study of Sun et al. (2019), 14 participants with mean age 57.9 (± 15.4) years [mean(SD)] presented significantly higher levels of prestin in their

blood before treatment compared to controls (p<0.001, statistical test not reported, type of treatment not reported). All measurements before treatment were conducted within seven days from the onset of HL and ranged from 190.30 to 9648.80 pg/mL, with a 1955.98 (± 2501.48) pg/mL [mean (SD)] average concentration. Half of ISSHL patients presented higher concentration compared to the average value for the control group and 35.7% of ISSHL participants had higher prestin level than the highest value detected in the control participants. Measurements were repeated at the end of treatment, within 4 – 11 days after the initial measurement, and ranged from 0 to 7610.45 pg/mL, with an average concentration of 1653.26 (± 1967.60) pg/mL [mean (SD)]. Prestin blood concentration before treatment did not correlate with treatment outcomes (**Table 2.2**). Six out of 10 participants who recovered from ISSHL had decreased blood prestin. All four participants that did not recover presented increased prestin level.

In the human NIHL study, Hana and Bawi (2018) revealed a significant difference in prestin blood level between the patient and control group immediately after noise exposure and before treatment (**Table 2.2**). In comparison to controls, NIHL patients' age, sex ratio, smoking habits and occupational exposure to noise [18.6 (± 7.6) months, mean (SD) and 87.0 (±7.6) dBA, mean (SD)] did not differ significantly. One month after treatment (no information on type of treatment was provided), mean prestin concentrations in the NIHL group were 55% lower than that initially observed [114 (±99,2) pg/ml, mean(SD)]. These values differed significantly from the ones before treatment (t = 4.3, p = 0.02) and from the control group (**Table 2.2**). Significant positive correlations were reported between prestin level and

severity of hearing loss (r = 0.971), otolin-1 level (r = 0.776), 8–OhdG (r = 0.556), and Cys/Cys genotype (r = 0.828).

The effect of the noxious agent (noise or drug) in rodent models has been verified in all relevant studies by means of histological and audiometric testing [Auditory Brainstem Responses (ABR) and/or Distortion Product Otoacoustic Emissions (DPOAEs)]. Liba et al. (2017) observed an increase in blood concentrations of prestin both in guinea pigs that had an increase in ABR thresholds and in mice that were found resistant to cisplatin according to their audiometric evaluation.

Dogan et al. (2018) exposed rats to low and high doses of amikacin (200 and 600 mg/kg/day, respectively) for 10 days and cisplatin (single dose of 5 and 15 mg/kg, respectively) for 3 days and conducted prestin measurements immediately after the end of the experiment. They report that their audiometric findings via DPOAEs showed significant changes at specific frequencies (4, 6 and 8 kHz). Mean prestin blood level were found to be 411.3 (± 73.1) pg/mL [mean (SD)] in the low-dose amikacin group and 512.6 (± 106.0) pg/mL [mean (SD)] in the high-dose amikacin group. Corresponding values for prestin in the cisplatin group were 455.0 (± 74.2) pg/mL [mean (SD)] in the low-dose group and 555.3 (± 47.9) pg/mL [mean (SD)] in the high-dose group (Dogan et al., 2018). Significant differences were found in blood prestin between the low and high amikacin groups, between the low and high cisplatin groups, and for all treatment groups compared to controls [377.0 (± 135.3) pg/ml, mean(SD)]. Prestin blood level were significantly correlated with the threshold changes in those frequencies where a significant threshold shift was detected in the

DPOAEs. In the study of Parham and Dyhrfjeld-Johnsen (2016), blood prestin concentration showed a linear negative relationship with DPOAE level change (r=0.563, p=0.01) and a linear positive relationship with ABR threshold change (r=0.46, p=0.036) at 14 days after exposure to noise. Naples et al. (2018) found that the increase in ABR threshold was recovered at day 7 and 14 in the guinea pigs that received diltiazem as otoprotectant after cisplatin exposure. Prestin blood concentrations were in accordance with these functional results, and no significant change in prestin level was observed in the diltiazem group.

Parham et al. (2019) included histological testing, ABR and DPOAEs to the assessment of cochlear damage due to exposure to 110 dB (low noise group) and 120 dB SPL (high noise group). In this particular study, agreement was observed between functional, histological and serological findings: significantly greater loss of OHCs was observed in the 120 dB SPL group and was associated with a greater extent of functional changes and decrease in prestin level compared to the 110 dB SPL group.

2.3.5. Variation in prestin blood level over time after cochlear damage

In the recent animal study by Parham et al. (2019), prestin level were measured at 4 h, 24 h, 48 h, 72 h, 7 and 14 days after 2 h of exposure to noise of 110 and 120 dB SPL. The study found a noise-level-dependent change of prestin over time; after an initial peak of prestin concentration right after the noise trauma (4 h), the overall (14 days) statistically significant decrease of prestin concentration in comparison to pre-exposure values was found to be less than 5% for the low-dose group (approximately 10 pg/ml) and more than 10% for the “loud” group (approximately 30 pg/ml). Two additional animal studies provide

information on change of prestin level over time; the first study by Parham and Dyhrfjeld-Johnsen (2016) evaluated blood prestin 14 days after noise exposure (rats). The second study was conducted by Liba et al. (2017) and evaluated blood prestin at 1, 3, 7, 14 days after one single dose of cisplatin at 8 mg/kg (rats and guinea pigs). The first study found that blood prestin concentrations in noise exposed rats were significantly below control level at day 14 after the noise trauma (Parham et al., 2019). The second study reports that prestin rose to a maximum value on day 7 (mice) and day 3 (guinea pigs) after cisplatin treatment and then declined back to or below baseline/control level on day 14 (Liba et al., 2017).

Naples et al. (2018) explored prestin blood level changes over time after exposure to ototoxic substances. Prestin level were measured at 1, 2, 3, 7, and 14 days post-cisplatin administration in 20 guinea pigs. Ten of them received treatment with diltiazem (as otoprotectant), while 10 received saline and served as controls. In this particular study, the diltiazem group had no significant change of prestin level before and after cisplatin. In the control group, the rise from baseline values reached a maximum at day 2 post-cisplatin administration and remained elevated at day 3 before trending back toward baseline at days 7 and 14. The mean percentage changes in prestin level for days 1 to 3 were statistically significant compared to baseline. A summary of the results of prestin concentration after trauma is provided in **Figure 2.2**. It should be noted that no specification of the time of day or point on circadian cycle was mentioned in any of the included studies.

A.

B.

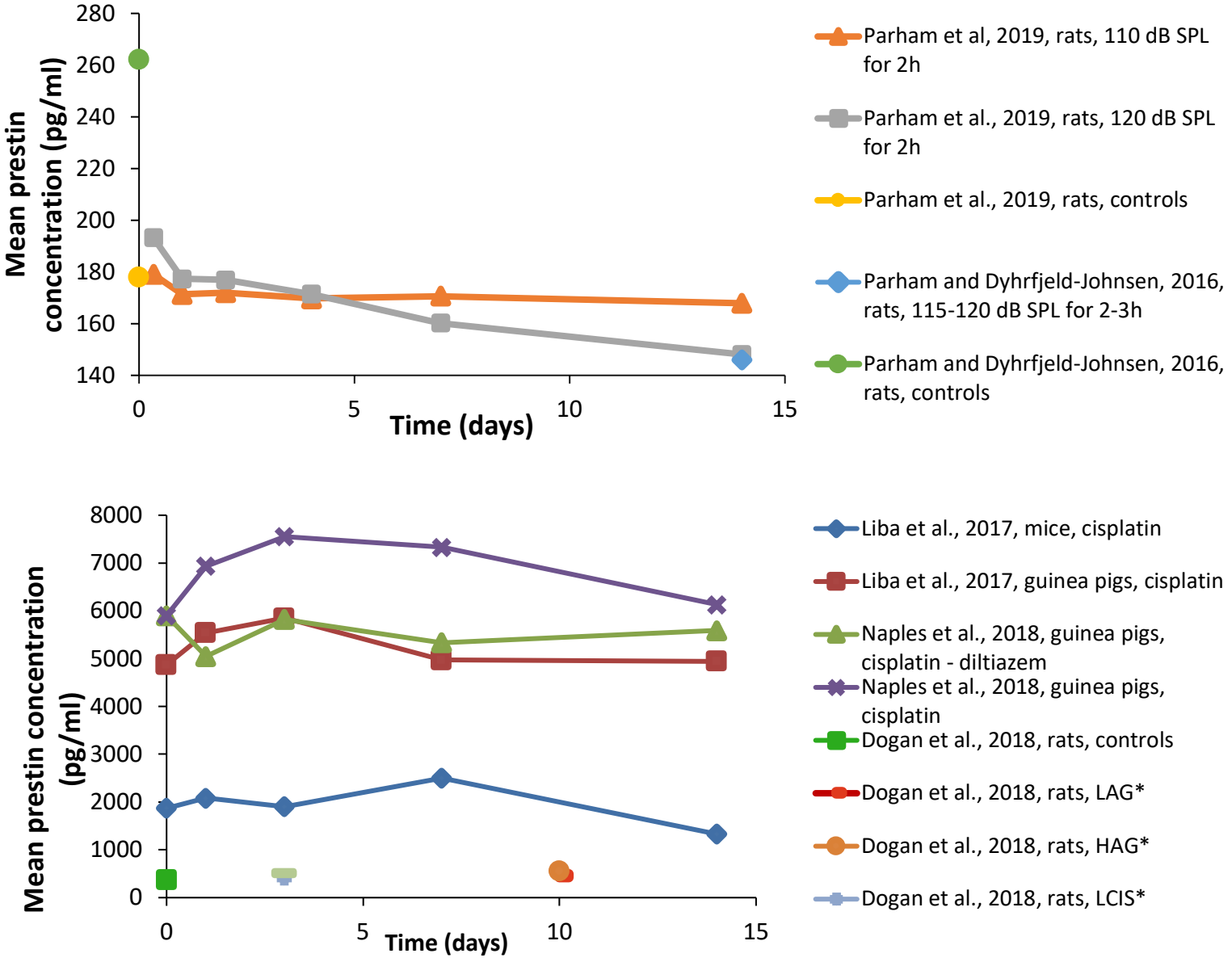


Figure 2.2. Average prestin concentrations as a function of time after trauma in the two NIHL rodent models over time (A) and in the three drug ototoxicity rodent models (B). (Data extracted from the original graphs via WebPlotDigitizer). * HAG = 600 mg/kg/day of amikacin (10 days), LAG = 200 mg/kg/day of amikacin (10 days), LCIS = 5mg/kg of cisplatin (3 days), HCIS = 15mg/kg of cisplatin (3 days).

2.4. DISCUSSION

It has been hypothesized recently that, apart from the changes due to normal prestin turnover within OHCs, blood prestin concentrations may also be related to cochlear damage (Parham, 2015). Building on this idea, prestin blood level have been measured in several rodent and human studies with NIHL, ISSHL, or drug-induced HL. The rationale for these studies is that these types of SNHL seem to be associated with OHC damage (Ryan et al., 2016; Tovi et al., 2018). ELISA is the only blood prestin detection method that has been identified in the present review (Table 1 and SM Table 1). A new electrochemical immuno-biosensor for circulating biomarkers of the inner ear (otolin-1 and prestin) has been recently proposed (Mahshid & Dabdoub, 2020). Nevertheless, no data from rodent or human studies are yet available, and further research is needed before validating the conditions under which this method can be used in research and clinical practice.

An *increase* in blood prestin concentrations in the case of OHC damage or loss may be explained by the release of prestin from the OHCs directly through the blood-labyrinth barrier or by means of phagosomes in the supporting cells (in the short term). Experimental data in guinea pigs have shown that the permeability of blood-labyrinth barrier increases after exposure to noise (4h of white noise at 120 dB SPL for 2 consecutive days; Ke et al., 2023). Another potential source of the increase in blood prestin level is the functional up-regulation of prestin expression in the residual OHCs (longer term) (Abrashkin et al., 2006; Liba et al., 2017). Although there is controversy concerning the correlation of prestin mRNA level to prestin protein level (Cheatham et al., 2005;

Liberman et al., 2002), data from 5- to 6-week-old wild-type CBA/CaJ mice show that prestin has been up-regulated by 32–58% within remaining OHCs after noise exposure (Xia et al., 2013). Similarly, in 5-week-old wild-type CBA/CaJ mice with diphtheria toxin-induced SNHL (intraperitoneal injections of diphtheria toxin, 50 ng/g for three days in a row) prestin up-regulation seems to be locally regulated by the steady-state transducer bias current with no involvement of centrally mediated efferent feedback (Roux et al., 2006; Song et al., 2015). Based on current evidence, it is difficult to conclude whether the increase of prestin blood level reflects cochlear damage directly, via passage of free prestin molecules to the circulation, or a cochlear compensation mechanism for temporarily or permanently damaged hair cells through up-regulation of the prestin gene in the remaining OHCs. It is also unclear how any up-regulation of the prestin gene may reflect to inner ear prestin level or to prestin's blood level. Better understanding of the pathophysiological mechanisms that are involved in noise-induced cochlear damage and prestin up-regulation may clarify the source of the post-noise exposure increase of prestin blood level observed in the studies mentioned in this review.

A *decrease* in blood prestin level in the case of OHC damage or loss may be the product of a dynamic equilibrium of cochlear function where the remaining, fewer OHCs release less prestin into circulation (Parham et al., 2019). Decreased blood prestin may also be a consequence of the disruption of the balance between the production of free radicals and the antioxidant defense system in the cochlea that can occur after exposure to intense noise or other noxious agents. Hana and Bawi (2018) observed a significant positive

correlation between prestin blood level and blood 8-OHdg. Nevertheless, evidence from age- and noise-related hearing loss rodent models shows that intracochlear reactive oxidative species (ROS) accumulation may affect, to some degree, OHCs' lateral wall and electromotility (Chen, 2006; Chen et al., 2009; Lee et al., 2019). OHC structure changes may decrease prestin's cellular, and thus free intracochlear, concentration, which may consequently have an effect on its blood concentration. Intracochlear ROS may lead to oxidation of different elements of the prestin molecule or to the formation of protein–protein cross-linkages (Berlett & Stadtman, 1997; Chen, 2006).

Over the studies reviewed here, a short-term increase in prestin blood level and a long-term decrease below baseline has been observed following trauma. This finding agrees with previous studies on prestin gene regulation. Prestin up-regulation has been shown in rats with verified HL (functional and histological assessment) after exposure to noise (10-20 kHz, 110 dB SPL for 4 h). In this animal model, prestin expression peaked at third post-exposure day (4.9 ± 0.3 folds of increase) and returned progressively to baseline four weeks after noise exposure (Chen, 2006). Similarly, in another rodent model, after monaural noise exposure, change of endocochlear prestin mRNA level was associated with the degree of hearing loss and differed among different parts of the cochlea (increased with a base-to-apex gradient (Mazurek et al. 2007). At first, exposed rats and guinea pigs presented moderate NIHL (15–25 dB DPOAE threshold shift), and prestin mRNA increased. One-week post-exposure, NIHL severity had increased (by about 30 dB) and prestin blood level tended to decline.

Interestingly, DPOAE decrease and prestin up-regulation was observed in the contralateral ear (non-exposed), as well (Mazurek et al., 2007).

Long-term prestin regulation in the case of SNHL remains unclear. Immunohistochemical staining of the cochlea of F344 rats with age-related hearing loss has indicated that prestin is reduced and that this age-related reduction may precede hair cell degeneration (Chen et al., 2009). Nevertheless, data concerning human prestin regulation and blood level is still missing.

2.4.1. Prestin blood level without hearing loss or noise trauma

It has been hypothesized that prestin is detected in the blood of "naïve" rodents due to its normal turnover in the OHC membrane (Parham & Dyhrfjeld-Johnsen, 2016). This may further imply that normal values differ per species and are correlated with the number of OHCs and the length of the cochlea (Liba et al., 2017). In human studies, prestin was found in the blood of people identified as of "normal hearing" (Hana & Bawi, 2018; Sun et al., 2019). However, there is a lack of information concerning their medical and noise exposure history and their full audiometric profile. This information is necessary in order to confirm that their hearing status was indeed healthy. Additional data on prestin level in people of different ages, who would have a full history and audiometric profiling, would be extremely useful in developing norms and determining which prestin level are part of the normal physiological turnover of OHCs and which indicate cochlear damage.

2.4.2. Relation of plasma prestin level to hearing loss

Prestin blood level changed significantly in rodents with acquired HL when compared to baseline measurements or controls (Dogan et al., 2018; Parham

et al., 2019; Parham & Dyhrfjeld-Johnsen, 2016). Using non-exposed “naïve” rodents and assessing the effect of noise or ototoxic agents on their hearing by means of histological and functional assessment allows the safe correlation of each change in prestin level with specific phase of cochlear damage.

Previous studies on intra-cochlear prestin expression indicate its base-to-apex gradient increase and its association with the degree of HL (Chen, 2006; Mazurek et al., 2007). Similarly, blood prestin level show an association to the cause and degree of cochlear damage. In the case of NIHL, prestin level have been associated with the levels of noise exposure and the degree of TTS and PTS that has been caused. In the study of Parham et al. (2019), 20 rats exposed to intense octave band noise at 120 dB SPL showed significant changes in prestin level when compared to the changes observed in the 110 dB SPL exposed group. In the case of ototoxicity, groups with high doses of cisplatin and amikacin presented both higher degrees of hearing loss and prestin blood level (Dogan et al., 2018).

A very interesting finding is that, in specific cases, prestin expression change precedes auditory findings and/or may have a higher predictive value than audiometric assessment. Liba et al. (2017) observed a rise in blood prestin level at day 2 post-cisplatin administration. This rise preceded the onset of significant ABR changes. This observation may be explained by the fact that early up-regulation of intra-cochlear prestin may maintain normal cochlear function. The findings of Parham et al. (2019) on NIHL suggest that an early rise of blood prestin is a better prognostic marker than ABR or DPOAEs threshold shifts. Nevertheless, it is not clear if, and under which conditions, prestin blood level

is indeed more sensitive than standard audiometric testing. Further research, combining histological examination of the cochlea, DNA expression determination, functional auditory testing and ELISA could clarify better the correlations and time sequencing among cochlear trauma, OHC loss, prestin gene expression, prestin protein endocochlear / blood level and auditory function.

In the human ISSHL study, Sun et al. (2019) found a significant difference in prestin blood level between patients and controls (Sun et al., 2019) However, only half of the ISSHL participants had higher level of prestin blood level when compared to controls. According to the authors, this finding suggests that only some of the ISSHL patients present true OHC damage and that prestin could serve as a means of their identification. However, before being able to generalize this claim, larger scale data on hearing phenotypes of ISSHL patients and controls are needed. Prestin level has shown some association with the degree of recovery, but more data are needed before being able to generalize this finding. In the NIHL study by Hana and Bawi, patients' prestin was significantly greater, both before and after treatment (1 month later) when compared to controls (Hana & Bawi, 2018). However, as mentioned before, methodological issues of those studies (small sample size, incomplete audiometric profiling and unclear timeline of measurements) do not allow their results to be generalized easily.

2.4.3. Variation in prestin blood level over time after cochlear damage

Recent evidence implies that there is a circadian regulation of auditory function and noise sensitivity (Basinou et al., 2017)**.** Quantitative real-time polymerase

chain reaction has revealed circadian regulation of various endocochlear transcripts, while specific neurotrophic factors that are associated with cochlear neurogenesis and homeostasis, such as brain-derived neurotrophic factor (BDNF), have also shown a circadian pattern (Singer et al., 2014). Current evidence from rodent and human studies does not clarify the effect of circadian regulation on prestin blood level.

Preliminary data from animal and human studies have shown that prestin blood level depend on the interval between the exposure to the ototoxic agent (drug or noise) and its measurement by means of ELISA. Two rodent models have shown that prestin presents an increase in the blood immediately after exposure to noise and then returns to baseline, or below baseline values, 14 days after (Parham et al., 2019; Parham & Dyhrfjeld-Johnsen, 2016). No data later than 14 days after trauma are available to date. No information on the specific time of the day that the ototoxic agent was applied, or the blood was drawn is available either.

To date, no human studies have assessed prestin blood level variation over time after exposure to noise or to an ototoxic agent in the absence of potential confounding factors after the initial time point, such as continuation of the exposure to the noxious agent (noise or ototoxic drug) or participants' therapeutic treatment for hearing loss. In order to assess the true change of blood prestin after cochlear damage, factors that may potentially affect its intracochlear regulation and concentration should be avoided after baseline measurement, and all variables but time should be held constant.

Consequently, longitudinal human studies focusing on prestin level over time are warranted.

In the two human models included in this review, prestin was measured before and after treatment for NIHL and ISSHL (Hana & Bawi, 2018; Sun et al., 2019). However, no specific timeline of participants' HL or the relation between day of onset of the HL or HL diagnosis and the day of prestin measurement was provided. No information on the specific time of the day that participants were exposed to noise, or that the blood was drawn, is available either.

It should be noted that if prestin level return to near-normal shortly after trauma, then they may be of little use in the diagnosis of established HL. Further research with multiple measurements during day- and night-time, for different hearing phenotypes, with and without exposure to noxious agents (e.g., noise), is needed to evaluate this. Long-term prestin gene functional expression and prestin protein release into regulation also needs further understanding.

2.4.4. Methodological pitfalls

Over the human studies reviewed here, there is great heterogeneity in primary endpoints, such as the mean prestin level and their range both in control and patient groups. There is also a lack of detail on important methodological issues. These differences could explain the heterogeneous results.

Age is another parameter that may be associated with the variability of prestin blood level. Data from rodent models show that prestin level in OHCs, evaluated semi-quantitatively by immunohistochemical staining, are reduced in the aging cochlea of F344 rats (Chen et al. 2009).

With regards to sample characteristics, other parameters may also affect prestin level and should be better clarified before drawing any conclusions. Different causes of HL are linked to different pathophysiological mechanisms and possibly different endocochlear and blood behavior. In a recent human study focused on ISSHL, Tovi et al. (2018) state that ELISA detected anti-prestin antibodies in the serum of only two out of 63 patients with unilateral ISSHL. These findings, along with the fact that ISSHL pathophysiology remains unclear and differs from NIHL, makes comparison of the two human studies reviewed here challenging (Greco et al., 2011).

Duration of the symptoms, age of onset, severity of HL, and interval between treatment and onset of HL, are all considered as SNHL prognostic factors. In the NIHL study by Hana and Bawi, it is described that patients were referred to the hospital because of NIHL, received treatment, and were tested for prestin blood level before and after this treatment. It is mentioned that tinnitus and HL were the main symptoms. However, information is lacking on whether acoustic trauma was acute or chronic, and on the specific kind of treatment patients have undergone. This information is important since different pathophysiological mechanisms could be involved (Le et al., 2017). Concerning the audiometric profile of patients, they have been classified according to their degree of HL as it was defined by average threshold at 0.5, 1, 2, 3, 4, 6 kHz in the pure tone audiogram. No further audiometric evaluation was conducted (Hana & Bawi, 2018). Similarly, in the ISSHL study of Sun et al. the prestin concentrations of 14 participants were included in the analysis, without any detail on their clinical variables being taken into account.

Hana and Bawi (2018) matched the two groups by age, sex, and occupational exposure. They asked patients and controls for a history of ototoxic drug usage and hearing-related family history, but they did not control for these in the analysis. They also included questions about work and disease history, however this information is not reported in the article. Participants of the profound hearing loss group were removed from the noisy environment and the study; however, no information is given concerning the continuation or not of noise exposure for the participants with mild, moderate, and severe HL (Hana & Bawi, 2018). If during their treatment, and thus participation in the study, participants remained working in the same noisy environment, this could be considered as a confounding factor. Sun et al. (2019) excluded participants with other than NIHL or ISSHL. Nevertheless, no particular measures were taken to exclude participants with age-related or overall-noise-exposure-related cochlear damage. This fact, along with the small sample size, does not allow strong conclusions about the relation of ISSHL pathophysiological pathways and blood prestin level changes to be drawn.

Understandably, the aforementioned factors were better controlled in the rodent models. In the animal studies presented in this review, all animals were healthy, 6-20 weeks of age and with no prior exposure to ototoxic agents or noise before undergoing the experimental procedures (exposure to cisplatin, aminoglycosides, or noise). In order to confirm cochlear damage, rodents exposed to noise or other noxious agents underwent functional or histological testing. Degree of cochlear damage and HL was evaluated by means of ABR and DPOAEs, while three studies also included histological examination of the

cochlea (Dogan et al., 2018; Parham et al., 2019; Parham & Dyhrfjeld-Johnsen, 2016). In two of them, Parham et al. used the same methodology and focused on the mean loss of OHCs in each group as a function of normalized distance from the apex, using the total length of each histological specimen. In the context of the third study by Dogan et al. a pathologist blinded to the groups scored the specimens for their OHC count (number of OHCs with an intact nucleus) according to the four-point scoring system for cisplatin-induced ototoxicity defined by Freitas et al. (Freitas et al., 2009). As a consequence, each deviation from baseline in the functional, histological or prestin level outcomes can be safely attributed to the HL originating from experimental interventions.

In conclusion, the discovery and validation of otologic biomarkers in human blood may be of great value to the prevention, early diagnosis, and prognosis of hearing loss. To date, there is some evidence that prestin blood levels change in the case of acquired HL in rodents, and that this change is correlated with the degree of cochlear damage, the region of the cochlea that is affected, and the time interval between onset of disease and prestin measurement. These proof-of-concept studies provide important insight on the matter and provide preliminary evidence that prestin may indeed serve as a valuable biomarker for HL. However, larger scale data are required in order to clarify the conditions under which blood prestin can be best used as a marker in the case of human subjects with SNHL. In human studies, specific methodological challenges have to be resolved before researchers are able to draw any conclusions. Future studies could be improved by larger samples, more detail on hearing phenotyping and clinical variables, prestin measurements at specific

time points during the course of cochlear damage, clear segregation of the effect that TTS has on prestin from the effect of permanent lesions, longer longitudinal experiments in unilateral and bilateral acquired HL, full audiometric profiling of participants, detailed quantification of all factors that could have led to OHC damage, and definition of the clinical and genetic variability of each HL case.

Note: Chapter II corresponds to the published paper Blood Prestin Levels in Normal Hearing and in Sensorineural Hearing Loss: A Scoping Review (Iliadou et al., 2021). Since its publication, more papers on human serum prestin have been published. I provide a comprehensive table of those papers below.

Table 2.3 Characteristics of included human studies from 20. All prestin blood level measurements were conducted by means of ELISA.

Author, Year, Journal	**Title**	**Population**	**Summary**	**Audiometric Assessment**	**Findings**
Turan et al. *J Chin Med Assoc.* 2023	Blood prestin levels in COVID-19 patients	45 patients diagnosed with COVID-19 (20F, mean age = 52, age range = 33–63 years, and 40 healthy volunteers (18F, mean age = 50, age range = 32–64	Comparison of serum prestin level in Covid-19 patients and healthy volunteers.	- No hearing tests were performed	Blood prestin was higher in COVID-19 patients when compared to healthy volunteers. It also showed a positive correlation with CRP and D-dimer.
Asli et al. *Eur Arch Otorhinolaryngol* 2023	Evaluation of the relationship between prestin serum biomarker and sensorineural hearing loss: a case-control study	2 groups each comprising 44 young adults (36±8 years) 44 old adults (63.1±8.6 years)	Investigation of the relationship between prestin serum levels and sensorineural HL in an Iranian population	Pure tone audiometry (no details are provided)	- Groups with SNHL had higher prestin levels (Mean = 182.29, SD = 71.24) compared to the control groups (Mean = 122.50, SD = 57.1) (P < 0.001). - Multinomial logistic regression between prestin level and SNHL: significant after controlling intervening variables (P < 0.001 and odds ratio = 1.017 and 95% CI OR: 1.01–1.024).

					- Ordinal logistic regression model: prestin level significantly associated with the degree of HL (P < 0.001 and Odds ratio = 1.009 and 95% CI and OR: 1.005–1.013) - The best cutoff point for the 20–50 group was the prestin content of 132.5 pg/ml (sensitivity: 75%, specificity: 70.05%), while for the group of ≥ 50 was as 130 pg/ml (sensitivity: 84.1%, specificity: 68.2%).
Jalali et al. *Eur Arch Otorhinolaryngol* 2022	Effect of cisplatin chemotherapy on the inner ear function and serum prestin level	52 adults (36F, mean age (SD) = 55.1 (12.9) years	Assessment of the correlation between serum prestin level and audiologic findings in patients after cisplatin chemotherapy.	- Pure tone audiometry - Speech audiometry (by GN Oto- metrics Astera, Madsen, Denmark) - Tympanometry (Zodiac 901 Middle Ear Analyzer, Madsen, Denmark) - DPOAE: Evoked responses for 2f1-f2, where f1 and f2 were 65 dB sound pressure level (SPL) and 55 dB SPL, respectively, (f2/f1=1.22), 1000–8000 Hz). SNR ≥ 3 dB -> "normal DPOAEs". Positive differences (baseline − post-intervention) greater than 14 dB at 750 Hz and/or greater than 7 dB at higher frequencies were considered "ototoxic"	- Significant positive correlation between prestin level after receiving more than 80 mg of cisplatin and the ototoxic changes in the DPOAE response (OR 1.003; 95%CI [1.000, 1.005]; P = 0.02)
Parker et al. *Hear Res* 2022	Age-related declines to serum prestin levels in humans	72 dults with normal hearing (50F, mean age =39.98, age range = 18-82)	Correlation of serum prestin level with age.	- Pure-tone audiometry conducted for standard frequencies (0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8 kHz), - Speech perception in noise testing utilizing QuickSIN (lists 1-4, binaurally via inserts at 75 dB HL with SNR loss being calculated for each list individually (25.5 – score = SNR loss),	- There was a significant negative correlation between average serum prestin level (pg/mL) and age (years) (r = −0.350, p = 0.008)

				and then averaged for one composite score to be used in our analyses. (Etymotic, Inc.). - DPOAEs (f1/f2 = 1.22, Mimosa Acoustics' HearID software).	
Parker et al. *Sci Rep* 2022	Noise exposure levels predict blood levels of the inner ear protein prestin	34 adults with normal hearing (23F, mean age = 20.26 , age range =18–24)	Correlation of serum prestin level with 3-week noise exposure.	- Tympanometry - Standard and extended-high frequency tympanometry - TEOAEs using HearID software (Mimosa Acoustics). A 50 dB SPL chirp served as the stimulus, band passed from 1 to 5 kHz, presented through an ER10C probe tip insert (Etymotic, Inc.) using a preset protocol (TE50_B2000_N60) within the HearID software.	- There was a significant negative correlation between average serum prestin levels (pg/mL) and average daily noise exposure level ($L_{Aeq, 8\,h}$ (dB) (r = − 0.455, p = 0.011)
Sadaat et al. *J Laryngol Otol* 2022	Assessment of prestin level changes as an inner-ear biomarker in patients with idiopathic sudden sensorineural hearing loss	50 adults [25 with idiopathic sudden sensorineural hearing loss (ISSHL) and 25 with normal hearing, 30F, 48.2 ± 14.6 years (range = 19–77)]	Comparison of the serum prestin level in unilateral ISSHL patients with controls, and exploration of any relationships between serum prestin levels and certain clinical features.	Pure tone audiometry (at day 0, + 14 and 30 for the ISSHL group)	- Geometric mean (GM) prestin level in the case and control groups at T0 was 227.7 pg/ml and 130.5 pg/ml, respectively. - GM prestin level in the case group demonstrated a downward trend at T1 and T2 (214.0 pg/ml and 180.1 pg/ml, respectively; $p < 0.001$).
Generotti et al. *Sci Rep.* 2022	Subclinical diagnosis of cisplatin-induced ototoxicity with biomarkers	8 adults (1F, mean age = 51.8 years)	Assessment of the relationship of ototoxic damage, serum prestin level, CDDP perilymph level, and inner ear morphology in the setting of cisplatin-induced ototoxicity in mouse and in human serum samples.	No hearing testing: samples obtained by the ITMAT biobank and were correlated with available medical history.	- Serum prestin level in human with a history of otological symptoms or signs were significantly lower (Median of differences = 328.5, p = 0.0156, mean and SEM was 728.8 ± 118.4 pg/mL for control and 423.4 ± 85.78 pg/mL cases).
Solis-Angeles et al. *Toxicol Appl Pharmacol* 2021	Prestin and otolin-1 proteins in the hearing loss of adults chronically exposed to lead	315 adults [168F, median age (IQR) = 42 (34–52)]	Evaluation of prestin and otolin-1 protein levels and their relationship with an increased hearing threshold in participants exposed to lead.	Pure tone audiometry	- Multiple linear regression models predicted an average decrease of 0.17 to 0.26 ng/mL in prestin level per decibel increase for the frequencies evaluated

Chapter III

Blood Prestin Level in Young and Middle-Aged Adults with Normal Hearing: Effects of Age, Sex, and Time of Day

Objectives: Recent evidence suggests that blood prestin level may be useful in the detection or evaluation of cochlear damage. Enzyme-linked immunosorbent assay (ELISA) is the method currently used to detect and quantify these level. To date, several controlled studies have measured prestin level changes in human subjects after trauma. Methodological limitations, and the fact that the reported plasma prestin level in both control and cases groups differ significantly among those studies, complicate the interpretation and generalization of the results. Before establishing blood prestin's value as a biomarker, specific aspects of its measurement by means of ELISA should be clarified: ranges of prestin level in the blood; test-retest reliability of prestin measurement; and relation of serum prestin level with factors such as the time of day, pure tone audiometric thresholds, age, and sex.

Methods: Eligible participants had to be over 18 years old and have normal hearing. Serum prestin level were evaluated four and six times in 24 hours (one blood sample every 6 and 4 hours, respectively), for 10 (Module 1a, seven female, age range: 26-36 years) and for 11 participants (Module 1b, six female, age range: 26 - 53 years), respectively. Increasing the number of measurements was decided due to newer evidence implying that four measurements may not be sufficient to detect any circadian effect on the auditory function. In Module 2, 40 participants (26 female, age range: 26-65 years) provided one serum sample at a single session. Prestin was measured

in all samples by means of ELISA. All samples were assayed in duplicates. Variation of serum prestin level throughout the day was estimated for the samples collected from the 21 participants of Module 1. Intraclass correlation (ICC) between duplicates of the same sample and among samples of the same participant collected in different time points was conducted. Moreover, in order to increase the sample size and thus the power of post-hoc analyses, such as relation of age, sex, PTA and EHF-PTA thresholds and OAEs amplitudes with blood prestin, we merged the samples of the 54 unique participants of this study, which were collected both in Phase 1 and 2, with the subset of seven unique participants of another ongoing experiment in our lab (5 females, age range: 20 – 38).

Results: Serum prestin level did not differ significantly across time of day. Median prestin level was 119.21 pg/ml (IQR = 86.39 pg/ml) in Module 1a and 127.95 pg/ml in Module 1b (IQR = 85.02 pg/ml). Test-retest reliability between duplicates of the same sample was excellent (ICC = 0.95, p = 4.80e-45, 95% CI 0.93-0.97). The test-retest reliability at different timepoints of the day was moderate both for the Module 1a samples (ICC = 0.57, p = 4.73e-06, CI = 0.26-0.84, k = 4, type = average) and for the Module 1b samples (ICC = 0.62, p = 4.60e-16, CI = 0.43 – 0.81, k = 6, type = single). Neither lifetime noise exposure, age, nor sex correlated with serum prestin level. Pure tone average thresholds and otoacoustic emissions amplitudes also showed no correlation with prestin level.

Conclusions: The lack of age effect on prestin blood level in our sample (adults up to 65 years old) may imply that prestin is not a good candidate biomarker for long-term cochlear integrity. However, its value in acute cochlear dysfunction and in samples with wider age ranges (and more variability in cochlear damage) are still to be assessed. Finally, although serum prestin level were unaffected by time of day and the test-retest reliability between duplicates of the same sample was excellent, the ICC and thus the test-retest reliability and agreement of their value across the day was moderate. Further longitudinal investigation of the reliability of blood prestin level measurement in larger samples is warranted.

3.1. INTRODUCTION

3.1.1. Background

Prestin is a protein situated in the lateral wall of OHCs and is responsible for their electromotility (Liberman et al., 2002). In vitro or in vivo deficiencies in the prestin molecule or the prestin responsible gene have been associated with 40-60 dB elevation of hearing thresholds in rodents and humans (Liu, 2003; Matsunaga & Morimoto, 2016; Mazurek et al., 2007; Mutai et al., 2013; Tang et al., 2005; Toth et al., 2007; Xia et al., 2013). Apart from the cochlea, it was shown recently that prestin can also be detected in the blood of rodents and humans (Dogan et al., 2018; Naples et al., 2018; Parham et al., 2019; Parham & Dyhrfjeld-Johnsen, 2016; Parker et al., 2021). Although the prestin-responsible gene is known to be expressed in other than OHC tissues (brain, kidney, skeletal muscle, heart, and liver), prestin level in those tissues have not

been found to exceed circulatory blood level (Naples et al., 2018; Zhang et al., 2021). These findings have led researchers to assume that blood level reflect cochlear prestin, while changes in prestin level may be a consequence of cochlear damage (Parham, 2015). Recent evidence supports that 1. Prestin blood level changes after cochlear damage (exposure to hazardous noise, exposure to ototoxic drugs or in idiopathic sudden hearing loss, ISSHL); 2. This change follows a specific time pattern with immediate increase and then decrease to baseline or below baseline level; and 3. This change is correlated with the degree of the damage Nevertheless, it should be noted that prestin was recently also found in cardiomyocytes, where it seems to be responsible for the amplification of cardiac motor functions (Zhang et al., 2021). Better understanding of the contribution of cardiac prestin to blood prestin levels is significant for the interpretation of the aforementioned findings.

Prestin level have been reported in humans with normal hearing and after cochlear damage. Hana and Bawi (2018) evaluated and compared prestin level in the plasma of workers with and without NIHL (cases and controls, respectively). According to the methodology described in the paper, participants of both groups were exposed to the same level of chronic occupational noise, without specific information being provided. In the cases group, prestin level were 169.0 pg/ml (SD=88.4 pg/ml) at the time of diagnosis of NIHL, while these level decreased to 114.0 pg/ml (SD=99.2 pg/ml) one month after a non-specified treatment regimen (Hana & Bawi, 2018). Controls' plasma prestin level differed significantly from those of the cases at both timepoints, with a mean of 100.9 pg/ml (SD=16.7 pg/ml). Sun et al. (2019) in their study on

ISSHL reported that healthy, normal hearing controls' plasma prestin level ranged between 85.40 to 1628.25 pg/ml. ISSHL patients' level differed significantly and ranged from 295.25 to 9648.80 pg/ml before treatment. In a more recent study (Parker et al., 2021), prestin was measured in 137 samples of serum from 33 adults with normal hearing. The mean prestin level was 250.20 pg/ml (SD=28.3 pg/ml, range: 11.76-1802.13 pg/ml). Blood prestin level has previously shown to have a weak negative correlation with age ($r = -0.350$, in 72 adults, 18-82 years old) (Parker et al., 2022a), and a moderate one with average daily noise exposure level ($L_{Aeq,8h}$(dB), measured by means of noise dosimeters for 3 weeks in 30 adults, 18-24 years old, $r = -0.455$) (Parker et al., 2022b). Another study investigating cisplatin-induced ototoxicity included 52 patients with normal hearing and measured prestin level in their serum before and after chemotherapy (Jalali et al., 2021). The mean baseline prestin level was 132.0 pg/ml (SD=29.5 pg/ml), while the mean change in prestin levels relative to baseline after the administration of different dosages of 20–40 mg, 40–80 mg and > 80 mg of cisplatin was 42.9 pg/ml, 97.8 pg/ml, and 396.9 pg/ml, respectively (Jalali et al., 2021). Finally, the relationship between serum prestin and hearing threshold in adults exposed to lead (BPb) was investigated in 315 participants (Solis-Angeles et al., 2021). Participants with normal hearing that were classified in group I (BPb < 10 μg/dL) showed a median prestin level of 11060 pg/mL. Participants in group II (BPb ≥ 10 μg/dL) had a median prestin level of 150 ng/mL (Solis-Angeles et al., 2021).

The discrepancy in blood prestin level across studies in participants with cochlear damage may be a consequence of the different pathophysiology

underlying idiopathic and noise- or drug-induced hearing loss. However, the fact that this variability is also found among controls with no cochlear damage suggests that the reliability of blood prestin measurement by means of ELISA may itself be limited and depend heavily on the setting of each experiment (population, storage and handling of samples, manufacturer, or type of kit). Possibly, additional sources of variation in blood prestin level measurement is the time of day that the measurement is taken (Basinou et al., 2017). Finally, factors such as age or sex may play a role in both normal ranges and changes of blood prestin after cochlear damage. Understanding these relations is necessary before future studies focusing on prestin blood level with or without hearing damage can be interpreted with confidence.

