University of Southampton

Faculty of Engineering and Physical Sciences

Institute of Sound and Vibration Research

Role of Innate Immune Function in the Neurobiological Substrates of Hearing Loss

DOI 10.5258/SOTON/T0038

by

Katie Louise Hough

ORCID ID 0000-0002-5160-2517

Thesis for the degree of Doctor of Philosophy

December 2021

University of Southampton

Abstract

Faculty of Engineering and Physical Sciences

Institute of Sound and Vibration Research

Thesis for the degree of Doctor of Philosophy

Role of Innate Immune Function in the Neurobiological Substrates of Hearing Loss

by

Katie Louise Hough

Macrophages are the most abundant immune cell within the cochlea. Their role in the pathophysiology of hearing loss is not well understood despite a body of work studying human and animal cochleae. The aim of this research was to investigate the role of innate immune function in the cochlea.

A multi-method approach, using analysis of mouse and human tissue to investigate the role of macrophages in cochlear health, was carried out. The current evidence for the distribution, morphology and function of macrophages following acute and chronic cochlear insults was reviewed. Findings support the hypothesis that cochlear macrophages contribute to the trajectory of hearing loss through macrophage activation. The effects of chronic middle ear inflammation were studied using the Junbo mouse model, a mutation that results in changes characteristic of otitis media. Increased macrophage activation and Il-1β expression was observed in the cochlea and middle ear of Junbo mice inferring that middle ear inflammation caused increased macrophage activation across regions of the inner ear, or cochlea. A method to analyse fibrotic tissue on explanted cochlear implants was developed. Histological analysis revealed an aberrant, unresolved wound healing response with evidence of ongoing inflammation and proliferation, on an implant 10 months after implantation. Cochlear implantation in the mouse was established as the basis of a model system for the experimental study of tissue responses to cochlear implantation. This model enables a combination of tissue analysis methods, alongside micro-computed tomography imaging, to investigate the tissue response and factors which influence the response.

This work has consolidated the evidence for cochlear macrophages as a key cell population in cochlear health, and that they may have a role in hearing loss. This is important as they may be amenable to manipulation that alters the course of hearing loss. It has established that an existing, partially characterised, mouse model warrants more detailed study that may be translationally relevant in understanding the consequence of early life middle ear inflammation. It reports the first published evidence for immune responses to an implant in a person undergoing subsequent re-implantation, and establishes a pipeline for human explant tissue analysis that will lead to new understanding of poor hearing performance with a cochlear implant. This research advances our understanding of the innate immune mechanisms involved in cochlear health and identifies a new putative therapeutic target that may lead to improved hearing outcomes with a cochlear implant.

Table of Contents

Table of Tables

Table of Figures

Table of Figures

List of Accompanying Materials

Title	Format	Place	Date
Quantitative analysis of Junbo mouse model-Dataset DOI: 10.5258/SOTON/D2060	Thesis dataset	Pure	December 2021
Investigating the tissue-electrode interface after cochlear implantation	Conference talk	Oticon Medical R&T Young Investigators Virtual Symposium	September 2021
Macrophages in the cochlea: an immunological link between risk factors and hearing loss DOI: 10.1002/glia.24095	Published peer reviewed review article	GLIA	September 2021
Inflammation at the tissue-electrode interface in a case of rapid deterioration in hearing performance leading to explant after cochlear implantation https://doi.org/10.1097/MAO.0000000000003014	Published peer reviewed research article	Otology & Neurotology	April 2021
Corroborating µCT and histological data to provide novel insight into the biological response to cochlear implantation at the electrode-tissue interface	Conference talk	Tomography for Scientific Advancement Symposium	September 2020
Early life inflammatory events; a risk factor for hearing loss in later life	Conference talk	Southampton Neuroscience Group Annual Conference	September 2019
Corroborating µCT and histological data to provide novel insight into the biological response to cochlear implantation at the electrode-tissue interface	Conference Poster	Tomography for Scientific Advancement Symposium	September 2019
Investigating immune priming in the auditory system as a cause of variable outcomes after cochlear implantation	Conference Poster	Wessex Immunology Group Spring Meeting	April 2019
Investigating immune priming in the auditory system as a cause of variable outcomes after cochlear implantation	Conference Poster	British Cochlear Implant Group Conference	April 2019

List of Accompanying Materials

Title	Format	Place	Date
Investigating immune priming in the auditory system as a cause of variable outcomes after cochlear implantation	Conference Poster	Southampton Neuroscience Group Seminar	February 2019
Emergence of synaptic and cognitive impairment in a mature-onset APP mouse model of Alzheimer's disease https://doi.org/10.1186/s40478-019-0670-1	Peer reviewed research article	Acta Neuropathologica Communications	February 2019
Investigating immune priming in the auditory system as a cause of variable outcomes after cochlear implantation	Conference Poster	Southampton Neuroscience Group Annual Conference	September 2018
Investigating the role of the immune response in cochlear health after cochlear implantation	Conference talk	University of Southampton Bioengineering Conference	January 2018

Research Thesis: Declaration of Authorship

Print name: Katie Louise Hough

Title of thesis: Role of Innate Immune Function in the Neurobiological Substrates of Hearing Loss

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

Hough, K., Sanderson, A., Grasmeder, M., Mitchell, T., Verschuur, C. A., & Newman, T. A. (2021). Inflammation at the Tissue-Electrode Interface in a Case of Rapid Deterioration in Hearing Performance Leading to Explant After Cochlear Implantation. *Otology & Neurotology*, *42*(4), e445–e450. https://doi.org/10.1097/MAO.0000000000003014

Hough, K., Verschuur, C. A., Cunningham, C., & Newman, T. A. (2021). Macrophages in the cochlea; an immunological link between risk factors and progressive hearing loss. Glia,1–20. https://doi.org/10.1002/ glia.24095

Signature: Date:

Acknowledgements

I would like to thank my supervisors Dr Tracey Newman and Professor Carl Verschuur for their ongoing encouragement, guidance and support over the last four years. Thank you, Tracey, for teaching me (by example) the skills required for good mentoring and leadership and giving me the opportunities to develop these skills.

I am grateful for the support and good times with my lab colleagues; Eleanor, Leafy, Rachel and Alan. I am particularly grateful to my lab partner and friend for life Alan for all of the laughter, great conversation and encouragement. I hope we can continue to 'do science' together! Thank you to all of my fellow PhD friends; Connor, Grace, Alan, Dani and Belle for the constant moral support and fun.

To the girls from home; Melissa, Georgia, Bec and Grace. I appreciate your patience, love and cheerleading from afar! Thank you for always welcoming me home with open arms and for organising such amazing and often fancy days out!

I am extremely grateful for my housemates who have been there to listen, encourage and motivate me to keep going. Gabs, thank you for always listening and cheering me on! A special shout out to the DD; Stef, Karen and Katrina for putting up with my moans, tiredness and difficult times particularly in the last 18 months where I've been working from home. I couldn't have got through it without you all.

I will be forever grateful to my family who have always been my biggest supporters. You have encouraged and allowed me to follow my passion which I am so grateful for. Thank you for always believing in me. I certainly wouldn't have got to this point in my life without the endless love and support you give me. Thank you to my Mum for being an excellent role model and for showing me what it means to be strong. To my sister, thank you for always being there whenever I needed you. For the cards of encouragement in the post, for driving 100 miles to see me for a picnic in the pandemic and for taking me on amazing holidays. And finally, thank you for your sacrifices and significant financial support over the years. Thank you to Bruce for caring for me like I was his sister. To my brother, thank you for your big cuddles which make everything better! I am so grateful for your unconditional love and encouragement and for the great fun we have together. Thank you to Steve for always welcoming me home, making me delicious dinners and for ferrying me to and from Southampton over the last 8 years! And thank you to my dad for always telling me he is proud.

And finally, to Em, thank you for being my rock, particularly when things have been really tough. I am grateful to the Bradshaws for being a second family and always welcoming us into their home! Thank you Em for your understanding, love and patience. Your calm, bubbly and positive outlook on life is a blessing to be around and I am grateful to know that I always have your unconditional support.

Contributors

Chapter 2- **My contribution:** A systematic literature search, distilled the relevant information and created all of the figures and tables. Wrote the text and edited the manuscript of the published review article titled 'Macrophages in the cochlea; an immunological link between risk factors and hearing loss'. Thanks to **Dr Colm Cunningham** for contributing to the drafting of the review article (Appendix C).

Chapter 3- **My contribution:** Contributed to the conceptual development and design of the project, which highlighted the utility of the Junbo mouse model for investigating macrophage biology. Carried out all of the immunohistochemical staining, image acquisition and qualitative and quantitative analysis of the H&E and immunohistochemically stained sections. Thanks to **Dr Derek Hood** and **Dr Pratik Vikhe** for providing the Junbo mouse tissue from MRC Harwell and contributing to conceptual development through regular meetings and discussion. Thanks to colleagues at the **Mary Lyon Centre** (MRC Harwell) for carrying out the animal handling and experiments. Thanks to the **Histology Department** (MRC Harwell) for processing and sectioning the mouse tissue.

Chapter 4- **My contribution:** Each stage of the experimental work: processed, embedded and sectioned the human explanted tissue. Stained the tissue using histology and immunohistochemistry with various markers. Carried out all image acquisition, processing and analysis. Produced the figures and contributed to the production of the manuscript for the research article titled 'Inflammation at the tissue-electrode interface in a case of rapid deterioration in hearing performance leading to explant after cochlear implantation'. The project planning and application for the EPSRC Doctoral Prize Award funding to carry out further human explant tissue analysis. Thanks to **Dr Alan Sanderson** for contributing to the study design and conceptual development for the explant tissue analysis. Thanks to **Dr Mary Grasmeder** for contributing her audiological perspective and insight about the patient with the explanted array.

Chapter 5- **My contribution:** Participated in all meetings with our collaborators at Oticon Medical and UCL. This involved intellectual contribution to experimental design and planning, and the design and optimisation of the electrode arrays. Contributed to the initial stages of protocol optimisation for surgical implantation. μCT image acquisition, processing and analysis of the mouse cochleae implanted with a CI. Thanks to **Dr Lucy Anderson** for contributing to the development of the mouse model, particularly with the optimisation of surgical technique, providing training for the surgical technique and for carrying out the cochlear implantation for the samples imaged by μCT. Thanks to our collaborators at Oticon Medical - **Dr Roger Calixto, Dr**

Martin Johansson, Dr Guillaume Tourrel, Dr Prabhakar Sidambaram, Dr Pierre Stahl for contributing to the development of the mouse model through regular meetings and discussion. Thanks to **Pierre Stahl** and **Guillaume Tourrel** for designing and producing the electrode arrays which required custom machining of molds for injection molding of the elastomer used to construct the soft material of the devices. Thanks to **Dr Orestis Katsamenis** who assisted in the μCT image acquisition and contributed to the discussion around methodological development. Thanks to **Dr David Chatelet** for providing training for Avizo and ImageJ software for the μCT image processing.

Experimental contributors: **Dr Shmma Quraishe** contributed practical advice and guidance for immunohistochemistry. **Dr Susan Wilson, Jenny Norman and Jon Ward** of University of Southampton Histochemistry Research Unit provided training on tissue processing, sectioning and histological staining.

Abbreviations

aABR	Acoustic auditory brainstem response
ABR	Auditory brainstem response
AD	Alzheimer's disease
AN	Auditory nerve
AOM	Acute otitis media
APES	Aminopropyltriethoxysilane
ARHL	Age-related hearing loss
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ASP	Animal stimulator platform
ATP	Adenosine tri-phosphate
AVA	Anterior vestibular artery
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BKB	Bamford-Kowal-Bench
BLB	Blood-labyrinth barrier
BM	Basilar membrane
BMI	Body mass index
CA	Cochlear artery
CBCT	Cone-beam computed tomography
CCL2	Chemokine C-C motif ligand 2
CCR2	Chemokine C-C motif receptor 2
CHL	Conductive hearing loss
CI	Cochlear implant
CNS	Central nervous system
CRP	C-reactive protein
Csf1	Colony stimulating factor 1
CSOM	Chronic suppurative otitis media
CT	Computerised tomography
CVA	Cochlear-vestibular artery