3.1.2. Objectives

The present study addresses the following research questions for adults with normal hearing:

i. Do serum prestin level change during the day?
ii. Are serum prestin level associated with by age or sex?

Data were also analyzed to determine the ranges of serum and plasma prestin level in adults with normal hearing in our experimental setting, and on the reproducibility of serum prestin measurement by means of ELISA in different samples of the same participant (test-retest reliability). Finally, post-hoc correlations between total lifetime exposure to noise, PTA threshold, OAE amplitude, and serum prestin level, were conducted. These insights would be

useful for the design of future studies assessing serum prestin level in normal hearing and in hearing loss.

3.2. MATERIALS AND METHODS

3.2.1. Study Design

This was a two-module prospective cross-sectional study approved by the Institutional Review Board of the General Hospital of Athens “Hippokrateion” in September 2020 (37°/17-9-2020) (**Figure 3.1**). Module 1a of the study was conducted in October 2020 and focused on test-retest variability of serum prestin level and its variation during the day; the first 10 recruited participants were included in this module and provided one serum sample every 6 hours (four times in 24 hours). Including 10 participants in Module 1 of the project and thus testing 40 serum samples twice was considered efficient for reaching a conclusion with regards to the variability between two serum prestin measurements of the same sample and its variation in different samples taken in different time points during the day. Module 2 (conducted in 12.2020) focused on the effects of age and sex on blood prestin level; the 40 recruited participants provided one blood sample at a single session at noon.

The first prestin assays of the samples of Module 1a and 2 had to be repeated due to systematic errors that affected the reliability of the experiment (inadequate dilution and miscalculations of prestin level by the initial laboratory who performed the ELISA assay). It was thus decided to repeat the ELISA for these 80 samples (four serum samples collected from 10 participants and one serum sample from 40 participants) 18 months after their collection (05.2022).

To be able to confirm the reliability and relevance of the new prestin measurements in the old samples, we compared the results of the repeated ELISA with the results of ELISA assays of fresh serum samples. Eleven more participants were recruited in 2022 and provided serum samples every 4 hours (seven of the 11 had also provided blood in 2020). In 2022 (Module 1b), we decided to collect blood at additional time points compared to 2020 since, when determining diurnal changes of a protein, four time points can miss the peak expression and it is thus preferrable to have six time points (minimum for assessing circadian effects) (Meltser et al., 2014; Park et al., 2016). The new blood samples were analyzed within the appropriate timeframe, within two months from their collection (03-05.2022), in a new laboratory (Zoology-Marine Biology Unit, Department of Biology, National and Kapodistrian University of Athens) where the assays of the old samples from Module 1a (2020) were also conducted. I was trained and performed the assays myself with the help and supervision of laboratory personnel (Professor Skarlatos G. Dedos). Prestin values of Module 1a and 1b blood samples were compared and results were evaluated to determine if the Module 1a samples were biologically intact and should be taken into account in our analyses.

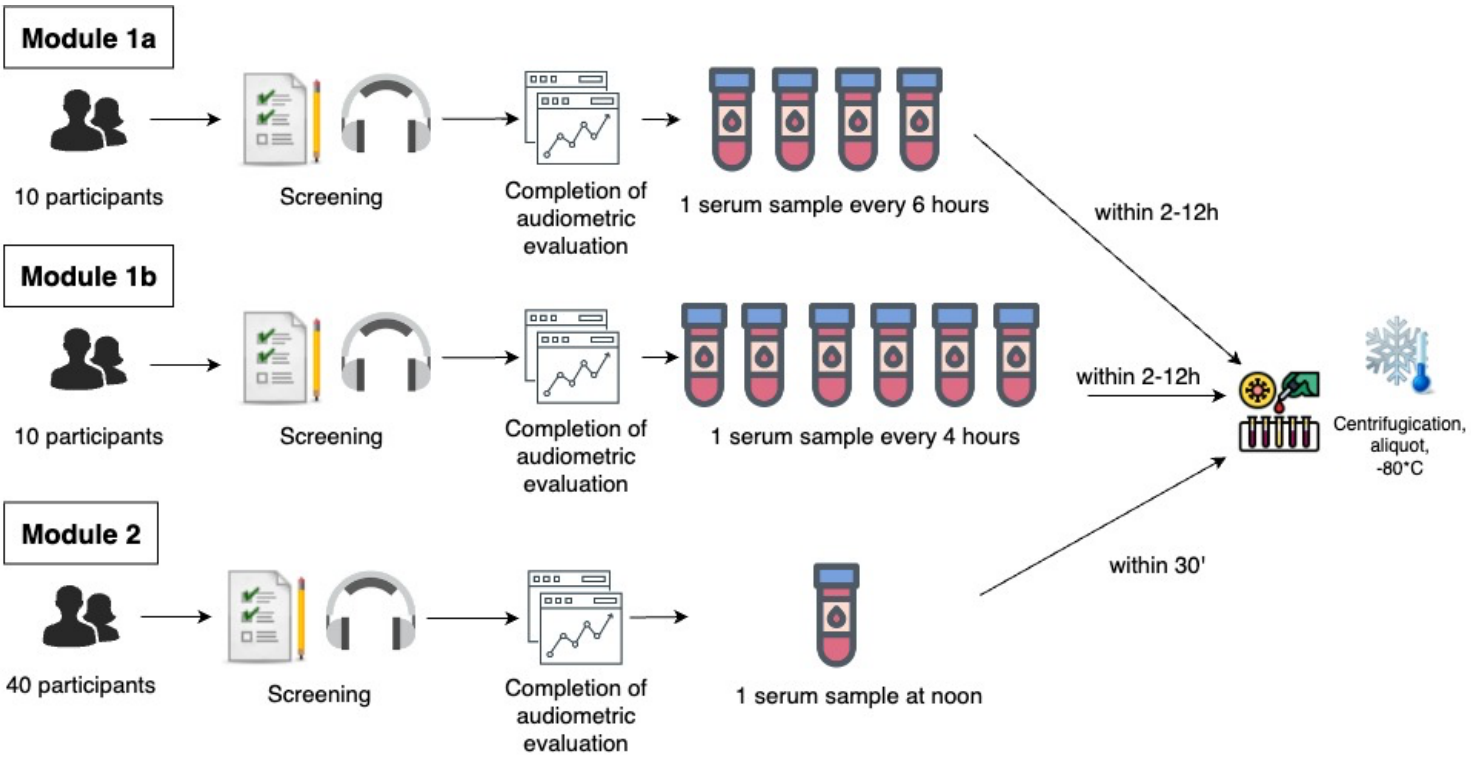


Figure 3.1. The two Modules of the study. Module 1a included 10 participants from whom a serum sample was collected every 6 hours. The morning sample was taken between 6.30 and 8.00 am, the noon–early afternoon sample was taken between 12.30 and 14.00 pm, the afternoon-evening sample was taken between 18.30 and 20.00 pm and the night sample was taken between 0.30 and 2.00 am. Module 1b includes 11 additional participants recruited in March 2022. Module 2 included 40 participants from whom one serum and one plasma sample were collected once per day (noon – early afternoon).

3.2.2. Setting

All study procedures were performed by means of dedicated clinical research equipment located at the Oto-neurology laboratory of the 1st University ENT Department, at the General Hospital of Athens “Hippokrateion”, in Athens, Greece. All procedures, but for the serum sampling during Module 1, were

completed in a single session lasting 30'. Serum sampling during Module 1 was completed in 24 hours. The study was performed from October 2020 to June 2022.

3.2.3. Participants

Participants were selected according to the following criteria; males and females older than 18 years old with no hearing complaints (no self-reported hearing loss, loss of speech perception, tinnitus or other hearing disorder), no other ear pathology (no abnormality in otoscopy or tympanometry, no air-bone gap in audiogram), no hearing loss history and symmetrical PTA thresholds (0.5-8 kHz) within normal limits (air conduction thresholds ≤25 dB HL for 0.25 – 8 kHz). PTA was performed according to the British Society of Audiology (2018) guidelines. Candidates were excluded according to the following criteria: participants with hearing complaints, hearing loss history, abnormal tympanometry or PTA, exposure to hazardous noise levels (80 dBA) during the last 72 hours (Melnick, 1991), exposure to ototoxic agents, such as cisplatin, aminoglycosides, loop diuretics, antimalarials, and non-steroidal anti-inflammatory drugs, acetaminophen, or aspirin in high doses, during the last 12 months.

3.2.4. Recruitment

Participants were recruited from the General Hospital of Athens "Hippokrateion" workforce. All participants were volunteers that received no compensation for their participation to the study. All participants had to read and understand the

information sheet and sign the study informed consent form. All participants were aware of the reasons why part of the study had to be repeated and why Module 1b had to be conducted in 2022. All participants were allowed to withdraw at any time.

3.2.5. Variables

During Module 1a, serum prestin level were assayed in duplicates for each of the four serum samples. The averages of these two measurements of the same sample were designated “Morning”, “Noon”, “Afternoon,” and “Night” and were used for the analyses (**Table 3.1**). In Module 1b, serum samples were collected every 4 hours [“Serum 1-6”, where 1 corresponds to the morning sample (8 a.m.) and 6 to early morning sample (4 a.m.)]. Analysis of Module 1a,b measurements explored the serum prestin level distribution, its variation during the day, and its test-retest reliability. During Module 2, serum prestin level were measured only once, at noon (“Serum”). Analysis of Phase 2 measurements explored serum prestin level distributions, and the relation of serum prestin level with age and sex.

PTA thresholds per ear are reported as PTA5, which is the pure tone average of 0.5 – 4 kHz, PTA7, which is the average for 0.5 – 8 kHz, and extended high frequency pure tone average (EHF-PTA) which includes frequencies over 8 kHz (9, 10, 11.2, 12.5, 14, 16 kHz). Amplitudes of transient-evoked and distortion-product otoacoustic emissions (TEOAEs and DPOAEs) were reported per frequency (0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10 kHz). Lifetime noise exposure

units were estimated according to the noise exposure structured interview (NESI version 1; Guest et al., 2018). One NESI unit is equivalent to one working year (2080 hrs) of exposure to 90 dBA. These variables were included in the post-hoc exploration of possible factors affecting serum prestin level.

Table 3.2 Variables of interest in Modules 1 and 2. Additionally, amplitudes of transient-evoked and distortion product otoacoustic emissions (TEOAEs and DPOAEs) were reported per frequency (0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10 kHz), per ear side. Lifetime noise exposure was estimated according to the Noise Exposure Structured Interview (NESI).

Study Module	Description	Variable	Unit
Module 1a	Serum prestin level were measured four times per day (once every 6 hours).	Morning	pg/ml
		Noon	
		Afternoon	
		Night	
Module 1b	Serum prestin level were measured 6 times per day (once every 4 hours)	Serum 1-6	pg/ml
Module 2	Serum prestin level were measured only once, at noon	Serum	pg/ml
All Modules	Pure tone average threshold of 0.5 – 4 kHz	PTA5	dB HL
	Pure tone average threshold of 0.5 – 8 kHz	PTA7	dB HL
	High-frequency average threshold (>8kHz)	EHF-PTA	dB HL

3.2.6. Data sources

Medical history

All participants were interviewed for basic demographics, medical history, and otological history.

Retrospective noise exposure history

All participants were asked to recall the level and duration of lifetime noise exposures using NESI (Guest et al., 2018).

Otomicroscopy

Visual assessment of the ear canal and tympanic membrane was conducted to exclude outer and middle ear conditions that could potentially interfere with the audiometric results. Normal otoscopic outcomes were defined as the absence of apparent abnormalities of the pina, external auditory canal, and tympanic membrane.

Tympanometry and middle ear muscle reflex (MEMR)

Ear drum mobility (compliance), middle ear volume, and *a*coustic reflex amplitude and latency were measured*,* with a GSI TympStar Version 2 tympanometer, to exclude middle ear pathology that could interfere with the audiometric results. Tympanometry results were considered within the normal range when middle ear pressure values ranged from -140 to +40 daPa, peak compensated static acoustic admittance fell between 0.3 and 1.8 ml, and acoustic equivalent volume (Vea) measured between 0.8 and 2.1 cm, as defined by Le Prell et al. (2012).

PTA

Thresholds from 250 Hz to 16 kHz were tested, using the British Society of Audiology procedure . Both air and bone conduction were measured. The whole procedure was conducted with an Amplaid A321 audiometer (EN 60645-1, ANSI S3.6) using TDH39 (PTA) and HDA300 headphones [extended high-frequency PTA (EHF-PTA)], while for bone conduction measurements B81 was

used. Bone conduction was evaluated to exclude any conductive hearing disorder that could confound the audiometric results. Frequencies of 12.5 kHz, 14 kHz and 16 kHz were tested for thresholds up to 70 dB HL, 40 dB HL, and 40 dB HL, respectively. If a participant did not respond to any pure tone above these thresholds, a “no response” (“99”) would be noted for this participant in that frequency.

OAEs

There were no OAE amplitude criteria for study enrollment; however, TEOAE and DP amplitudes were measured. OAEs were collected using the Interacoustics Titan Abris 440 device. Maximum residual noise was set to 30 dB SPL. TEOAEs were recorded in the range 1-5 kHz. DPOAEs were tested between 0.5 and 10 kHz, at an f2: f1 ratio of 1.22, with levels f1: 65 dB SPL; f2: 55 dB SPL.

Blood preparation and blood prestin measurement

In Modules 1 and 2, all blood samples were collected and handled by the first author, who is a medical doctor with training and considerable experience in drawing blood from patients. Blood was drawn once and was directly collected using SST (serum) tube. The samples were then centrifuged for 15 minutes at 1000 g within 2 hours of collection. Due to practical reasons, and since, according to the ELISA kit manual by MyBioSource, samples can remain to clot overnight at 2-8°C before centrifugation, we decided to store seven serum samples overnight at 4°C: three afternoon samples for 11-12 hours, and four night samples for 5-6 hours (exclusively samples collected in 2020). The resulting supernatant was stored in a –80°C freezer until time of assay. ELISA

was performed at the Department of Biology of the National Kapodistrian University of Athens (NKUA), and the samples were stored in ice during transport. Prestin level were measured using the Human Prestin (SLC26A5) MBS282125 ELISA Kit (MyBioSource, San Diego, California) as described in the manufacturer's instruction manual. The selected kit has a detection range of 15.6 pg/mL – 1000 pg/ml, high sensitivity, and excellent specificity. According to the kit manual, duplication of all standards and specimens, although not required, is recommended. In our study, all samples were run in duplicates through ELISA Kits of the same LOT number (n=5; one kit for Module 1a, two kits for Module 1b samples, two kits for Module 2 samples). All samples were processed in the same plate included in the ELISA kit. The optical density was measured at 450 nm and 540 nm FlexStation 3 Multi-Mode Microplate Reader, in ELISA mode.

3.2.7. Statistical analyses

Comparison between Module 1a and Module 1b samples

Statistical analyses were performed using R (RStudio, 2022.02.03). Initially, mean prestin level in blood samples collected in Module 1a and 1b were compared in order to determine if the Module 1a results were biologically plausible. Subsequently, and based on the results of this process, we proceeded with the originally designed statistical analysis for our paradigm.

Variation of blood prestin level during the day

To test for differences in serum prestin level during the day a two-level linear mixed effect model was used [repeated measurements of prestin level at four and at six times points (fixed factor) within the same subject (random factor)] for the Module 1a and Module 1b samples, respectively.

Correlation of prestin level with age, sex, PTA, and EHF-PTA average thresholds, and OAE amplitudes

For these the analyses, we combined the samples of the 54 unique participants of this study with a subset of baseline samples of seven unique participants of another ongoing study in our lab (five females, age range: 20 – 38 years) to evaluate prestin level before and after exposure to music. Their baseline serum prestin level value was considered adequate to be included in the present analyses since the recruitment criteria (adults with normal hearing) and ELISA methods, timeline and materials are exactly the same in this experiment.

Spearman correlation was calculated to evaluate the relation of serum level with age and sex. Linear regression was used to predict serum prestin values from age, year of blood collection, sex, PTA and EHF-PTA thresholds per frequency and average thresholds (PTA5, PTA7, EHF-PTA), OAE amplitudes, and total lifetime noise exposure in log units.

Intra-class correlation

Test-retest reliability for serum prestin measurement by means of ELISA was assessed by calculation of intra-class correlation coefficients (ICCs; McGraw & Wong, 1996). The ICC was chosen since it would reflect not only the degree of

correlation among our measurements but the level of their agreement, as well. According to our study's design, ICC estimates and their 95% confident intervals (CI) were calculated based on the xfollowing models: 1) ELISA's test-retest reliability for the same sample of serum was based on running each specimen twice and was evaluated by conducting one-way, absolute-agreement, random-effects model based on average rating [ICC (1,1)] and 2) in the case of serum prestin repeated measurements (four and six in the case of 2020 and 2022 samples respectively), reliability was evaluated by means of single-rating , absolute-agreement, two-way random-effects model [ICC(2,1)] (Koo & Li, 2016). Using a random effect model allows the generalization of our results to any measurement/raters that possess the same characteristics. Type selection depends on how the measurement protocol is assumed to be conducted in the actual application. Although, more conservative, and expected to give lower ICC values than the "average" ("mean of k raters") type, the type "single" was used in our analysis, since, at a future clinical or research context, we assume the use of a single measurement/rater. ICC values were interpreted as follows: <0.5, poor reliability; 0.50-0.75, moderate reliability; 0.75-0.90, good reliability; and >0.90, excellent reliability (Koo & Li, 2016).

3.3. RESULTS

3.3.1. Participants

In Modules 1a and 2, 56 candidates were recruited and screened for their eligibility. Candidates that fulfilled the inclusion criteria were then allocated to the two study modules according to chronological order. The demographic

characteristics of the 50 included participants are reported in Table 2. Their age distribution is provided in Supplementary Material 1. Their log-transformed lifetime noise exposure scores are reported in **Figure 3.2**. In Module 1b (2022), 11 participants were recruited. Seven of them were also part of the Module 1a (2020) sample. Their demographics are reported in **Table 3.2**.

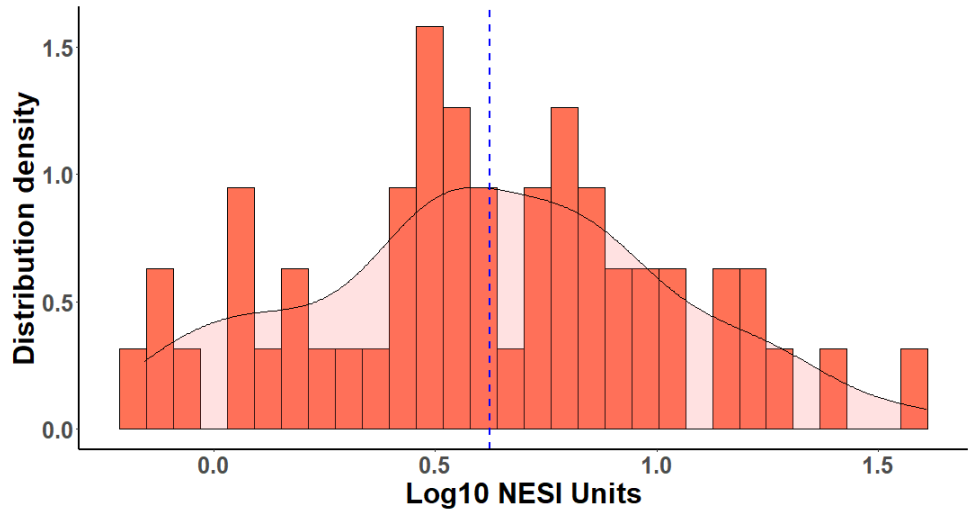


Figure 3.2. Log-transformed life-time noise exposure units of the 50 participants included in the study in 2020 (Modules 1a and 2), according to the noise exposure structured interview (NESI).

Table 3.2. Participants' Basic Demographics.

	Module 1a N = 10	Module 1b N = 11	Module 2 N = 40	Module 1a and 2 N = 50
Age (yrs)				
Range	26 – 36	26 – 54	25 - 65	25 - 65
Median (IQR)	30 (4,25)	30 (4)	43 (18,25)	38,5 (21,75)
Sex N (%)				
Female	7 (70%)	6 (54,5%)	26 (65%)	33 (66%)
Male	3 (30%)	5 (45,5%)	14 (35%)	17 (34%)
Education N (%)				
Secondary	10 (100%)	11 (100%)	8 (20%)	8 (16%)
Post-secondary non-tertiary education			12 (30%)	12 (24%)
Tertiary education (Bachelor's, Master's or Doctoral level)			20 (50%)	30 (60%)
Smoking habits N (%)				
Smokers	1 (10%)	0 (0%)	3 (7,5%)	4 (8%)
Non-smokers	9 (90%)	11 (100%)	37 (92,5%)	46 (92%)

*Participants in Module 1a and Module 2

3.3.2. PTA and OAEs

The PTA profiles of the Module 1a and 2 participants are presented in **Figures 3.3a and b.** PTA profiles per different age groups for the same participants are presented in Supplementary Material 3.2-3.5. DP amplitudes across different age groups are presented in Supplementary Material 6. PTA and OAE profiles of the Module 1b sample (N=11) are presented in Figure 3.4 and Supplementary Material 3.7 and 3.8.

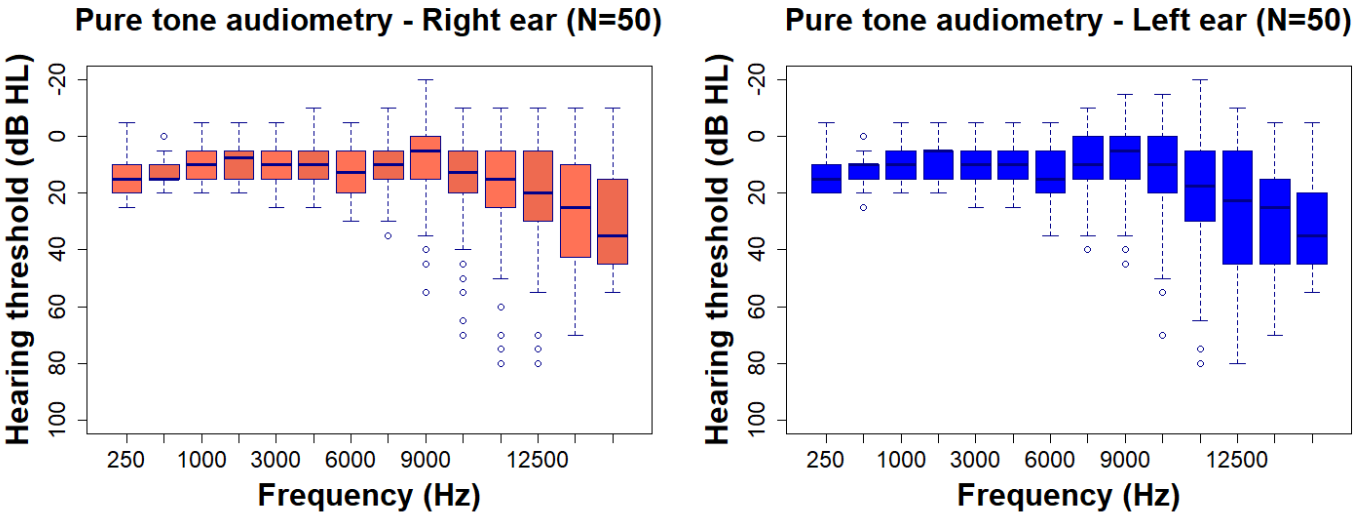


Figure 3.3. Pure tone threshold boxplots of the Module 1a and 2 samples (2020, N=50) of the right (A) and left (B) ear. Interquartile ranges are larger in high frequencies (<9 kHz). At 12.5kHz, one participant gave no response (right ear), at 14 kHz, seven participants gave no response (right and left ear), at 16 kHz, 17 participants gave no response (right ear and left ear).

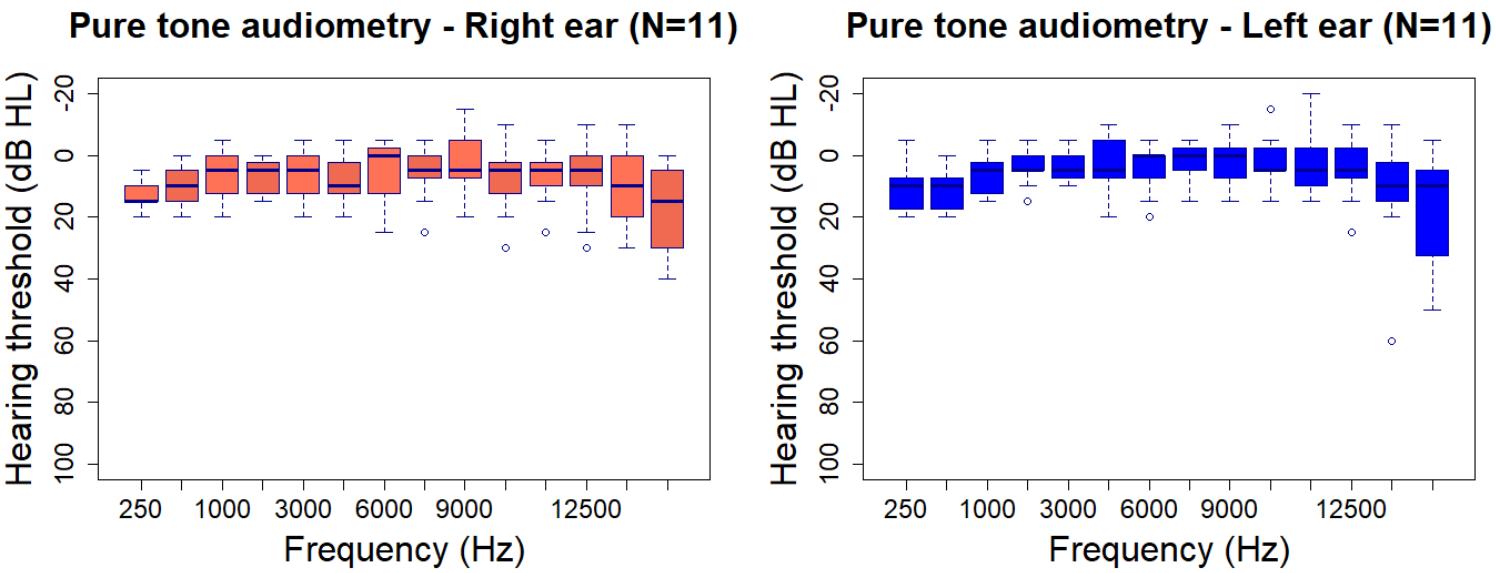


Figure 3.4. Pure tone thresholds boxplots for the eleven participants entering the study in 2022 (Module 1b).

3.3.3. Comparison between samples collected in Module 1a and 1b.

Median serum prestin was 119.21 pg/ml (Phase 1, n = 10, IQR = 86.39 pg/ml) in Module 1a, and 127.94 pg/ml in Module 1b (n = 11, IQR = 84.56 pg/ml) **(Figure 3.5A)**. Serum prestin was not significantly different based on the year of sampling (p = 0.39). For the seven participants who provided blood both in Module 1a (2020) and 1b (2022), median prestin serum was 105.84 pg/ml (IQR = 70.1 pg/ml) in 2020 and 159.0 pg/ml (IQR = 95.1 pg/ml) in 2022 **(Figure 3.5B)**. However, a paired Wilcoxon test revealed no statistically significant difference between these values (V = 9, p = 0.47). Moreover, in all 2020 and 2022 assays, the ICC of sample duplicates was excellent (ICC = 0.95, p = 4.80e-45, 95% CI 0.93-0.97). This finding is compatible to the intra-assay precision reported by MyBioSource (CV < 8%). We concluded that our results do not differ significantly between 2020 and 2022 and we decided to proceed with the statistical analysis as originally designed, including the 2020 samples.

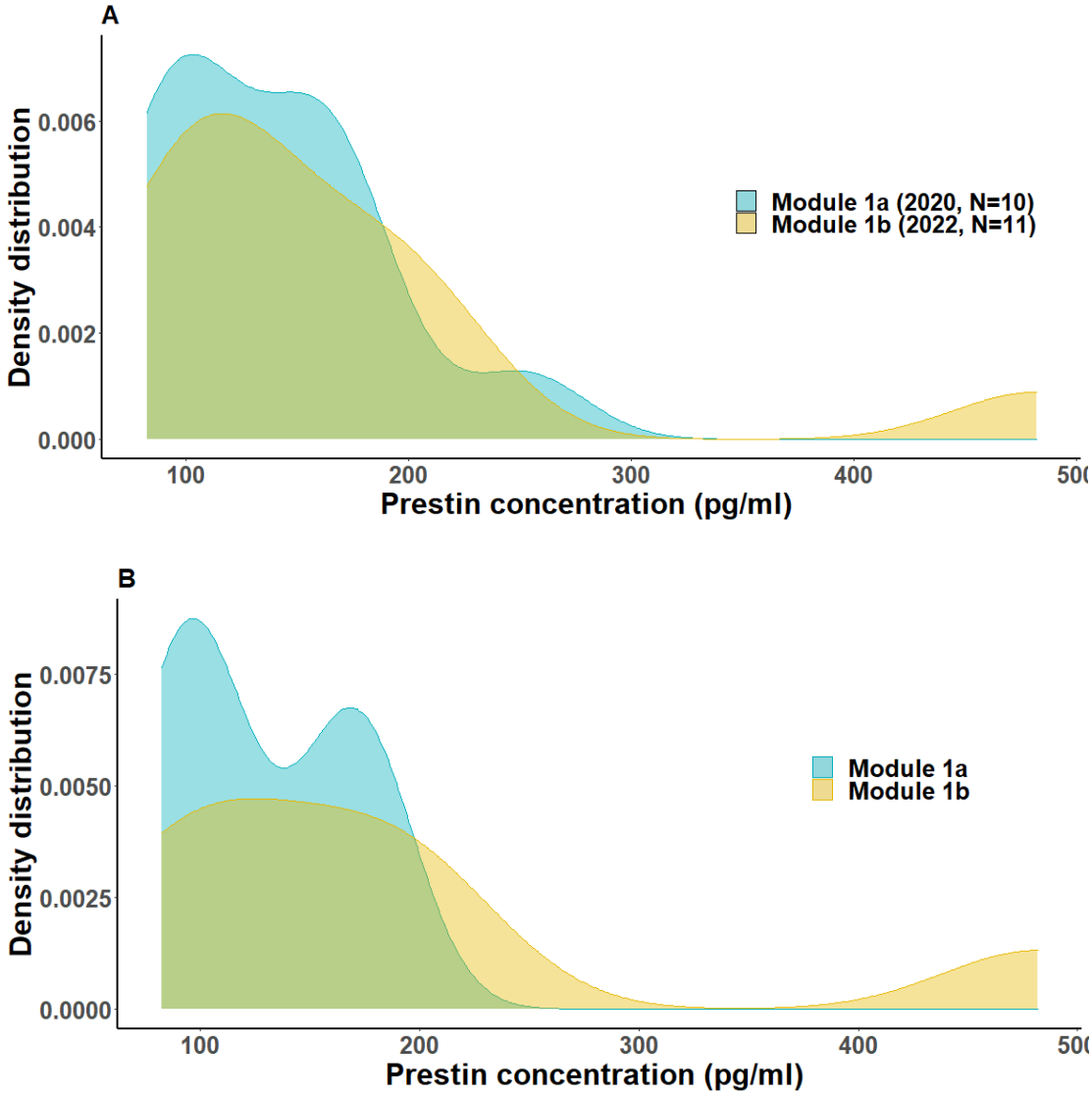


Figure 3.5. Serum prestin level distributions for: A. Participants of Module 1a (N=10, 2020) and new participants of Module 1b (N=11, 2022); and B. Participants that participated both in Modules 1a and 1b (N=7).

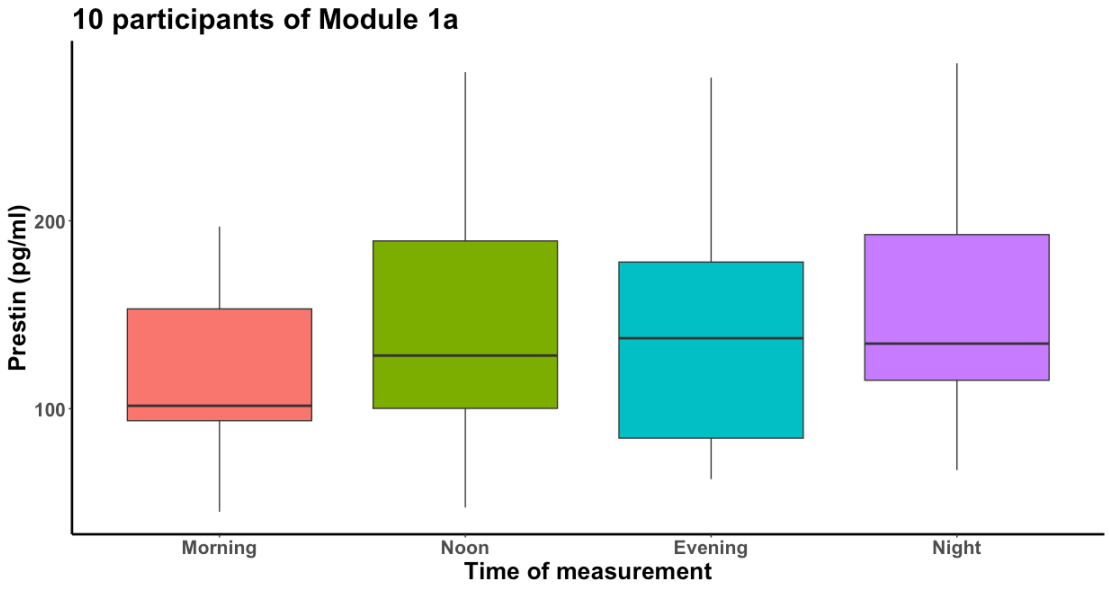
3.3.4. Serum prestin level: variation with time of day, and test-retest reliability

The distribution of serum prestin level at the different measurement points is presented in **Figures 3.6A and 3.6B** shows the mean and variability at each measurement point, in Module 1a and Module 1b, respectively. The prestin

level range in the serum samples was 45.12 - 362.42 pg/ml and 41.68 - 806.60 pg/ml, in Module 1a and 1b, respectively.

No statistically significant difference was found in mean serum prestin level across the day in our sample either in Module 1a (N=10) or in Module 1b (N=11). It should be noted that, although one morning sample was a high outlier (prestin level = 806.60 pg/ml), the range of morning serum prestin level was narrower compared to the rest of the day, and the distribution more normal. In a post-hoc analysis, the ICC for all four measurements across time of day, and thus the reliability and agreement of "Morning", "Noon", "Afternoon", and "Night" blood prestin level level, was found to be low to moderate (ICC = 0.57, p = 4.73e-06, CI = 0.26 - 0.84, k = 4, type = single). The ICC for the 2022 samples was also found to be moderate (ICC=0.60, p = 4.60e-16, CI = 0.42 - 0.79, k = 7, type = single).

A.



B.

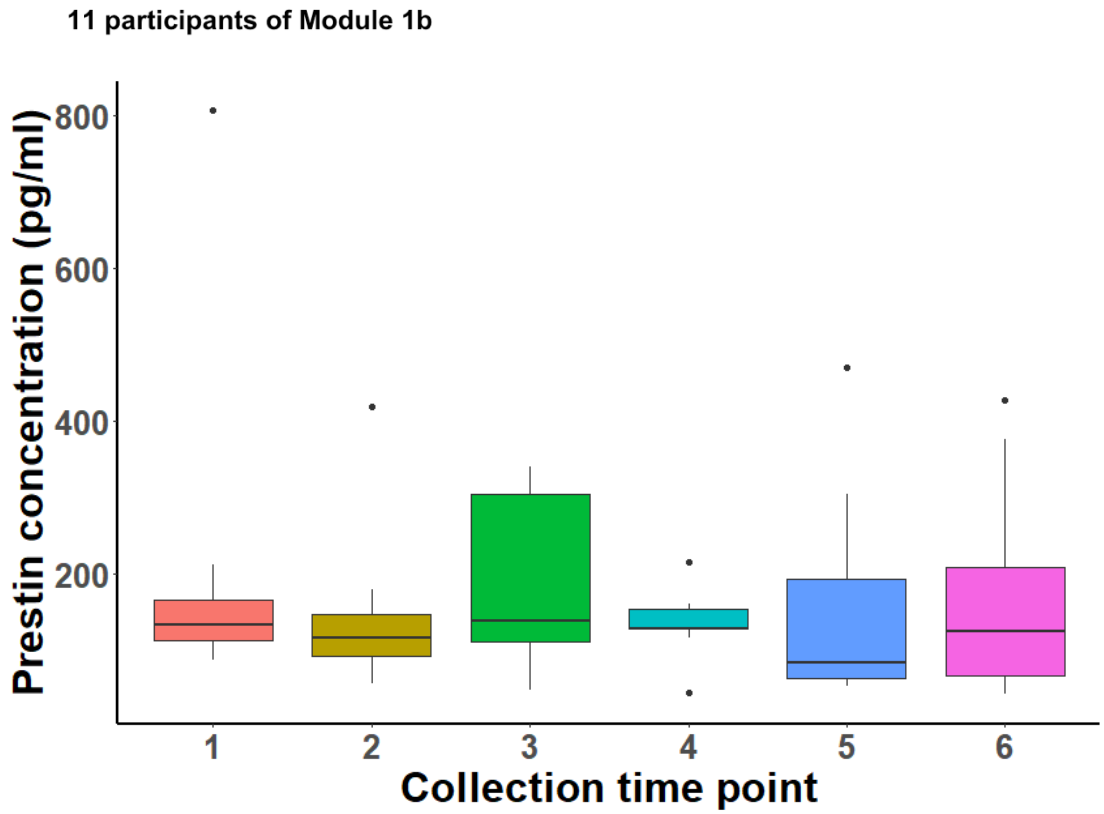


Figure 3.6. Distribution of serum prestin level across the day for participants in Module 1a (A) and in Module 1b (B).

3.3.5. Serum prestin level: relation to age and sex

Five samples from the Module 2 were found significantly hemolyzed, so their assays were not considered reliable, and were not included in the analysis (Ni et al., 2021). As a result, a total of 56 serum samples were analyzed for their relation to age and sex. Distribution of ages of the 56 participants merged sample included in the analysis is provided in **Figure 3.7**. The distribution of their serum prestin level per sex is provided in **Figure 3.8** respectively. In males (N = 20), serum prestin level ranged from 34.42 to 309.84 pg/ml (median = 149.01 pg/ml, IQR = 71,78 pg/ml). In females (N = 36), the range of serum prestin level was 37.65 – 479.00 pg/ml (median = 137.60 pg/ml, IQR = 56.94

pg/ml). No statistically significant difference between males' and females' serum prestin was found (**Figure 3.8**).

No significant correlation was found between serum prestin and age [$r_{(54)}$ = -0.04, S = 30461, p = 0.76 (Supplementary Material 9)].

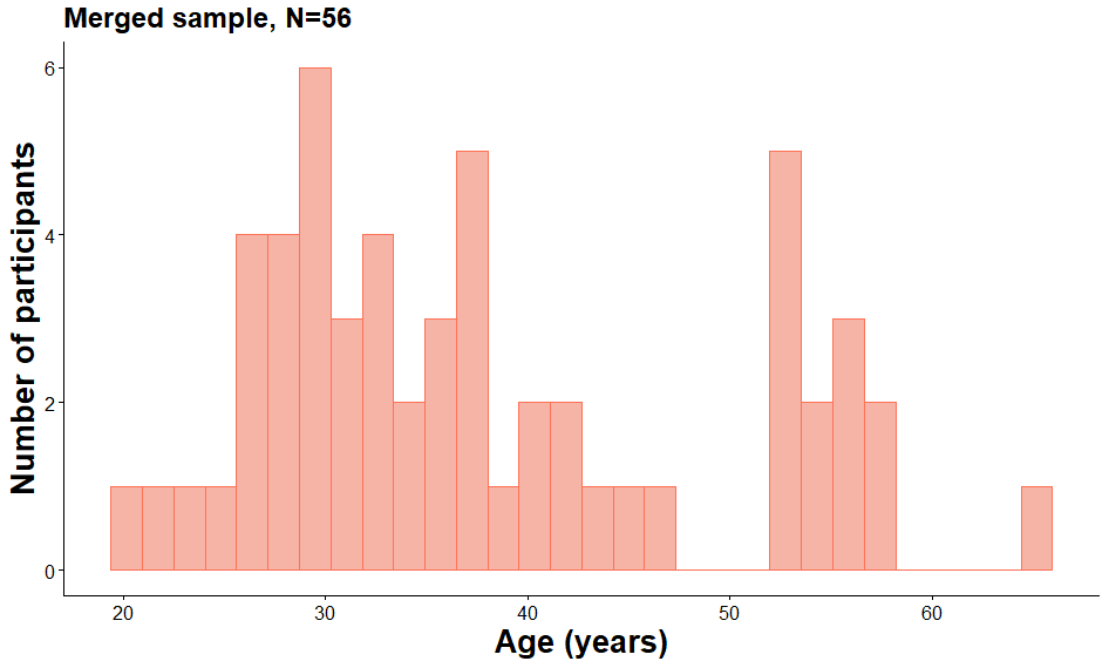


Figure 3.7. Age distribution for the combined sample of 56 unique participants in 2020 and 2022.

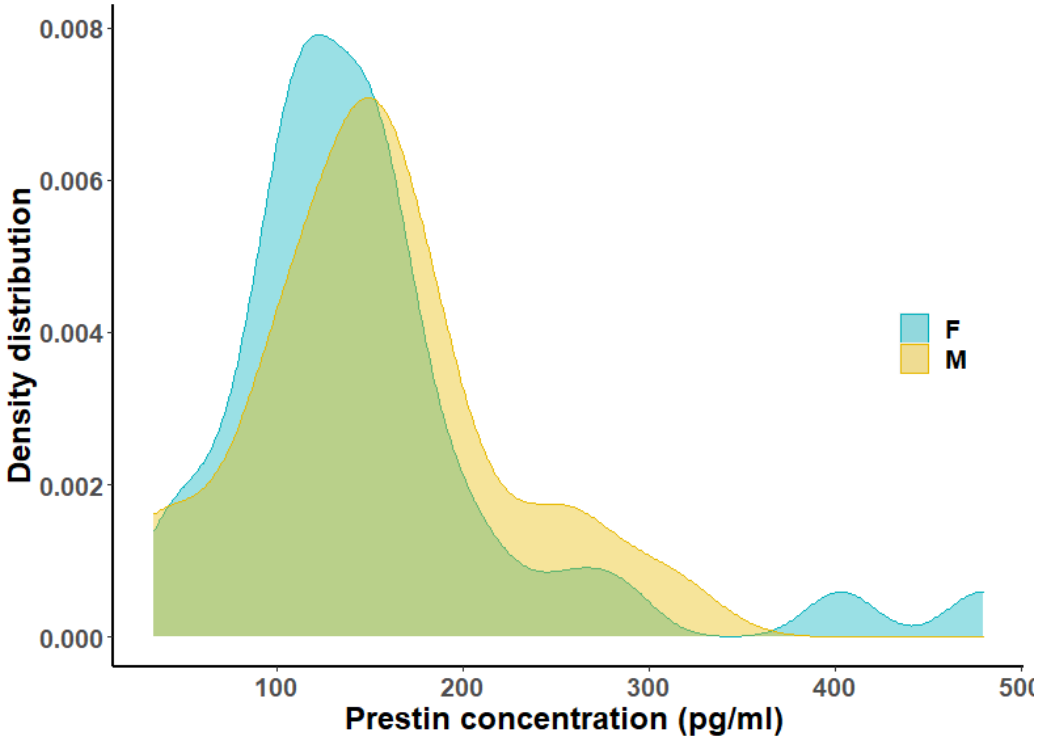


Figure 3.8. Serum prestin level for the merged sample of 56 participants per sex (R^2 = -0.01, $F(1,54) = 0.31$, $p = 0.581$).

3.3.6. Post-hoc analyses: relation of serum prestin level to audiometric thresholds, OAE amplitudes, and lifetime noise exposure

These analyses were conducted using the merged sample of 56 participants. No significant correlation was observed between PTA5, PTA7, or EHF-PTA of the left or right ear and serum prestin level. Similarly, OAE amplitudes of the left and right ear were not significantly correlated to serum prestin level at any of the tested frequencies. Although blood prestin is not ear specific, in our analysis both PTA and OAEs were analyzed separately per ear side, since averaging them for the two ear sides does not have a clinical or physiological basis. No statistically significant correlation was found between serum prestin and NESI log units ($r_{(54)} = 0.07$, $p = 0.62$).

3.4. DISCUSSION

This is one of the first observational studies designed to explore the effects of time of day, age, and sex on prestin blood level in adults with normal hearing. The data analyses showed that serum prestin level did not differ significantly across the day, and that there was no significant effect of age or sex on this level.

3.4.1. Serum prestin level: variation with time of day, and test-retest reliability

Before establishing a blood prestin measurement methodology for research or clinical purposes, its test-retest reliability under the same experimental conditions, along with the factors that may affect it, should be explored. Although, in a recent study by Parker et al. (2021), reliability of prestin measurement by means of ELISA was estimated using a “global” ICC across five sessions (“mean of five raters”) and was found to be excellent (ICC = 0.98), the majority of previous studies have focused on single measurements of serum or plasma prestin, without testing for reproducibility (Davies, 1992; Hana & Bawi, 2018; Sun et al., 2019). Hence, the reliability of a single prestin measurement was for the first time investigated in our study.

Moreover, animal models have shown that the auditory system is involved in circadian entrainment (Goel, 2005), that there is a diurnal sensitivity to noise and ototoxic agents (Halberg et al., 1958; Meltser et al., 2014), and that dysregulation of the circadian clock of male CBA/CaJ mice by constant light

was related to greater loss of OHCs, reduction of synaptic ribbons, and greater PTS after high-intensity noise exposure (Yang et al., 2020). Furthermore, OAEs have been hypothesized to be influenced by the time of day (Bell, 1992; Cacace et al., 1996; Haggerty et al., 1993). Based on these data that show some circadian effect on the auditory system, and on the hypothesis that intracochlear and blood prestin level may be related to the OHC function and number, we hypothesized that blood prestin level may also exhibit diurnal variation. Our sample consisted of healthy young and middle-aged adults with no known reasons for dysregulation of their circadian cycles according to their self-reported medical and social history, so assessing additional parameters related to circadian rhythms (oral temperature resting-pulse-rate, actigraphy) was considered out of the scope of our study and thus not included in our design and methods.

In contrast to the above hypothesis, repeated measures analysis showed that serum prestin variation during the day (and thus its diurnal variation) was non-significant. Moreover, the ICC of sample duplicates was excellent in all assays. The results suggest that a single serum prestin sample can be taken at any time of the day and be analyzed via ELISA, without biasing the results. However, when the ICC was computed to across the four (2020) or six (2022) measurements from the same participant for one day, there was only a moderate absolute agreement. It should be noted that a moderate ICC could relate to the lack of variability among the sampled participants or their small number (Koo & Li, 2016; Lee et al., 2012). The moderate reliability we observed may thus reflect the small size of our sample or its homogeneity with regards

to hearing phenotypes (exclusively adults with no evidence of recent cochlear damage). If the measurement error is large compared to the effect of trauma, however, then this would severely limit the application of prestin as a biomarker. Further studies will be needed to assess reliability in a more heterogenous in respect to hearing sensitivity population.

3.4.2. Relation of serum prestin level to age and sex

Although in a recent observational study, blood prestin showed a weak negative correlation with age ((r = −0.350, in 72 adults, 18-82 years old) (Parker et al., 2022a), in our sample of adults up to 65 years old, no statistically significant relationship between age and serum prestin level was found. A protein that does not reflect the age-related decline of cochlear function may show insensitivity to long-term cochlear damage and thus be of limited use in the evaluation and monitoring of permanent hearing loss. However, prestin may be a useful biomarker of acute trauma, as suggested by rodent studies (Dogan et al., 2018; Parham & Dyhrfjeld-Johnsen, 2016) .

In a recent human study (F:M = 23:11), no difference in serum prestin level between males and females with normal hearing was observed ($t_{(148)}$ = –0.08, p = 0.96; Parker et al., 2021). In our sample (F:M = 36:20), there was no sex difference in serum prestin level, implying that the normal level of the protein in the blood is similar for the two sexes.

3.4.3. Relation of prestin level to audiometric thresholds, OAE amplitudes, and lifetime noise exposure

Parham and Dyhrfjeld-Johnsen (2016) observed that serum prestin level was significantly lower in 21 noise exposed rats in comparison to the six control ones, and that there was a statistically significant moderate negative linear relationship between serum prestin level and the change in DPOAE level due to exposure (Parham & Dyhrfjeld-Johnsen, 2016). Parker et al. (2021) assessed the relation between TEOAEs and serum prestin level for five different sessions and their average (Parker et al., 2021). In each separate session TEOAEs magnitudes and serum prestin level had a statistically non-significant positive weak to moderate correlation, while when both TEOAEs magnitudes and serum prestin level were averaged for each participant across the five sessions and then correlated, the relation was statistically significant and stronger than for any intra-session pairwise comparison, but still moderate and with no clarification if it remained statistically significant after correction for multiple comparisons (Parker et al., 2021). In our study, post-hoc analyses revealed no correlation of serum level with the OAE amplitudes of our participants. This finding cannot be compared with the one in the rodent model by Parham and Dyhrfjeld-Johnsen (2016), since in our human study to our knowledge no serum prestin measurement was taken after acute trauma (within exclusion criteria). Furthermore, our results may be a consequence of the homogeneity of our sample, which consists of 50 adults with age-normal hearing. Future studies involving larger samples with wider age range and varied hearing phenotypes are warranted to resolve this issue.

Similarly, no correlation between serum prestin level and PTA thresholds was observed. As discussed previously, this latter finding may relate to the fact that our sample consisted exclusively of adults with normal hearing. Results may have differed if correlation between prestin blood level and PTA thresholds were sought in adults with hearing disorders.

Based on the hypothesis that, apart from acute or brief noise exposure, overall lifetime noise exposure may also have an effect on OHC number (Wu et al., 2020), an effort has been made to accurately collect this information from our sample through their lifetime noise exposure estimated using the NESI (Guest et al., 2018). The NESI is designed to minimize this risk of recall bias through its structured reporting procedures (Guest et al., 2018). In our study, serum prestin level were not significantly correlated with the total lifetime noise exposure as measured by NESI. Our finding does not agree with the results of Parker et al. (2022) which showed that serum prestin level of 30 adults with normal hearing had a statistically significant moderate negative relation with their noise exposure level over a 3-week period (Parker et al., 2022b). Further investigation on the effect of chronic and acute exposure to noise of various intensity is warranted.

3.4.4. Limitations

There are several limitations in our study that should be considered before generalizing the results. First of all, it should be underlined that ELISA is a method that is heavily dependent on several factors, such as referencing, type

of antibodies, and target protein epitope (Aydin, 2015; Imtiaz & Yunus, 2019; Saracevic et al., 2019). Thus, we cannot claim that our results reflect “normal prestin ranges” that would apply to different experimental settings. In the present study, to minimize the possibility of systematic error, all blood collection, storage, and handling procedures were held according to MyBioSource ELISA kit manual, with no exceptions, and results were consistent for all our participants. Samples of Module 1b (2022) underwent only one freeze-thaw cycle, while the samples of 2020 were thawed three times. Different dilution factors have been tested prior to the assays of samples of interest (1:50, 1:10, 1:5, 1:4, 1:2, 1:1). Moreover, our sample consisted of adults with an age range of 25 to 65 years old. Participants outside this age range may present different results. Similarly, we included only candidates with normal hearing and with no hearing disorders. The evaluation of reliability and variation during the day has been conducted only for serum samples and only in a small number of participants. Finally, due to an initial systematic error we needed to repeat the 2020 sample assays in 2022, 18 months after the initial blood collection. However, the comparison of the serum prestin level for the samples of 2020 with the ones for the 2022 samples showed no statistically significant differences, thus allowing us to use these measurements in our analyses. This may also have an implication for the use of older blood samples for such serum prestin measurements.

3.4.5. Interpretation and generalizability

To conclude, our study is the first to assess the reliability of a single serum prestin level measurement. Moreover, blood prestin measurement by means of ELISA is evaluated for its extent of reproducibility and its correlation with various factors. Our findings suggest that serum prestin measurement by means of ELISA presents clinically and statistically non-significant differences during the day, between males and females, and between participants of different age, up to 65 years old. Serum prestin measurements of different samples of the same participant showed moderate reliability. Before understanding the value of prestin as hearing biomarker, improvement of the experimental settings and longitudinal studies with regards to prestin measurement in serum, and in populations with various hearing phenotypes (normal hearing, hearing loss or other hearing disorders) is warranted.

Note: During Module 1b and 2, we also collected blood using EDTA (plasma) tubes. The samples were then centrifuged for 15 minutes at 1000 g within 30 minutes, and then stored at -80°C until time of assay, similarly to serum samples. No prestin was detected in any of the plasma samples collected (1:50, 1:10, 1:5, 1:4, 1:2, 1:1 dilution was tested in three different plates; in two of them, both serum and plasma samples were assayed and prestin was detected only in the serum ones). Hence, plasma samples were not further analyzed.

Chapter IV

Exposure to noise or music in clinical trials: A scoping review on ethical and methodological considerations

Objective: To summarize methodological and ethical aspects of previously published experimental paradigms using high-level noise or music exposures.

Methods: This is a scoping review, conducted according to PRISMA-Scr guidelines. Four major databases (Medline, Central, Web of Science and Scopus) and two trials registries (Clinicaltrials.gov and EU Clinical Trials) were searched by two reviewers independently. Extracted items were author and year of publication, study design and purpose, population, setting timeline and material, selected battery test, and effect of noise / music on participants' hearing.

Results: Thirty-four studies were included in the review. All studies involved young and middle-aged adults with normal hearing. White or narrow band noise ($NBN_{0.5\text{-}4kHz}$) was used in NIHL studies, and pop music in MIHL ones. More prominent temporary threshold shifts (TTSs) and reduction in distortion product otoacoustic emissions (DPOAEs) were found at 1-8 kHz, with maximum average TTS ~21.5 dB at 4 kHz after NBN and ~11.5 dB at 6 kHz after music exposure. In all studies testing for permanent hearing loss, participants recovered their hearing, with the exception of one participant in a MIHL study.

Conclusions: Experimental exposure paradigms can produce temporary changes to hearing without measurable long-term consequences. Methodological and ethical aspects identified in this review should be considered for the development of future paradigms.

4.1. Introduction

4.1.1. Noise and music induced hearing loss.

The leading cause of preventable hearing loss is overexposure to noise and music (Di Stadio et al., 2018b; Śliwińska-Kowalska & Zaborowski, 2017b). It is estimated that more than 1.1 billion people 16 to 25 years old are at risk of losing their hearing due to unsafe listening habits (Krug & World Health Organization, 2015). Additional to the general population, musicians and professionals of the music industry are exposed to high level of music occupationally and recreationally and are at high risk of developing hearing loss and related symptoms (Di Stadio et al., 2018b). Occupational or recreational noise and music can induce oxidative stress, metabolic exhaustion, and ischemia of cochlear hair cells (Ryan et al., 2016). These changes can lead to hearing loss and other audiological symptoms such as tinnitus (perception of a sound with no external source), diplacusis (perception of the same stimulus as of different pitch in the two ears), and hyperacusis (intolerance of everyday sounds) (Di Stadio et al., 2018b). Noise- or music-induced hearing damage can be temporary or permanent. The conditions under which a temporary lesion recovers completely or not are not yet clarified (Nordmann et al., 2020; Ryan et al., 2016).

4.1.3. PTS

Noise and music induced hearing loss (NIHL and MIHL) are associated with permanent cochlear dysfunction (Śliwińska-Kowalska & Zaborowski, 2017b). Investigation of PTS in humans is limited to observational studies, since causing permanent acoustic damage to volunteer participants under experimental conditions is unethical, with irreversible effects of varied degree on participants' communication capacity, overall wellbeing, and quality of life (Li et al., 2013). This important limitation explains the fact that although many rodent studies focus on PTS (He et al., 2021; Qian et al., 2021), human experimental studies focus exclusively on TTS (Engdahl, 1996; Kil et al., 2017; Quaranta et al., 2003). Observational studies of PTS in humans, either longitudinal or cross-sectional, present methodological limitations; estimation of the amount, type and duration of noise/music exposure is challenging (Guest et al., 2018), and confounding factors, such as exposure to other ototoxic factors (Kraaijenga et al., 2018), are difficult to control or eliminate. Moreover, for them to succeed, observational prospective cohort studies need long-term monitoring of participants, while cross-sectional ones present the issue of temporality, are unable to establish causality, and their sensitivity may be affected by between-subject variance in factors unrelated to noise exposure (Grimes & Schulz, 2002).

4.1.3. TTS

On the other hand, TTS is a reversible phenomenon that is observed after

exposure to high levels of noise or music (Ryan et al., 2016). The molecular and biochemical processes underlying TTS are distinct to those of PTS (Ryan et al., 2016). Although TTS and PTS are based on different pathophysiologic mechanisms (Ryan et al., 2016), TTS investigation may be of great value for the better understanding of NIHL and MIHL. Repeated cochlear insults leading to temporary loss of hearing sensitivity have been associated with permanent hearing disorders (Ryan et al., 2016). Moreover, TTS is indicative of exposure to acoustic energy capable of causing permanent lesions, and thus TTS may serve as a good proxy measure in otoprotectant-related trials (Kil et al., 2017). Finally, recent data support that particular otoprotective agents may have an effect on both TTS and PTS (Kil et al., 2017; Pourbakht & Yamasoba, 2003).

Studies investigating TTS may be observational or experimental. All PTS-related limitations are also evident in TTS observational studies (confounding factors, quantification of noise/music exposure). Additionally, since TTS is a dynamic phenomenon and often recovers rapidly, the time needed for the participant to reach the audiometric equipment, and thus the timeline of measurements, may also vary and confound the audiometric results (Kraaijenga et al., 2018; Melnick, 1991). However, experimental studies of TTS can avoid most of these limitations.

4.1.4. Using noise or music as an intervention

NIHL therapeutics and otoprotectants are currently under investigation (Kil et al., 2017; Le Prell et al., 2016; Schilder et al., 2019). Controlled, experimental, TTS studies testing otoprotective agents, role of potential NIHL biomarkers, or

hearing protection devices, could reliably evaluate and compare their effects on cochlear dysfunction and thus their effects on cochlear protection. However, designing a study that includes noise and/or music exposure as an intervention requires finding the balance between avoiding exposing participants to possibly hazardous levels of noise and at the same time reaching those levels that will be efficient to create a measurable change in the primary outcome. Hence, important ethical and methodological considerations arise. Particularly in the case of studies investigating NIHL or MIHL, developing and validating an intervention that enables researchers to reliably cause cochlear insult, and thus its pathophysiological sequelae, without the risk of causing permanent auditory dysfunction or damage to the participants, is of major priority. Additionally, the possibility of creating irreversible hearing damage that is not evident in the PTA should not be neglected. It is thus important to develop a validated approach that induces TTS without putting participants' long term cochlear integrity at risk.

In the current paper, we present a thorough review of literature identifying and analyzing previously used models. Ethical and methodological considerations of exposure to noise or music as interventions in clinical trials were considered, and intervention paradigms were assessed for their safety and efficacy.

4.1.5. Objectives

The main goals of this scoping review of the literature were:

(1) To document studies that describe noise and/or music exposure (> 80 dB A) as an intervention.

(2) To identify the methodological (type of noise/music, intensity levels, duration etc.) and ethical aspects of noise or music exposure in each study.

(3) To evaluate the effect that each intervention had on participants' auditory or other system, by attempting to align the diverse methodological aspects, discrepancies, and experimental conditions between studies in order to produce a general understanding.

The findings of this review were later used to develop a music-induced TTS paradigm and explore its impact on the hearing sensitivity of adults with normal hearing.

4.2. Methods

This scoping review followed PRISMA-SCRA guidelines (Tricco et al., 2018).

4.2.1. Review question

What are the methodological and ethical aspects of experimental exposure to high levels of noise and/or music in published research, and what are their effects on participants' auditory or other system?

Population: human, no limitation on condition under focus

Intervention: noise and/or music > 80 dB SPL

Comparator: not applicable

Outcomes:

(1) Ethical considerations of exposing participants to high-levels of noise or music; legislations, regulations, or pathophysiological mechanisms that were taken into account.

(2) Methodological aspects of noise and music exposure and of testing the exposure's impact on participants' hearing and health: target condition (e.g. TTS, noise-induced stress etc.), population (male to female ratio, age range and average, any particular characteristics, e.g. specific occupational population), type of noise / music, setting (exposure duration, levels, equipment, venue etc.), timeline of procedures (exposures or post-exposure measurements), audiometric or other involved measurements.

(3) Effect of noise or music exposure on participants' auditory or other system, such as TTS in PTA, decrease of OAEs amplitude or other, according to the particular tests that were chosen in each study.

4.2.2. Eligibility criteria

Inclusion Criteria:

(1) Study Samples: Adults or children with or without hearing loss

(2) Intervention: Noise or music equal or higher than 80 dB SPL

(3) Study type: Experimental studies, case reports, case series, methodological papers

(4) Publication type and language: English, French, Spanish or German-language journal articles. Publication year: all studies after 1947 (year of the establishment of Nuremberg Code)

Exclusion Criteria:

(1) No full text available

(2) Study designs: reviews, meta-analyses, observational studies

(3) Studies using noise or music < 80 dB SPL.

(4) Studies with unclear levels of noise or music exposure

4.2.3. Information Sources and search strategy

Medline, Cochrane Central Register of Controlled Trials (CENTRAL), and Scopus were searched for eligible studies by two reviewers independently. Grey literature was sought in Clinicaltrials.gov and EU Clinical Trials Register. Results were hand-searched.

4.2.4. Selection of sources of evidence and data charting

Literature was searched independently by the two first authors. In that stage of analysis, the authors identified duplicates or multiple reports of the same study by first examining the titles and abstracts of the yielded studies and then their full text. One single list of included papers was created by cross matching the two authors' lists. Any discrepancies were discussed and resolved by the two authors, with the help of the senior author. Data were then extracted from eligible studies by each author independently. Again, data extraction tables were cross matched for discrepancies.

4.2.5. Extracted Data Items and Data synthesis.

(1) Study identification: 1st author, year of publication.

(2) Study Methods: Target condition, population [number of patients included, demographic characteristics, type of hearing loss (NIHL, MIHL, other), other condition tested], type of noise / music, setting, timeline, audiometric or other involved measurements. Ethical considerations of exposing participants to high levels of noise or music (legislations, regulations etc.). Type of noise / music, setting (exposure duration, levels, equipment, venue etc.), timeline of procedures (exposures or post-exposure measurements), audiometric or other involved measurements.

(3) Study results: effect of exposure on participants' hearing

The studies included in this review and the corresponding extracted data items are presented in the form of tables in **Supplementary Material 4.1-4.3** and text in the main manuscript, in alignment with our research question and study objectives.

4.2. Results

4.2.1. Selection of sources of evidence

Procedures followed for the selection of included studies are presented in **Figure 4.1** (Page et al., 2021).

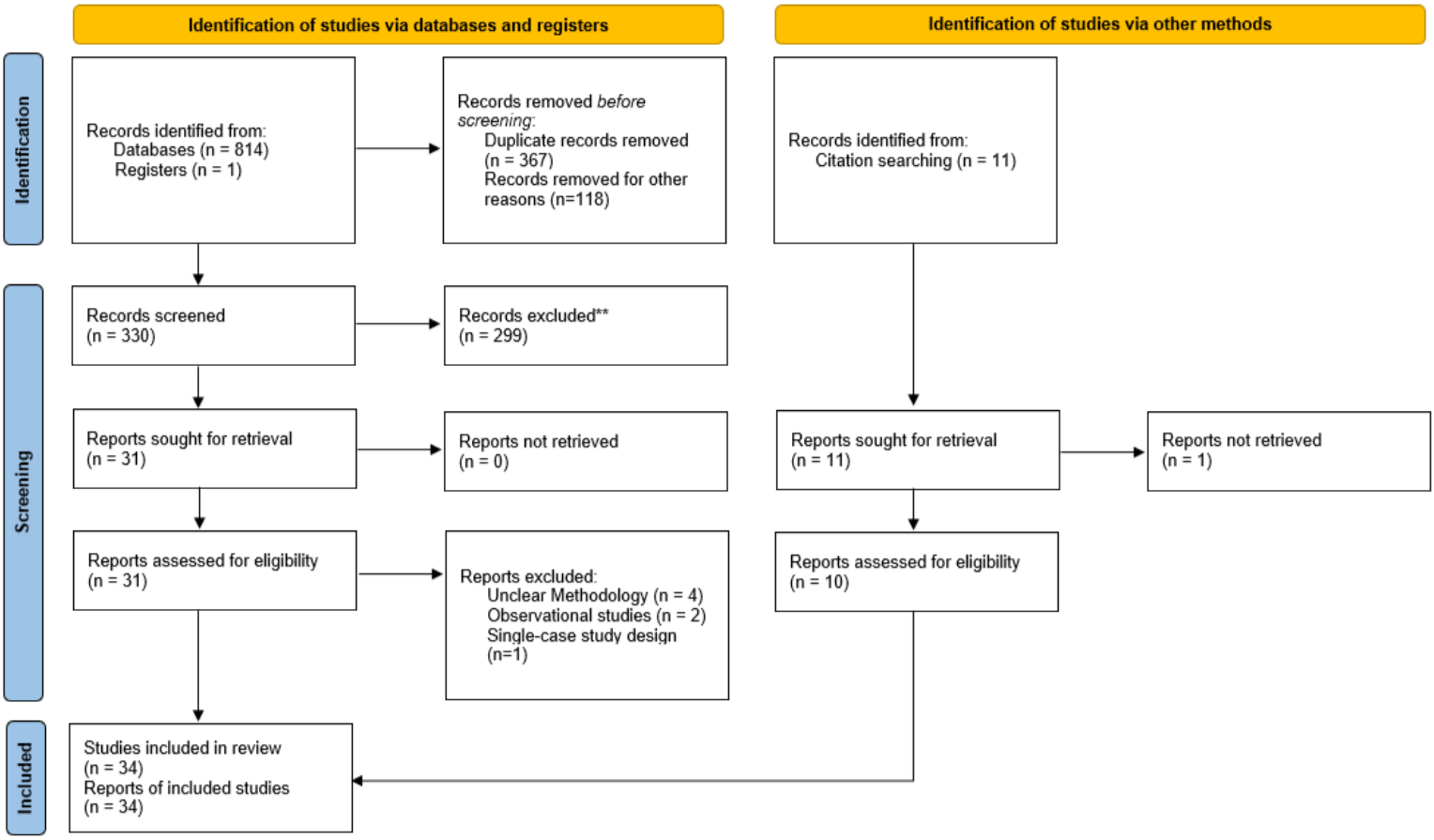


Figure 4.1. PRISMA flowchart.

4.2.2. Characteristics of sources of evidence

Thirty-four studies were included in this review. According to our inclusion criteria, all of them were interventional, either cross-sectional or longitudinal. They concerned adults.

Twenty-three studies involved noise as primary study intervention and 11 of them concerned NIHL (**Supplementary Material 4.1**); 11 studies involved music as primary study intervention and 10 of them concerned MIHL (**Supplementary Material 4.2**). The rest of the studies concerned the effects of noise or music on stress or discomfort levels and on cognitive or

cardiovascular parameters, such as memory performance, blood pressure and heart rate variability. Their data are presented in **Supplementary Material 4.3**.

4.2.3. Synthesis of results

Population

We did not identify any interventional study assessing the effects of hazardous noise or music on children. The majority of studies included young healthy participants in the 2nd-4th decade of their life, with normal hearing and no reported particular source of chronic noise or music exposure, while a few of them include people in their 5th and 6th decade of their life (Andrén et al., 1981; Cavaliere et al., 2004, 2008).

Among included studies a lack of representation of specific populations that are more prone to chronic exposure to noise or music, such as workers or professional musicians, is observed. In one of the studies included in our review, the sample consisted of musicians and non-musicians with normal hearing (Kikidis et al., 2019). The study aimed to assess the effects of brief exposure to music on auditory brainstem responses (ABRs) and DPOAEs, which were considered indirect indicators of synaptic function. No difference in temporary DPOAE signal-to-noise ratio (SNR) shift between musicians and non-musicians was eventually observed, while no difference was observed in ABR before and after exposure for either musicians or non-musicians. However, it should be taken into account that the sample was small (four musicians, six non-musicians) and that no PTA was performed, so the aforementioned result should be interpreted with caution.

Frequency spectrum of noise / music

White or narrow band noise (NBN) was chosen in all NIHL related studies, while the bandwidth of the NBN ranged from 0.5 to 4 kHz (**Supplementary Material 4.1**). Playlists consisting of mainstream pop-rock songs were selected in all studies included in this review (**Supplementary Material 4.2**). Participants' preference and pleasure were taken into account in most cases by asking them to select the playlists they would be exposed to (Kikidis et al., 2019; Kil et al., 2017; Le Prell et al., 2012, 2016).

Experimental setting

The experimental setting varied significantly in both noise and music exposure paradigms (**Supplementary Material 4.1 and 4.2**). According to our inclusion criteria, all studies included noise or music > 80 dB SPL (maximum value 115 dB SPL (Lichtenhan & Chertoff, 2008)); and the duration of exposure ranged from 3' to 26 h (Lightfoot, 1955; Mills et al., 1979). Although in most studies, levels and duration of exposure were fixed for all participants, in two studies participants were asked to select the music levels that they would prefer to be exposed to (Kikidis et al., 2019; Lee et al., 1985). Noise / music was delivered via headphones, earphones, or free-field monitors, monaurally or binaurally, in one non-stop session or in multiple consecutive sessions (Engdahl, 1996; Manson et al., 1994).

Audiometric assessment

Standard PTA (0.25 – 8 kHz) was the audiometric test used in most studies. Whenever the step size was not stated, we considered that a 5 dB step size

was applied. Nevertheless, a 2 dB step was used in specific cases where more accuracy on detecting TTS was needed (Kil et al., 2017; Le Prell et al., 2012, 2016). Békésy audiometry was used in two studies (step size 1 dB) (Engdahl, 1996; Moshammer et al., 2015a). Other studies included in our review also used DPOAEs, TEOAES, contralateral suppression, and ABR (33 and 44 clicks/second, at 90 dB nHL; Engdahl, 1996; Kikidis et al., 2019; Kil et al., 2017; Prell, 2019). Decrease of DP amplitudes (at 1-8 kHz, with unequal primaries) was reported in all included studies, while no particular effect of temporary cochlear dysfunction on the other measures was demonstrated (see *Impact on hearing* section and **Supplementary Material 4.1 and 4.2**).

Impact on hearing

TTS. Temporary changes in hearing sensitivity were detected in post-exposure PTA in multiple studies (**Figures 4.2-4.5**). More prominent TTSs were found in the region of 4-8 kHz. Maximum average TTS was ~21.5 dB after NBN (Quaranta et al., 2004) and ~11.5 dB after music exposure (Krishnamurti & Grandjean, 2003).

DP amplitude shift. In one study by Fetoni et al. (2009), reduced DP amplitudes were observed 1 and 16 hours after 10' of white noise at 90 dBA, but not after 7 and 14 days *(*Fetoni et al., 2009*)*. Similarly, significant decrease at 2, 3, 4, and 6 kHz, for f1 levels ranging from 35 to 55 dB SPL, was observed after 4 h of exposure to music at 100 dBA (Le Prell et al., 2016) and after 2 h of music exposure at a night club (Lavg = 92.5-102.8 dBA, with a mean of 98.1 dBA, for DPOAE methods see (**Supplementary Material 4.2** in Kramer et al, 2006). Exposure to music more intense than 83 dBA for 30' also caused a

statistically significant decrease in DPOAE SNR measurements at 2-6 kHz in one study (Kikidis et al., 2019) Finally, in their study about the otoprotective effect of magnesium (Mg), Attias et al. (2004) observed a DP amplitude shift of up to 4 dB, with complete recovery in the Mg group at 15' and 50% recovery (2 dB) in the non-intake and placebo sessions at 30' (Attias et al., 2004).

In all studies testing for permanent hearing loss, participants completely recovered their hearing according to PTA, with the exception of one participant in Kil et al. (2017):

(1) Kil et al. (2017): 80 out of 81 participants returned to baseline PTA thresholds after 4h of exposure to 100 dBA via ER6i earphones (one participant was 6 dB, hence it had not returned to within 4 dB of baseline at 6 and 8 kHz one week after exposure; the definition of TTS recovery in this particular study was the return of threshold to within 4 dB from baseline).
(2) Le Prell et al. (2016): All 100 participants' TTS returned to baseline after 4 h of exposure to music of 100 dBA.
(3) Le Prell et al. (2012): All 33 participants' TTS returned to baseline after 4 h of exposure to music of 97, 99, and 100 dBA.
(4) Kramer et al. (2006): All participants returned to baseline after 2 h at Lavg range of 92.5 to 102.8 dBA.

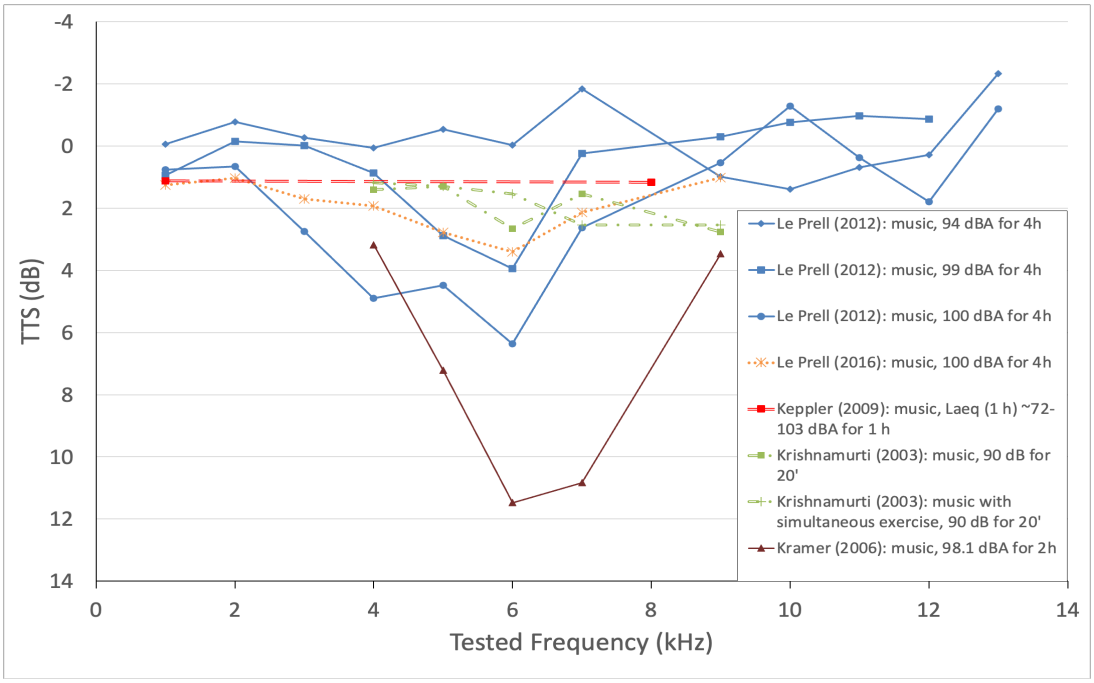


Figure 4.2. Music-induced TTS in terms of PTA across studies (2'-20' post-exposure). Data were obtained through WebPlotDigitizer. Le Prell et al. (2012; 2016) exposed participants to music for 4 hours **at coupler levels** of 97-100 dBA and Kramer et al. (2006) for 2 hours at 92.5 to 102.8 dBA (**free field**, mean exposure levels = 98.1 dBA).

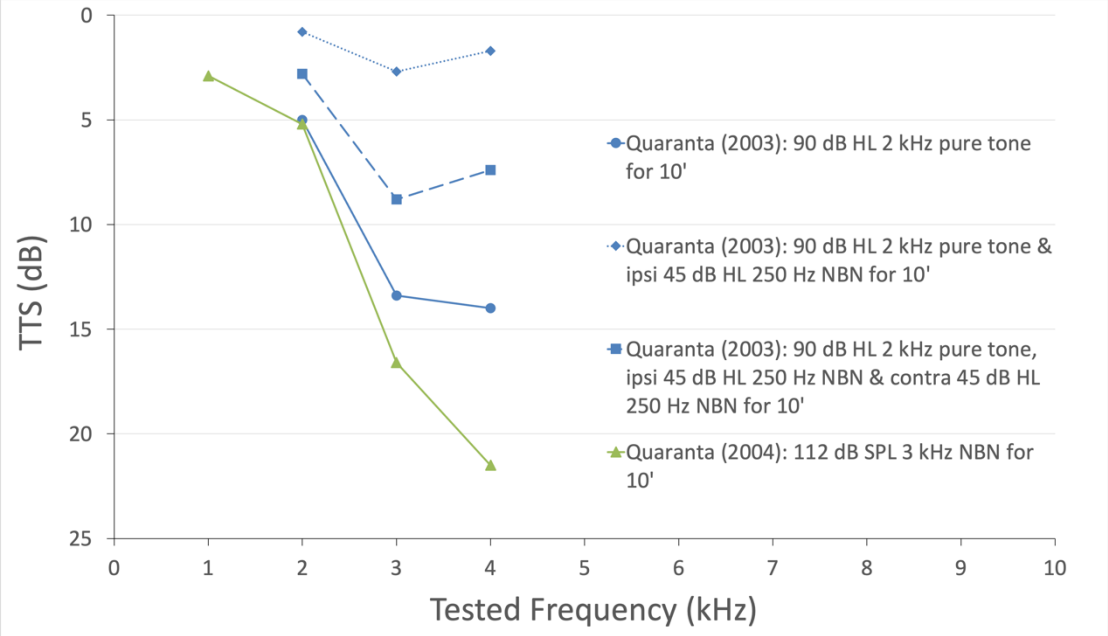


Figure 4.3. Noise-induced TTS across studies (2'-20' post-exposure). Studies conducted by Quaranta et al. using 2 kHz pure tones(Quaranta et al., 2003) and 3 kHz NBN(Quaranta et al., 2004) Data were obtained through WebPlotDigitizer .

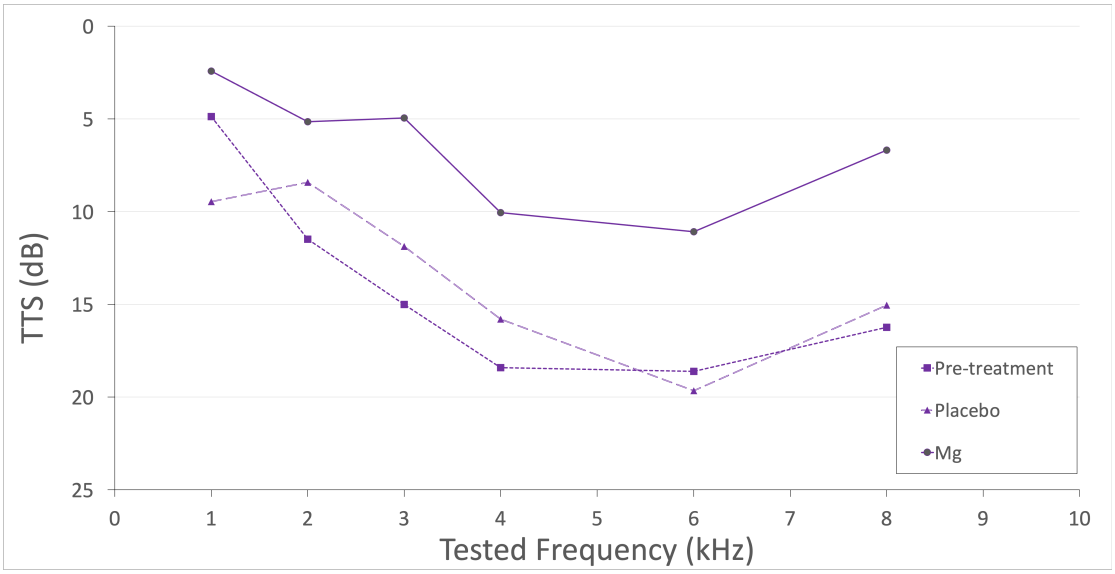


Figure 4.4. Noise-induced TTS across studies (2'-20' post-exposure). Study by Attias et al. using white noise(Attias et al., 1994, 2004). Data were obtained through WebPlotDigitizer.