Abbreviations

DAB	Diaminobenzidine
DAM	Disease associated microglia
dB	Decibel
DEX	Dexamethasone
DPX	Dibutylphthalate polystyrene xylene
eABR	Electric auditory brainstem response
EDTA	Ethylenediaminetetraacetic acid
EI	Electrode impedance
ENU	N-ethyl-N-nitrosourea
ET	Eustachian tube
FBGCs	Foreign body giant cells
FBR	Foreign body reaction
FDA	Food and drug administration
GWAS	Genome wide association studies
H&E	Haematoxylin & Eosin
HDACs	Histone deacetylase
Iba1	Ionised calcium binding adapter molecule 1
IHCs	Inner hair cells
IL-1β	Interleukin 1-beta
IL-6	Interleukin 6
KLH	Keyhole-limpet hemocyanin
LMIC	Low to middle-income country
LPS	Lipopolysaccharide
LW	Lateral wall
MA	Mastoid antrum
MAUDE	Manufacturer and User Facility Device Experience
MH	Modiolar hugging
MyD88	Myeloid differentiation primary response
NET	Neutrophil extracellular trap
NFkβ	Nuclear factor-kβ
NICE	National Institute of Health Care Excellence

NIHL	Noise-induced hearing loss
NRT	Neural response telemetry
NTHi	Non-typeable *Haemophilus influenzae*
OC	Organ of Corti
OCT	Optimal cutting temperature
OHCs	Outer hair cells
OM	Otitis media
OME	Otitis media with effusion
OSL	Osseous spiral lamina
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PEDF	Pigment epithelium growth factor
PEDOT	Poly(3,4-ethylenedioxythiophene)
PFA	Paraformaldehyde
PTMs	Post-translational modifications
PVM/Ms	Perivascular macrophage-like melanocytes
PVMs	Perivascular macrophages
rAOM	Recurrent acute otitis media
RBC	Red blood cell
RC	Rosenthal's canal
REVS	Recording of electrode voltages
RT	Room temperature
SA	Stapedial artery
SEM	Scanning electron microscopy
SGNs	Spiral ganglion neurons
SL	Spiral ligament
SNHL	Sensorineural hearing loss
SPL	Sound pressure levels
SR	Synchrotron radiation
SR-SIM	Super-resolution structured illumination microscopy
ST	Scala tympani

Abbreviations

SV	Stria vascularis
TBI	Traumatic brain injury
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
USAIS	University of Southampton Auditory Implant Service
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor-2
WBC	White blood cell
WT	Wild-type
α-SMA	Alpha-smooth muscle actin
μCT	Micro-computerised tomography

Chapter 1 A role for the innate immune system in driving hearing function

1.1 Hearing loss and cochlear implantation

Hearing loss is a debilitating disability that affects 11 million people across the UK, which is around one in six of us. In the next 15 years this figure is expected to increase to around 15.6 million people across the UK: one in five people. Hearing loss has significant social, emotional and economic costs to the individual and society. It restricts a person's ability to connect to, and communicate with, the world around them and is associated with reduced educational attainment, unemployment/underemployment and reduced income (Emmett and Francis, 2015; He *et al.*, 2018; Scholes *et al.*, 2018) across a lifetime.

Hearing is the perception of sound waves travelling through the outer and middle ear to the inner part of the ear, the cochlea. Here, the sound vibrations are converted to neural signals through the action of specialised sensory cells, called hair cells, which are induced in the auditory nerve. The auditory nerve transmits this sound information to the brain, allowing sound to be heard. Dysfunction in any part of this sound transduction pathway can cause hearing loss. It has been estimated that around 900,000 people in the UK have severe to profound hearing loss and could benefit from cochlear implantation. Cochlear implants (CIs) are an implanted auditory prosthesis that function to bypass the damaged hair cells of the inner ear and directly stimulate spiral ganglion neurons (SGNs) that transmit sound information from the auditory nerve to the brain, enabling perception of sound. The total number of people in the UK with CIs in 2021 was around 19,000 people (Cullington, 2021). A small but significant group of these people with a CI have sub-optimal performance with their implant. This project aims to investigate the role of innate immune function and the local tissue response at the tissue-electrode interface in driving hearing performance outcomes following cochlear implantation.

1.2 Anatomy of auditory system

The auditory pathway is subdivided into the central (regions from the cochlear nucleus in the brainstem to the auditory cortex) and peripheral systems (the outer, middle and inner ear (cochlea)). Figure 1.1 illustrates the pathway, morphology and cellular components of the auditory system which are essential for hearing. The outer, middle and inner ear have different roles in sound transduction and balance. The outer ear is important for the localisation of sound.

It consists of the pinna (or auricle), the visible part of the ear, and the external acoustic meatus (ear canal) which is an S shaped tube extending from the pinna to the tympanic membrane. The skin lining the meatus has ceruminous glands and hair follicles to prevent the passage of foreign materials by the production of ear wax and the presence of hairs (Musiek and Baran, 2018). The middle ear is an air-filled space which communicates with the nasopharynx via the Eustachian tube, which has a role in equalising the air pressure between the outer and middle ear. The transmission of sound to the inner ear requires air-borne vibrations to be converted to vibrations in the cochlear fluids, resulting in a difference in impedance between the two mediums. The middle ear therefore serves as an impedance transformer and ensures efficient transmission of sound energy from air (outer ear) to fluid (cochlea) by two mechanisms: a reduction in the area of the oval window compared to the tympanic membrane and the lever formed between the malleus and incus (Musiek and Baran, 2018). The inner ear comprises the cochlea and the vestibular apparatus consisting of the semi-circular canals, the vestibule and the Otolith organs called the utricle and saccule. The vestibular apparatus, part of the vestibular system, is essential for balance, equilibrium and motion. The cochlea plays an essential role in hearing and auditory transduction by converting sound waves into electrical impulses which are induced in the auditory nerve and transmitted to the brain (Musiek and Baran, 2018).

Sound waves move through the air as vibrations from the source to the listener. The vibrational sound waves are collected and funnelled into the external acoustic meatus (ear canal) by the pinna. The vibrations move through the ear canal to reach the tympanic membrane, which separates the outer and middle ear, causing it to vibrate. The vibrations from the tympanic membrane cause movement of the three ossicles in the middle ear (malleus, incus and stapes). The ossicles form a chain between the tympanic membrane and oval window (Anthwal and Thompson, 2016). The malleus is connected to the centre of the tympanic membrane and allows the transfer of sound energy from the malleus, to the incus then finally to the stapes. The stapes footplate is in contact with the oval window of the cochlea, therefore movement of the stapes causes the footplate to hit the oval window. Here the vibrational waves are converted to fluid-filled vibrations in the scala vestibuli of the cochlea. The organ of Corti is positioned on the basilar membrane, a strand of connective tissue running from the base to the apex of the cochlea. It vibrates differently at the basal end compared to the apical end, depending on the frequency of sound vibrations. These structural features are important for mechanotransduction, the conversion of mechanical energy (vibration) to electrical energy.

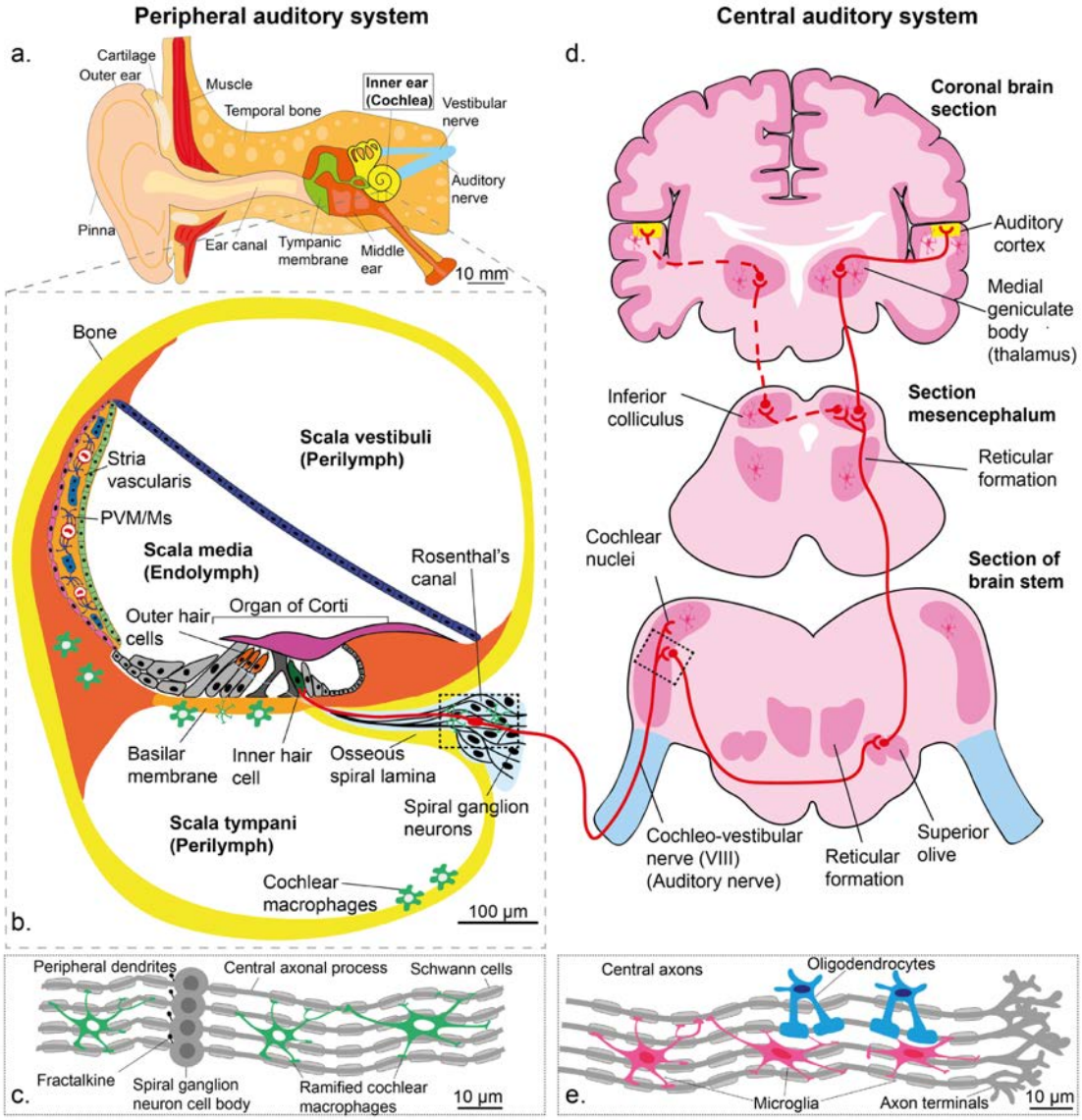


Figure 1.1 **Overview of the organisation and anatomy of the auditory system, highlighting the distribution of macrophages**

The peripheral and central regions of the auditory system (left and right columns). The peripheral auditory system (**a**) consists of the outer, middle and inner part of the ear or cochlea. A high magnification (**b**) cross-section of a cochlear turn to identify the organ of Corti, stria vascularis and Rosenthal's canal. An illustration (**c**) of the cellular organisation of a region of the cochlear auditory nerve showing SGNs, which have peripheral and central axonal processes. Peripheral dendrites synapse with the hair cells. Centrally (**d**) the pathways project from the cochlear nucleus in the brainstem to the auditory cortex. The ascending primary auditory pathway relays signals from the cochlea along axons of SGNs to the cochlear nucleus in the brain stem. The auditory fibres cross the midline and relay to the superior olivary complex and inferior colliculus. The final relay is the medial geniculate body in the thalamus before the signal is relayed to the auditory cortex. An illustration (**e**) of the cellular

organisation of a region of the distal part of the auditory nerve, with centrally projecting axons of SGNs with axon terminals that synapse in the cochlear nuclei. Function within the auditory pathway relies on resident glial cells including non-myelinating and myelinating Schwann cells, oligodendrocytes and specialised sub-populations of macrophages.

The cochlea is located within a bony labyrinth and enclosed within the bony labyrinth is a membranous labyrinth. The spiralling tunnel that forms the cochlea is subdivided into three distinct channels called scalae. The scala vestibuli and scala tympani (the upper and lower chambers) are filled with perilymph, a fluid with a high sodium and low potassium ion concentration, similar to the composition of interstitial fluid. The scala media (or cochlear duct) is separated from the scala vestibule by Reissner's membrane (specialised epithelial cells) and contains endolymph, a fluid with a high potassium and low sodium ion concentration. Reissner's membrane is a rigid structure of connective tissue which vibrates in response to the movement of fluid in the scale vestibule and scala tympani (Raphael and Altschuler, 2003). The maintenance of the difference in ionic gradient between cochlear fluids is essential for establishing the endocochlear potential (Patuzzi, 2011), which hair cell function relies on.