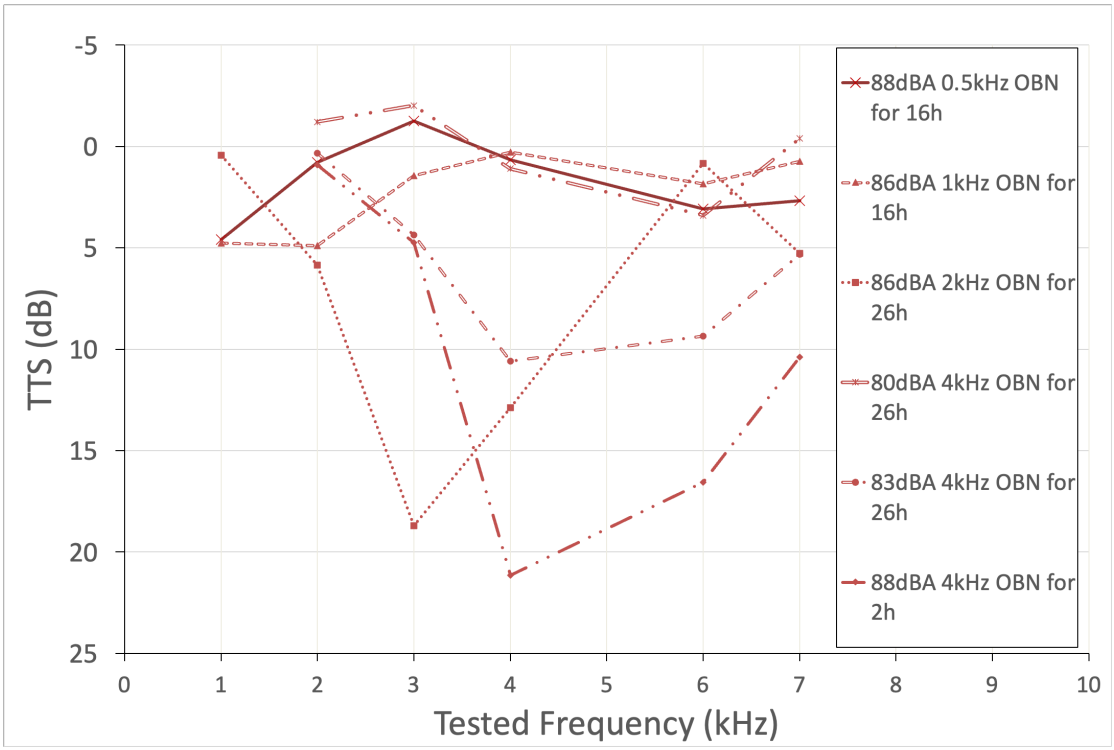


Figure 4.5. Study by Mills et al.(Mills et al., 1979) using OBN of 0.5-4 kHz. Data were obtained through WebPlotDigitizer.

Impact on other systems

All effects of loud sounds on systems other than the auditory were temporary, with no permanent dysfunction or handicap being reported in any of the included studies (**Supplementary Material 4.3**). The experimental setting varied significantly across studies. Unedited or isolated hospital noises, such as continuous positive airway pressure (57-94 dBA for 140') or non-invasive ventilation equipment (60-110 dBC for 20') caused discomfort to healthy adults (Cavaliere et al., 2004, 2008). Discomfort was also created to participants by traffic (85.6 dBA for 60'), helicopter (three sessions of 20' with 5' breaks, at 77, 81 and 86 dBA, respectively), fan (80 dBA for 20'), and chainsaw sounds (five sessions of 2' exposure at 105 dB). Combining loud sounds with other stressors seemed to intensify its effect on participants' annoyance (Reinhardt et al., 2012). Tinnitus patients were also more annoyed by noise of the same type, duration and levels (fan-like sound at 80 dBA for 20' binaurally) when compared to non-tinnitus controls (Hébert & Lupien, 2009). Magnetic resonance imaging (MRI) noise reduced the perception of pain level in young adults, something that needs to be taken into account when functional MRI is used in studies assessing the mechanisms of pain perception (Boyle et al., 2006). Cardiovascular effects of loud noise include increase of diastolic and mean arterial blood pressure in adults with essential hypertension, while no change was found in healthy ones (broad-band noise; Andrén et al, 1981), and decrease of heart rate variability in healthy females (10' of music of 70-80 dBA with or without white noise (90 dBA); Roque et al., 2013). In a study testing for effects of noise in human performance, participants had to complete an arithmetic task of two different levels of difficulty. During participants' effort to

complete the difficult level, their exposure to a recorded mix of noises (traffic, office machinery, and babble, for 90'at 90 dBA peaks) caused significantly increased heart rate, norepinephrine, and cortisol levels compared to pre-exposure values (Tafalla & Evans, 1997). Finally, the gastrointestinal system may be affected by exposure to loud sounds. Exposure to traffic (87.4 dBA), hospital (91.3 dBA), and babble noise (85.6 dBA) for 60' through headphones decreased gastric myoelectric activity and increased the incidence of bradygastria in young healthy individuals (Castle et al., 2007).

Ethical aspects

Although all studies considered in the review were approved by a responsible Ethics Committee, 12 of them did not include any hearing assessment after the noise or music exposure (**Supplementary Material 4.3**), while special considerations about the safety of the levels and duration of exposure were reported in only in a few of them. Local health and safety regulations (Attias et al., 1994), OSHA limits (Kil et al., 2017; Le Prell et al., 2012, 2016), and participants' maximum comfortable level (Kikidis et al., 2019) were taken into account in the experimental design of those few studies.

4.3. Discussion

4.3.1. Considerations on the Population of the study

Most previous studies focusing on TTS involved young or middle-aged healthy people with normal hearing, which affects the generalizability of their results.

No older adults or people with established hearing loss or hearing loss susceptibility factors, such as family history or concurrent ototoxic medication, have ever been assessed. Their findings should be interpreted considering this limitation. In cases where the scope of the study is the creation and testing of a music-induced TTS paradigm, and not the analysis and understanding of TTS-related factors, age range could be omitted from the inclusion criteria. Nevertheless, more insight on the age effect on TTS is needed. In the future, this need could be addressed through study designs with larger samples and better representation of all age groups, and/or age correction of the degree of NIHL. Moreover, specific populations with chronic exposure to noise and music, such as musicians or workers, are under-represented in current TTS literature. Selecting specific populations that have a chronic exposure to high-level sound may be beneficial and clinically highly relevant, since these are the populations that present higher incidence of NIHL and MIHL. They are also the most adequate populations to assess the effect of repeated exposure to less intense sounds on the “toughening” or “conditioning” of the cochlea, and thus on one’s vulnerability to noise. Finally, a reliable method for collecting information about lifetime noise exposure and for identifying all factors that are needed to reach a safe estimate of noise exposure over the lifespan is important for the description of participants in NIHL and MIHL studies (Guest et al., 2018; Spankovich et al., 2019). Although noise exposure history taking can be heavily affected by recall bias, user-friendly structured interviews could help in reducing this risk. The Noise Exposure Structured Interview could be a valid option (Guest et al., 2018).

4.3.2. Considerations on frequency spectrum of noise / music

Previous studies, show that the maximum cochlear insult is mostly happening one-half to one octave higher in characteristic frequency than the frequency of the exposure stimulus (Ward, 1965), and at the extended high frequencies (Hunter et al., 2020; Lough & Plack, 2022). According to this, when compared to white noise, exposure to octave band noise (OBN), NBN, and pure tones limits the region of the affected area, yet still results in a broad enough region of damage to be able to measure the effect (Engdahl, 1996). With regards to the optimal selection of frequency in the case of human cochlea, early studies on NIHL have shown that tones or narrowband noises in the range of 1-4 kHz had a greater temporary effect on hearing (up to 60 dB or greater TTS) than stimuli of lower frequency (H. Davis et al., 1946; Gittleman et al., 2019; Mills et al., 1979; Zakrisson, 1975).

Nevertheless broad-band noise, OBN, or NBN may be unpleasant to listen to (Lindgren & Axelsson, 1983). Especially in the case of studies on MIHL, use of music that is generally acceptable and pleasant is a valid solution. Studies included in this review used popular pop-rock music at fixed levels and duration, or at levels selected by participants according to their own preferences. Levels and duration of exposure have to be high enough to create a measurable effect on the cochlea, and also safe enough in order not to create permanent cochlear dysfunction. The influence of factors other than the physical properties of the fatiguing sound energy on TTS have been assessed in several studies. The findings of Hörmann et al. (1970) suggest that there is an emotional effect on noise-induced TTS, since participants who were exposed to hazardous noise

as punishment had higher TTSs at 4 kHz than those participants who received it as reward, and higher than those who did not receive any emotional influence, neither reward nor punishment. Moreover, Chüden and Strauss (1974) observed that disc-jockeys developed less TTS after exposure to music than after exposure to steady-state noise of equal energy. These findings were further supported by Lindgren and Axelsson (1983) who evaluated TTS data from 10 participants exposed five times to noise and five times to music of equal energy and observed that noise-induced TTS was generally higher, although there was variability between sessions and participants.

4.3.3. Experimental setting

Across NIHL and MIHL studies there is a large heterogeneity with regards to the equipment and setting, and the use of binaural or monaural exposure. Current standards, according to National Institute for Occupational Safety and Health (NIOSH) suggest a permitted daily noise “dose” based on free-field levels of sound (Śliwińska-Kowalska & Zaborowski, 2017b). Sound pressure levels at the tympanic membrane may vary due to the distance from the sound source and the resonance of the external ear. Delivering the audio material via headphones eliminates distance from source and effect of pinna (Ward, 1965), although the effect of ear canal resonance remains significant. Paradigms may thus vary and adapt to each use case and clinical query of interest. Binaural and free-field exposure may be more adequate for studies mimicking real life listening conditions. In studies that concern the development and validation of music-induced TTS, monaural delivery could be a valid and safer option since one side is kept unexposed. Although it has been hypothesized that it may be

more dangerous due to lack of contralateral middle ear muscle reflex (MEMR) and of the protective effect of the efferent system (Engdahl, 1996; Rajan, 1995), there is currently no evidence that contralateral exposure has a protective effect on TTS (Quaranta et al., 2003).

4.3.4. Audiometric assessment

No consensus has been reached with regards to the hearing assessment before and after music exposure either. Any TTS test battery should be quick, efficient, easily repeatable without learning effect, and able to detect even subclinical cochlear dysfunction.

To date, the principal audiometric test used in everyday clinical and research practice for the identification of temporary and permanent changes of hearing sensitivity due to noise or music overexposure has been PTA (0.25-8 kHz) (Mehrparvar et al., 2014). Although PTA is the gold standard in hearing loss assessment, clinical experience and research evidence suggest that this specific audiometric method is not efficiently sensitive and cannot detect early or small changes in auditory function (Mehrparvar et al., 2014). Moreover, PTA is not sufficiently reliable since its standard test-retest variability (±5dB) could be misinterpreted as a significant effect of hearing loss due to noise.

The 6-dB up, 2-dB down modified PTA procedure, limited to those frequency regions that are proven to be more affected by high-volume noise and music, conducted before and after the exposure (instead of the 10-dB up, 5-dB down method, in the whole 0.25 – 8 kHz standard PTA range) could be a valid option

in order to detect quickly TTS smaller than 5 dB. Additionally, temporary and permanent NIHL and MIHL could be explored by a set of complementary behavioral, neurophysiological and electrophysiological tests (Le et al., 2017). Extended high-frequency PTA (EHF-PTA > 8 kHz) seems to be able to detect acute and chronic cochlear dysfunction earlier than standard PTA . Nevertheless, its value in TTS evaluation remains to be clarified; evidence suggests that EHF-PTA thresholds are not affected by exposure to music, which tends to cause elevation of thresholds in the 3-6 kHz region (Kil et al., 2017; Le Prell et al., 2012, 2016; Schmuziger et al., 2007). Although there is lack of agreement across studies, OAEs (especially DPOAEs) also seem valuable for the detection of TTS or PTS (B. Davis et al., 2005; Engdahl, 1996; Kramer et al., 2006). Previous evidence has established that DPOAEs elicited with unequal f_1 and f_2 levels are considerably more sensitive to reductions in emission levels induced by brief exposure to noise (Sutton et al., 1994).

4.3.5. Impact on other systems

No permanent effects of noise or music > 80 dB sPL on systems other than the auditory system were reported in any of the included studies (**Supplementary Material 4.3**). Apart from one study using music (Roque et al., 2013), the rest of the studies used mostly what is considered unpleasant noise, such as traffic, hospital noise, or babble (Lindgren & Axelsson, 1983; Pouryaghoub et al., 2016). This may explain the association of the exposure with increased annoyance and discomfort. The presence of tinnitus, and the combination of high-level sounds with other stressors, made participants more vulnerable to noise and increased their annoyance. People with essential hypertension

presented higher blood pressure after exposure to noise, when compared to healthy volunteers. Future studies targeting TTS may choose to use as unique stressors isolated sounds that are not considered unpleasant, such as music, and involve healthy participants with no cardiovascular comorbidities or risks.

4.3.6. Ethical aspects

Previously published experimental studies on TTS raised controversy about the safety for participants' hearing, and the interpretation and generalizability of their results (Themann et al., 2015). In response to Moshammer et al. (2014), two independent Letters to the Editor (Themann et al., 2015; Verbeek, 2015) underline the fact that TTS-related research raises particular concerns: First, the relation between TTS and PTS is yet to be clarified, so the results of experiments causing temporary cochlear dysfunction cannot be translated with confidence to permanent NIHL (Verbeek, 2015); Second, TTS may completely recover but the exposure may not be safe in the long-term, since it may be linked to cochlear synaptopathy which is not detectable by PTA (Themann et al., 2015); Finally, current noise protection guidelines are suboptimal due to the unclarified individual susceptibility to noise (skin or eye color, age, sex etc.) (Henderson et al., 1993; Themann et al., 2015; Verbeek, 2015). Nevertheless, testing for temporary hearing changes after noise exposure under presumed safe conditions is still a valuable tool for future studies in the hearing domain, such as clinical trials testing the efficacy and effectiveness of otoprotective agents or specific listening habits.

In order to decrease safety concerns, all interventional studies involving music and noise exposure should take into account findings of previous literature. Local and international health and safety regulations should also be respected. Even studies focusing on other than NIHL or MIHL conditions, such as stress or memory, should monitor participants' hearing after exposure to hazardous levels of noise or music. Selecting exposure levels, duration, and setting based on results of previous paradigms raise the likelihood of safely creating TTS without causing PTS or other suprathreshold issues. A summary of the risks of participating in the study should be presented in the study protocol. These risks should also be explained in plain language during informed consent procedures. Participants should be aware of the overall "dose" of noise or music they shall receive during the study. They should also acknowledge the fact that they may need to avoid any further exposure from other sources outside the study a few days before and after their participation to the study, in order not to exceed the permissible levels per day or week.

Moreover, in TTS interventional studies, the cut-off point that defines post-exposure PTS should be strict. In Kil et al. (2017), one participant presented an elevation of PTA threshold of 6 dB at 6 and 8 kHz at one week after exposure; the definition of TTS recovery in this particular study was return of threshold to within 4 dB from baseline within a week. Although the PTS definition was clear, the set cut-off point of threshold shift and time of measurement were much lower than those proposed by NIOSH and OSHA. According to NIOSH, a standard threshold shift is defined as "an increase of 15 dB in hearing threshold

level (HTL) at 500, 1000, 2000, 3000, 4000, or 6000 Hz in either ear, as determined by two consecutive audiometric tests," and OSHA permits a confirmation with a retest within 30 days of the initial test, allowing employers to avoid reporting injury if the retest shows recovery within this 30-day window (Ryan et al., 2016). In the case where a PTS is detected, we suggest that the interventional study is paused and a revision of the methods or decision to end the study is considered.

4.3.7. Conclusion

This scoping review provides a comprehensive overview of methodological and ethical aspects of the experimental use of noise and music > 80 dB SPL. Included papers presented high methodological heterogeneity and relatively small sample sizes. Review of the literature suggests that TTS can be safely evaluated in the context of an interventional clinical study, using white and NBN or music, under specific experimental conditions. Previous studies using noise and music at up to 100 dBA in-ear level for 4 hours caused detectable TTS without causing permanent hearing disorder to any participants, with the exception of one controlled study, where one out of 81 participants had not returned to within 6 dB of baseline at 6 and 8 kHz one week after exposure. Paradigms of shorter duration or lower intensity should be tested for their efficacy and safety in larger samples. Hearing tests that have been proven adequate for the detection and longitudinal monitoring of TTS are PTA thresholds at 1-6 kHz, and DPOAEs with unequal primaries at 1-8 kHz. Monaural exposure may be a safe option since one ear is spared and there is currently no evidence that the lack of MEMR and contralateral suppression

affects thresholds, while monaural hearing testing may significantly reduce testing time. Tinnitus may increase the annoyance caused to participants during their exposure to noise or music. In non-tinnitus related studies, excluding patients with tinnitus should be considered. Participants with essential hypertension may experience some temporary increase of their blood pressure due to exposure to noise. This should be taken into consideration when they are being included in noise or music exposure paradigms. Our findings have direct implications for the design of new music paradigms aiming at creating safely and reliably temporary cochlear dysfunction.

Chapter V

Development and validation of an efficient and safe music exposure paradigm

Abstract

Purpose: To develop a time-efficient music exposure and testing paradigm, that safely creates temporary cochlear dysfunction that could be used in future temporary threshold shift (TTS) studies.

Method: A 30-minute audio compilation of pop-rock music tracks was created. Adult volunteers with normal hearing were then exposed to this music material monaurally through headphones for 30 min at 97 dBA or 15 min at 100 dBA. Levels were measured from the ear of a manikin and are considered to provide an equivalent daily noise dose based on a 3-dB exchange. We assessed the changes in their hearing, by means of distortion product otoacoustic emission (DPOAE) testing, and standard and extended high-frequency pure-tone audiometry before and after exposure. There were 17 volunteers in total. In a first trial, eight volunteers [four females; median age=31 years (IQR=4.25)] were included. Although TTS was observed in all eight participants for at least one frequency, a large variation in affected frequencies was observed. To address this issue, the audio material was further remastered to adjust levels across the different frequency bands. Fourteen adults [nine newly recruited and five from the first trial; seven females; median age=31 years (IQR=5)] were exposed to the new material.

Results: All but 2 out of 17 participants presented clinically significant TTS or decrease in DP amplitude in at least one frequency. Statistically significant average TTS of 7.43 dB was observed at 6 kHz. There were statistically significant average DP amplitude shifts of -2.55 dB at 4 kHz, -4.97 dB at 6 kHz, and -3.14 dB at 8 kHz. No participant presented permanent threshold shift.

Conclusions: A monaural music paradigm was developed and shown to induce statistically significant TTS and DP amplitude shifts, without evidence of permanent loss. This realistic and time-efficient paradigm may be serve as a useful option for experimental studies of temporary music-induced hearing loss.

5.1. Introduction

TTS has long been investigated as a proxy of NIHL and MIHL. Previously, paradigms of noise or music exposure with sound levels up to 100 dBA in-ear level and lasting up to 4 hours caused detectable TTS without causing any permanent hearing disorder to the participants (Kramer et al., 2006; Le Prell et al., 2012). Being able to create safely and reliably detectable TTSs under controlled laboratory conditions, using stimuli that are pleasant to participants, may facilitate future studies on TTS and its relation to participants' characteristics, hearing loss biomarkers, or effect of otoprotective agents. The aim of this study is the development and validation of: (i) a new music exposure paradigm, briefer than previous examples and with real-world validity, in order to achieve temporary cochlear dysfunction without participants being at risk of permanent hearing loss or other hearing disorder; and (ii) a test battery which

is brief yet capable of reliably detecting temporary changes in cochlear function as measured by TTS and DPOAE shifts. Such a paradigm could safely and efficiently be used by researchers in future interventional TTS studies.

Concerning the selected audio material used in experimental settings, it should be pleasant and at levels easily acceptable to the average listener. Researchers should also be able to document in detail the dynamic range and exposure levels of each participant's exposure. In our case, we selected pop-rock music regarded as pleasant by participants, to mimic regular music exposure and to reduce drop out risk. Music was delivered monaurally through headphones at levels compatible with the Greek legislation (Y.A. Υ2/Οικ. 15438/2001, 2001) and the in-ear exposure levels did not exceed the recommended daily exposure limits of the National Institute for Occupational Safety and Health (NIOSH) standards, which allow up to 15 min at 100 dB and up to 30 min at 97 dBA for U.S. workplace exposures (National Institute for Occupational Safety and Health. Division of Biomedical and Behavioral Science, 1998). Taking into account that NIOSH standards and permitted daily noise "dose" are based on the hazard associated with repeated noise exposure during five workdays for 40 work years, and not on one single exposure as in our experiment, we considered that our paradigm was safe for our participants. Moreover, NIOSH standards concern free-field levels of sound. In our study, music was delivered via headphones, hence levels were lower than free-field equivalent levels. Since assessing the efficacy of our paradigm in creating TTS does not require exposure and thus insult of both ears, only monaural exposure was considered. Monaural delivery of noise/music was chosen in multiple

previous studies (Attias et al., 2004; Bhagat & Davis, 2008; Keppler et al., 2010; Quaranta et al., 2003, 2004).

Concerning the optimal test battery, this had to be quick yet efficient. In our case, we selected hearing tests that have previously been proven to detect temporary changes in cochlear function reliably (Kikidis et al., 2019; Kil et al., 2017; Le Prell et al., 2011, 2012). We thus decided to use a previously tested modified PTA method, the 6 dB down, 2 dB up method, instead of the 10 dB down, 5 dB up method, to be able to detect TTS less than 5 dB (Kil et al., 2017; Le Prell et al., 2016). We chose to test 1, 3, 4, 6, 8, 10, and 12.5 kHz of the exposed ear to focus on frequencies that are more prone to be affected quickly, to avoid missing short-term TTS, and to be comparable to previous literature (Kil et al., 2017; Le Prell et al., 2012). DP amplitude measurement (1-8 kHz) with unequal primaries was also selected, since the measurement is quick and sensitive to detection of temporary cochlear dysfunction (Le Prell et al., 2012).

5.2. Methods

5.2.1. Audio material

A 30-minute compilation of 2-3 min excerpts from pop-rock music tracks was created. Short-term audio levels (such as the sound pressure level which would yield the same energy to the instantaneous sound signal, within a duration of 1s, namely $L_{eq,1s}$) in pop-rock music may fluctuate considerably across tracks, and along the time-course of any single song (e.g., between different chorus, verse, or bridge parts of a song, albeit much less than in other musical genres). Additionally, dynamic ranges across frequencies (especially for frequencies

<200 Hz and >3-4 kHz) also show significant variability, as observed by measurements of the long-term average spectrum (LTAS) of different music tracks (Hill et al., 2021; Le Prell et al., 2011). Level variation between consecutive parts (whose durations may be of the order of several seconds, mostly following the musical structure of the track, e.g., intro, verse, chorus, etc.) of music tracks is about 5 dB. The average level (i.e., over the whole duration of a track) between different music tracks may differ by 15 dB. The dynamic range of within bands of the LTAS of a track is also typically around 15 dB.

To achieve a relatively low variability of exposure time (e.g., “constant” level; Le Prell et al, 2011) under such variations of level, we followed a low-moderate nonuniform compression scheme of the audio material which would avoid over-compressing (Réveillac, 2017). The nonuniform compression scheme comprised of a 3:1 compression of peak levels ($L_{eq,1s}$ > -6 dB_{max}) and a 2:1 compression over the rest (the lowest parts) of the dynamic range, for each music track, with appropriate makeup gain value (again, applied individually on each track). Thus, we achieved a roughly constant average level between tracks, and at the same time, we avoided severe distortions due to clipping. Finally, the mastering level of the whole audio material was adjusted to obtain an average level of 100 dBA, measured on a BK4128 HATS with TDH-39 headphones. The BK4128 HATS microphones' calibrations were conducted using a BK4228 pistonphone calibrator. The BK4128 output was continuously sampled at 44.1 kHz using a National Instruments USB-6251 and LabView 2010 software, and voltage values were converted to SPL using the HATS microphone sensitivity values obtained from the calibration. Subsequently, the

whole length of the sampled audio material was analysed by computing the Leq SPL at 1 s consecutive intervals, from which all audio material statistics were calculated. During a small informal pilot study, conducted with five naive normal-hearing listeners prior to the main investigation, the audio material was delivered in lower intensity, and the above compression scheme achieved high acceptability of the processed audio without any complaints regarding sound quality compared to the original material. **Figure 5.1** shows the evolution of instantaneous SPL of the 15-min long audio material. **Figure 5.2** shows the distribution of SPLs and **Figure 5.3** shows the cumulative distribution of SPL. **Table 5.1** shows the main statistics of the SPL distribution. **Figure 5.4** shows the 95th, 50th and 5th percentiles of the 1/3-octave LTAS of the audio material.

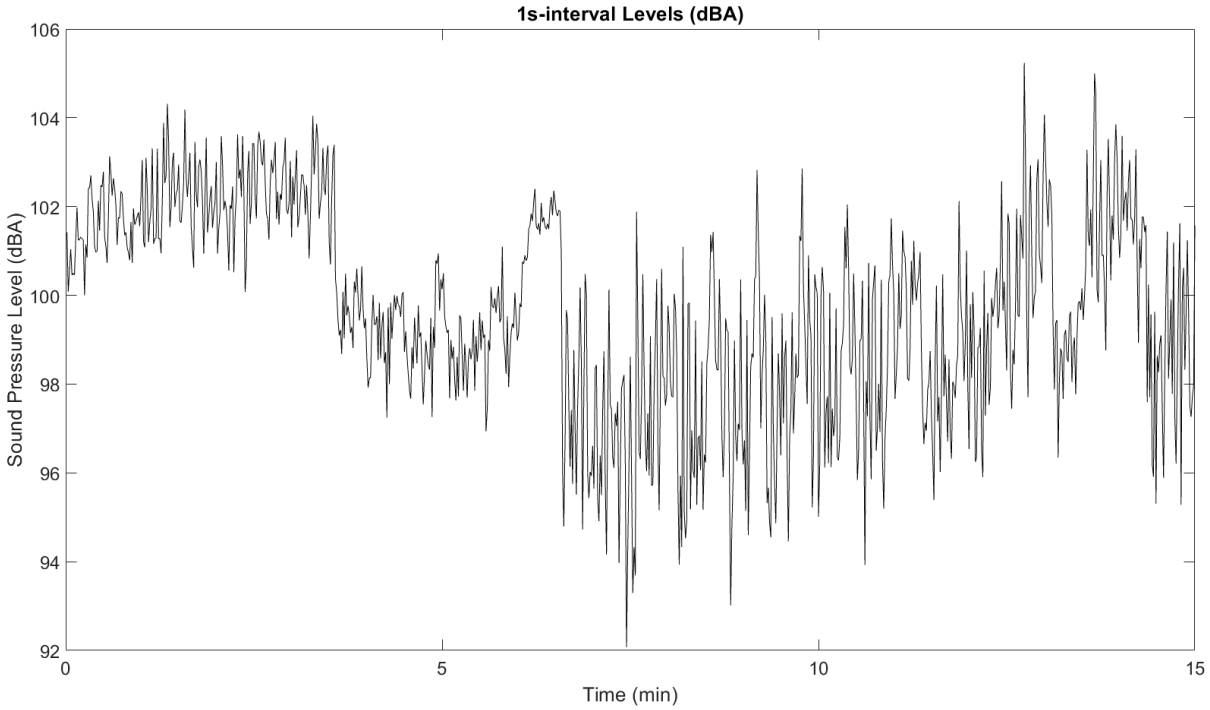


Figure 5.1. Sound pressure levels (dBA) of 15 min of the audio material, measured on a BK4128 HATS with TDH-39 headphones. The levels reported here are HATS measured levels. The free field equivalent level (FFE) transformation, used to

adjust for individual ear canal amplification, is conservatively assumed to be 5 dB, although individual measurements are often greater than 5 dB.

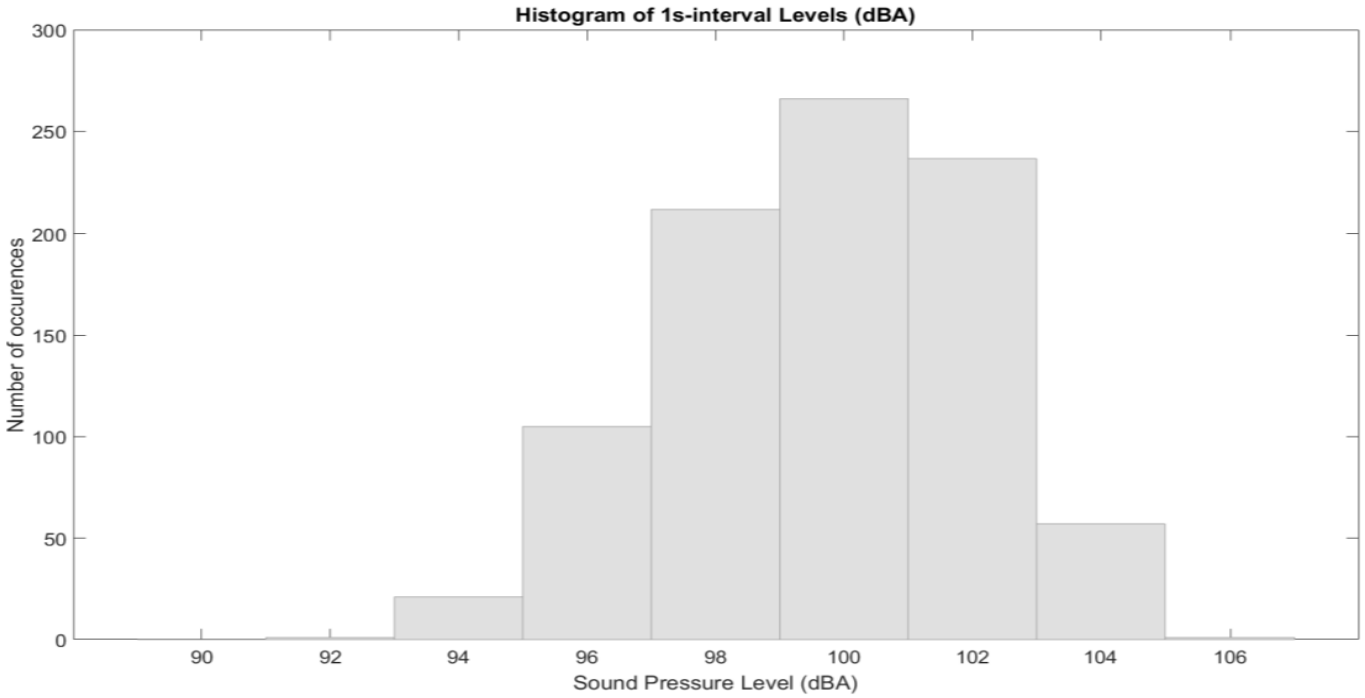


Figure 5.2. Histogram of SPL (dBA) of the 15-min audio material.

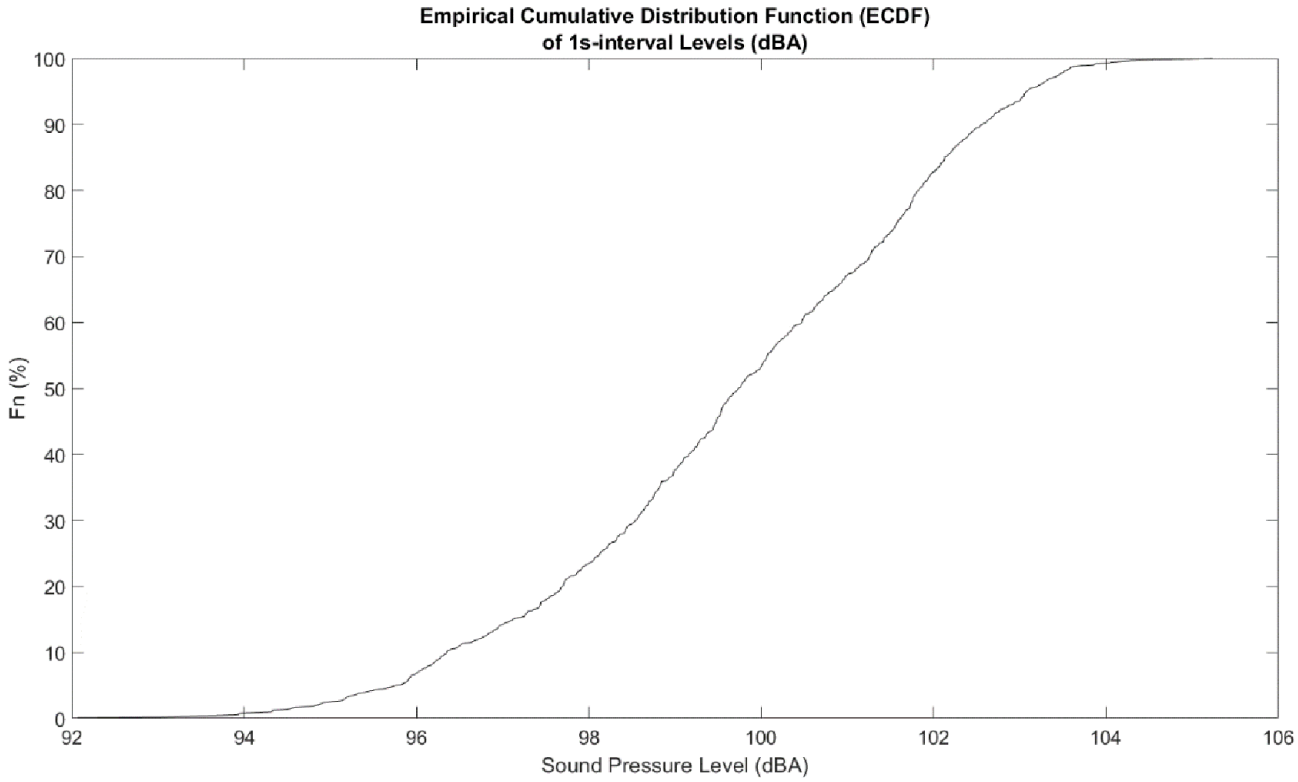


Figure 5.3. Empirical cumulative distribution of SPL (dBA) of the 15-min audio material.

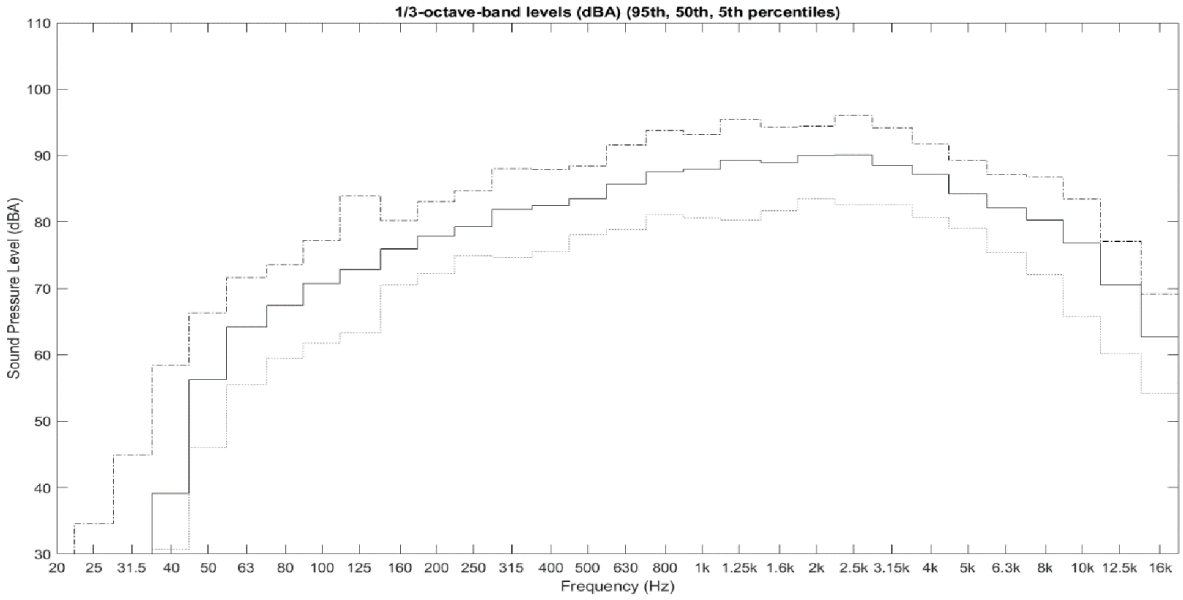


Figure 5.4. 1/3-octave LTAS of the 15-min audio material.

Table 5.1: Leq,1s SPL (dBA) statistics of the 30' audio material

Mean	Median	SD	IQR
99.68	99.73	2.29	3.38 (98.12-101.5)

If the 100 dBA exposure level was chosen, then the initial 15min of the 100 dBA audio material was played, while in the 97 dBA exposure the full 30min length of the audio material was used.

5.2.2. Participants

Participants were recruited by the 1st Otorhinolaryngology Department of the National and Kapodistrian University of Athens and underwent medical and hearing loss history, otomicroscopy, tympanometry, and PTA. Screening PTA was performed according to the British Society of Audiology (2018) guidelines. The inclusion criteria included no self-reported current or previous history of hearing loss, no loss of speech perception, tinnitus or other hearing disorder, no other ear pathology (no abnormality in otoscopy or tympanometry, pure tone thresholds within normal limits in both ears (≤ 25 dB HL for 0.5 – 8 kHz) and symmetric across ears (no more than 15 dB difference between the ears at any frequency). Candidates with middle ear pathology (abnormal otomicroscopy or tympanometry), with previous or current inner ear pathology, asymmetry in pure tone audiometric thresholds >15 dB at any of the tested frequencies, radiotherapy or ingestion of ototoxic substances during the last 12 months, or exposure to hazardous noise during the last 72 h were excluded. Tympanometry was considered normal when middle ear pressure values ranged from −140 to +40 daPa, peak compensated static acoustic admittance from 0.3 to 1.8 ml and acoustic equivalent volume (Vea) from 0.8 to 2.1 cm (Le

Prell et al., 2012). Candidates fulfilling criteria received oral and written explanations of the study purpose and procedures and were asked to sign the relevant consent form.

5.2.3 Participants' assessment

Included participants underwent:

(1) Medical and hearing loss history: Lifetime noise exposure was evaluated using a recently developed instrument that attempts to estimate lifetime recreational, occupational and fire-arm noise exposure based on self-report, the Noise Exposure Structured Interview (NESI; (Guest et al., 2018).The full interview lasted 10 min on average, while the collected data concerned participants' age, sex, and NESI units.

(2) Hearing testing:

a. PTA and extended high frequency PTA using Interacoustics Affinity audiometer (EN 60645-1, ANSI S3.6), and TDH39 and HDA 300 headphones (for >8 kHz). Findings of previous studies show that more pronounced TTS may be found at 1-8 kHz (Kil et al., 2017; Le Prell et al., 2012, 2016), while extended high frequency PTA has been associated with the early diagnosis of NIHL (Mehrparvar et al., 2014; Schmuziger et al., 2007). Hence, tested frequencies in our study were 1, 3, 6, 8, 10, and 12.5 kHz [with the addition of 4 kHz after the further manipulation of our audio material (see below)]. The signal level was varied in a 6 dB

down, 2 dB up manner (Kil et al., 2017; Le Prell et al., 2016). The whole procedure lasted approximately 5 min. Collected data included PTA thresholds before and after music exposure per frequency.

b. DPOAEs using Interacoustics Titan. The frequency ratio of primary tones, f1:f2, was 1.22, and their levels were 65 and 55 dB SPL, respectively. Maximum residual noise was set to 30 dB SPL. The geometric mean of the pair was swept from 8 to 1 kHz. Data collection was terminated after three such sweeps, lasting 1 min. The DPOAE-related endpoints were the DP amplitude before and after music exposure per frequency.

5.2.4. Procedure

All participants were advised not to expose themselves to further high-level noise or music 72 h prior and during study procedures. At the day of the experiment, participants had to confirm their adherence to this advice, otherwise their participation would be postponed to another day. A medical history was taken and baseline PTA and DPOAE testing occurred just before music exposure. Participants were subsequently exposed to the audio material at 100 dBA or 97 dBA (exposures that both provide an equivalent daily noise dose based on the 3-dB exchange rate), according to their preference for 15 min or 30 min respectively. The audio material was provided by means of headphones (Focal Spirit Professional) to the left ear connected to the same laptop, always under the same conditions, in an audiological booth. The

contralateral (right) ear was sealed. Caution was taken not to exceed the overall acoustic energy that would result in PTS if repeated, according to previous studies' findings and national and European legislation. Immediately after music exposure, participants were asked to rate their comfort level during the experimentation and the degree of aural fullness, on scales from 1 to 10. For safety reasons, they were also asked if they experienced any tinnitus or other symptoms. Two minutes after the end of the music, they underwent DPOAE testing. At 3 – 4 min after the end of music exposure, PTA was performed. PTA and DPOAEs were repeated later, within 24h, to ensure that PTA and DPOAEs returned to baseline. All post-exposure PTA and DPOAEs testing was conducted unilaterally (left ear). In our study, the return of threshold to within 4 dB of baseline was used as a conservative cut-off point for clinically significant PTA threshold change in healthy adults. The same cut-off point has been used in previous studies using the same PTA methods (Kil et al., 2017). However, this was not used as a criterion for categorical data analysis, but only for purposes of safety characterization (i.e., PTS identification).