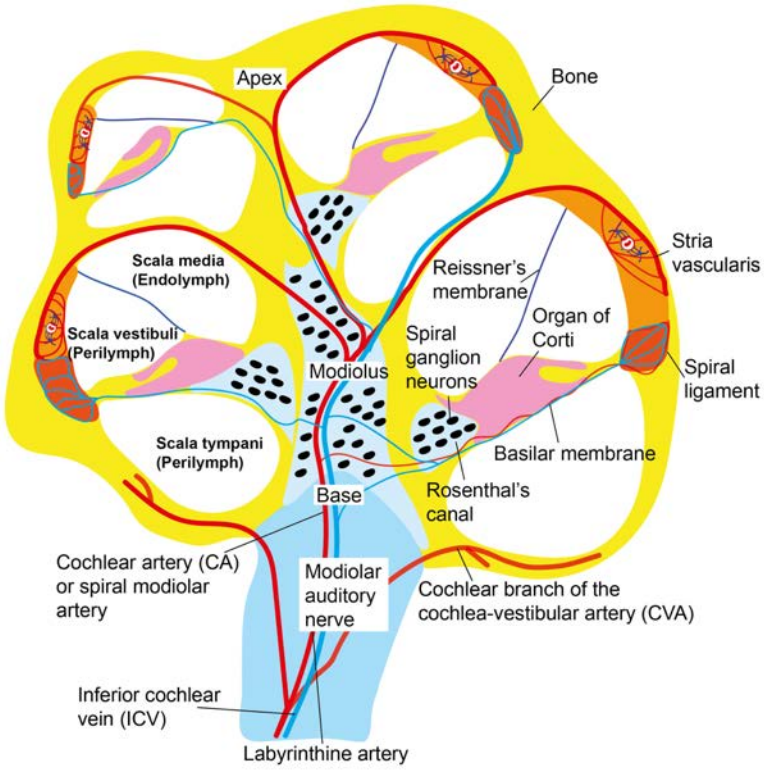


Figure 1.2 **Labelled schematic of the cochlea showing key regions and structures**

Mid-modiolar view of a mouse cochlea showing the cochlear turns with key regions labelled including organ of Corti, stria vascularis and Rosenthal's canal containing the SGN cell bodies. The blood supply to the cochlea has been labelled.

The cochlea is well vascularised, reflecting the energetic demand of the high firing rates and the need for fidelity in the transmission of sound, perceived by the hair cells, to the central auditory pathway neurons. The cochlea is supplied by the labyrinthine artery which has three branches within the internal auditory canal: the cochlear-vestibular artery (CVA), the cochlear artery (CA) (or spiral modiolar artery) and the anterior vestibular artery (AVA) (Mei *et al.*, 2018, 2020). The CVA divides into cochlear and vestibular branches which run in opposite directions. As shown in Figure 1.2, the spiral ganglion neurons in the modiolus and the blood vessels in the lateral wall are supplied, or arise from, the CA. Anatomical variability in the human arterial supply to the cochlea has been reported based on micro-computerised tomography (μCT) and synchrotron imaging of human temporal bones (Mei *et al.*, 2018, 2020). This variability may explain the apparent relative vulnerabilities of the base and apex of the cochlea to damage, as is seen with high frequency loss (basal cochlea) with age. The major capillary beds in the cochlea run within the bone and are located in the stria vascularis, the spiral ligament, the spiral ganglion, spiral limbus and fewer at the osseous spiral lamina and basilar membrane (Salt and Hirose, 2018).

Control of movement of substances and cell traffic between the sub-compartments of the cochlea is regulated through the blood-labyrinth barrier (BLB), which includes all cochlear barriers that exist between cochlear fluid and the blood. The structure and function of the blood-labyrinth barrier has many similarities to the blood-brain barrier (BBB) which separates the central nervous system (CNS) from the blood. The BBB is pivotal for maintaining homeostatic conditions in the brain for normal function (Varatharaj and Galea, 2017), acting as a regulator for the traffic of substances across the barrier in a controlled and controllable manner.

The BLB is composed of endothelial cells linked by tight junction proteins, a basement membrane and pericytes and perivascular macrophages wrapping around the basement membrane (Figure 1.3a). The BBB structure is more complex, including two basement membranes; the basement membrane one is shared between endothelial cells and pericytes and the basement membrane two is formed by the wrapping of the astrocyte endfeet (Figure 1.3b). Similar to the BLB, tight junctions are positioned between endothelial cells, and perivascular macrophages wrap around the endothelial cells. The function of BLB and BBB is to control the movement of solutes and immune cells from the circulation. Additional barriers in the cochlea include the blood-perilymph barrier which is found in all the capillary beds. The intrastrial fluid-blood barrier (aka blood-endolymph barrier) is the strial barrier with an essential role in maintaining the endocochlear potential (Shi, 2010), at +80mV relative to the blood plasma, required for functional hearing.

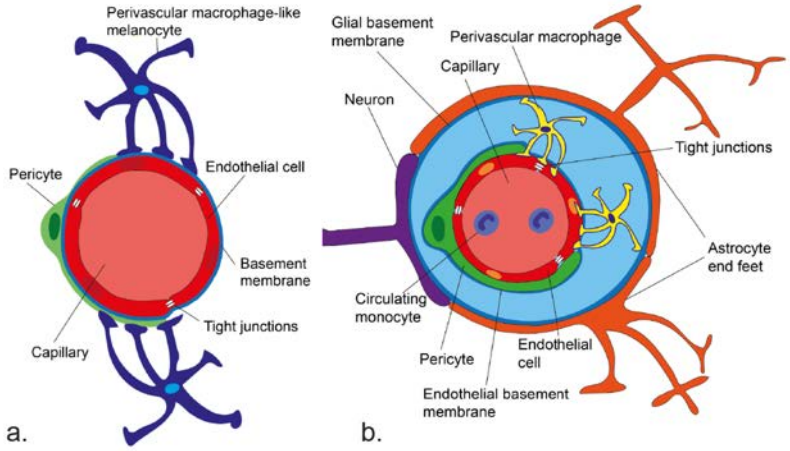


Figure 1.3 **Schematic of the blood-labyrinth barrier (BLB) and blood-brain barrier (BBB), which control the movement of solutes and immune cells from the circulation into the cochlea and brain**

a. Composition of the BLB consisting of endothelial cells connected by tight junction proteins, basement membrane, pericytes and perivascular macrophage-like melanocytes.

b. Composition of the BBB consisting of two basement membranes, endothelial cells connected by tight junction proteins, pericytes and perivascular macrophages.

The stria vascularis (SV) is a vascular, metabolically active epithelium that forms the barrier between the intrastrial fluid and the blood. It regulates the ionic composition of the cochlear fluids and comprises layers of marginal, intermediate and basal cells as indicated in Figure 1.4. The marginal cells are polarised epithelial cells, forming one layer to line the scala media fluid space (Raphael and Altschuler, 2003) and therefore are in contact with endolymph. A main role of these cells is to maintain ionic composition of endolymph to regulate the endocochlear potential. These epithelial cells are connected to each other by tight and adherens junctions forming a diffusion barrier between the cells (Raphael and Altschuler, 2003). Marginal cells are densely packed with mitochondria to provide adenosine tri-phosphate (ATP) for the Na^+/K^+/ATPase transporter in the basolateral membrane, actively transporting K^+ ions from the intrastrial space into the marginal cells (Figure 1.4). Intermediate cells are lateral to marginal cells and are melanocytes derived from the neural crest. It was shown that in a mouse mutant *viable dominant spotting* - which has a primary neural crest defect and therefore no melanocytes - had an endocochlear potential of ~0 mV, indicating intermediate cells are vital for normal strial development and functioning (Steel and Barkway, 1989). The basal cells are squamous cells adjacent to the fibrocytes in the spiral ligament and are important for maintaining the barrier

between the stria vascularis and spiral ligament (Raphael and Altschuler, 2003). There is a dense capillary network within the stria vascularis, predominantly within the intermediate cells which provide the oxygen and nutrients for the active transport of K^+ ions.

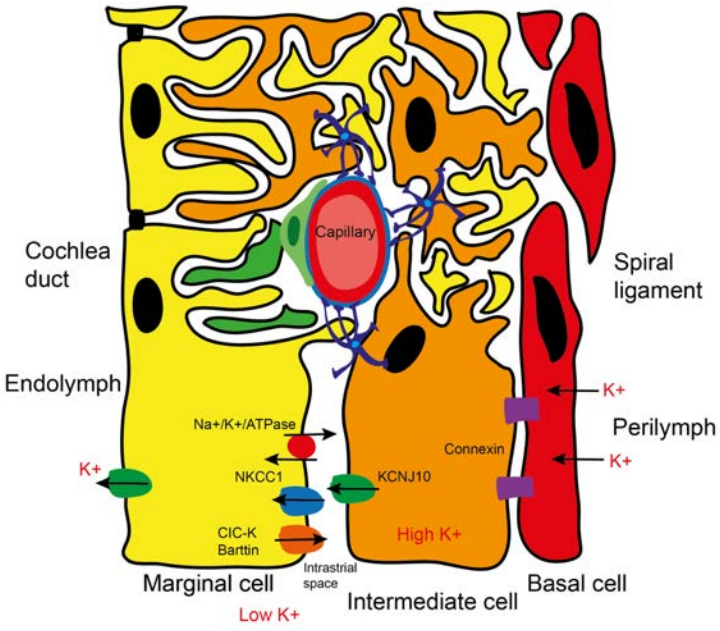


Figure 1.4 **Labelled schematic highlighting the structure and function of the stria vascularis**

Schematic showing the three layers of cells in the stria vascularis: basal, intermediate and marginal cells. The junctions between cells and the transporters, required for the movement of K^+ ions across the stria to generate the endocochlear potential, are highlighted. PVM/Ms are found amongst the intermediate cells and are in contact with capillary walls.

Perivascular resident macrophage-like melanocytes (PVM/Ms) are located amongst the intermediate cells in the stria (Figure 1.4) and control the permeability of the intrastrial fluid-blood barrier (Zhang *et al.*, 2012). PVM/Ms express markers characteristic of macrophages (CD68, F4/80 and CD11b) (Shi, 2010) and melanocytes (GSTalpha4, GST and Kir4.1) (Zhang *et al.*, 2012). These melanocytes have a role in homeostasis (Ohlemiller *et al.*, 2009; Murillo-Cuesta *et al.*, 2010). PVM/Ms, located around the blood vessels of the stria, are branched cells making contact with the vessels through cytoplasmic processes (Zhang *et al.*, 2012). PVM/Ms synthesise pigment epithelium growth factor (PEDF). PEDF contributes to the regulation of the intrastrial-barrier by altering the expression of tight and adhesive junction proteins in the vasculature. Tight control of this barrier is necessary for maintaining the endocochlear potential (Zhang *et al.*, 2012, 2013). Depletion of PVM/Ms in mice caused hearing loss and a significant reduction in endocochlear potential (Zhang *et al.*, 2012). Cochlear pericytes, located in the stria vascularis, have multiple cytoplasmic branches which attach to the abluminal strial capillary wall and are embedded in the basement membrane

(Shi, 2016). There is evidence that pericytes and PVM/Ms are integral in maintaining the intrastrial fluid-blood barrier (Neng *et al.*, 2013).

The organ of Corti is the sensorineural end organ for hearing (Raphael and Altschuler, 2003). It comprises of inner and outer hair cells with 3-4 rows of stereocilia on the apical surface of each cell, supporting epithelial cells, a basement membrane containing the basilar membrane, nerve endings and the tectorial membrane (Raphael and Altschuler, 2003) (Figure 1.5). The inner hair cells (IHCs) convert stereocilia movement into electrical impulses along the auditory nerve to the brain. The apical portion of IHCs are bathed in endolymph whereas the basolateral portion, where the nerve terminals are located, are bathed in perilymph. Outer hair cells (OHCs) enhance performance of the cochlea by increasing the sensitivity and selectivity of sound. Efferent neurons pass through the supporting cells and form nerve terminals at the basal end of the OHCs.