5.2.5. Statistical analysis

A three-level linear mixed effect model was used to reflect the multilevel structure of data (repeated measurements of PTA thresholds and DPOAE levels at different frequencies, before and after exposure, within the same participant) of cochlear regions corresponding to tested frequencies nested into participants. Age, Sex, NESI units, and the interaction between Exposure and Frequency were modelled as fixed factors. Random effects were modelled by

a random intercept of Frequency within Participant to account for individual differences in thresholds for each frequency for each participant, before exposure. A random slope of Exposure within Participant was also fitted to account for differences in the magnitude of the effect of music exposure for each individual.

Statistics were computed using R statistical language. The linear mixed models were created using the lme4 package and fitted by the restricted maximum likelihood method and t-tests using Satterthwaite's method (Bates et al., 2015). Model selection was based on backward stepwise regression. Deviation from homoscedasticity or normality was verified by visual inspection of both residual and random effect plots, and the Kolmogorov-Smirnov test. Analysis of variance tables (using the Kenward–Rogers method for estimating degrees of freedom), marginal means and significance testing of their differences (using Tukey's HSD method to adjust p-values for multiple comparisons) were calculated via the lmerTest package.

The structural equation of the final model selected was:

$$[\text{PTA threshold or DPOAE level}]_{tij} = \beta_0 + \beta_1[\text{Exposure}]_{tij} + \beta_2[\text{Frequency}]_{tij} + \beta_3[\text{Exposure}] \times \text{Frequency}]_{tij} + u_{0j} + u_{0i|j} + u_{1i} \times [\text{Exposure}]_t + \varepsilon_{tij}$$

where, u_{0j} is the random intercept for Participant (capturing individual differences in threshold for each participant, before exposure), u_{1i} is the random slope of [Exposure] for each Participant (capturing differences in the magnitude of the effect of music exposure for each individual irrespective of frequency),

$u_{0i|j}$ is the random intercept of Frequency nested within Participant (capturing individual differences in threshold for each frequency for each participant, before exposure), and ε_{tij} is the residual (unexplained) error for each participant.

5.3. Results

5.3.1. Population

Seventeen volunteers with normal hearing participated to the study. Initially, audio material was tested in eight volunteers that fulfilled the inclusion criteria [four females; median age = 31 years (IQR = 4.25); $PTA_{1\text{-}8kHz}$ = 4 dB HL and $PTA_{1\text{-}12.5kHz}$ = 2.63 dB HL]. DPOAE average amplitudes for these eight volunteers were 7.14 dB SPL (1 kHz), 13.16 dB SPL (1.5 kHz), 10.11 dB SPL (2 kHz), 5.82 dB SPL (3 kHz), 7.74 dB SPL (4 kHz), 1.28 dB SPL (6 kHz), and -7.83 dB SPL (8 kHz). The range of lifetime noise exposures was 1.46 to 66.93 NESI units (median = 13.48, IQR = 8.3). One NESI unit is equivalent to one working year (2080 hrs) of exposure to 90 dBA. Two participants were exposed to 97 dBA for 30 min and six participants were exposed to 100 dBA for 15 min, according to their preference. Although TTS larger than 4 dB was observed in six out of eight participants for at least one frequency, a large variation in affected frequencies was observed (**Supplementary Material 5.1**).

Music material was then further manipulated digitally to adjust levels across the different frequency bands. Fourteen adults (nine newly recruited and five that were also exposed to the initial audio material; seven females; median age = 31 years; IQR = 5 years) met the inclusion criteria. Their PTA average before exposure was 3.87 dB for 1-8 kHz and 4.44 dB for 1-12.5 kHz. DPOAE average

amplitudes for these fourteen volunteers were 3.34 dB SPL (1 kHz), 8.35 dB SPL (1.5 kHz), 6.95 dB SPL (2 kHz), 4.33 dB SPL (3 kHz), 5.16 dB SPL (4 kHz), 3,10 dB SPL (6 kHz), and -6.19 dB SPL (8 kHz). NESI units ranged from 1.46 to 219.90 (median = 12.40, IQR = 29.92). All 14 participants were exposed to 100 dBA for 15 min, according to their preference (**Supplementary Material 5.1**). Their data were included in our analyses.

5.3.2. TTS in standard and extended high frequency PTA.

TTS larger than 4 dB was observed in at least one frequency in six out of eight participants in the first trial, and in twelve out of fourteen participants in the second one (**Supplementary Material 5.1**). Time of baseline measurements ranged between 08.00 and 18.30, so four participants had to return the following day to repeat the hearing test and assess recovery. Estimated marginal means of PTA threshold for each frequency before and after exposure for the 14 participants of trial 2 are presented **Figure 5.5A** and **Table 5.2**. There is a statistically significant PTA threshold shift of 7.43 dB at 6000 Hz [($t_{(114.9)}$ = -4.31, 95% CI: (4.06, 10.80), p < 0.001)]. For the PTA analysis, the Akaike information criterion (AIC) for the null and the selected model were 2006 and 1980 respectively ($x^2_{(20)}$ = 66.53, p < .001). The adjusted and conditional intraclass correlations (ICCs) for the selected model were 0.829 and 0.718, respectively. For particular participants, for some frequencies a reduction of threshold was observed following music exposure (up to 14 dB for standard audiometry and up to 16 dB for extended high frequency audiometry). These data were included in the analysis. Within 24h, all participants' pure tone thresholds recovered at all tested frequencies (within 4 dB from baseline, see

Figure 5.6, and **Supplementary Material 5.2 and 5.3**). There was statistically significant decrease of pure tone thresholds when compared to baseline ones at 8000 Hz [4.57, $t_{(99.5)}$ = 2.58, 95% CI: (1.02, 8.11), p = 0.03)], 10000 hHz [5.57, $t_{(99.5)}$ = 3.15, 95% CI: (2.03, 9,11), p = 0.006)], and 12500 Hz [5.43, $t_{(99.5)}$ = 3.06, 95% CI: (1.89, 8.97), p = 0.006). After Bonferroni correction for multiple comparisons only the 10000 Hz statistical significance survived.

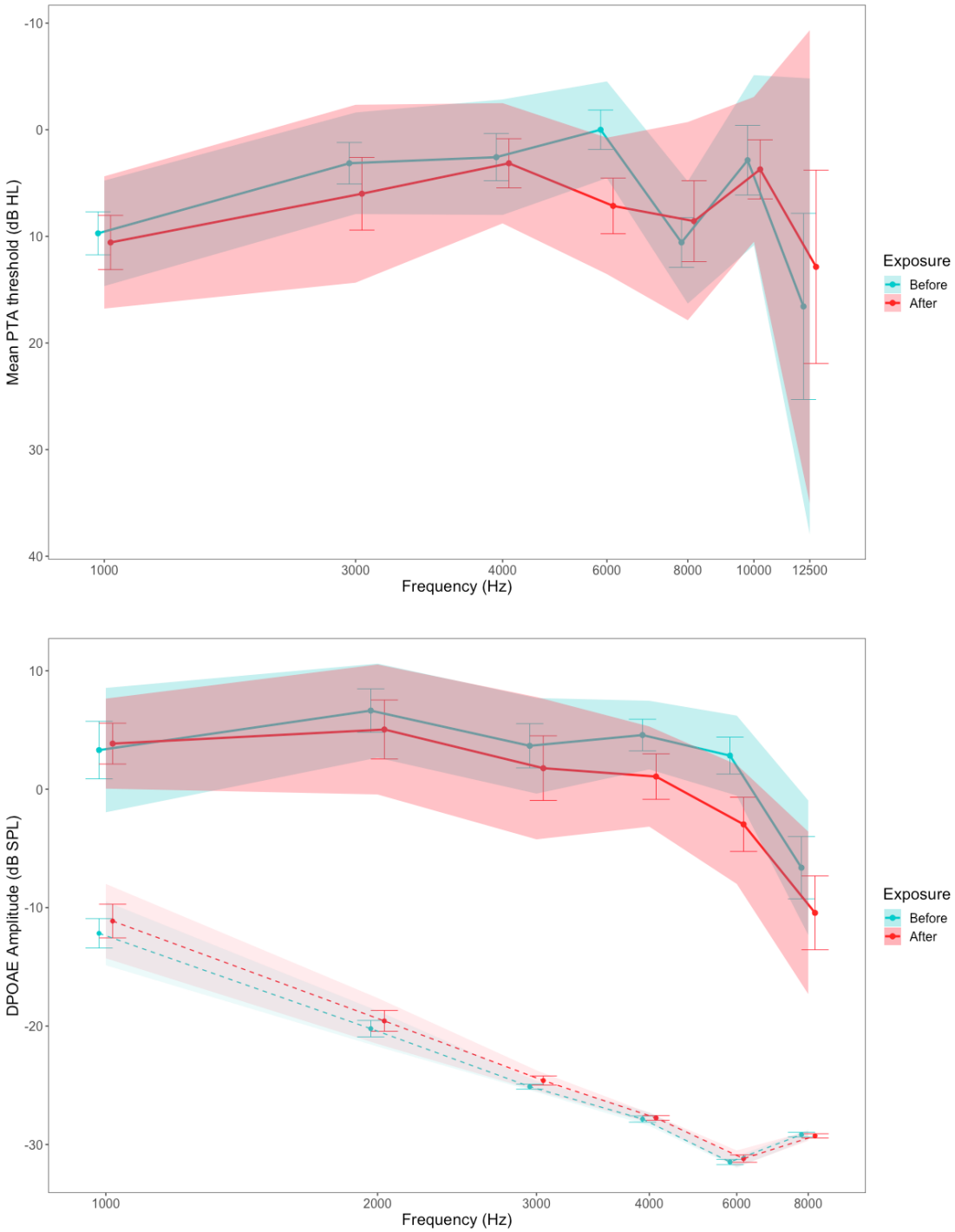


Figure 5.5. Participants' mean pure tone audiometry thresholds (A) and DP amplitudes and noise floor levels (solid and dashed lines respectively) (B) before and immediately after music exposure per frequency. Error bars show 1 standard error and the shaded area the 95% confidence intervals.

Table 5.2. Estimated marginal means of PTA threshold and DPOAE temporary amplitude shifts for each frequency.

Frequency (Hz)	Estimated marginal means of PTA temporary thresholds shifts (dB HL) (95% CI)	p-value	Estimated marginal means of DPOAEs temporary amplitude shifts (dB SPL) (95% CI)	p-value
1000	0.143 [−3.26, 3.54]	.99	1.66 [−0.22, 3.56]	.0795
2000	—	—	−1.54 [−3.44, 0.362]	.1223
3000	−3.00 [−6.4, 0.4]	.19	−1.66 [−3.56, 0.24])	.0833
4000	−2.71 [−6.11, 0.68]	.26	**−2.55 [−4.45, 0.65]**	**.0087***
6000	**7.43 [4.06, 10.80]**	**.0001*****	**−4.97 [−6.87, −3.07]**	**< .0001*****
8000	−0.29 [−3.69, 3.11]	.98	**−3.14 [−5.04, −1.24]**	**.0014****
10000	−0.71 [−2.69, 3.59]	.91	—	—
12500	−2.86 [−0.54, −6.26]	.26	—	—

Bold font corresponds to statistically significant values.
* Statistically significant at the .05 level, ** statistically significant at the .01 level, **statistically significant at the .0001 level

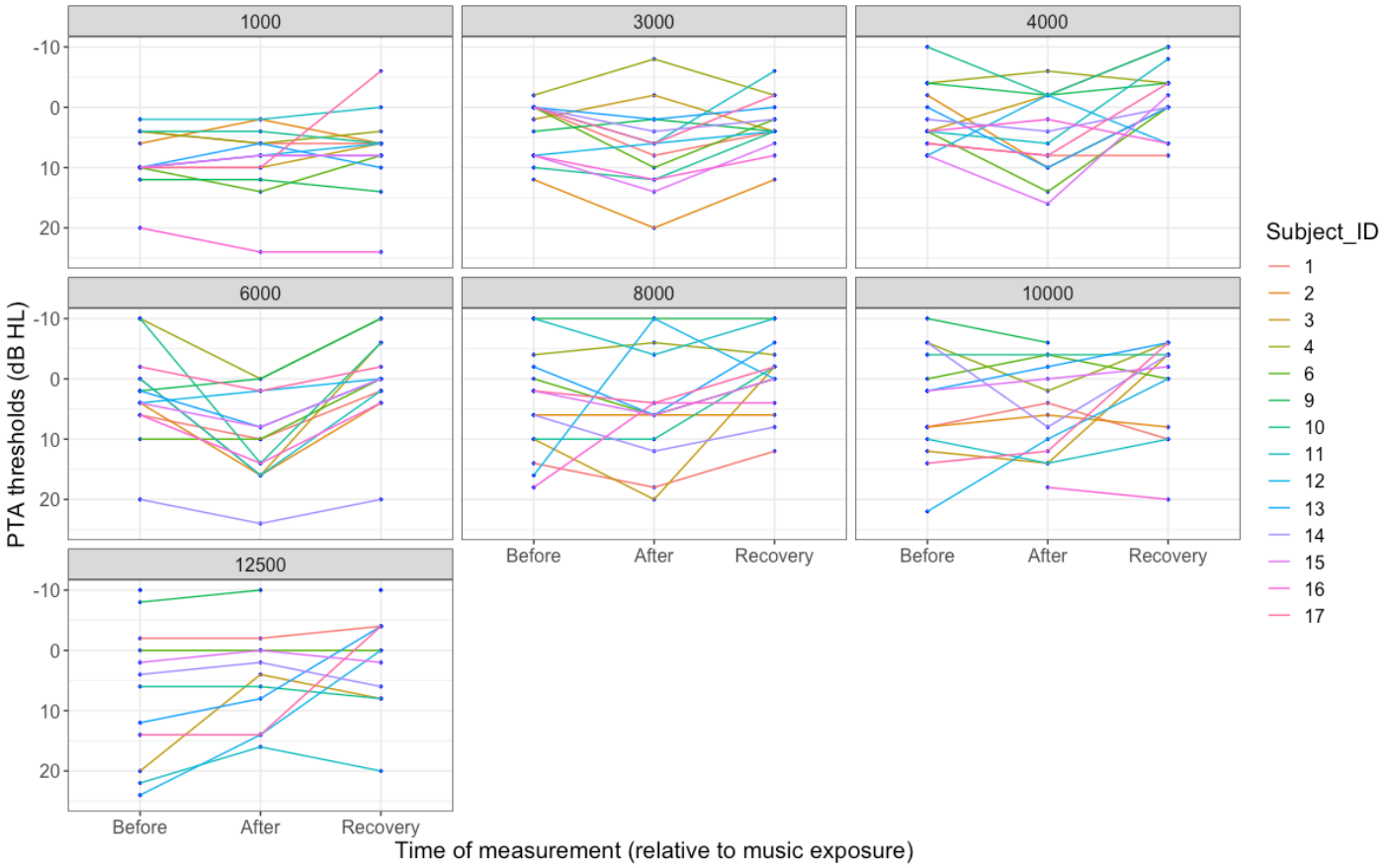


Figure 5.6. Pure tone audiometry threshold change, per frequency, per subject.

5.3.3. DP amplitude shift

DP amplitude shift was reliably observed in all 17 participants in at least one frequency. DP amplitude shifts for the 14 participants of trial 2 per frequency are presented in **Figure 5.5B and Supplementary material 4**. The difference between the estimated marginal means of DPOAE levels for each frequency before and after exposure are reported in **Table 5.2**. For the DPOAE analysis, the AICs for the null model and the selected model were 1060 and 1017 respectively ($x^2_{(6)} = 54.54$, $p < .0001$). Adjusted and conditional ICCs for the selected model were 0.90 and 0.64 respectively. A deviation from normality was noted in both tails of the residual distribution, but not of the random effects, in the DPOAE data. Linear mixed models are considered robust regarding distribution assumptions, but the estimates, although unbiased, may be imprecise (Schielzeth et al., 2020).

There was a statistically significant DP amplitude shift of -2.55 dB at 4 kHz [($t_{(92)} = 2.68$, 95% CI: (-4.45, -0.65), $p = .0087$)], -4.97 dB at 6 kHz [($t_{(92)} = 5.23$, 95% CI: (-6.87, -3.07), $p < .0001$)], and -3.14 dB at 8 kHz [($t_{(92)} = 3.30$, 95% CI: (-5.04, -1.24), $p = .0014$)]. Although no formal DPOAE test-retest reliability analysis was performed, the 90% CIs of the Standard Error of Measurement (Demorest & Walden, 1984) between the pre-exposure and recovery DP amplitudes for all frequencies were calculated. These were narrower than those reported by a recent meta-analysis on DPOAE test-retest variability (Reavis et al., 2015). We are hence confident that no permanent DP amplitude shift occurred.

5.4. Discussion

TTS has long been used as an early audiometric marker of traumatic noise exposure, since it may be indicative of sound energy high enough to create cochlear insult, and at the same time it can safely be tested in both experimental and observational studies (Lindgren & Axelsson, 1983; Ryan et al., 2016). Nevertheless, its use as outcome measure has been limited by its high variability. Human studies have shown that similar exposures may lead to different degrees of TTS or affect different frequencies (Kil et al., 2017; Kramer et al., 2006; Le Prell et al., 2011, 2016; Lee et al., 1985; Lindgren & Axelsson, 1983). This variability may be linked with differences in the methods used, or participants' individual vulnerability to noise. Use of one single standardized and validated exposure and hearing assessment paradigm could eliminate part of this variability. In this technical report, we present the development and validation of an experimental model that safely creates a measurable temporary cochlear dysfunction as evidenced by TTS at 6 kHz. In our study, although the degree of recovery showed variability per individual participant and per frequency, the average recovery threshold shifts showed uniform directionality (elevation in comparison to the baseline, see Supplementary material 5.2 and 5.3). There was statistically significant decrease of pure tone audiometry thresholds at 8000, 10000 και 12500 Hz, but after correction only the 10000 Hz statistical significance survived. This phenomenon may be explained by a learning effect that may occurred after the first two audiograms. It could also be a result of the fact that participants were aware that their hearing was being tested to confirm full recovery, and this knowledge may have increased their attention and alertness during the procedure.

Our paradigm had a shorter duration than previous ones that were effective in demonstrating TTS. Le Prell et al. (2012; 2016) exposed participants to music for 4 hours at coupler levels of 97-100 dBA and Kramer et al. (2006) for 2 hours at 92.5 to 102.8 dBA (free field, mean exposure levels = 98.1 dBA). Other short paradigms did not create any clinically or statistically significant TTS: Krishnamurti and Grandjean (2003) exposed participants to music of 90 dB SPL (estimated in-ear levels) for 20 minutes and detected TTS of 1-6 dB, but no change in participants' DP amplitudes. Reduction of exposure time may lead to higher recruitment and lower drop-out rates and save resources.

Our paradigm was efficient in creating temporary cochlear dysfunction that was evident in PTA and DP amplitude shift in all participants. We calculated mean TTS value and mean DP amplitude shift per frequency, and we analyzed our results by a mixed-effects linear model to take into account the hierarchical structure of data and the repeated measurement of the outcome variables at each level. The frequency region with higher TTSs was 3-6 kHz, while the maximum TTS obtained in our experiment was 24 dB (at 6 kHz). The same frequencies were also those most affected by noise and music in previous studies (Kramer et al., 2006; Krishnamurti & Grandjean, 2003; Le Prell et al., 2012; Ryan et al., 2016). Although our exposure lasted only 15 min and included lower levels of music than other studies, our maximum TTS was slightly higher than those from other studies assessing music-induced TTS. Exposure to music at 100 dBA coupler level for 4h was reported to cause

immediate TTS up to 13 dB (Le Prell et al., 2012; Ryan et al., 2016), while in another paradigm of 2h of music exposure at a nightclub (93-103 dBA) maximum TTS of 14 dB was found at 4 kHz (Kramer et al., 2006). Mean TTS and DP amplitude shifts in our study were compatible to those reported in previous studies. No TTS was detected in EHF-PTA. This finding is in agreement with previous studies (Le Prell et al., 2012).

Apart from efficient, our paradigm is also safe. Our exposure "dose" was lower than the upper Leq 15-min sound levels limit during a music event according to WHO guidelines (World Health Organization, 2022). We conservatively estimate that the delivered-to-the ear sound levels are 5 dB lower than would be case for the same levels specified in the free field (Shaw, 2005), which practically means that participants were exposed for 15 or 30 min to free-field equivalent music of 95 dBA or 92 dBA (less than 1/3 of the maximum permissible dose) respectively . Moreover, we asked them to avoid exposure to noise > 80 dB SPL three days before, and 7 days after the music exposure, so that their weekly exposure dose would remain lower than the weekly permissible dose, which according to the recent WHO guidelines equals 18.75 min per week at 101 dBA or 37.5 min per week at 98 dBA (World Health Organization & International Telecommunication Union, 2019). Previous rodent (mice) studies using cochlear functional assays and confocal imaging have shown that noise exposures capable of inducing temporary pure tone threshold elevations of ~40–50 dB 24 hours post-exposure may lead to (permanent) rapid synaptic deficits and decreased evoked potential amplitude (Kujawa & Liberman, 2009, 2015). Researchers hypothesize that in humans a similar

neurodegenerative noise-induced phenomenon would add to difficulties in hearing in noisy environments, tinnitus, hyperacusis, and other perceptual anomalies commonly associated with inner ear damage (Kujawa & Liberman, 2009). Although many studies have attempted to identify signs of cochlear synaptopathy in humans, methods and findings across studies present high heterogeneity (Bramhall et al., 2019). It is also proven that much higher levels are required to produce cochlear synaptopathy to primates than in rodents (Valero et al., 2017). Furthermore, in all previous study paradigms, levels of exposures were higher and/or longer than ours (Bramhall et al., 2019; Wang et al., 2021). In a recent commentary about justification of modification of current regulation of occupational noise exposure based on research findings on noise-induced cochlear neuropathy in rodents, authors conclude that these findings cannot be directly translated in humans, and that humans seem to be less susceptible to TTS and probably cochlear synaptopathy (Dobie & Humes, 2017). Levels and duration of exposure chosen in our paradigm, based on methodological aspects, ethical considerations, and audiometric results of previous studies, were considered tolerable by all participants. Most participants characterized the listening experience as comfortable, answering 6 or higher to the question “How comfortable was listening to this music in this setting for you?”. Moreover, although all participants presented measurable and reliable temporary changes of their auditory function, no PTS or other permanent hearing disorder (i.e., tinnitus) was observed in any of them. This study hence provides some assurance for the future reproduction of the same paradigm in larger samples. Nevertheless, if, in the future, a clinical test is proven sensitive to cochlear synaptopathy and neurodegeneration in humans,

this should be included as part of the pre- and post-exposure assessments to ensure synaptic and neural integrity.

One of the limitations of our study is the fact that no formal test-retest reliability analysis for DPOAEs was conducted. However, the 90% CIs of the Standard Error of Measurement between the pre-exposure and recovery DP amplitudes for all frequencies were calculated and were found to be narrower than the test-retest variability reported by the meta-analysis of Reavis et al (2015). Although measurements were performed in a sound-treated room, in compliance with the ANSI/ASA S3.1-1999 (R2018) standard for environmental noise, no real-time noise monitoring was employed during the measurements. Thus, we cannot exclude the possibility of variability, especially at lower frequencies [as can also be indirectly seen by the fact that the DP noise floors were higher and more varying at lower frequencies (e.g., 1 kHz)]. This may possibly also explain the larger PTA shifts that were observed in some of our participants compared to the expected test-retest reliability limits of +/- 5 dB, as commonly assumed in PTA measurements (Le Prell et al., 2012; Ryan et al., 2021; Schlauch & Carney, 2007). However, observations of larger test-retest differences may be observed by chance, as shown by Schlauch and Carney (2007). The authors estimated that, when thresholds of six frequencies are measured, 14% of the people tested would be expected to have at least one threshold differing by 15 dB or more. To conclude, there are some extreme values in our data. However, as the analysis has to take into account the above factors in calculating the F statistic, we chose not to exclude these extrema. Additionally, the use of mixed

effect models also takes into account intrasubject variability for the estimation of expected mean values.

5.5. Conclusion and implications

A brief, safe, and pleasant music exposure and testing paradigm, showing consistent and reliable effects on PTA thresholds and DP amplitudes for adults with normal hearing, was created. In the future, our paradigm may be used to further assess TTS degree and time of recovery function. It could also be useful in studies that correlate TTS with participants' characteristics and habits, with progressive and permanent types of hearing loss, or with subjective impressions such as listening comfort and post-exposure aural fulness or tinnitus. Finally, it may be a useful instrument for measuring objectively the effect of otoprotective agents or ear protection devices.

Chapter VI

Blood prestin levels following music exposure that induces temporary threshold shifts: a pilot study.

Abstract

Objectives: To determine if blood prestin level change after exposure to high levels of music, and if this change is associated with music-induced temporary threshold shift (TTS) and/or a decrease in distortion product otoacoustic emission (DPOAE) amplitude.

Methods: Participants were exposed to pop-rock music at 100 dBA for 15 minutes monaurally through headphones. Pure tone audiometry, DP amplitude, and blood prestin level were measured before and after exposure.

Results: Fourteen adults [nine women; age range: 20-54 years, median age=31 (IQR=6.75)] with normal hearing were included in the study. Prestin level seemed to increase shortly after exposure to music, then return to baseline within 1 week. All participants presented TTS or a decrease in DP amplitude in at least one frequency after music exposure. There was a statistically significant average threshold elevation at 4 minutes post-exposure. Statistically significant DPOAE shifts were observed at 4 kHz and at 6 kHz at 2 minutes following exposure. Mean baseline serum prestin level [mean: 140.00 pg/ml, 95% CI: (125.92, 154.07)] progressively increased following music exposure, reaching a maximum at 2 hours [mean: 158.29 pg/ml, 95% CI: (130.42, 186.66)] and returned to pre-exposure level at 1 week [mean: 139.18

pg/ml, 95% CI: (114.69, 163.68)]. However, after correction for multiple comparisons, mean prestin level showed no statistically significant increase from baseline at any timepoint. No correlation between maximum blood prestin level change and average TTS or DP amplitude shift was found. However, in an exploratory analysis, TTS at 6 kHz (the frequency at which maximum TTS occurred) decreased significantly as baseline blood prestin level increased.

Conclusions: The results suggest that blood prestin level may change after exposure to high-volume music, although statistical significance was not reached in this relatively small sample after correction. Baseline serum prestin level may also predict the degree of one's TTS. These findings thus suggest that the role of baseline serum prestin level as a proxy marker of cochlear susceptibility to music exposure should be further explored.

6.1. Introduction

Overexposure to noise and music is one of the primary causes of sensorineural hearing loss (Di Stadio et al., 2018a; Śliwińska-Kowalska & Zaborowski, 2017b). MIHL may affect a wide range of people: people attending music events or using personal listening devices (Kähäri et al., 2011; Welch & Fremaux, 2017), music students and tutors, and musicians and professionals of the music industry (Di Stadio et al., 2018a). It has been estimated recently that 1.1 billion teenagers and young adults are at risk of hearing loss due to overexposure to recreational noise (le Clercq et al., 2016).

Although due to ethic constrictions, studies on the permanent effects of music should be strictly observational, temporary - and thus reversible - changes, can be assessed at an experimental level. Although some authors have expressed their concerns on the conduction of interventional TTS studies (Henderson et al., 2006; Themann et al., 2015; Verbeek, 2015), assessing temporary hearing changes after exposure to noise or music under conditions safe for the participants is still considered a valuable way to investigate the mechanisms of noise damage in humans reliably (see Discussion in Chapter IV and V). To date, the most commonly assessed effects of noise overexposure are temporary threshold elevation in PTA, TTS, and the temporary decrease in DP amplitude. Both outcomes have been studied previously in observational and experimental settings (Keppler et al., 2010; Kil et al., 2017; Kraaijenga et al., 2018; Le Prell et al., 2012). Although these temporary changes may relate to different pathophysiological mechanisms than the permanent ones, such as PTS, temporary changes have been proven to serve as a useful proxy for permanent changes (Ryan et al., 2016). Repeated episodes of TTS seem to be associated with permanent hearing disorders in both rodents and humans (Wang & Ren, 2012; Ryan et al, 2016), and particular otoprotective agents may have an effect on both TTS and PTS (Kil et al, 2017; Pourbakht & Yamasoba, 2003). On the other hand, it should be noted that TTS as an outcome measure in clinical studies may present high variability. Previously published human research has indicated that comparable exposures can result in varying level of TTS or impact distinct frequencies (Kil et al., 2017; Le Prell et al., 2011, 2016). Combining TTS with another outcome measure, would add value to the findings of any clinical study. Otoacoustic emissions (especially DPOAEs) have

also been proven valuable for the detection of temporary or permanent noise-induced hearing changes (B. Davis et al., 2005; Engdahl, 1996; Kramer et al., 2006). Previous evidence has established that DPOAEs elicited with unequal f2/f1 levels are considerably more sensitive to reductions in emission levels induced by brief exposure to noise (Sutton et al., 1994).

Apart from TTS and decrease of DP amplitude, newly proposed sensorineural hearing loss markers have emerged (Hana & Bawi, 2018; Parham, 2015; Parham et al., 2019; Solis-Angeles et al., 2021). Prestin is an OHC protein, responsible for electromotility (Liberman et al., 2002), which can be detected in the blood by means of enzyme-linked immunosorbent assay (ELISA). It has also been found in cardiomyocytes, where it is also considered to be responsible for the amplification of cardiac motor functions (Zhang et al., 2021). The way prestin exits the cochlea and enters blood circulation remains unknown. The fact that prestin molecules have been found in phagosomes of supportive cells, suggests the involvement of phagocytosis (Abrashkin et al., 2006). It is also possible that due to its small size, prestin may cross the labyrinthine-blood barrier with no involvement of other cellular mechanisms. To date, this information is not verified in humans (Parham et al., 2019). Blood prestin level was found to be decreased in participants with sensorineural hearing loss due to chronic exposure to lead, and acutely increased after a dose of more than 80 mg of cisplatin (Jalali et al., 2022; Solis-Angeles et al., 2021). Moreover, blood prestin level has previously shown to have a weak negative correlation with age ($r = -0.350$, in 72 adults, 18-82 years old) (Parker et al., 2022a), and a moderate one with average daily noise exposure levels ($L_{Aeq,8h}$(dB), measured by means of noise dosimeters for 3 weeks in 30

adults, 18-24 years old, r = −0.455) (Parker et al., 2022b). This decline in serum prestin level has been linked with two hypotheses: 1. The Hidden OHC Damage Hypothesis: a lower number of intact OHCs with normal prestin production and turnover results in lower prestin level in circulation; 2. The Environmental Downregulation Hypothesis, according to which prestin expression is downregulated as part of the natural dynamics of OHCs responding to loud environmental conditions and the decreased need for cochlear amplification in loud environments (Parker et al., 2022b). In addition to these chronic changes, blood prestin level may vary after a single exposure; in a rodent study serum prestin level increased immediately after 2 hours exposure to noise and then decreased to near or below baseline at 14 days (Parham et al., 2019). However, this temporal pattern has never been assessed in humans.

The study presented here is the first experimental study to evaluate blood prestin level in adults who have been exposed to music, and their association with other markers of temporary hearing dysfunction (TTS and decrease of DP amplitudes). Based on previous evidence showing a temporal pattern of blood prestin level after noise exposure, we hypothesized that:

H1. Prestin concentration changes over time immediately after exposure to music (95 dB FFE).

Moreover, and to confirm that our music exposure paradigm is efficiently creating temporary cochlear dysfunction, we hypothesized that:

H2a. PTA threshold (average for higher frequencies ≥ 10 kHz) is different in adults with normal hearing before and after music exposure.

H2b. PTA threshold (average for lower frequencies ≤ 8 kHz) is different in adults with normal hearing before and after music exposure.

H3. OAEs amplitude is different in adults with normal hearing before and after music exposure.

Finally, to explore any possible correlation between serum prestin level and degree of cochlear dysfunction evident in TTS, we hypothesized that:

H4a. The degree of average pure tone TTS for higher frequencies immediately after music exposure is a predictor of the maximum serum prestin concentration shift after music exposure, adjusted for age, sex and self-reported lifetime noise exposure.

H4b. The degree of average pure tone TTS for lower frequencies immediately after music exposure is correlated to maximum serum prestin level shift after music exposure, adjusted for age, sex and NESI score.

In order to assess their time course of the blood and hearing variables, measurements were made at multiple timepoints before and after exposure.

The hypotheses, methodology, and primary statistical design were pre-registered (https://doi.org/10.17605/OSF.IO/6ZX2Shttps://osf.io/nuw6d/?view_only=dbef375ddf51471093553f27f041764e).

6.2. Materials and methods

6.2.1. Study Design

This was an experimental, longitudinal study which involved multiple measurements of the concentration of blood prestin in adults after exposure to music.

6.2.2. Setting

The study was conducted at the 1st University Department of Otolaryngology and Head and Neck Surgery of the General Hospital "Hippokrateion", Athens, Greece. Recruitment started in November 2021 and was completed in May 2022.

6.2.3. Participants

Inclusion Criteria: Adults with no hearing complaint, normal baseline PTA, and willing to listen to loud music. PTA screening was carried out following the guidelines provided by the British Society of Audiology (2018). Individuals meeting the inclusion criteria, which included the absence of self-reported current or previous hearing issues, speech perception loss, tinnitus, or other hearing disorders, as well as no observable ear abnormalities during otoscopy, no abnormal tympanometry results, no air-bone gap greater than 10 dB in PTA, and symmetrical pure tone thresholds of 25 dB HL or lower for frequencies ranging from 0.5 to 8 kHz in both ears, underwent detailed explanations of the study's purpose and procedures. They were then requested to provide both oral and written consent for participation.

Exclusion Criteria: Candidates with middle ear anomalies identified through abnormal otomicroscopy or tympanometry, those with ongoing or past inner ear disorders, individuals displaying threshold asymmetry exceeding 15 dB at any

of the tested frequencies, were excluded from participation. Moreover, since other causes that may lead to OHC dysfunction, such as ototoxic substances (aminoglycoside antibiotics, macrolide antibiotics, salicylates, chemotherapeutic agents such as cisplatin, loop diuretics, antimalarials, non-steroidal anti-inflammatory drugs, acetaminophen and aspirin in high doses, and quinine) or radiotherapy have been linked to sensorineural hearing loss of varied degree and changes of blood prestin level, candidates having been exposed to them within the past 12 months were also excluded (Naples et al., 2018; Shi et al., 2021; Solis-Angeles et al., 2021; Yang et al., 2014). Moreover, to avoid exceeding the weekly permissible noise exposure limits, participation of otherwise eligible candidates that were exposed to hazardous noise within the preceding 72 hours was postponed to a new date. Tympanometry results were considered within the normal range when middle ear pressure values ranged from -140 to +40 daPa, peak compensated static acoustic admittance fell between 0.3 and 1.8 ml, and acoustic equivalent volume (Vea) measured between 0.8 and 2.1 cm, as defined by Le Prell et al. (2012).

6.2.4. Audio Material

The audio material used in this study was previously developed and validated for its safety and efficiency in a previous pilot study (Please see more details in Chapter IV).

6.2.5. Clinical assessment and sessions

Entry to the study: All candidates were recruited by the 1st University Otolaryngology and Head and Neck Surgery Department in Hippokrateion

Hospital and underwent medical and hearing loss history taking, physical examination, otomicroscopy, tympanometry, and PTA. Those who fulfilled the inclusion criteria received oral and written explanations of the purpose and procedures of the study. If they agreed to take part, they were asked to sign the relevant consent form.

Music exposure: Participants were exposed to the audio material at 100 dBA for 15 minutes, by means of headphones (FOCAL Spirit Professional), to the left ear. The headphones were connected to the same laptop, always under the same conditions, in an audiological booth. The contralateral (right) ear was sealed.

Pre- and post-exposure assessments: Before music exposure, participants' demographics were collected (age, sex). Their lifetime noise exposure was evaluated with the help of a structured interview (noise-exposure structured interview or NESI). One NESI unit is equivalent to one working year (2080 hours) of exposure at 90 dBA (Guest et al., 2018).

DPOAEs for the left ear were measured before and at 2 minutes post-music exposure using Titan by Interacoustics. The frequency ratio of the primary tones, f2/f1, was 1.22, and their levels were 65 and 55 dB SPL, respectively. Maximum residual noise was set to 30 dB SPL. The geometric mean of the pair was swept from 8 to 1 kHz, with measurements conducted at two points per octave. Data collection was terminated after three such sweeps (Guest et al., 2019). The whole procedure lasted 1 minute. Collected endpoints included DP amplitude shift from baseline, per frequency. DPOAEs were measured again at

2 and 4-8 hours right before PTA and extended high-frequency PTA (EHF-PTA).

PTA and EHF-PTA were performed before and post-music exposure at three time points (4 minutes, 2 hours, 4-8 hours), immediately after DPOAE measurement. Only the exposed (left) ear was tested. Tested frequencies followed the following order: 1, 3, 4, 6, 8, 10, and 12.5 kHz. The signal level was varied in a 6-dB up, 2-dB down manner (Kil et al., 2017); each provided tone lasted approximately 3 seconds. Following a satisfactory positive response by the participant, the level of the tone was reduced in 6-dB steps until no further response occurred. Then, the level was increased in 2-dB steps until a response occurred. After the first response using an ascending approach, the tone was decreased by 6 dB and another ascending 2-dB series was initiated until the participant responded again. This procedure was repeated until the participant responded at the same level on two out of two, three or four (i.e., 50 % or more) responses on the ascent. This was considered the hearing threshold level for that particular frequency. Since conductive hearing loss was an exclusion criterion for our study, only air conduction data were collected. The whole procedure was performed with an Affinity audiometer (EN 60645-1, ANSI S3.6, Interacoustics) using TDH39 (for ≤ 8 kHz) and HDA 300 (for > 8 kHz) headphones. The duration of the PTA procedure was approximately 3 minutes. Collected endpoints included PTA thresholds per frequency (dB).

Finally, blood sampling for prestin measurement by means of ELISA was performed before and after music exposure at different time points (baseline

and then at 20 minutes, and at 2 hours, 4-8 hours, 24 hours and 1 week following music exposure, right after the completion of PTA, EHF-PTA, and DPOAEs). Serum samples were collected in Serum Separator Tubes (SST). The samples were then centrifuged for 15 minutes at 1000 g within 2 hours of collection. The resulting supernatant was stored in a –80°C freezer until time of assay. ELISA was performed in the Department of Biology of the National Kapodistrian University of Athens, and the samples were stored in ice during transport. Prestin level were measured using the Human Prestin (SLC26A5) MBS282125 ELISA Kit (MyBioSource, San Diego, California) as described in the manufacturer's instruction manual. All samples were assayed in duplicates, as recommended by the manufacturer. The selected kit has a detection range of 15.6 pg/mL – 1000 pg/ml, high sensitivity, and excellent specificity. The optical density was measured at 450 nm and 540 nm FlexStation 3 Multi-Mode Microplate Reader, in ELISA mode. Collected data include serum prestin concentration in pg/ml. The pre- and post-exposure procedures are detailed in **Figure 6.1**.

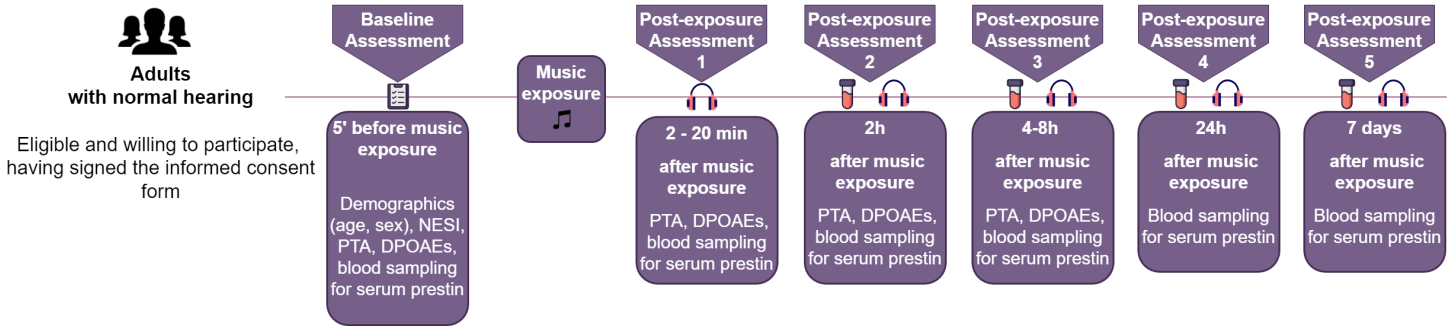


Figure 6.1. An illustration of the experimental procedure. According to our original protocol, PTA and DPOAEs would be conducted at five times points after music exposure. During the study, we decided to stop measurements at the point where PTA thresholds and OAE amplitude returned to baseline.