The organ of Corti is positioned on the basilar membrane, a strand of connective tissue that has structurally distinct regions and ultrastructural organisation that governs its ability to move in response to sound. The medial portion where the IHCs are positioned, the arcuate zone, is enclosed in the bony osseous spiral lamina and therefore has restricted ability to move in response to sound. The lateral portion where the OHCs are positioned, the pectinate zone, can move freely in response to sound vibrations. In the process of mechanotransduction, sound vibration causes movement of the pectinate zone resulting in deflection of the stereocilia on the hair cells towards the taller row; this opens stretch sensitive cation channels in the tip links. This causes an influx of potassium ions (K^+) to move down a concentration gradient from the endolymph into the hair cells, depolarising the cells. Voltage-gated calcium channels open leading to an influx of calcium ions (Ca^{2+}). Calcium ion influx causes the release of the neurotransmitter, glutamate, from glutamatergic synapses at the base of the cell. This propagates the signal to afferent neurons through action potentials. Neurons transmit this information through the auditory pathway to the brain. Movement of stereocilia in the opposite direction, towards the shorter row, closes the ion channels to stop this process.

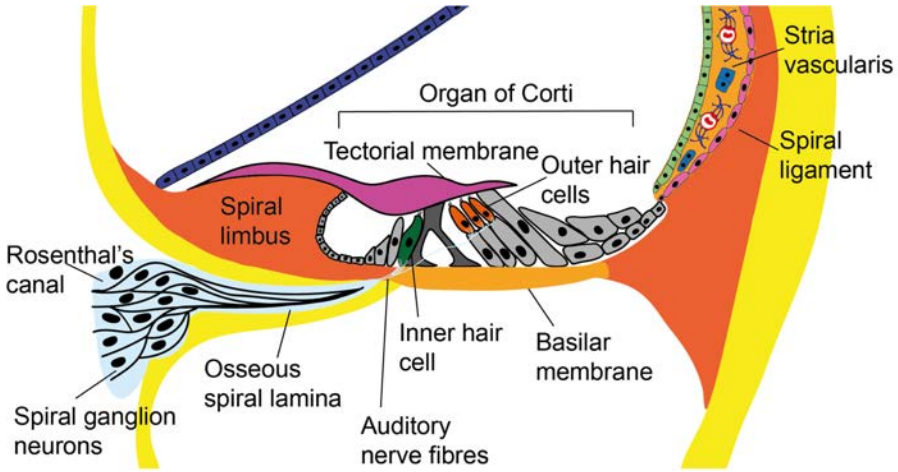


Figure 1.5 **Key regions of the cochlea including organ of Corti, Rosenthal's canal and stria vascularis**

Specific locations along the length of the cochlea can detect specific frequencies of sound due to the variable stiffness of the basilar membrane. While the base of the cochlea has maximal response to high frequency signals, the apical end has maximal response to low frequency signals. Therefore, hair cell damage in different cochlear regions is associated with reduced sensitivity to a specific frequency.

Spiral ganglion neurons (SGNs) transmit sound information from the cochlea to the brain. The cell bodies of these neurons reside in Rosenthal's canal (Figure 1.5) in the modiolus: the central bony axis of the cochlea. While peripheral axons extend through the osseous spiral lamina to form synapses with the hair cells, central axons are bundled in the auditory nerve and project through to synapse onto cells in the cochlear nucleus within the brainstem (Figure 1.1d). Two populations of SGNs exist in the cochlea. Type I neurons are large, myelinated cells which innervate a single IHC (Glueckert *et al.*, 2005; W. Liu *et al.*, 2015). They represent 90 - 95% of the afferent population. Type II neurons are small, bipolar, and unmyelinated cells which innervate multiple OHCs. They represent 5 - 10% of the afferent population. During embryonic development in rodents, SGNs begin to innervate cochlear hair cells including mis-targeted connections with both inner and outer hair cells (Echteler and Nofsinger, 2000). During the first postnatal week, retraction and pruning of excessive axons takes place to result in mature innervation patterns (Echteler, 1992; Echteler and Nofsinger, 2000; Echteler, Magardino and Rontal, 2005). Auditory glial cells, satellite and Schwann cells, are positioned around SGNs and their axons. Type I SGNs somas and axons are myelinated by satellite and Schwann cells respectively whereas type II SGNs are ensheathed by non-myelinating satellite and Schwann cells (Brown *et al.*, 2017). The central axons of the SGNs form the main component of the auditory nerve, which transmit signals from the peripheral axons projecting from the hair cells in the cochlea to the brainstem, to travel through the auditory pathway ending in the auditory cortex (Figure 1.1d,e). An adequate

population of SGNs are required for the precise coding of acoustic sound information and are required for effective hearing with a cochlear implant.

1.3 Types of hearing loss

The two main types of hearing loss are conductive and sensorineural hearing loss (Figure 1.6). Conductive hearing loss (CHL) occurs when sound waves cannot pass from the outer ear, through the middle ear to the inner ear. The most common cause of conductive hearing loss in children is otitis media, which is inflammation or infection in the middle ear. In adults otosclerosis, a condition of abnormal bone growth around the stapes in the middle ear, is the most common cause of conductive hearing loss. Conductive hearing loss can occur alongside sensorineural hearing loss (SNHL), referred to as mixed hearing loss. SNHL occurs when there is loss or damage of sensory hair cells in the inner ear and/or dysfunction of the auditory nerve that connects the cochlea and the brain. SNHL includes a variety of hearing disorders including age-related hearing loss (ARHL), sudden deafness, noise-induced hearing loss (NIHL) and Meniere's disease (Okano, 2014). There are multiple possible causes of SNHL including age (presbycusis), genetics, loud noise, a benign tumour, infection, otitis media (OM) and ototoxic medication. SNHL is typically permanent, whereas conductive hearing loss can be temporary or permanent but more commonly temporary. While conductive hearing loss is at worst moderate in severity, SNHL or mixed can be any severity, including profound. There are no effective medical or pharmacological treatments for SNHL, although acoustic hearing aids and cochlear implants (surgical implants which sit in the inner ear and stimulate the auditory system electrically) are able to reduce and manage the effects of hearing loss.