6.2.6. Statistical analysis

R was used for the statistical analysis (R core team, 2023). To test hypotheses 1, 2a, and 2b, a two-level linear mixed effect (LME) models was constructed to account for repeated measurements of serum Prestin level, PTA threshold (average for higher frequencies ≥ 10 kHz), and PTA threshold (average for lower frequencies ≤ 8 kHz) respectively, at different time points within the same participant. Time was modelled as a fixed factor and Participant as a random factor (random intercept) to account for individual differences in Prestin level for each participant before exposure.

To test hypothesis 3, a two-level linear mixed effect models was constructed to account for repeated measurements of OAE level at the tested frequencies, at different time points within the same participant. Time and Frequency were modelled as fixed factors and Participant as a random factor (random intercept) to account for individual differences in OAE levels for each participant before exposure.

In the above LME models, deviations from homoscedasticity or normality were assessed by visual inspection of residual plots, and the Shapiro-Wilk test. Analysis of variance tables (using the Kenward–Rogers method for estimating degrees of freedom), marginal means and significance testing of their differences were calculated via the lmerTest package (Kuznetsova et al., 2017). Dunnett's correction was used for controlling the family-wise error rate.

To test hypotheses 4a and 4b, simple linear regression models were fit to explore the average PTA threshold shifts for lower and higher frequencies following music exposure as predictors for the maximum prestin serum level observed. Age, sex, and NESI were used as covariates.

After reviewing and analyzing the data, the following exploratory post-hoc analyses were also performed: 1) qualitative descriptive analysis on the post-exposure prestin response among participants (responders and non-responders) 2) analysis of mean TTS per frequency, before and at 4 minutes after music exposure (the only time point when TTS was observed) with a simple linear regression model. 3) Baseline serum prestin level as a predictor of the magnitude of TTS at 6 kHz (the frequency where maximum PTA threshold shift occurred).

6.2.7. Deviations from the original pre-registered statistical analysis plan

Our sample size deviated from that in the pre-registration. Our initial sample size calculation was based on the statistical model required for the primary hypothesis. At the time this was one-way ANOVA, repeated measures, within-subject factor, to detect a medium effect size (Cohen's f) of 0.25 with a significance level of 0.008 (0.05 Bonferroni corrected for six hypotheses), and 80% power (1 – beta). Based on this, the required sample size was estimated to equal 27 participants in total. Due to resource and time constraints, we decided to stop recruiting in May 2022 (n=14) and proceed with the analysis of

our data. We did not perform post-hoc power analysis since this is now considered conceptually wrong and misleading in the statistics literature.

ANOVA was initially planned for the analysis of hypotheses 1 (one-way repeated measures), 2a (one-way), 2b (one-way) and 3 (two-ways). Due to partially missing data, LME models were used instead to maximize the use of available data and at the same time account for both repeated measures and random effects of individual participants.

Finally, we initially panned on repeating PTA and DPOAEs at five time points (2 minutes, 2 hours, 4-8 hours, 24 hours and 7 days). During the study, we decided that we should be measuring PTA and DPOAEs only until they return to baseline. All our participants had returned their PTA and DPOAEs in baseline levels by 4-8 hours post-music exposure.

6.3. Results

6.3.1. Participants

Fourteen participants were included in the study [nine women; age range: 20-54 years, median age=31 years (IQR=6.75 years)].

H1. Prestin concentration before and after music exposure

Out of possible 84 serum samples (14 participants, one session before and five sessions after music exposure), we managed to gather 81, since three sessions were missed by the participants. We were able to measure prestin level in 77

of them (15 samples at baseline and at 20 minutes, 11 at 2 hours, 13 at 4-8 hours, 10 at 24 hours and nine at 7 days post-exposure). Haemolysis prevented accurate prestin measurements in four of them (each in different participant and time of day), while four measurements (3 in the same participant) with values > 450 pg/ml were considered outliers (> 3 standard deviations from the mean) and were not included in the analysis.

Serum prestin level at baseline [mean: 140.00 pg/ml, 95% CI: (125.92, 154.07)] increased gradually following music exposure, reached a maximum at 2 hours [mean: 158.29 pg/ml, 95% CI: (130.42, 186.66)], and returned to pre-exposure level at 1 week [mean: 139.18 pg/ml, 95% CI: 114.69, 163.68] (**Figure 6.2**). According to the LME model, the overall Time effect is not statistically significant, although the uncorrected serum level at 4-8 hours is significantly different to the baseline [b = 18.40; $t_{(48.66)}$ = 2.61; 95% CI: (4.26, 32.55); p = 0.012, uncorrected]. All other differences were non-significant. Using Dunnett's correction to control for multiple comparisons (for five contrasts comparing with the baseline) the difference of the marginal mean at 4-8 hours and the baseline is 18.40 pg/ml and is not statistically significant [$t_{(54.3)}$ = 2.47; 95% CI: (3.78, 33.02); p = 0.069].

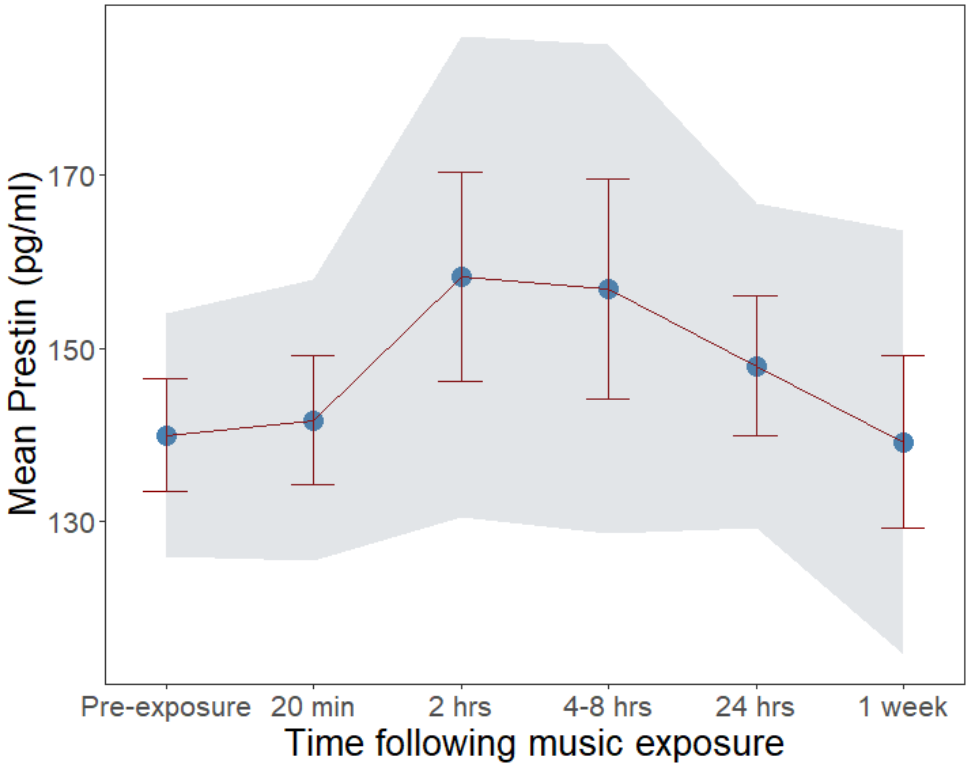


Figure 6.2. Mean prestin level before and at specified intervals following music exposure. Error bars show 1 standard error and the gray-shaded area the 95% confidence intervals.

Post-hoc analysis of the post-exposure prestin response among participants (responders and non-responders): Although all participants experienced a TTS in PTA and a temporary reduction in the amplitude of the DPOAEs immediately in the initial (up to 5 minutes) post-exposure measurements, only four of the 14 participants (three with baseline values > 150 pg/ml) showed a sharp increase (arbitrarily defined as > 50% increase from baseline) 2 – 8 hours following exposure, followed by decreased values in the subsequent measurements (**Figure 6.3**). Moreover, four participants (with baseline values < 125 pg/ml) showed no change in serum prestin level following exposure to music.

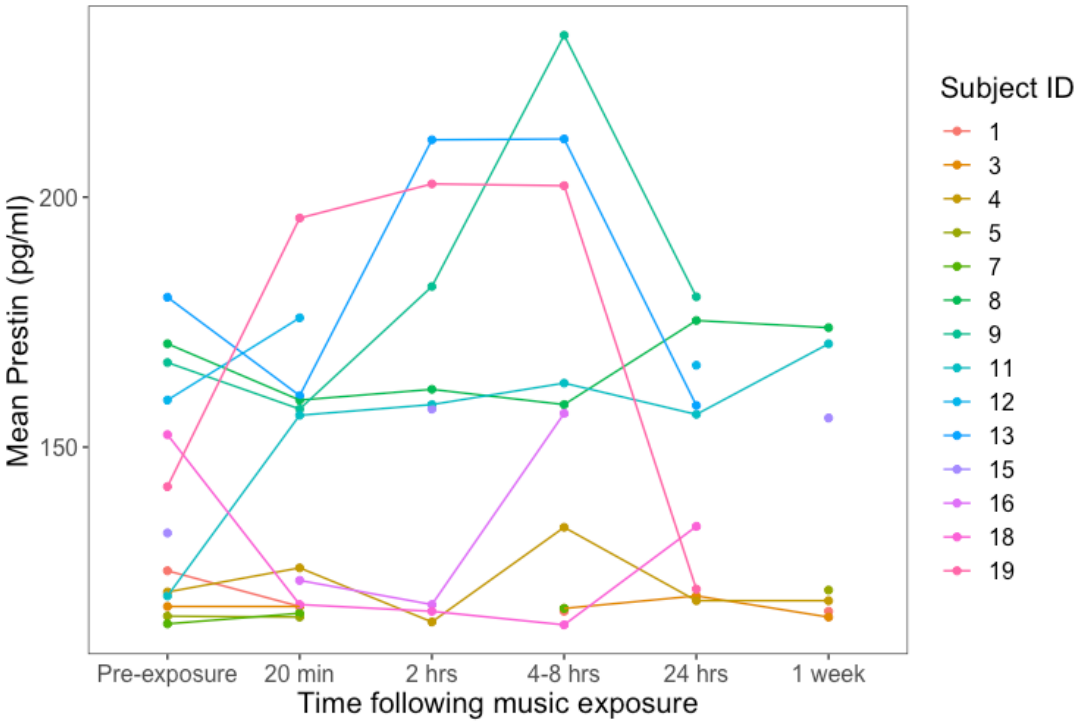


Figure 6.3. Spaghetti plots of serum prestin level in different participants across the different time points.

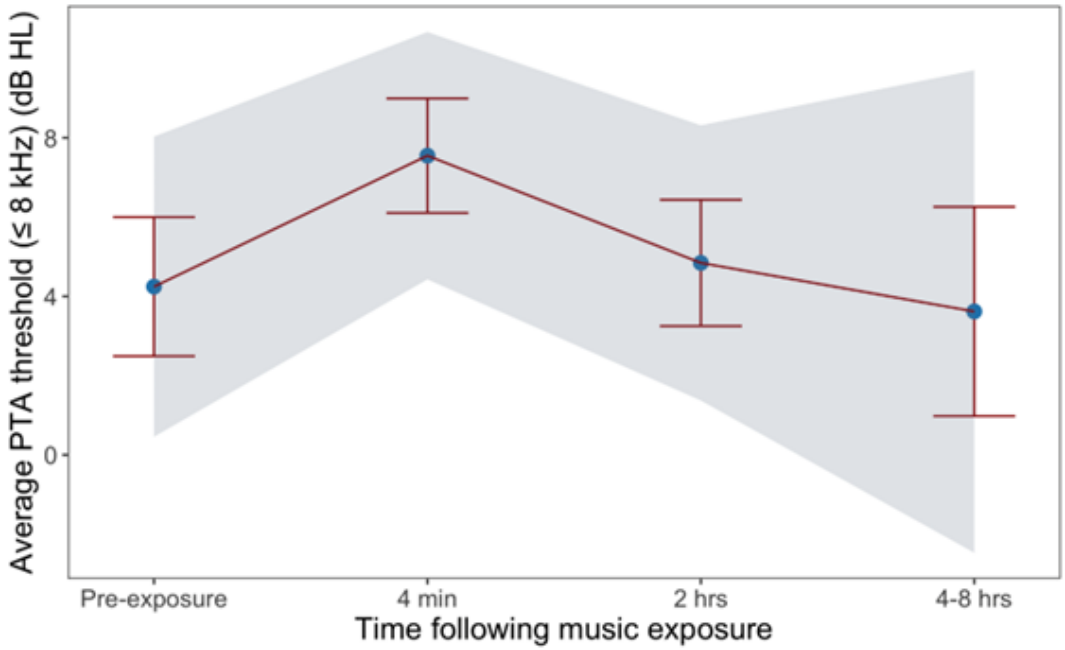
H2. Average PTA thresholds for standard and extended high frequency audiometry before and after music exposure.

Extended high frequency PTA thresholds: There was no statistically significant elevation of the average PTA threshold in extended high frequency audiometry (≥ 10 kHz) at any time point.

Standard PTA thresholds: The overall Time effect was statistically significant in the model of PTA threshold in standard audiometry (≤ 8 kHz) [($F_{(3,\ 36.33)}$) = 7.28; $p < 0.001$]. More specifically there was a statistically significant average threshold elevation at 4 minutes post-exposure [b = 3.31, $t_{(36.14)}$ = 3.51, 95% CI (1.41, 5.20), p = 0.001, uncorrected]. Using Dunnett's correction to control for multiple comparisons (for three contrasts comparing with the baseline) the

difference of the marginal mean at 4 minutes and the baseline is 3.31 dB and is statistically significant [$t_{(39.2)}$ = 3.36; 95% CI: (1.34, 5.271); p = 0.005]. The threshold elevation at 2 and 4-8 hours post-exposure was statistically not significant (**Figure 6.4**).

Figure 6.4. Mean pure tone thresholds before and at specified intervals



following music exposure. Error bars show 1 standard error and the gray-shaded area the 95% confidence intervals.

H3. Distortion product otoacoustic emissions amplitudes before and after music exposure

Both Time and Frequency effects were significant in the model [$F_{(4, 379.65)}$ = 2.90, p = 0.021; and $F_{(6, 377.97)}$ = 55.90, p <0.001 respectively]. According to the LME model, the DPOAE levels (across frequencies) at 2 minutes were significantly different from the baseline [b = -2.15; $t_{(377.97)}$ = -2.93; 95% CI: (-0.71, -3.59); p

= 0.004, uncorrected]. Using Dunnett's correction to control for multiple comparisons (for four contrasts comparing with the baseline) the difference of the marginal means at 2 minutes following music exposure and the baseline is -2.15 dB [$t_{(388)}$ = -2.89, 95% CI (-0.32, -3.98,), p = 0.015]. There was no statistically significant shift at any later time point, with levels returning to pre-exposure levels, on average, and for each participant individually.

At 2 minutes following music exposure, the maximum DPOAE shift of -4.07 dB is observed at 6 kHz [($t_{(91)}$= -3.54; 95% CI: (-1.79, -6.35); p < 0.001]. A statistically significant shift of -2.52 dB was also observed at 4 kHz [($t_{(91)}$ = -2.20, 95% CI: (-0.24, -4.80), p = 0.031)]. The DP amplitude shifts observed are presented in **Figure 6.5**.

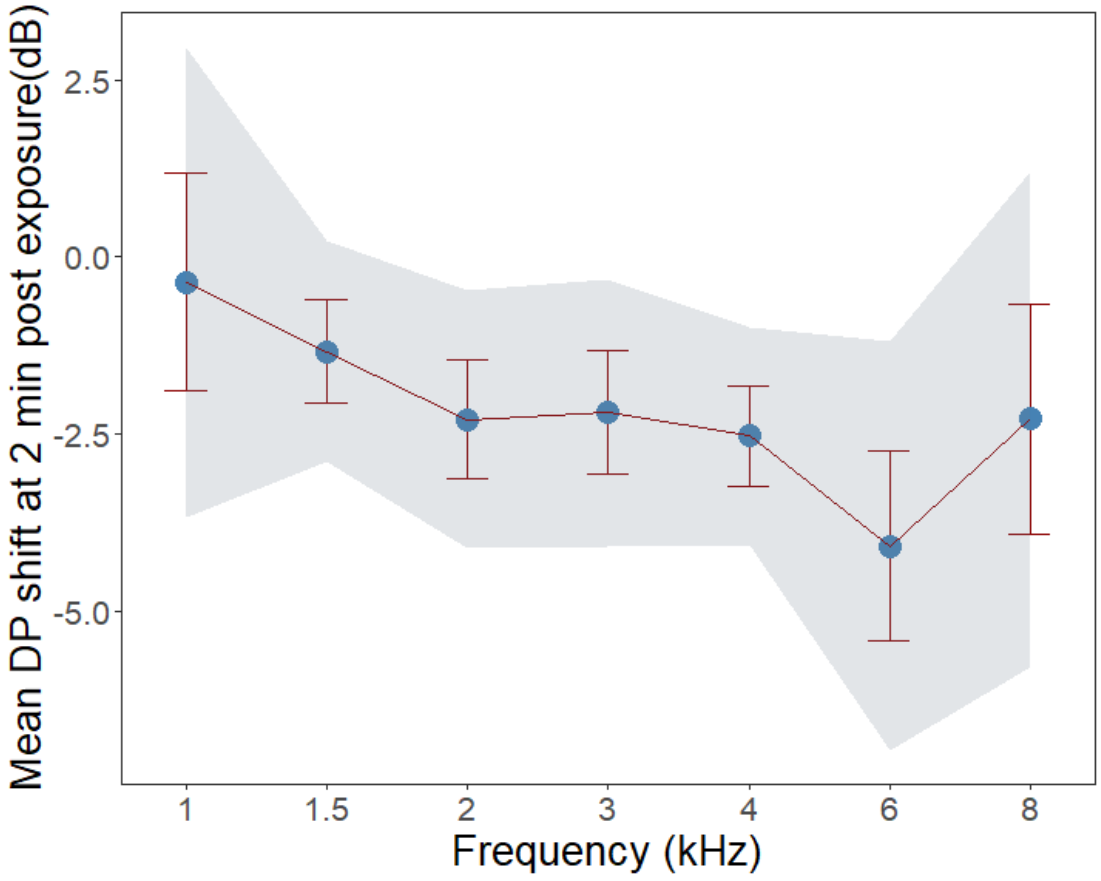


Figure 6.5: Mean shift of distortion product otoacoustic emission amplitude 2 minutes after music exposure. Error bars show 1 standard error and the gray-shaded area the 95% confidence intervals.

Both of the above models deviated from normality. Although linear mixed effect models are robust when distributional assumptions are objectively violated and the β parameter estimates are considered unbiased (Verbeke & Molenberghs from their book 'Linear Mixed Models for Longitudinal Data, 1st Ed, Springer; Schielzeth et al 2020; Knief & Forstmeier, 2021), robust linear mixed models were also fitted that do not make assumptions on normality to estimate 95% confidence intervals and marginal means. The results were comparable to our first estimates.

H4. Average TTS at lower and higher frequencies as a predictor of maximum serum prestin shift

Neither average temporal threshold shift at lower [1-8 kHz, $F_{(4,9)} = 0.75$, $p = 0.582$] nor at higher frequencies [10-12.5 kHz, $F_{(4,8)} = 0.47$, $p = 0.758$] was a significant predictor of maximum serum prestin level shift.

Post-hoc analysis of mean PTA threshold shift per frequency: Since TTS was observed at 4 minutes post-exposure, it was further explored which frequencies were mostly affected, by using a simple linear regression model.

Frequency was significant in the PTA threshold shift model at 4 minutes following music exposure [$F(6, 91) = 5.13$, $p < 0.001$)] (**Figure 6.6**). The

maximum TTS of 9.571 dB was observed at 6 kHz [($t_{(91)}$ = 6.03, 95% CI: (6.42, 12.72), p < 0.001)]. The following statistically significant TTSs were also observed: 3.57 dB at 3 kHz [($t_{(91)}$=2.25, 95% CI: (0.42, 6.72), p = 0.027], 3.71 dB at 4 kHz [($t_{(91)}$ = 2.34, 95% CI: (0.57, 6.86), p = 0.021)], and 3.29 dB at 8 kHz [($t_{(91)}$ = 2.07, 95% CI: (0.14, 6.44), p = 0.041)].

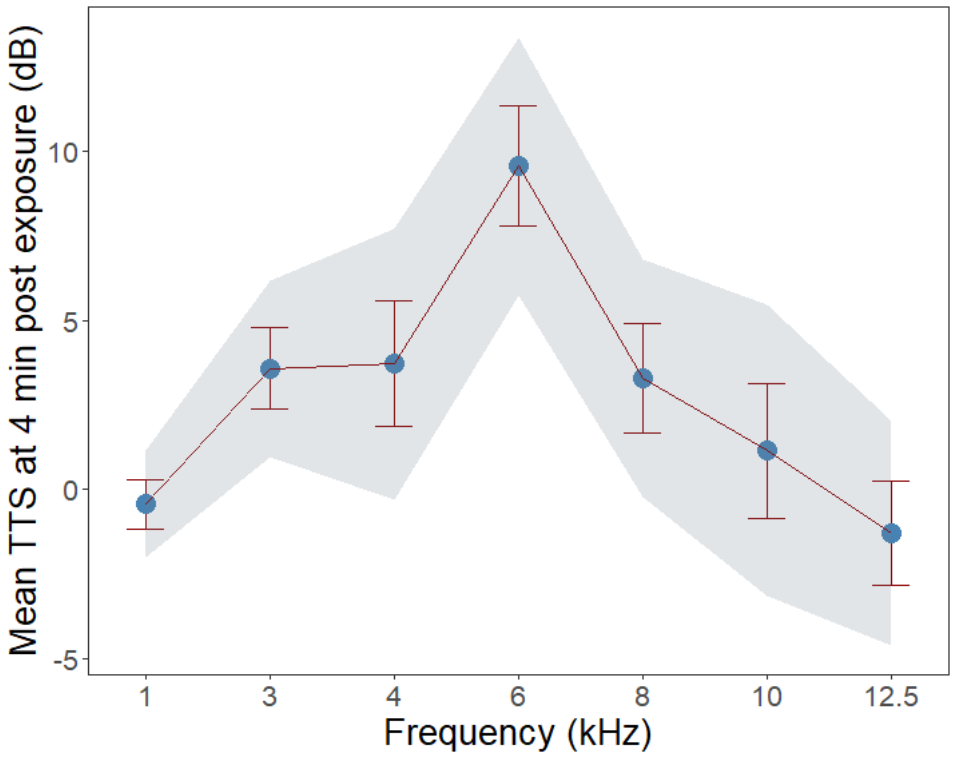


Figure 6.6. Mean PTA threshold shifts immediately at 4 minutes after exposure to music. Error bars show 1 standard error and the gray-shaded area the 95% confidence intervals.

Exploratory post-hoc analysis

Baseline serum prestin level as a predictor of the magnitude of TTS at 6 kHz: Since the maximum PTA threshold shift occurred at 6 kHz, it was further explored as to whether baseline serum level could serve as a predictor of the magnitude of this shift, and thus justify the use of the latter as a possible

biomarker of cochlear susceptibility to short-term and therefore long-term cochlear damage.

A fixed effect linear model was used to explore baseline serum prestin as a predictor of PTA threshold shift at 6 kHz (where the maximum TTS was observed), with age as a covariate. Baseline serum prestin level effects were significant [Adjusted R^2 = 0.401, $F_{(2,10)}$ = 5.02, p = 0.031]. More specifically, the model showed a significant decrease of TTS with increasing prestin concentrations at baseline (β= -0.24; $t_{(10)}$ = -3.15; 95% CI: (-0.36, -0.12); *p* = 0.010; **Figure 6.7**). In contrast, baseline serum prestin is not a significant predictor of DP amplitude shift at 6 kHz (where the max DPOAEs shift was observed).

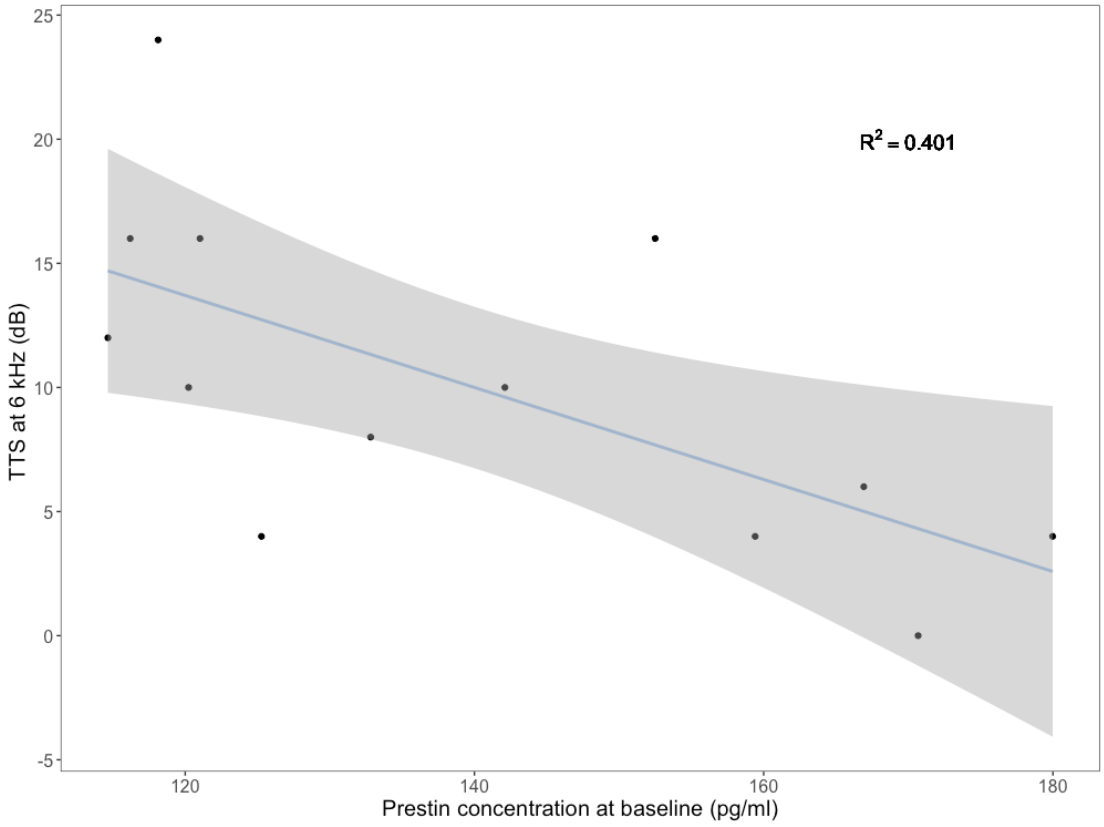


Figure 6.7. Scatter plot of prestin concentration at baseline and TTS at 6 kHz

with a fitted regression line (solid blue) representing the best linear fit. The grey-shaded area around the regression line indicates the 95% confidence interval ($p = 0.031$, $R2 = 0.401$).

6.4. Discussion

In our study, 14 participants were exposed to music for a short period of time, in a setting that has been previously shown to create reliably and safely create temporary cochlear dysfunction in a similar population (TTS and decrease of DP amplitude) (Iliadou et al, Development and validation of an efficient and safe loud music exposure paradigm, in press in Journal of Speech, Language, and Hearing Research). We hypothesized that serum prestin level of adults with normal hearing would change after our music exposure paradigm, and that this change will correlate with changes in PTA and DP amplitude. Our hypothesis was based on previous evidence showing that individuals with higher daily noise exposure level (chronic exposure) were found to have lower prestin level (Parker et al., 2022b). Moreover, in rodents, prestin concentration has been found to increase immediately after noise exposure (acute exposure) and then return back to near baseline 2 weeks later (Parham et al., 2019).

In our study, after exposure to the audio material, participants' blood prestin level were measured at several time points after their exposure by means of ELISA. The fact that our patients did not have any heart conditions, and the only intervention they underwent was the exposure to music, gives us confidence that all observed changes in blood prestin level is not linked with temporary changes in other systems where prestin is being expressed, such as the heart. Serum prestin increased soon after music exposure, reaching its

maximum concentration at 2-8 hours, and then decreased back to near the pre-exposure baseline by 24 hours post-exposure. According to the model, a mean increase of 11.38 pg/ml and 18.40 pg/ml is expected in serum prestin, at 2 and 4-8 hours respectively. However, none of the changes were statistically significant after correction, so should be regarded as trends only. Blood prestin level returned to baseline level in all participants with available data at day 7 following exposure. This time course of change of blood prestin level is in agreement with previous animal studies showing a post-exposure initial increase and then decrease to baseline of blood prestin over time. In a recent animal study by Parham et al. (2019), prestin level were measured at 4 h, 24 h, 48 h, 72 h, 7 days, and 14 days after 2 h of exposure to noise of 110 and 120 dB SPL. After an initial peak of prestin concentration after the noise trauma (4 h), prestin level returned to near or below baseline by day 14 post-exposure. The degree of its increase was shown to be correlated to the animal's degree of hearing loss, damaged cochlear region, and degree of recovery. Although our study was under-powered, our findings may suggest that serum prestin could serve as the first non-auditory test to assess noise-induced cochlear insult, although further investigation is warranted. Moreover, although no statistically significant relation between TTS or DP amplitude shift (maximum or on average) and prestin level change was found, prestin level at baseline was a good predictor of TTS at 6 kHz (where the maximum TTS shift was observed in our sample): an elevation of the mean baseline prestin level by 43.48 pg/ml corresponded to a mean reduction in TTS at 6 kHz of 10 dB. This exploratory finding may show some utility of baseline level as a predictor of subclinical

cochlear dysfunction or susceptibility to noise, and it warrants further investigation.

Regarding the generalizability and safety of our paradigm, the audio material used in our paradigm consists of pop commercial music, and its duration and intensity mimics regular listening habits at fitness clubs, nightclubs, at live music events, and while using personal listening devices (Dudarewicz et al., 2015; Kähäri et al., 2011; Schmidt et al., 2011; Tronstad & Gelderblom, 2016) making our lab findings comparable to real-world exposure conditions. Confirming the presence of temporary cochlear dysfunction by means of TTS or DPOAEs was considered important for confirming that our music exposure is capable of creating temporary cochlear changes. The selected music exposure paradigm was successful in the current study, since all participants presented TTS and decrease of DP amplitude in at least one frequency. Music was presented monaurally through headphones at sound levels compliant with Greek regulations outlined in the " Protection of Public Health from Music Sounds in Entertainment and other venues" (Υ.Α. Υ2/Οικ. 15438/2001 (ΦΕΚ 1346/Β` 17.10.2001)), and these levels were lower than the standards established by the National Institute for Occupational Safety & Health (NIOSH) or the World Health Organization for exposures lasting 15 minutes (Śliwińska-Kowalska & Zaborowski, 2017b; WHO, 2022). It is important to note that NIOSH standards and the permitted daily noise "dose" are designed to assess the risks associated with repetitive noise exposure over 40 years of working five days a week, rather than a single exposure, as in our experiment. Furthermore, NIOSH standards pertain to sound levels in open environments, whereas in our study,

music was delivered through headphones, resulting in lower sound levels compared to open environments. We conservatively estimated that the delivered-to-the ear sound levels are 5 dB lower than the free field levels (Shaw, 1966), which practically means that participants were exposed for 15 minutes to free-field equivalent noise of 95 dBA (less than 1/3 of the maximum permissible dose). Regarding the WHO guidelines for safe listening, the weekly exposure limit of 1.6 Pa^2 h (Pascal squared hours) per 7 days is recommended as the reference exposure. This limit is equivalent to 80 dBA for 40 hours a week and translates to a maximum exposure of 101 dBA for 18.75 min within a week (using a 3-dB exchange rate). This exposure is more than the 100 dBA for 15 minutes that we have used in our paradigm, provided, that we had informed our participants not to be exposed to >75 - 80 dBA for the rest of the week, and checking for no high exposure during the preceding 72 hours. As our primary aim was to evaluate the effectiveness of our paradigm in inducing TTS, which does not necessitate exposure to and potential harm to both ears, we exclusively considered monaural exposure. The choice of monaural noise/music delivery aligns with the practices of numerous previous studies (Attias et al., 2004; Bhagat & Davis, 2008; Keppler et al., 2010; Quaranta et al., 2003, 2004).

Statistically significant TTSs were observed at 3 kHz, 4 kHz, 6 kHz and 8 kHz, while statistically significant DP amplitude shifts were recorded at 4 kHz and at 6 kHz. Our findings are consistent with previous studies showing that TTS and DP amplitude shift is more prominent in the 3-6 kHz region (Engdahl, 1996; Le Prell et al., 2012; Moshammer et al., 2015b). Regarding the risk of music-

induced cochlear synaptopathy in our sample, this was also taken into account before designing and conducting our study. Prior animal studies utilizing cochlear functional assessments and confocal imaging have demonstrated that exposure to noise levels capable of causing temporary pure tone threshold increases of approximately 40–50 dB 24 hours post-noise can lead to rapid and lasting synaptic deficiencies, as well as reduced evoked potential amplitude (Kujawa & Liberman, 2009, 2015). However, it has been established that significantly higher noise levels would be required to induce cochlear synaptopathy in primates compared to rodents (Valero et al., 2017). In a recent commentary discussing the rationale for modifying current regulations governing occupational noise exposure based on research findings related to noise-induced cochlear neuropathy in rodents, the authors conclude that these findings cannot be directly extrapolated to humans. They suggest that humans appear to be less susceptible to TTS and likely cochlear synaptopathy (Dobie & Humes, 2017). Moreover, in the case of human studies, numerous studies attempting to identify signs of cochlear synaptopathy have yielded high heterogeneity in their methods and results (Bramhall et al., 2019). In those studies, noise exposures were either of greater intensity or duration than those used in our research (Bramhall et al., 2019; Wang et al., 2021). According to the current understanding of noise-induced damage, and the available audiometric tests, the safety of our study protocol (in terms of pure tone thresholds and DPOAE) is supported by the fact that, despite all participants exhibiting measurable and consistent temporary changes in their auditory function, none of them experienced nor reported any lasting hearing disorders such as threshold shifts (PTS). Nevertheless, if, in the future, any clinical tests

are validated as sensitive to cochlear synaptopathy and neurodegeneration in humans, they should be incorporated into both pre- and post-exposure assessments to ensure the preservation of synaptic and neural integrity.

Limitations

The main limitation of this study is the small sample size, which reduces the power of our analysis, increases the chance of type 2 error, and limits the extrapolation of our findings. The lack of statistical significance may be a consequence of the small sample size and the choice of two-tailed rather than one-tailed tests (although the literature suggests that prestin level increase following acute noise exposure in rodents (Parham et al., 2019)). Additionally, the fact that only some participants responded to music exposure with an elevation in serum prestin while others did not, should be taken into account in the statistical analysis plan when replicating this study. In the future, we plan to involve larger samples that will provide sufficient power to our analyses. We also chose to deliver the audio material only to one ear side, as was previously done in multiple published studies, for safety and time saving reasons. In the future, we would like to explore if exposure in both ears would alter the observed effect size. Another limitation is the limited range of age in our sample. Recently, blood prestin was shown to be significantly lower in people over the age of 50 years when compared to younger adults (Parker et al., 2022a). In the present study, the sample was small and consists mainly of young adults with normal hearing, so our findings cannot be generalized for older adults. Finally, although the ELISA kit used in this study has an excellent specificity and high sensitivity, ELISA itself may be affected by multiple technical factors

(refrigerated transport and storage, numerous preparation and wash steps of the assay, haemolysis of the samples). In order to address them, all procedures were duplicated and followed the ELISA kit manual guide, while samples with high haemolysis were not included in the analysis (Ni et al., 2021).

Despite the aforementioned limitations, we consider the results of this analysis useful for better understanding prestin's behaviour after music exposure, and for optimizing the design of future studies. The effect size estimated here and observed drop-out rate may be used in obtaining a more accurate sample size calculation. Moreover, it seems that prestin level show no change from baseline at 20 minutes or 7 days after the music exposure at the levels and durations tested in the present study.

6.5. Conclusions

Our study is the first human study assessing the changes of blood prestin concentration after brief exposure to high-level music, and its correlation to TTS and DP amplitude decrease. Our findings suggest that serum prestin level may show detectable change after music overexposure, and could thus serve in the future as a useful and relatively easy to perform test to indicate noise- or music-induced temporary cochlear dysfunction. Moreover, baseline prestin may predict the degree of one's TTS, providing individuals with a pre-exposure measure of their vulnerability to music. However, future studies with larger samples and higher study power are required to confirm these non-significant findings.

Chapter VII
Generalizability of results

The main aim of my thesis was the determination of the role of a specific blood marker (prestin) in NIHL, focusing on temporary MIHL. To achieve this aim, I conducted five discrete small-scale projects, that each added a separate piece of knowledge.

As a result, this PhD project provides the following:

1. Mapping and understanding of current knowledge on blood prestin level in normal hearing and in SNHL (Chapter II).

The scoping review on blood prestin level in normal hearing and sensorineural hearing loss has facilitated the understanding of the evidence that have led our and other research teams to further explore the role of prestin as a blood biomarker. Moreover, the summarized information and the critical appraisal of previous studies provided in the review are essential for future researchers wishing to assess prestin or other blood biomarkers in hearing loss.

Seven studies focusing on measuring prestin blood level by means of ELISA in rodents and human subjects with normal hearing and noise-induced, drug-induced, or idiopathic sudden hearing loss were found eligible and were included in the analysis. According to these proof-of-concept studies, prestin could be detected in the circulation of subjects with normal hearing, however its level showed high variability across studies. Following cochlear damage, blood prestin level seemed to initially rise and return 14 days later to near or

below baseline level. Prestin blood level and their temporal variation showed correlation with the degree of cochlear damage, however methodological weaknesses, such as small sample size, lack of detailed audiometric phenotyping, insufficient exclusion of confounding factors and short follow-up did not allow for robust conclusions. There was also high heterogeneity among published studies, making it impossible to pool the existing data. In the future, confounding factors that may affect prestin concentration level should be addressed. We now know that prestin is also expressed in cardiomyocytes. Hence, any measurement of bloodstream prestin should be held under conditions where no change in the cardiovascular function occurs, to ensure that the origin of blood prestin level changes is cochlear and not myocardial. Moreover, important prognostic SNHL factors such as participants' duration of the symptoms, or detailed audiometric phenotyping must be thoroughly documented and taken into account. Different pathophysiological mechanisms may be associated to the different types (e.g., ISSHL, NIHL, and ototoxicity SNHL) and stages or degrees of SNHL. Blood prestin level may also show variation when measured at different points of SNHL time course, or in participants with different PTA thresholds or DPOAEs.

2. Understanding of ranges of normal blood prestin level, their diurnal variation and their correlation with age, sex and lifetime noise exposure (Chapter III).

During an observational study, serum prestin level were evaluated in multiple time points throughout a day. Prestin was measured in all samples by means

of ELISA. All samples were assayed in duplicates, with excellent test-retest reliability. Serum prestin level did not differ significantly across time of day. The test-retest reliability at different timepoints of the day was moderate. Neither lifetime noise exposure, age, nor sex correlated with serum prestin concentration. Pure tone average thresholds and otoacoustic emissions amplitudes also showed no correlation with prestin concentration.

During this preliminary study, it was possible to standardize the procedures of collecting blood sample and measure blood prestin by means of ELISA, reaching a satisfying level of reliability. Matters such as selection of the most convenient type of blood sample (plasma or serum) or the most adequate ELISA kit, proper assay of the blood samples (dilution, wavelength), and management of hemolytic samples were specified. Regarding the blood sample type, serum was selected in this project, since the timeframe for its storage is wider (2 hours) than the one for plasma (30 minutes), and because although multiple dilutions of plasma were tried out, prestin was not identified in any of our plasma samples (see Note in Chapter III). The ELISA kit that was chosen (MBS282125, MyBioSource, San Diego, California) was selected due to its good detection range of 15.6 pg/mL – 1000 pg/ml, its high sensitivity, and excellent specificity. Moreover, it was the ELISA kit that was most frequently used in previously published studies, so our findings could be comparable to literature ones. Since the completion of our experiments, ELISA kits, such as the MBS167508 ELISA kit (MyBioSource, San Diego, CA, detection range: 10–3000 pg/mL, sensitivity: 4.87 pg/mL) with wider range of detection have been produced and are now commercially available. These kits have been used in

other published studies (Parker et al., 2021, 2022a, 2022b). In the future they could serve as a viable alternative to the kit that was used in this project. Several dilutions (1:50, 1:10, 1:5, 1:4, 1:2, 1:1) were tested for serum samples, and the 1:2 was selected since it was consistently the best fit to the standard curve. The optical density was measured at 450 nm and 540 nm for more accurate results. Samples were assessed for hemolysis according to a standard chart and those that were considered severely hemolysed, and thus their optical density non-reliable, were not included in the study (Ni et al., 2021).

According to the experience gained during this project, the experimental setting was defined and followed whenever prestin assays were needed. Moreover, the gained insights about blood prestin level in normal hearing were also significant. No age-related decline on prestin blood level in our sample of adults up to 65 years old was observed. However, the role of blood prestin level in acute cochlear dysfunction and in samples with wider age ranges (and more variability in cochlear damage) are still to be assessed. Finally, although serum prestin level were unaffected by time of day and the test-retest reliability between duplicates of the same sample was excellent, the ICC and thus the test-retest reliability and agreement of their value across day was moderate. Further longitudinal investigation of the reliability of blood prestin level measurement in larger samples is warranted.

3. Understanding of current knowledge on experimental use of noise and music in clinical studies (Chapter IV)

Testing for temporary hearing changes after noise exposure under safe conditions is a valuable tool in hearing research. The scoping review on previously published noise exposure paradigms provided essential insights and critical appraisal on methodological and ethical aspects of exposing participants in high levels of noise. The effect of hazardous noise on hearing, but also on stress levels, emotional status, and cardiovascular function were examined.

Thirty-four studies were included in the review. Experimental setting varied significantly. Eleven studies assessing hearing loss used white or narrow-band noise [($NBN_{(0.5\text{-}4\ kHz)}$, up to 115dBA, duration range:3'-24h)], and ten used pop music (up to 106dBA, duration range:10'-4h). Thirteen studies concerned stress, working memory, or cardiovascular parameters. More prominent TTSs were found in the region of 4-8 kHz, with maximum TTS ~21.5 dB at 4 kHz after NBN and ~11.5 dB at 6 kHz after music exposure All participants recovered their pure tone thresolds, with the exception of one participant in one MIHL study who had not returned within 6 dB from baseline within 7 days after exposure. High levels of music and noise caused significant reduction in distortion product otoacoustic emissions (DPOAEs) amplitude at 1 to 6 kHz, as well. In the 13 non-hearing loss studies, exposure to various noises and real-life sounds was associated with temporary stress, bradygastria, and cardiovascular changes. Noise-induced subjective stress was shown to be higher for participants with tinnitus. Noise (100dBA, 10') increased diastolic and

mean blood pressure only in participants with hypertension. The findings of the review guided the next step of the project, the creation of a paradigm that could be well tolerated by the participants and would not put them in risk of permanent hearing loss, or other distress. Based on previous experiments, it was decided i. To use a short exposure paradigm (15 to 30 minute), since it seemed sufficient in creating detectable hearing changes, ii. To expose participants monaurally, since it would reduce testing time and thus help with recruitment and retention, and iii. To use hearing tests that have been proven adequate for the detection and longitudinal monitoring of noise-induced hearing changes (PTA thresholds at 1-6 kHz, and DPOAEs with unequal primaries in the same frequential region). Including participants with tinnitus was avoided, since music exposure might cause increased distress to them. Participants with essential hypertension were also excluded, since evidence about the effect of music in cardiovascular function remains to be clarified.

4. A new, safe, reliable and time-efficient pop-rock music exposure and testing paradigm for future use in clinical studies (Chapter V)

A 30-min audio compilation of pop-rock music tracks was created. Seventeen adult volunteers with normal hearing were then exposed to this music material monaurally through headphones for 30 min at 97 dBA or 15 min at 100 dBA. Levels were measured from the ear of a manikin and were considered to provide an equivalent daily noise dose based on a 3-dB exchange. We assessed the changes in their hearing, by means of DPOAEs, PTA, and EHF-PTA. All 17 participants presented TTS > 4 dB or decrease in DP amplitude in

at least one frequency. Statistically significant average TTS of 7.43 dB was observed at 6 kHz. There were statistically significant average DP amplitude shifts of -2.55 dB at 4 kHz, -4.97 dB at 6 kHz, and -3.14 dB at 8 kHz. No participant presented permanent threshold shift.

The realistic and time-efficient music exposure paradigm developed and used in this PhD project was found reliable, pleasant, and safe in adults with normal hearing. It was successful in causing elevation of PTA threshold or decrease in DP amplitude shift to all participants in at least one frequency. Considering that the physical characteristics of the audio material to which participants are exposed may be a source of variability for the TTS, using the same paradigm could help reducing the variability across studies. In the future, our music paradigm could be used to explore aspects of temporary hearing loss that are now partially understood (time course of threshold elevation and threshold recovery, correlation of the degree or time course of hearing loss with factors such as age and sex). It may also be considered a viable option for experimental studies of temporary music-induced hearing loss.

Moreover, during this pilot study, some interesting aspects of audiometric data collection and analysis were explored. PTA thresholds and DPOAE levels were measured at multiple time points (before and after exposure to music), across a set of frequencies in different subjects. The dataset had therefore a hierarchical architecture with repeated measurements across both frequencies and time. A three-level model with random effects associated with both the

intercept and slope for subjects and with individual frequencies within subjects: Level 3 Subjects Level 2 Frequency ((cochlear locations corresponding to test frequencies)) Level 1 Time: repeated measures was selected. The selected mixed effect models would thus take into account the intrasubject variability for the estimation of expected mean values.

No formal test-retest reliability analysis for PTA thresholds and DPOAE measurement was conducted. Although, PTA measurements were performed in a sound-treated room, in compliance with the ANSI/ASA S3.1-1999 (R2018) standard for environmental noise, no real-time noise monitoring was employed during the measurements. To address this limitation, the pre-exposure and recovery data were analysed and the SEm (standard error of the measurement) and the 90% CIs were computed, to answer which of our findings are statistically significant and do not lay within the range of our test-retest variability. This deeper understanding of the audiometric data is significant for their appropriate handling in other hearing research studies in the future.

5. Understanding of blood prestin level before and after exposure to music in adults with normal hearing (Chapter VI)

We conducted the first human study assessing the changes of blood prestin concentration after brief exposure to music (15' at 95 dB FFE). Fourteen adults [nine women; age range: 20-54 years, median age=31 (IQR=6.75)] with normal hearing were exposed to the previously developed and validated audio material. All participants presented clinically significant TTS or a decrease in

DP amplitude in at least one frequency after music exposure. There was a statistically significant average threshold elevation at 4 minutes post-exposure. Statistically significant DPOAE shifts were observed at 4 kHz and at 6 kHz at 2 minutes following exposure. Mean baseline serum prestin level progressively increased following music exposure, reaching a maximum at 2 hours and returned to pre-exposure level at 1 week. However, after correction for multiple comparisons, time did not show any effect on mean prestin level. No correlation between maximum blood prestin level change and average TTS or DP amplitude shift was found. It is recommended that in future studies, blood prestin should be measured directly after 2 hours post-exposure, since our measurement at 20 minutes did not show any change from baseline in any of the participants.

Exploratory analysis also provided some interesting findings. TTS at 6 kHz (the frequency at which maximum TTS occurred) decreased significantly as baseline blood prestin level increased. If this finding is confirmed in the population, it may mean that blood prestin level may be used as a biomarker of one's susceptibility to noise-induced TTS and thus vulnerability to noise. Moreover, although average blood prestin level showed a time pattern of initial increase and then decrease, only four of the 14 participants showed a sharp increase (arbitrarily defined as > 50% increase from baseline) 2 – 8 hours following exposure. Another four participants showed no change in serum prestin level following exposure to music at all. If this finding is consistent in larger samples, it might be useful to understand which factors differentiate responders from non-responders. This knowledge would be useful for the

design of blood prestin research studies, and in the long-term for the use of blood prestin as biomarker for vulnerability in NIHL.

Despite its limitations, such as its sample size, the results of this small-scale study are useful for optimizing the design of future ones. The observed effect size and the missing data rate may be used in obtaining a more accurate sample size calculation in the future. Moreover, although statistical significance was not reached, the results suggest that the role of serum prestin level as a proxy marker for temporary or permanent cochlear dysfunction after exposure to music should be further explored, and that future studies with larger samples are required to reject or not reject these non-significant findings.

Future implications

Our future aim is to build on the outcomes of this PhD project and design future larger-scale studies that will evaluate:

1. Factors, such as age, sex, or lifetime noise exposure that may affect degree of TTS.
2. Factors, such as age, sex, or lifetime noise exposure that may affect blood prestin level.
3. Changes of blood prestin level in temporary and permanent SNHL

In the long term, determining the basis for the association between prestin blood level and NIHL will inform potential interventions and provide exploitable results and impact in research, public health, and everyday clinical practice. If blood prestin level are proven to rise after exposure to music, serum prestin could serve as the first non-auditory test to assess noise-induced cochlear

insult. As a new assessment tool, blood prestin may thus complement current audiometric tests and inform prevention strategies at both an individual (adoption of better listening habits and avoidance of those environments that lead to increase of blood prestin level), and collective level (redefinition of current noise exposure guidelines and health policies, which include referral to specialists, and PTA and OAEs as screening methods).

Moreover, since recent research, both in animals and in humans, suggests significant variations among individuals in their susceptibility to noise exposure, exploration of the association of the sources of this variation with blood prestin level may be useful. Although limited, there is evidence that factors like previous noise exposure patterns (Bidelman et al., 2017; Kujawa & Liberman, 1999; Niu & Canlon, 2002), circadian rhythm (Basinou et al., 2017; Meltser et al., 2014), melanin level (Barrenäs & Lindgren, 1990), or concurrent alcohol consumption or smoking (Dengerink et al., 1992; Ms & Rh, 1978; Upile et al., 2007) may play a role in one's vulnerability to noise. It is imperative to gain a deeper understanding of these factors and their association with blood prestin level to pinpoint individuals who may be especially prone to risks in their work or recreational environments. This new knowledge would change the current one-size-fits-all regulations about noise and music overexposure (occupational and recreational), and design of public health policies.

Finally, blood prestin level could be a secondary outcome measure in clinical trials assessing the efficacy or effectiveness of otoprotective substances or safe

listening habits (attending a festival wearing earplugs), and in studies with focus on other than NIHL (i.e., ototoxicity).

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Supplementary material

Chapter II

Supplementary material 2.1. Characteristics of included animal studies. All prestin blood level measurements were conducted by means of ELISA.

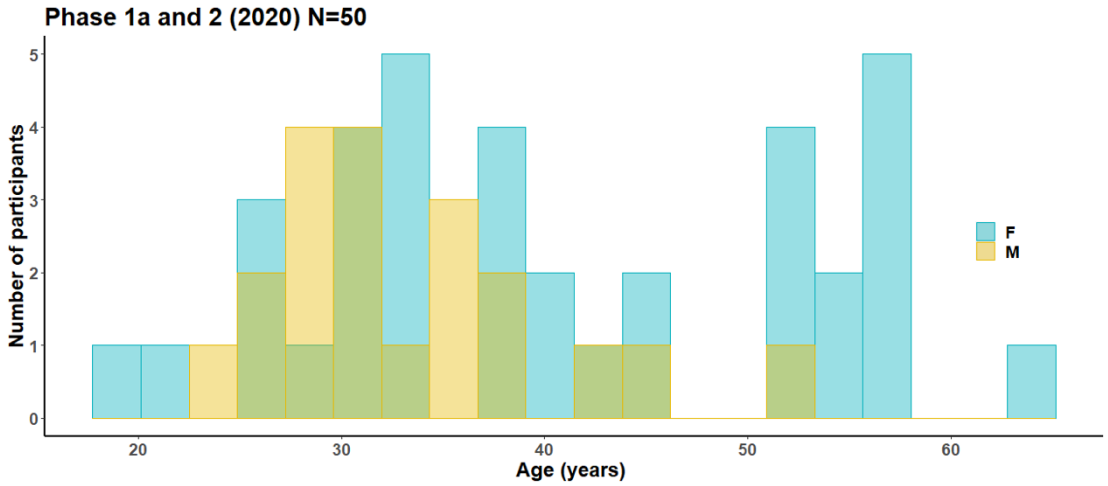
Author, Year, Journal	Title	Population	Summary	Audiological Assessment	Findings
Parham et al. *Hearing Research* 2019	Noise-induced trauma produces a temporal pattern of change in blood levels of the outer hair cell biomarker prestin	Male Wistar rats (6-9 weeks old)	Measurement of prestin blood levels at 4 h, 24 h, 48 h, 72 h, 7 and 14 days in 20 rats exposed to intense octave band noise for 2 h at either 110 or 120 dB SPL. Comparison with 26 naïve male rats.	Auditory brainstem responses (Tone-pips with 2 ms duration, 300 repetitions at a rate of 20/s, from 10 to 90 dB SPL with a 5 dB step, at 8, 16, and 24 kHz) & Distortion product otoacoustic emissions (80 /70 dB SPL, with an f2/f1 ratio of 1.2. At 4, 8, 16, 24, and 32 kHz) Day 0 & 14 [Histological evaluation of hair cell loss was held at day 14]	110 dB SPL group: Statistically non-significant, <5%, rise of prestin concentration at 4 hours post exposure, then a statistically significant gradual decrease to 10pg/ml (compared to baseline) at 14 days after exposure. 120 dB SPL group: 6 subjects presented increased prestin levels at 4 hours post exposure (22.8 ± 9 pg/mL) and 4 subjects decreased ones (17.3 ± 7 pg/mL). A statistically significant 10% decrease of prestin levels (compared to baseline) was observed at day 14 after exposure.
Dogan et al. *American Journal of Otolaryngology* 2018	Utilizing prestin as a predictive marker for the early detection of outer hair cell damage	Male Wistar rats (16–20 weeks old)	Measurement of prestin blood levels in 35 rats that received: 200 mg/kg/day of amikacin for (10 LAG), 600 mg/kg/day of amikacin for 10 days (6 HAG), one single dose of 5 mg/kg (9 LCIS), one single dose of 15 mg/kg (10 HCIS) and 10 controls.	Distortion product otoacoustic emissions (70/70 dB SPL, with an f2/f1 ratio of 1.22. in 2001, 3154, 4003, 6298 and 7998 Hz) [Histological assessment of stria vascularis, organ of Corti, spiral ganglion according to 4-point scoring system for cisplatin-induced ototoxicity defined by Freitas et al.]	Dose-dependent cochlear damage and increase of prestin blood concentration.
Naples et al. *Otology & Neurotology* 2018	Prestin as an Otologic Biomarker of Cisplatin Ototoxicity in a Guinea Pig Model	Guinea pigs	Measurement of prestin blood levels performed at days 0, 1, 2, 3, 7, and 14 post-cisplatin administration, in two groups of guinea pigs (one treated with diltiazem and one control)	Auditory brainstem responses - tone bursts of 4, 8, 16, 24, and 32 kHz, 5-ms (2-ms rise/fall time), delivered at a rate of 21/s - click-evoked	Rise in blood prestin levels (25.6%) at day 2 post cisplatin administration (precedes onset of significant ABR changes) in the control group. No prestin concentration rise in the diltiazem group.
Liba et al. *Otology & Neurotology*	Changes in Blood Prestin Concentration	Mice Guinea pigs	Measurement of prestin blood levels in 30 mice and 10 guinea pigs at 1, 3,	Auditory brainstem responses - Clicks - Rate of 21/s	Blood prestin concentrations rise, peak on days 7 (mice) and 3 (guinea pigs),

2017	After Exposure to Cisplatin		7, 14 days after 1 single dose of cisplatin at 8 mg/kg. (five mice were sacrificed at each time point, all guinea pigs were tested at each time point)	- Step of 5 dB	decline back to or below baseline / control levels 14 days after treatment.
Parham and Dyhrfjeld-Johnsen *Otology & Neurotology* 2016	Outer Hair Cell Molecular Protein, Prestin, as a Blood Biomarker for Hearing Loss: Proof of Concept.	Male Wistar rats	Measurement of prestin blood levels in 21 rats of 6-11 weeks old at day 14 after intense octave band noise for 2 to 3 hours and six controls.	Auditory brainstem responses - Tone-pips (5 ms duration at a rate of 21/s, 2 ms rise-fall time) at 8, 16, and 24 kHz from 90 dB SPL in steps of minimum 5 dB. Distortion product otoacoustic emissions - 70/70 dB SPL, f1:f2=1.2 - 4, 8, 16, 24, and 32 kHz	Noise-exposed rats demonstrated statistically significant decrease in prestin concentrations 14 days post-exposure

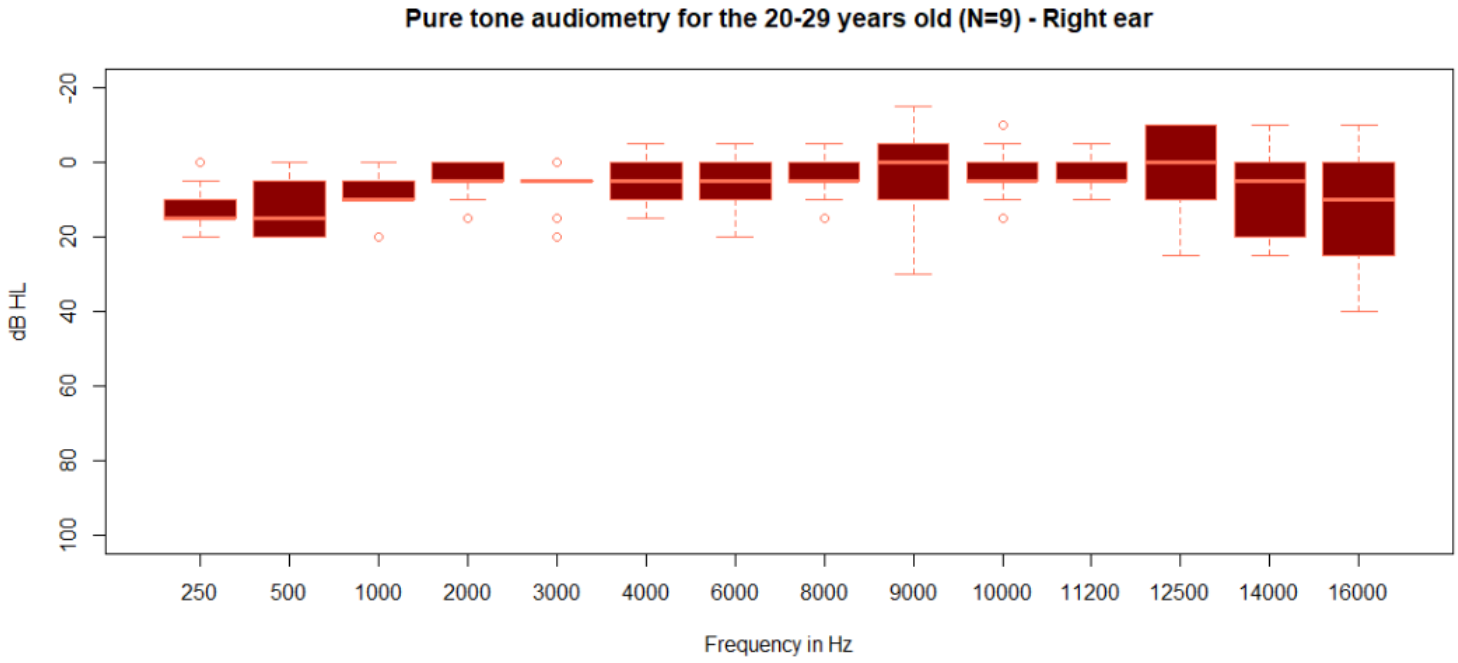
LAG, low aminoglycoside group; HAG, high aminoglycoside group; HCIS, high cisplatin; LCIS, low cisplatin

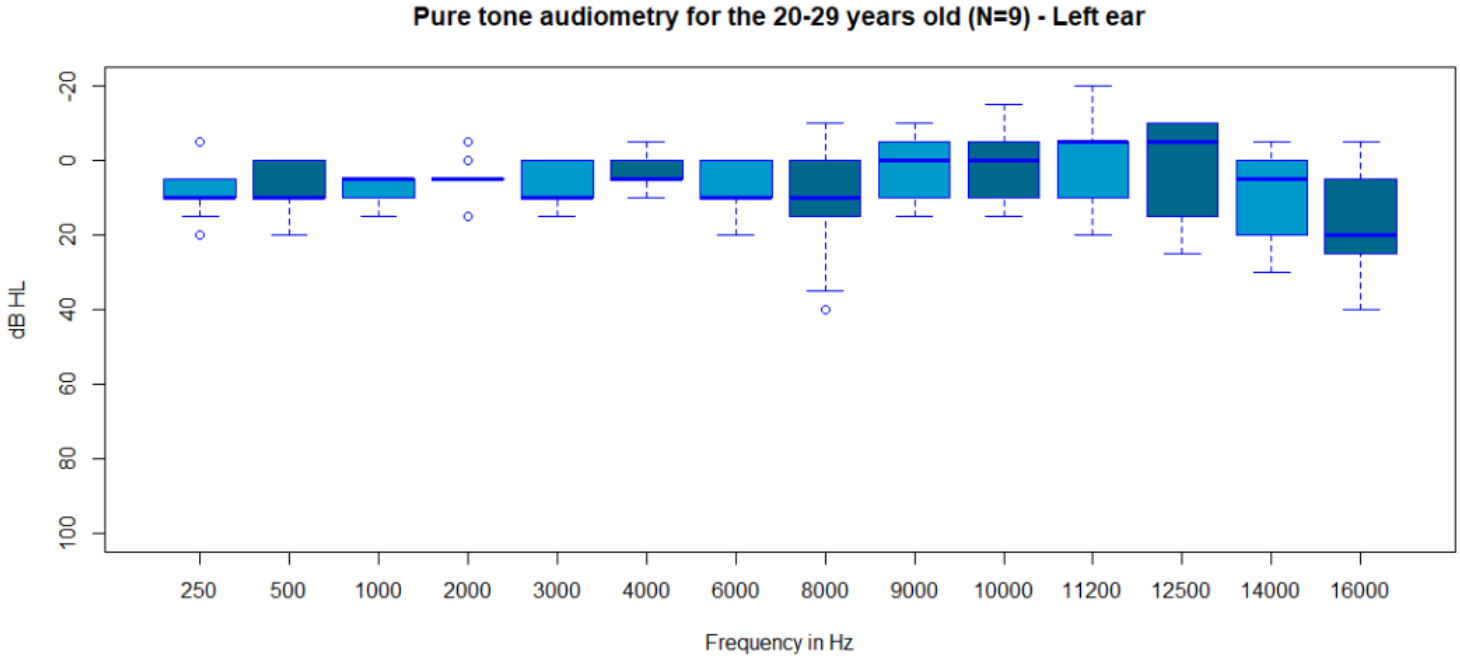
Chapter III

Supplementary material 3.1. Age distribution per sex. The upper right graph corresponds to the age distribution of participants of Module 1a (2020, N=10).

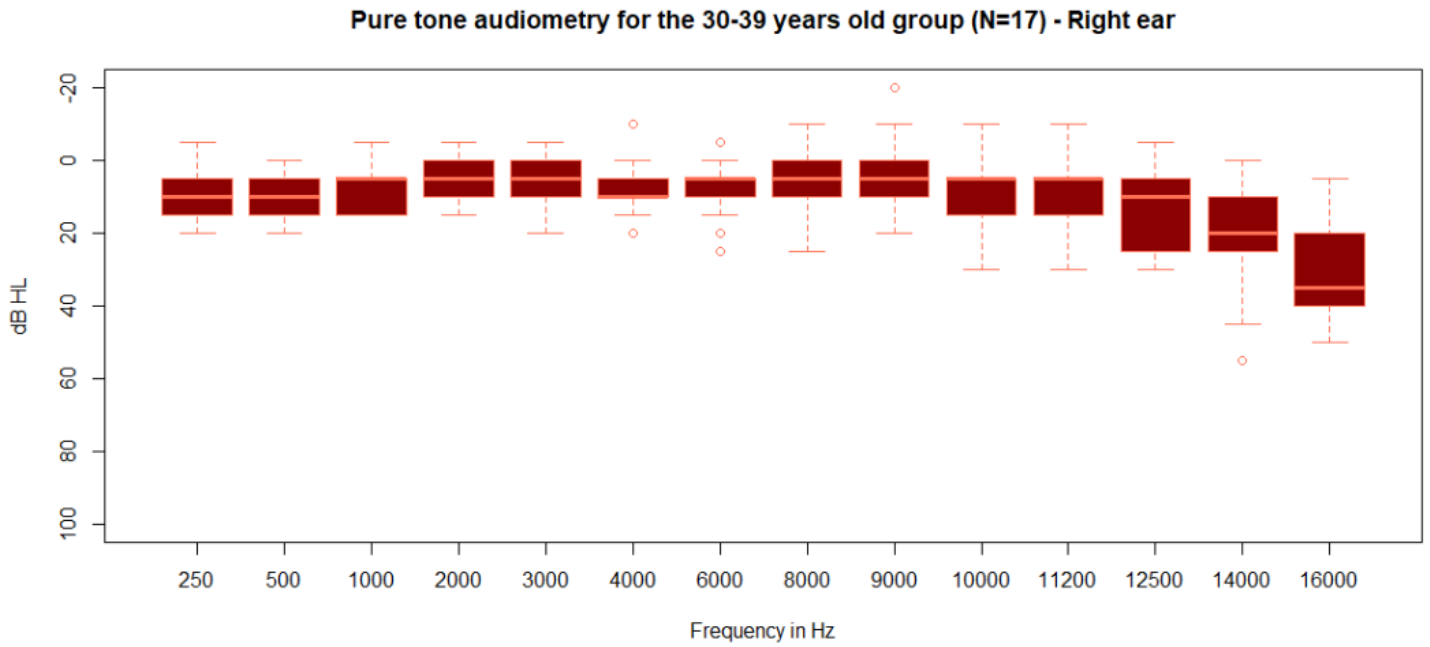


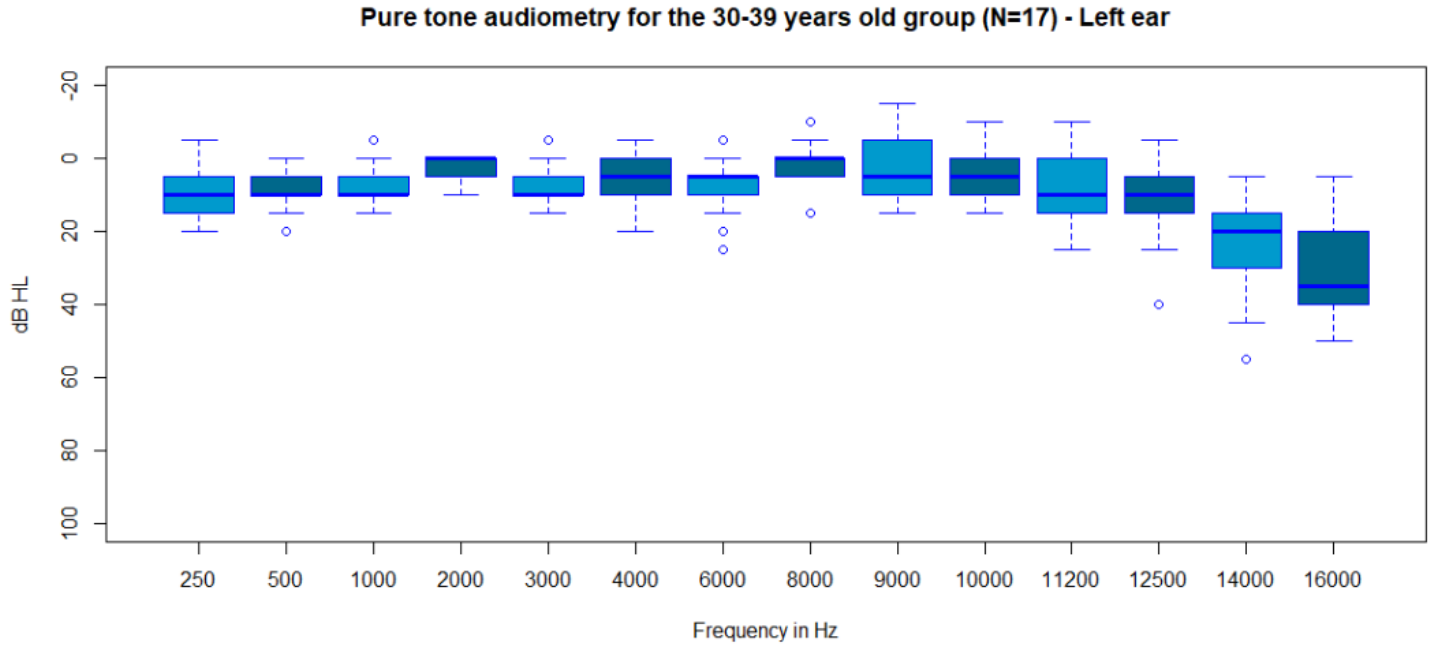
Supplementary material 3.2. Pure tone threshold boxplots of the Module 1a and 2 (2020, N=50). Samples of the right (A) and left (B) ear for the 20-29 years old.



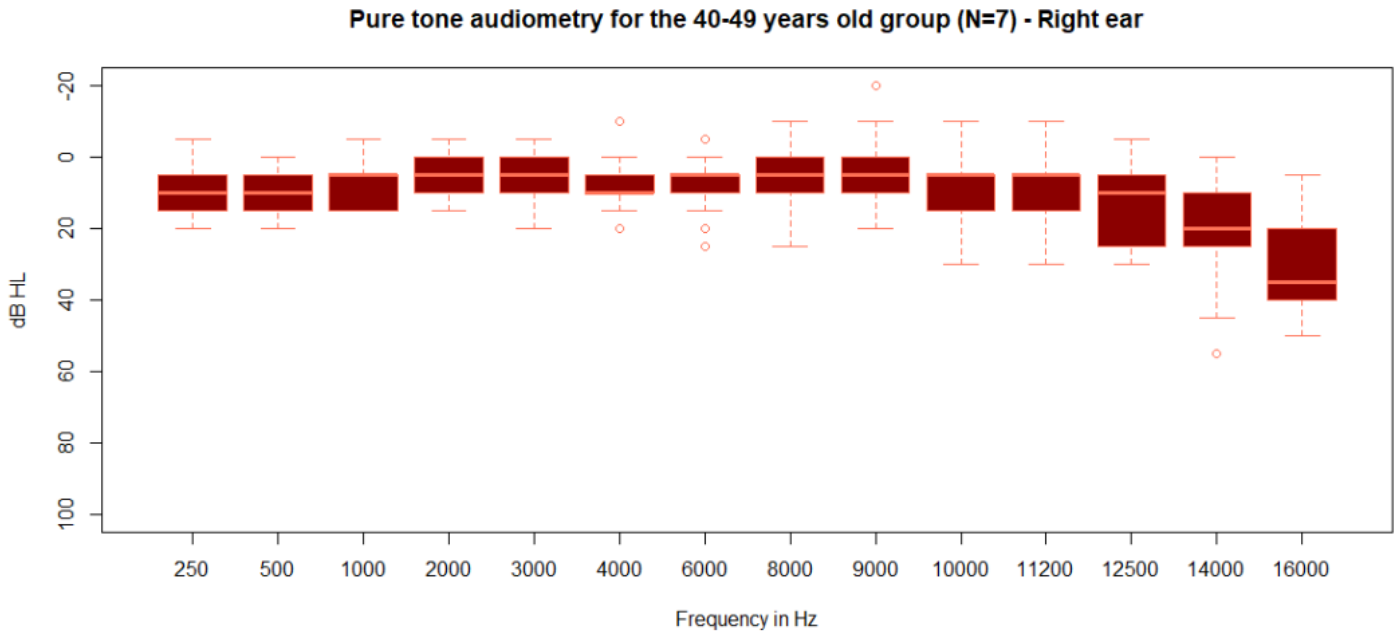


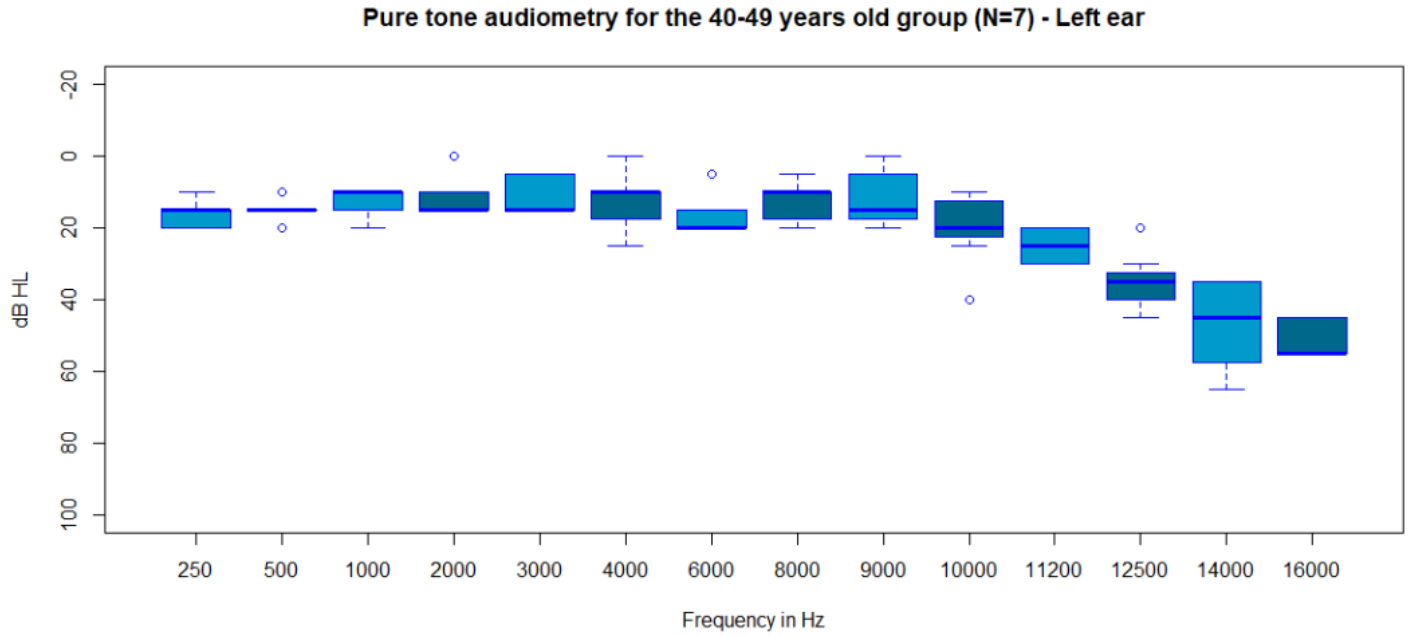
Supplementary material 3.3. Pure tone threshold boxplots of the Module 1a and 2 (2020, N=50). Samples of the right (A) and left (B) ear for the 30-39 years old.



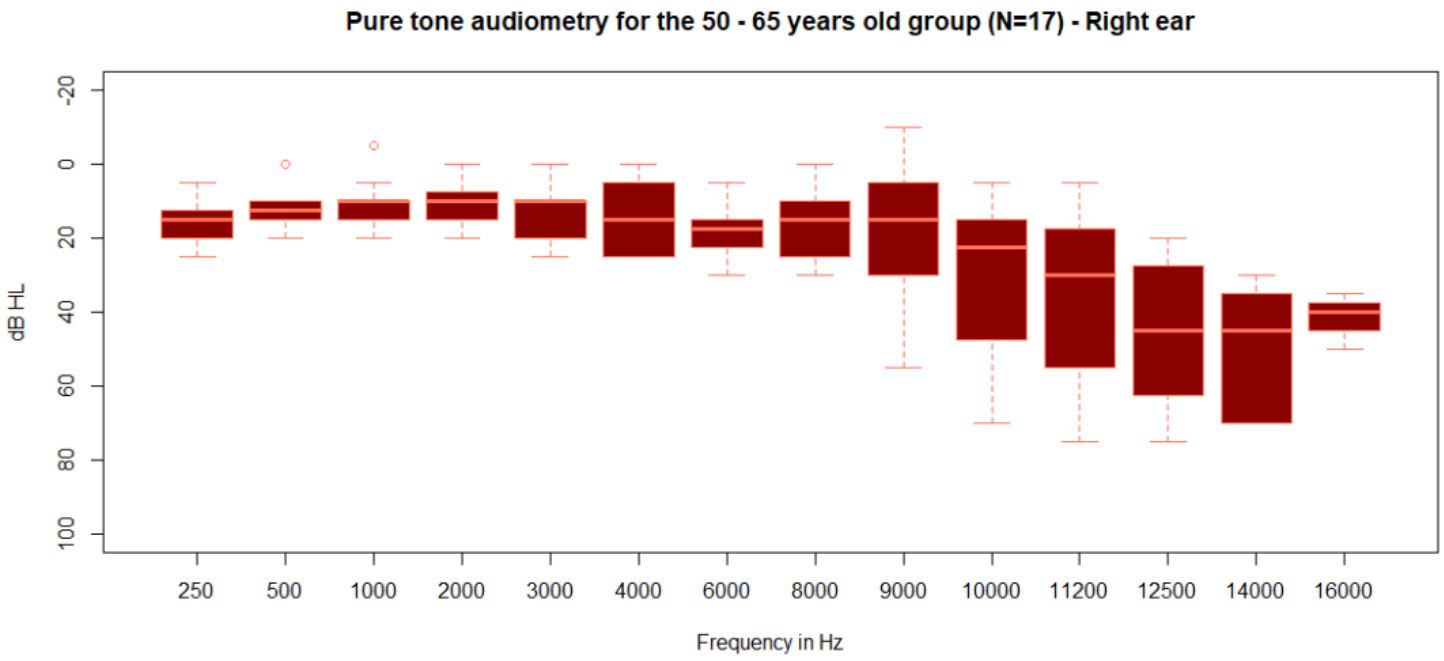


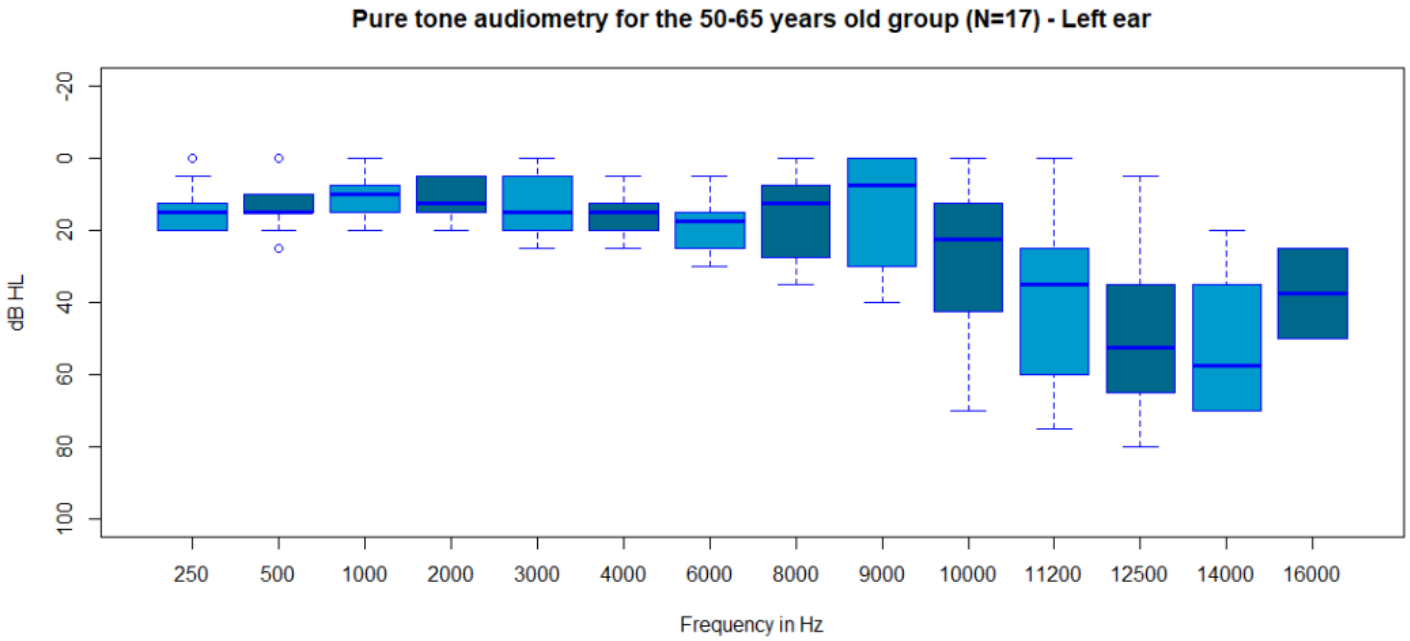
Supplementary material 3.4. Pure tone threshold boxplots of the Module 1a and 2 (2020, N=50). Samples of the right (A) and left (B) ear for the 40-49 years old.





Supplementary material 3.5. Pure tone threshold boxplots of the Module 1a and 2 (2020, N=50). Samples of the right (A) and left (B) ear for the 50-65 years old.



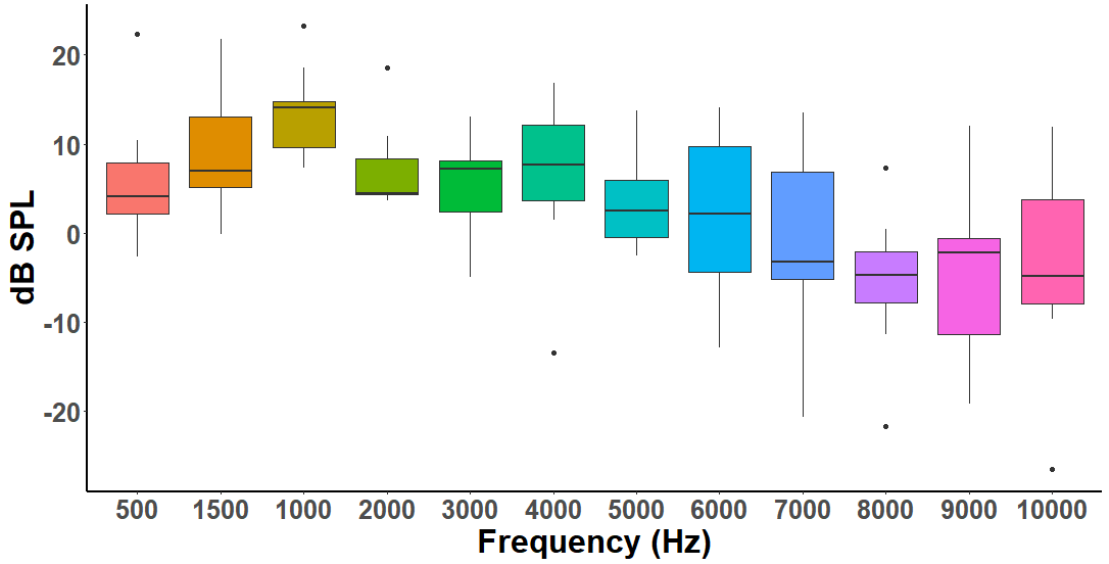


Supplementary material 3.6 i. Averaged Distortion Product Otoacoustic Emissions amplitude for the right ear, per frequency, per age group (top table). ii. Averaged Distortion Product Otoacoustic Emissions amplitude for the left ear, per frequency, per age group (bottom table).

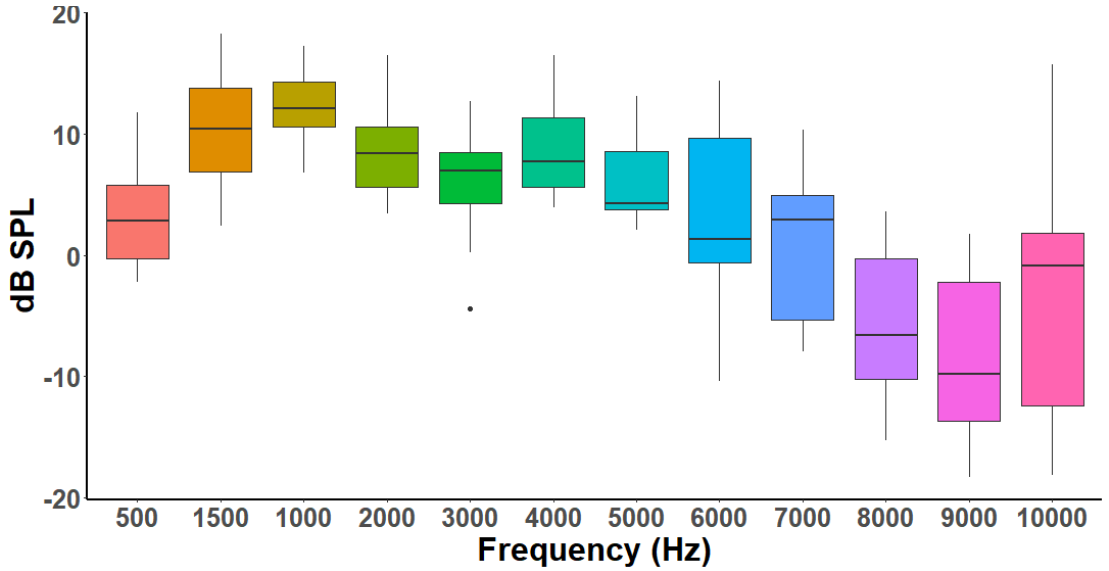
						i. Right Ear							
Age group	Age (years)		0.5kHz	1kHz	1.5kHz	2kHz	3kHz	4kHz	5kHz	6kHz	7kHz	8kHz	9kHz	10kHz
20-29	mean	27.22	3.56	9.21	13.97	10.04	7.90	7.00	4.06	4.82	-0.86	-5.09	-5.59	-5.26
	SD	1.09	10.19	8.31	4.97	4.58	3.90	6.10	5.63	6.37	10.55	9.63	10.71	12.04
30-39	mean	34.29	-0.25	7.19	11.62	7.95	6.29	5.86	3.38	2.99	-0.24	-4.83	-4.58	-4.29
	SD	3.00	6.65	7.29	4.52	4.78	3.73	5.25	5.67	5.58	8.28	8.35	10.39	11.53
40-49	mean	42.86	0.44	5.29	6.83	6.29	4.06	7.53	4.54	3.16	0.26	-5.51	-9.24	-10.80
	SD	2.19	4.16	3.09	4.74	4.49	4.26	4.18	5.69	8.44	9.75	8.73	16.31	18.42
50-65	mean	55.12	2.35	6.09	7.51	4.90	-1.41	-1.60	-3.65	-7.35	-13.88	-16.15	-16.72	-15.61
	SD	3.06	5.70	4.76	6.31	5.90	10.15	11.03	10.20	11.32	11.04	11.18	12.28	14.67

						ii. Left Ear							
Age group	Age (years)		0.5kHz	1kHz	1.5kHz	2kHz	3kHz	4kHz	5kHz	6kHz	7kHz	8kHz	9kHz	10kHz
20-29	mean	27.22	-0.02	6.23	11.52	8.54	7.18	6.51	4.56	2.19	-4.99	-10.43	-8.17	-8.03
	SD	1.09	7.10	6.77	6.37	5.55	4.83	6.55	6.07	10.13	14.06	11.28	8.24	15.62
30-39	mean	34.29	2.66	6.78	9.48	8.46	7.18	5.71	4.62	2.67	-1.16	-3.96	-3.78	-2.06
	SD	3.00	3.69	7.69	5.21	3.94	3.68	4.15	5.99	4.86	7.69	8.52	8.66	11.24
40-49	mean	42.86	0.73	5.91	9.99	9.83	7.30	9.50	8.84	4.13	0.17	-5.29	-7.31	-8.26
	SD	2.19	6.20	9.84	8.40	9.96	11.46	12.69	13.30	16.14	18.92	15.76	15.38	14.79
50-65	mean	55.12	2.85	4.08	6.56	4.56	-0.66	1.18	-3.19	-8.14	-13.22	-16.76	-16.88	-20.67
	SD	3.06	5.93	9.19	7.60	6.97	9.53	6.32	8.86	10.54	7.85	10.00	5.50	9.94

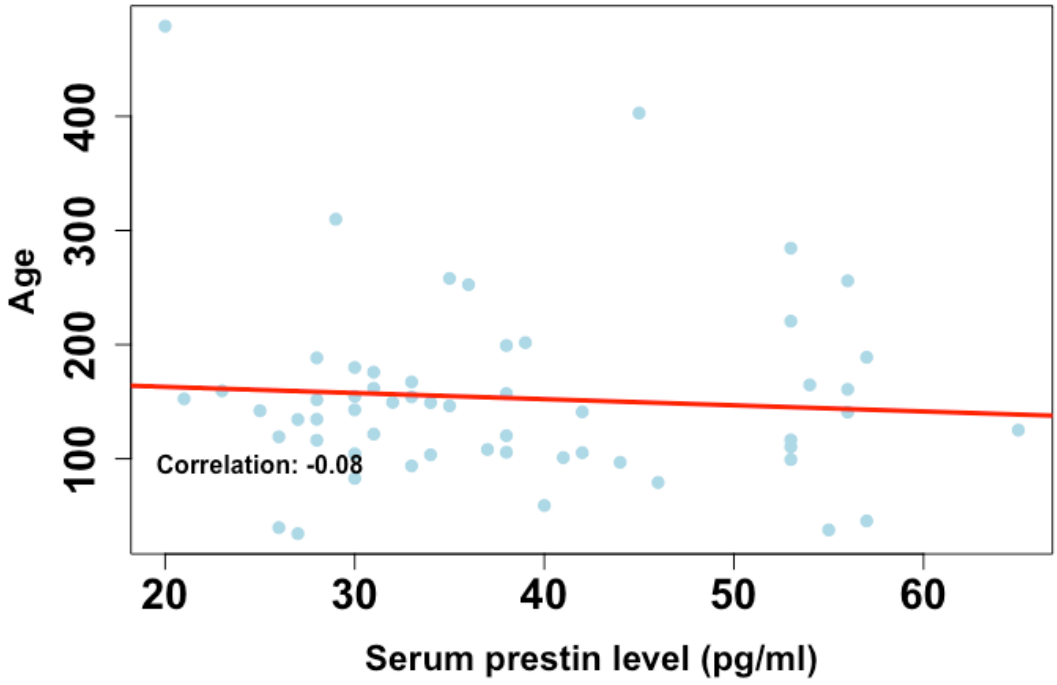
Supplementary material 3.7. OAE amplitudes boxplots for the eleven participants having provided blood samples in 2022 (Module 1b, right ear)



Supplementary material 3.8. OAE amplitudes boxplots for the eleven participants having provided blood samples in 2022 (Module 1b, left ear)



Supplementary material 3.9. Relation between serum prestin levels and participants' age (merged sample of 56 participants, $r_{(54)} = -0.04$, S = 30461, p = 0.76).



Chapter IV

Supplementary Material 4.3. Studies focusing on temporary and permanent noise-induced hearing loss.

STUDY ID	POPULATION	INTERVENTION	DURATION – LEVELS OF EXPOSURE	AUDIOMETRIC OR OTHER MEASUREMENT	IMPACT OF EXPOSURE ON HEARING
LIGHTFOOT (1954)	24 adults	2 kHz or white noise	3' at 105 dB SPL monaurally	Pure tone audiometry (0.125-8 kHz)	Temporary threshold shift (TTS) up to 22 dBA: 0-80'': thresholds rapidly returned toward normal; 80''-5': returned toward normal in patterns characterized by bounces and plateaus; 9': thresholds still elevated
MILLS (1978)	60 participants (37F, mean age=20.8±2.3 yrs)	Octave-band noise centred at 4, 2, 1, or 0.5 kHz	16-24 h at 75, 80, 83 and 88* dB via loud-speakers binaurally *stopped after 1-2 hours to avoid PTS	Pure tone audiometry (0,25- 8 kHz)	Threshold shifts increased for all levels of noise for about 4-12 h and then either decrease or level off to a plateau or asymptote. (TTS and exposure duration relation: simple exponential function with a time constant of 2.1 h). Recovery to within 5 dB of pre-exposure thresholds was achieved within 24 h or less (Recovery: simple exponential function with a time constant of 7.1 h.
SUTTON (1993)	14 participants (7F, age range=19-48 yrs, mean= 30.4, SD= 7.3 yrs)	2.8 kHz	3' at 105 dB SPL	Monaurally (best ear), Input / output functions were obtained for primaries at 3.636 kHz (f1), 1 and 4.400 kHz (f2). at a geometric mean of 4 kHz (DPOAE = 2.872 kHz), in 5-dB steps for two general stimulus conditions.	1. DPOAEs elicited with unequal primaries were more sensitive to reductions in emission levels 2. recovery of DP amplitudes over the first 15 min post exposure appeared to be roughly linear in log time. 3. DPOAE and behaviorally measured TTS had similar recovery time course.
MANSON (1994)	28 healthy participants (mean age=26, SD=2.6 years)	1/3 octave band-filtered noise with 2000 Hz centred frequency	3 sessions of 10' at 104 dB SPL monaurally through earphones	PTA (0,25- 4 kHz) after each session VO2 peak, core temperature, heart rate during experiment and 24-48h later	TTS: High-cardiovascular-fitness group: 5.8±1.42 dB, Low-cardiovascular-fitness group: 8.2±2.09 dB, Medium-cardiovascular-fitness group: 6.59±2.09 dB No testing for PTS
ENGDAHL (1996)	8 (3F) (mean age=28 yrs, age range: 25-33 yrs)	1. Tested ear: Narrow band noise (NBN) focused on 2 kHz 2. Contralateral ear: 2 kHz centred 1/3 octave band noise of 60 dB SL	3 sessions of 10' at 105 dB SPL (1 every 48h) monaurally, with insert earphones	Bekesy audiometry: 1dB step. Contralateral stapedius reflex at 2kHz 226Hz tympanometry	TTS was observed in all patients: 1 min after the noise exposure (14.7 dB, SD=3.8) and at 22 min (4.1 dB, SD=1.9) DPOAEs: greatest change (6.8 dB) reduction measured at 2' after noise exposure, at 3kHz. No testing for PTS.
QUARANTA (2003)	16 healthy adults (10F, age range=20–30 yrs)	10' of one of the 3 conditions: 1. 2kHz tone at 90dB HL* 2. 2kHz tone at 90dB HL* and ipsilateral 45 dB HL 250 Hz NBN 3. 2kHz tone at 90dB HL* and contralateral 45 dB HL 250 Hz NBN *right ear		At 2' after the exposure: PTA (250 ms duration, 25 ms rise/fall time, 50% duty cycle, (0.125-8 kHz, up to 120 dB SPL). Click TEOAEs and contralateral suppression	TTS: PTA: 2kHz: up to 5dB SPL 3kHz: up to 12.2dB SPL, [$TS2_{Group\ A} > TTS2_{group\ B}$ (p=0.012)], 4 kHz: up to 12.6dB SPL, [$TS2_{Group\ A} > TTS2_{group\ B}$ (p=0.003), $TTS2_{Group\ C} > TTS2_{groupB}$ (p=0.013)]. TEOAEs: TEOAE amplitude=7.06 dB (SD=4.3 dB). In all subjects, CAS caused suppression of >0.5 dB [ES= 1.32 dB (SD= 0.8 dB)]. No testing for PTS
QUARANTA (2004)	20 healthy adults (age range=20-30 yrs)	NBN masker centred at 3 kHz with a bandwidth of 775 Hz	10' at 112dB SPL unilaterally (right ear)	At 2' after exposure: PTA (0.25-8kHz) B12 measurement	$TTS2_{before\ treatment}$=2.9 dB (SD=2.0) at 1 kHz, 5.2 dB (SD=2.3) at 2 kHz, 16.6 dB (SD=4.7) at 3 kHz, and 21.5 dB (SD=5.9) at 4 kHz
ATTIAS (2004)	20 healthy males (Mean age = 21 yrs, age range= 16-37 yrs)	White noise	10' at 90 dB SL, monaurally	PTA immediately after 3 sessions of noise exposure (1 with no prior medication, 1 after 10 days of placebo treatment and 1 after 10 days of 122mg Mg) DPOAEs immediately and after 15' and 30'	PTA: TTS up to 25 dB, mostly in high frequencies (increasing by approximately 50% between 1 and 6 kHz) and statistically significant lower in post-Mg. DPOAEs: up to 4 dB amplitude shift, with complete recovery in the Mg session at 15' and 50% recovery (2dB) in the non-intake and placebo sessions at 30' No testing for PTS

LICHTENHAN (2008)	33 healthy adults (29F, age range:20-38, median age=28 yrs): 27 in the noise group, 6 controls	narrow-band noise (NBN) centred at 2 kHz	15' at 115 dB SPL, binaurally	PTA at 4kHz [30''-60'' post exposure and at the end of the experiment (1-1.5 h)] CAP at 75–125 dB pSPL in 10 dB steps, ascending or descending (60'' post exposure)	PTA: threshold shift ranged from -5 dB (thus improvement, 1 subject) to 30 dB. CAP: neurons contributing to the CAP during the aforementioned TTS seemed to be fewer in number, shorter in latency, and poorer in synchrony than before noise exposure
FETONI (2009)	20 males (mean age= 26.4 yrs range=23-28 yrs)	White Noise	15' at 90 dB HL presented symmetrically in open field	Before and 1h, 16h, 7 and 21 days after exposure: PTA (0.125-8kHz) DPOAEs: 70 dB SPL f2:f1 = 1.22 1-6kHz). Oxidative stress markers measurement	No TTS in PTA. DPOAEs: reduced amplitudes 1 and 16 h after exposure compared with the baseline values ($p<0.05$) No PTS observed
MOSHAMMER (2014)	125 males	200 – 500 Hz	20' at 100 dBA	Bekesy audiogram at 4 kHz starting 30 s after exposure for at least 10 min + Follow-up audiograms every 3 to 5 years	Mean TTS2 = 16 dB (range: 0-38 dB) PTS prediction: Using 14 dB TTS as a cut-off had 82% sensitivity and 53% specificity to predict 20 dB or higher levels of PTS.

Supplementary Material 4.4. Studies focusing on temporary and permanent music-induced hearing loss.

STUDY ID	POPULATION	INTERVENTION	DURATION – LEVELS OF EXPOSURE	AUDIOMETRIC OR OTHER MEASUREMENT	IMPACT OF EXPOSURE ON HEARING
LENDGRIN (1983)	10 adolescents (1F, mean age= 16.2 yrs, range = 16-17 yrs)	Music and Noise with level-, frequency-, and time-distribution characteristics, measured in octave-band steps, equal to those of the music	Binaurally, for 10' over the headphones at Leq = 106 dBA at each session (5 sessions of music, 5 sessions of noise, in randomised order)	Computerized sweep-frequency audiometer (type Bekesy) in the frequency range 1-8 kHz through inserts	TTS_{max} = 20 dB, one subject improved thresholds after exposure
LEE (1985)	16 healthy adults	Rock or Fusion music	3 hours at their usual preferred maximal listening level (90-104 dB SPL) binaurally	Standard Pure tone audiometry (PTA, 0.25-8 kHz)	9 volunteers exposed at 90-92 dB SPL: no TTS. 6 volunteers exposed at 98-99 dB SPL: TTS=10 dB at least 1 frequency 1 volunteer exposed at 103-104 dB SPL: TTS=30 dB at 4kHz and lower in all other frequencies
KRISHNAMURTI (2003)	9F (mean age=22, SD=5yrs)	Light-rock music	20' at 90 dB x 3 sessions with > 6days distance binaurally	Standard PTA and DPOAEs (2-8 kHz) on one ear, before and 2' after exposure	TTS of 1-6 dB No change in DPOAEs. No testing for PTS.
KRAMER (2006)	31 normal hearing participants (17F, age range=19-29, mean age=22 yrs)	Live music	2h at Lavg range of 92.5 to 102.8 dBA (mean=98.1 dBA) (open field, in a night club)	PTA (1,2,3,4,6,8 kHz) DPOAEs (frequency sweep method): f2 varied at six points between 2 and 8 kHz (2, 3, 4, 5, 6, and 8 kHz) and f2/f1=1.2. L1/L2=60/ 50 dB SPL. DPOAEs (ratio sweep): f2=4 kHz, f1 varied so that the f2/f1 was swept between 1.1 and 1.3. L1/L2=60/45 dB SPL. (starting ear=right)	TTS: Highest TTS found at 4 kHz and averaged 14.1 dB for those having PTA first and 9.8 dB for those having PTA second. Mean TTS across all participants and frequencies = 7.6 dB HL. DPOAEs 3-4 kHz: amplitude differences were -3.46 dB SPL for those who had DPOAE testing first and -2.97 dB for those who had DPOAE testing second. DPOAEs 5–8 kHz: amplitude differences were -5.63 dB for those who had DPOAE testing first and -6.30 dB for those who had DPOAE testing second. PTS: no PTS was observed; however, the recovery rate was

					steeper in the NAC-treated group.
BHAGAT (2008)	20 healthy adults (16F, age range:18-38yrs)	1 extended rock song	30' at 85 dB C ± 3 dB monaurally through MP3 earbuds (arbitrarily chosen ear)	Standard PTA (0.25-8 kHz), DPOAEs and SSOAEs within 10'-15'	PTA: No TTS nor PTS observed. DPOAEs: levels in half-octave bands centred from 1.4 to 6.0 kHz significantly reduced (averaged differences ranged from 0.08 to 0.47 dB) SSOAEs: Highly variable
KEPPLER (2009)	"Noise exposure group: 21 (11F, 19-28yrs) Control group: 28 (14F, 19-28yrs)"	Music (consisted of 17 songs from the CD Afrekening Volume 37 (PIAS, Brussels, Belgium), which is a compilation CD from the hit lists of a popular Flemish radio station.)	1h of LAeq from 76.87 to 102.56 dBA for the earbuds and from 71.69 to 97.36 dBA for the supra-aural headphones of an iPod 2GB	Standard PTA (0.25-8 kHz), DPOAEs and SSOAEs at 1h post-exposure, at 1 random ear per participant to obtain an equal number of left and right ears per sex	TTS of ~1 dB (3-6 kHz spared)
LE PRELL (2012)	33 normal-hearing (20F, mean age=20.9 yrs; age range=18–27)	1. "pop music playlist" 2."rock music playlist"	4h at 94, 99 and 100 dBA binaurally with ER61	PTA & EHF-PTA (5 dB HL down, 2dB HL up, for 0.25-8 kHz and 12.5-16 kHz) DPOAEs (DPOAES growth (input-output) with increasing stimulus level (L1=25 to 65 dB SPL, with stimulus levels decreasing in 5 dB steps within frequencies) were obtained at each of the six f2 frequencies. At 15 minutes, 1.15 min, 2.15 min, and 3.15 min post-music exposure. Then 24-h and 1-week post music exposure	TTS up to 7 dB HL in PTA. No TTS in EHF-PTA. No PTS was observed.
LE PRELL (2016)	70 young adults (38F)	1. "pop music playlist" 2."rock music playlist"	4h at 100 dBA Binaurally with ER61	PTA (6 dB HL down, 2 dB HL up, for 0.25-8 kHz) DPOAEs (f1 levels range: 25 to 65 dB SPL.) At 15 minutes, 1.15 min, 2.15 min 3.15 min, 24-h and 1-week post music exposure	PTA or EHF-PTA: TTS at 2, 3, 4, and 6 kHz (mean=3.7±4.6 dB, range: -4 to 20 dB). DPOAEs: significant difference between placebo and treatment group No PTS was observed
KIL (2017)	83 young adults (age range=18-31 years)	2 different playlists containing 63 rock songs or 69 pop songs.	4h at 100 dBA binaurally with ER61	Standard PTA: Modified Westlake procedure (6 dB HL down, 2 dB HL up, for 0,25-8 kHz) at 15 min, 1.25 h, 2.25 h, and 3.25 h post-noise exposure, 1 day later and 1 week post music exposure. Heart rate, blood pressure, respiration and temperature.	Larger TTS was observed at 4 kHz of the placebo group (mean=4.07 dB, SE=0.90 dB). 1 out of 81 participants did not return to within 4 dB from baseline (thresholds elevation of 6 dB at 1 week post-exposure).
KIKIDIS (2019)	4 musicians, 6 non-musicians	56 wav files with pop – rock songs	30' at maximum acceptable comfortable level (>83 dBA) through headphones	Before and within 5' after exposure: "PTA (0.25-8 kHz) HF-PTA (9-14 kHz) DPOAEs: 1-6 kHz (f2:f1=1.22) Auditory Brainstem Response (ABR): 33 and 44 clicks/second, at 90 dB nHL.	Statistically significant decrease in the DPOAE signal to noise ratio (SNR) measurements after noise exposure in all 10 participants, with the exception of 1 kHz No testing for PTS

Supplementary Material 4.3. Studies focusing on other than noise- or music-induced hearing loss.

STUDY ID	POPULATION	TARGET CONDITION	INTERVENTION (NOISE / MUSIC)	DURATION – LEVELS OF EXPOSURE	AUDIOMETRIC OR OTHER MEASUREMENT	IMPACT OF EXPOSURE ON HEARING OR OTHER SYSTEM
REINHARDT, (2012)	Study 1: 9 adults (8F) (age average=25.4 yrs) Study 2: 30 adults (15F) (age average=24.7 yrs, SD=4.6 yrs)	Noise-induced stress	White noise , as part of Manheim Multicomponent Stress Test (cognitive (mental arithmetic), emotional (affective pictures), acoustic (white noise) and motivational stressors (loss of money))	5' at 78-93 dB (ascending)	No audiometric test mentioned. Skin Conductance Level (SCL) and number of Skin Conduction fluctuation (NSCF) per minute during experiment (study 1). Saliva cortisol levels following baseline conditions (T1), after stress induction (T2) as well as 10, 20, 30, and 40 min after cessation of stress (T3–T6) (Study 2). Heart rate was continuously being measured throughout Study 1 and 2.	No testing for temporary (TTS) nor permanent threshold shift (PTS). Significant changes in electrodermal activity, subjective stress, heart rate, and salivary cortisol levels
HEBERT (2017)	20 Adults with tinnitus (10F) 20 Controls (9F)	Noise-induced stress	Low-frequency flat spectrum hissing noise (125–2000 Hz) with additional sound pressure in the 31.5–125 Hz range, resembling a fan	20' with a 2' pause at 80 dBA binaurally	Pure tone audiometry (0.5-8 kHz), only as inclusion criterion. Tinnitus Reaction Questionnaire (TRQ), Hyperacusis Questionnaire, a 10-point Likert-type stress scale, a 10-point Likert-type tinnitus intensity scale, salivary cortisol, at (0, +10', +20', +30', +40' and +60' post-noise exposure	No testing for TTS nor PTS Noise changed significantly cortisol response, subjective stress, and tinnitus intensity for both groups. The tinnitus group had lower overall cortisol levels, but higher subjective stress.
ANDREN (1981)	9 male adults with essential hypertension (mean age=42, range 29-56 yrs)	Essential hypertension	Broad-band noise	60' at 40 dBA, then 10' at 100 dBA binaurally	No audiometric test mentioned Blood pressure, heart rate, total peripheral resistance, stroke volume, heart failure monitored in multiple occasions during 3 different experiments on 3 separate days.	No testing for TTS nor PTS Diastolic and mean arterial blood pressure significantly increased. The increase was resistant to metoprolol, while propranolol accentuated it.
FUTATSUKA (1996)	6 healthy adults with no previous exposure to noise / hand-arm vibration (age range: 21-52 yrs)	Noise, hand-arm vibration and weight-induced changes in peripheral circulation	Chain-saw noise with and without use of ear plugs	5 sessions of 2' exposure at 105 dBA, 1 session of 2' exposure at 65 dBA (between 18.00-20.20)	No audiometric test mentioned Finger blood pressure, after each session	No testing for TTS nor PTS Vibration, noise and finger grip exercise induced 29.7 mmHg of increases on average in finger blood pressure, compared to 9.5 mmHg by vibration alone.
ROQUE (2013)	40 healthy females	Heart rate variability	Group 1 (n=21): music Group 2 (n=19): music and white noise	Between 08.00-12.00: Group 1 (n=21): music (2 sessions of 5') + quiet pause (70-80 dBA). Group 2: (n= 19) music and white noise auditory stimulation (90 dB)	No audiometric test mentioned. Heart rate variability, during 10' after exposure.	No testing for TTS nor PTS. Slight decrease of global heart rate variability in both groups.

POURYAGHOUB (2016)	100 healthy males (age range: 20-40 yrs)	Noise-induced stress	Recorded industrial noise	20' at 90 dBA	No audiometric test mentioned.	No testing for TTS nor PTS Mean salivary cortisol differed significantly between cases and controls and between cases before and after exposure.
ATTIAS (1994)	24 (12F) [mean age (SD) =27.1 (5.7) yrs]	Pain	1. White noise 2. MRI scanner noise	72' (6 blocks of 12') at 80-90 dB	No audiometric test mentioned	No testing for TTS nor PTS. Both MRI scanner noise and white noise significantly reduced unpleasantness ratings, while the ability to localize pain was not significantly affected.
CAVALIERE (2004)	10 healthy subjects (2F) (age range=23–49 yrs, median=27yrs)	Noise - induced discomfort and sleep disruption in patients of the ICU	Noise of NoN-Invasive Ventilation (NIV) with a helmet, a nasal mask, and a facial mask.	2h at 60-110 dB	No audiometric test mentioned. Noise exposure levels and subjective evaluation of noise according to a visual analog scale (VAS)	No testing for TTS nor PTS. Similar discomfort for patients using NIV helmet and masks, although helmet is much louder.
CAVALIERE (2007)	8 healthy volunteers (4F), (age range=27–52 yrs, mean=37)	Noise - induced discomfort and sleep disruption in patients of the ICU	CPAP noise	140' at 57-94 dBA	No audiometric test mentioned. Noise exposure levels and subjective evaluation of noise (VAS)	No testing for TTS nor PTS. Subjective discomfort mirrors the objective noise measurements.
ANDREN (1983)	13 (8F), (mean age =44 yrs, age range: 21-59	Effects of loud noise on cardiovascular system	Broad band noise	10' at 100 dBA	No audiometric test mentioned. Hemodynamic parameters measured.	No testing for TTS nor PTS. Exposure to noise caused a significant increase in systolic, diastolic and mean arterial pressure. There was no significant change in heart rate, stroke volume or cardiac output.
CASTLE (2007)	21 males (age range=22-71 yrs, mean= 44 yrs)	Gastric myoelectric activity (GMA) and autonomic nervous system function	1.Unedited hospital noise at 87.4 dBA 2. Conversation babble at 91.3 dBA 3. Traffic noise at 85.6 dBA	60’ through stereophonic headphones.	No audiometric test mentioned after exposure. blood pressure, GMA	No testing for TTS nor PTS Loud noise may decrease the overall percentage of three cycle per minute (CPM) activity, especially in younger participants and increase the incidence of bradygastria.
LJUNGBERG (2004)	54 participants (27F, mean age =25 yrs, range: 19-30)	Cognition	Noise from a helicopter (dominant frequency at 21 Hz)	20’ of exposure 5’ stopped x 3 times (at 77, 81 and 86 dBA) through loudspeaker	No audiometric test mentioned. Short-term memory performance testing	No testing for TTS nor PTS No change in reaction times, but higher annoyance in noise group.
TAFALLA (1997)	33M (mean age=21.4, range:18-31 yrs)	Performance effort	Random bursts of intermittent background noise consisting of traffic, office machinery, and babble	For 90’at 90dBA peaks: Noise bursts were 15’’ to 1’ apart, lasting 3’’ to 5’’ through loudspeaker	No audiometric test mentioned	No testing for TTS nor PTS Noise increased heart rate, norepinephrine, and cortisol under high effort. Blood pressure did not change significantly. Reaction time slowed significantly.

Chapter V

Supplementary Material 5.1. TTS per frequency for the 17 participants of the pilot study. All participants were exposed to 15' or 30' of the same pop-rock playlist, at 100 or 97 dBA respectively. Levels of exposure, self-reported comfort level during music exposure (1 = extremely uncomfortable, 10 = very comfortable), any type of tinnitus/buzzing in the ears, and aural fullness immediately after music exposure (1 = no aural fullness, 10 = sensation of complete blockage) are also reported. Trial ID corresponds to the part of the

ID	TRIAL ID	TINNITUS DURATION (MIN)	AURAL FULNESS	COMFORT LEVEL	EXPOSURE (DBA)	TTS1	TTS3	TTS4	TTS6	TTS8	TTS10	TTS12.5
1	1	1	3	7	97	10	6	n/a	0	2	0	-8
2	1	0	2	5	97	-8	12	n/a	26	14	4	6
3	1	0	10	3	100	-2	2	n/a	-4	0	-2	-6
4	1	0	10	1	100	4	0	n/a	0	0	6	24
5	1	0	8	6	100	6	10	n/a	0	-2	2	-8
6	1	0	8	6	100	0	2	n/a	-10	2	-2	-4
7	1	0	5	7	100	0	-2	n/a	-8	12	2	2
8	1	0	4	3	100	-4	2	n/a	8	8	0	8
ID	**TRIAL ID**	**TINNITUS DURATION (MIN)**	**AURAL FULNESS**	**COMFORT LEVEL**	**EXPOSURE (DBA)**	**TTS1**	**TTS3**	**TTS4**	**TTS6**	**TTS8**	**TTS10**	**TTS12.5**
1	2	0	10	1	100	2	8	2	4	4	-4	0
9	2	0	5	1	100	0	-2	2	-2	0	4	-2
10	2	0	4	1	100	0	2	8	24	0	0	0
11	2	0	6	4	100	0	6	2	16	6	4	-6
3	2	0	8	2	100	0	-4	-6	16	10	2	-16
12	2	0	3	2	100	-2	-2	-10	-2	-26	-12	-10
2	2	0	2	5	100	-4	8	12	12	0	-2	4
6	2	0	8	6	100	4	10	10	0	6	-4	0
13	2	0	10	6	100	-4	2	10	6	8	-4	-4
4	2	0	10	1	100	2	-6	-2	10	-2	8	-4
14	2	0	9	4	100	-2	4	2	4	6	14	-2
15	2	0	7	1	100	-2	6	8	4	4	-2	-2
16	2	0	5	4	100	4	4	-2	8	-14	-12	2
17	2	0	6	7	100	0	6	2	4	2	-2	0

study (1=first part and initial audio material, 2=second part with further manipulated audio material).

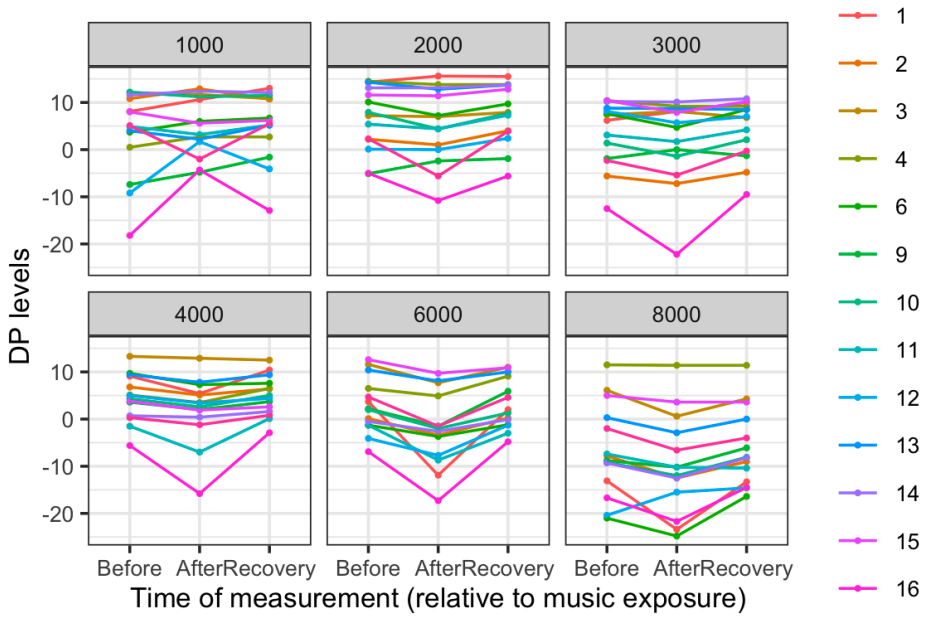
Supplementary Material 5.2. Ultimate PTA threshold shift per frequency for the first part of the experimental study. PTA thresholds have returned within 4 dB from baseline for all participants.

ID	1-KHZ ULTIMATE THRESHOLD SHIFT	3-KHZ ULTIMATE THRESHOLD SHIFT	6-KHZ ULTIMATE THRESHOLD SHIFT	8-KHZ ULTIMATE THRESHOLD SHIFT	10-KHZ ULTIMATE THRESHOLD SHIFT	12.5-KHZ ULTIMATE THRESHOLD SHIFT
1	-4,00	-4,00	-12,00	0,00	0,00	-8,00
2	-2,00	2,00	4,00	4,00	2,00	-2,00
3	2,00	2,00	-2,00	2,00	-12,00	-10,00
4	0,00	-2,00	-2,00	0,00	-2,00	-6,00
5	2,00	2,00	-10,00	-16,00	-4,00	-4,00
6	0,00	2,00	-10,00	2,00	-2,00	-4,00
7	0,00	-2,00	-8,00	0,00	2,00	2,00
8	-6,00	0,00	-4,00	0,00	0,00	0,00
AVERAGE TTS	-1	0	-5,5	-1,25	-2,25	-4

Supplementary material 5.3. Ultimate PTA threshold shift per frequency for the second part of the experimental study. PTA thresholds have returned within 4dB from baseline for all participants. The average shift for participants 1-17, per frequency is presented in the row "Average change".

ID	1-KHZ ULTIMATE THRESHOLD SHIFT	3-KHZ ULTIMATE THRESHOLD SHIFT	4-KHZ ULTIMATE THRESHOLD SHIFT	6-KHZ ULTIMATE THRESHOLD SHIFT	8-KHZ ULTIMATE THRESHOLD SHIFT	10-KHZ ULTIMATE THRESHOLD SHIFT	12.5-KHZ ULTIMATE THRESHOLD SHIFT
1	2	4	2	-4	-2	2	-2
9	2	0	0	-12	0	-2	-4
10	2	-6	0	4	-12	0	2
11	-2	-6	-12	2	0	0	-2
3	-4	-6	-12	2	0	0	-2
12	0	-4	-8	-4	-16	-22	-24
2	-10	-8	2	-4	-6	-8	-32
6	-6	-12	-4	-10	0	0	0
13	0	0	0	-2	-4	-8	-16
4	4	2	0	0	0	0	0
14	-2	2	-2	0	2	2	2
15	-2	2	-10	-4	-2	-4	0
16	4	0	2	-2	-2	-10	-4
17	-16	-2	-10	0	-4	-20	-18
AVERAGE CHANGE	-2	-2.43	-3.71	-2.43	-3.29	-3.57	-7.14

Supplementary material 5.4. DP amplitude at different time points before and after music exposure, per frequency, per individual.



Relevant Publications – Conference Presentations

Conferences

1. Biomarkers In Noise-Induced Hearing Loss

10.2019 Oral Presentation, Panhellenic ENT Congress – Larissa, Greece

2. Prestin as Biomarker in Sensorineural Hearing Loss

03.2022 Invited talk, Round table presentation on “Sensorineural Hearing Loss”, Panhellenic ENT Congress – Thessaloniki, Greece

3. Music Induced Hearing Loss: Development of a temporary threshold shift paradigm and role of prestin as a biomarker.

06.2023 Invited talk, NKUA – UoPitts Collaboration Meeting – Hippokrateion General Hospital, Athens, Greece

4. Blood prestin levels following music exposure that induces temporary threshold shifts.

09.2023 Poster presentation, 58th Inner Ear Biology Workshop & Summit - UCL Great Ormond Street Institute of Child Health London, London, UK

5. Blood prestin levels following music exposure that induces temporary threshold shifts.

09.2023 Poster Presentation, Basic Auditory Science 2023 - Dyson School of Design Engineering at Imperial College London, London, UK

6. Music Induced Hearing Loss: Development of a temporary threshold shift paradigm and role of prestin as a biomarker.

11.2023 29th Panhellenic Congress of Otoneurology, Audiology, and Otology, Electra Palace Thessaloniki

7. Blood prestin levels following music exposure that induces temporary threshold shifts.

11.2023 Poster presentation, UK Hearing Conservation Association Conference, Sheffield, UK

Publications

1. Iliadou, Eleftheria; Kikidis, Dimitrios; Pastiadis, Konstantinos; Plack, Chris J.; Bibas, Athanasios. Blood Prestin Levels in Normal Hearing and in Sensorineural Hearing Loss: A Scoping Review. Ear and Hearing: September/October 2021 - Volume 42 - Issue 5 - p 1127-1136, doi: 10.1097/AUD.0000000000001045
2. Iliadou, Eleftheria; Pastiadis, Konstantinos; Dimitriadis, Dimitrios; Plack, Chris J.; and Bibas, Athanasios. Development and validation of an efficient and safe loud music exposure paradigm. In press, in the Journal of Speech, Language, and Hearing Research.

Pending publications

1. Serum Prestin Levels in Young and Middle-Aged Adults with Normal Hearing: Effects of Age, Sex, and Time of Day: will be soon submitted for publication in the Ear and Hearing Journal.
2. Exposure to noise or music in clinical trials: A scoping review on ethical and methodological considerations: submitted for publication in the Noise and Health Journal, status: under review.
3. Blood prestin levels before and after exposure to loud music: a longitudinal study: submitted for publication in the Ear and Hearing Journal, status: Accepted for publication with minor revision.