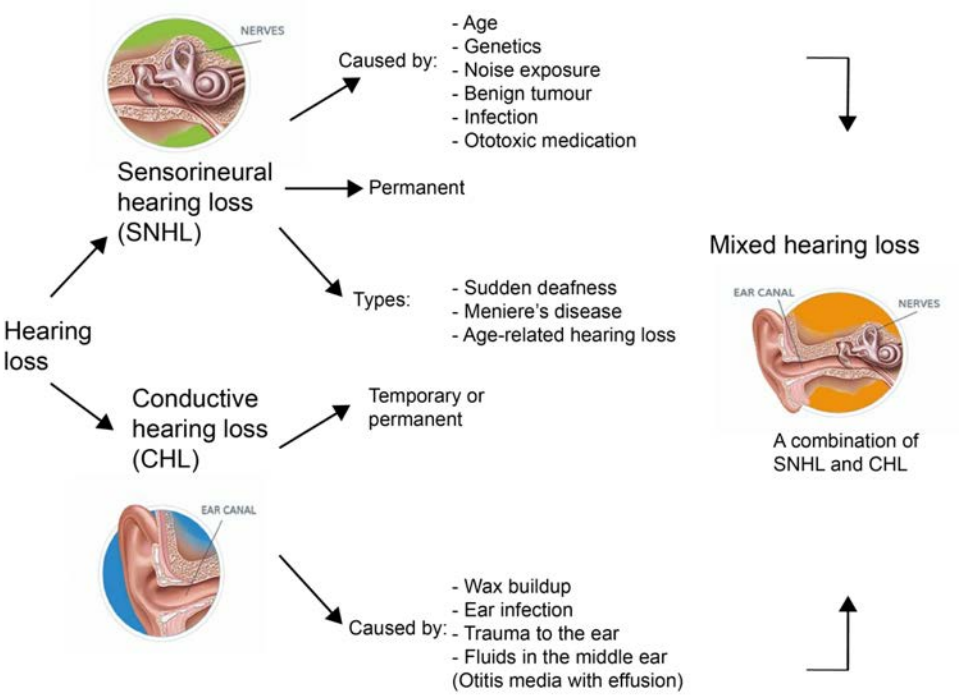


Figure 1.6 **Schematic showing the different types of hearing loss and the varying characteristics of these**

Summary of the types of hearing loss: sensorineural, conductive and mixed hearing loss including causative factors and prognosis.

Age-related hearing loss (ARHL) or presbycusis is the single biggest cause of hearing loss. It is a progressive, degenerative condition that affects 71.1% individuals over 70 years old worldwide (Action on Hearing Loss, 2015) and is the greatest contributor in terms of modifiable mid-life factors non-familial dementia (Livingston *et al.*, 2017). ARHL is caused by a combination of genetics (Wells, Newman and Williams, 2020) and the accumulation of environmental factors which are known to drive hearing loss over time. Many of the known drivers of hearing loss are associated with changes in immune function indicating a role of inflammatory processes in the progression of hearing loss, particularly with aging (Verschuur *et al.*, 2012; Verschuur, Agyemang-Prempeh and Newman, 2014). Lifestyle factors such as smoking and high-fat diet are associated with reduced hearing acuity in older individuals (Fransen *et al.*, 2008; Dawes *et al.*, 2014). Chronic conditions with an inflammatory component including type 2 diabetes (Mitchell *et al.*, 2009; Gupta *et al.*, 2019), cardiovascular disease (Gates *et al.*, 1993; Hutchinson, Alessio and Baiduc, 2010) and dementia are also associated with hearing loss. Although much is known about the correlates and predictors of hearing loss, evidence about the mechanisms that mediate the effect of these factors on the auditory system is more limited.

Noise exposure is the second most common cause of hearing loss. Noise-induced hearing loss (NIHL) is a health burden and of growing concern in the industrialised world. Overexposure to a

loud sound such as an explosion, or prolonged exposure to moderate sound in the work place, can cause permanent damage to the peripheral and central auditory pathway which can lead to SNHL (Fetoni *et al.*, 2015; Liberman and Liberman, 2015). 25% of American adults suffer from NIHL according to the Centers for Disease Controls (Liberman, 2017). To prevent the increasing number of people affected by NIHL, ear plugs or other protective devices are recommended for people who often engage in loud noise activities.

Otitis media (OM), middle ear inflammation, can cause hearing impairment, whereby the severity is determined by the type and duration of OM. Otitis media can cause conductive or SNHL. Conductive hearing loss is associated with acute otitis media (AOM) or otitis media with effusion (OME), due to the increased stiffness and mass of tympanum caused by middle ear effusion (Cai and McPherson, 2017). This conductive hearing loss may either be transient or permanent. SNHL can be a result of either type of OM and the hearing loss is often permanent. The exact mechanisms that cause SNHL associated with OM are not well understood and will be discussed in detail in Chapter 3.

1.4 Therapeutic interventions for hearing loss

Currently there is no cure for any type of hearing loss. Two electronic devices are available as therapeutic intervention: hearing aids and cochlear implants (CIs). Acoustic hearing aids use a microphone to pick up sounds and transform them into an electric current, boosted in size by an amplifier. A loudspeaker then turns the boosted electric current into a louder sound, which flows through a tube and ear mould into the person's ear. The sound waves are transmitted to the cochlea where they are converted to electrical impulses and sent to the brain to be processed; to be perceived as sound. Along with the conventional behind the ear hearing aids, bone-anchored hearing aids are also available. These are surgically implanted under the skull and function through bone conduction of sound vibrations. Bone-anchored hearing aids are suitable for people with chronic ear infections who require continual drainage from their ears or for people with middle or outer ear malformations. Hearing aids are worn by around 2 million people in the UK; however, they are not beneficial for everyone due to the severity of their hearing impairment. For people with severe to profound deafness cochlear implantation is recommended. A CI is a neural prosthesis that functions by directly stimulating residual SGNs of the auditory nerve, bypassing the hair cells, sending the sound information directly to the brain. For a CI to function, a functional auditory nerve and an implantable cochlea are essential.

1.4.1 Cochlear implants

Cochlear implants (CIs) are the most successful neuro-prosthesis, allowing individuals with severe to profound deafness the ability to access speech, music and environmental sound. CIs consist of two main components: a sound processor worn externally behind the ear, and an implanted receiver-stimulator which is surgically recessed into the bone of the skull and secured under the skin, as shown in Figure 1.7. The batteries powering the device are housed in the external processor unit. The electrode array projecting from the receiver-stimulator unit is inserted into the cochlea through an incision made in the round window, or just beside the round window, via a cochleostomy. The process begins when the sound processor detects and captures environmental sound and converts it into a digital code. The processor transmits the digitally coded sound through a communication coil on the outside of the head to the implant. The digitally coded sound is converted into an electrical impulse by the implant which is transmitted along the electrode array in the cochlea. The auditory nerve is stimulated by the electrode, sending impulses to the brain where they are interpreted as sound.

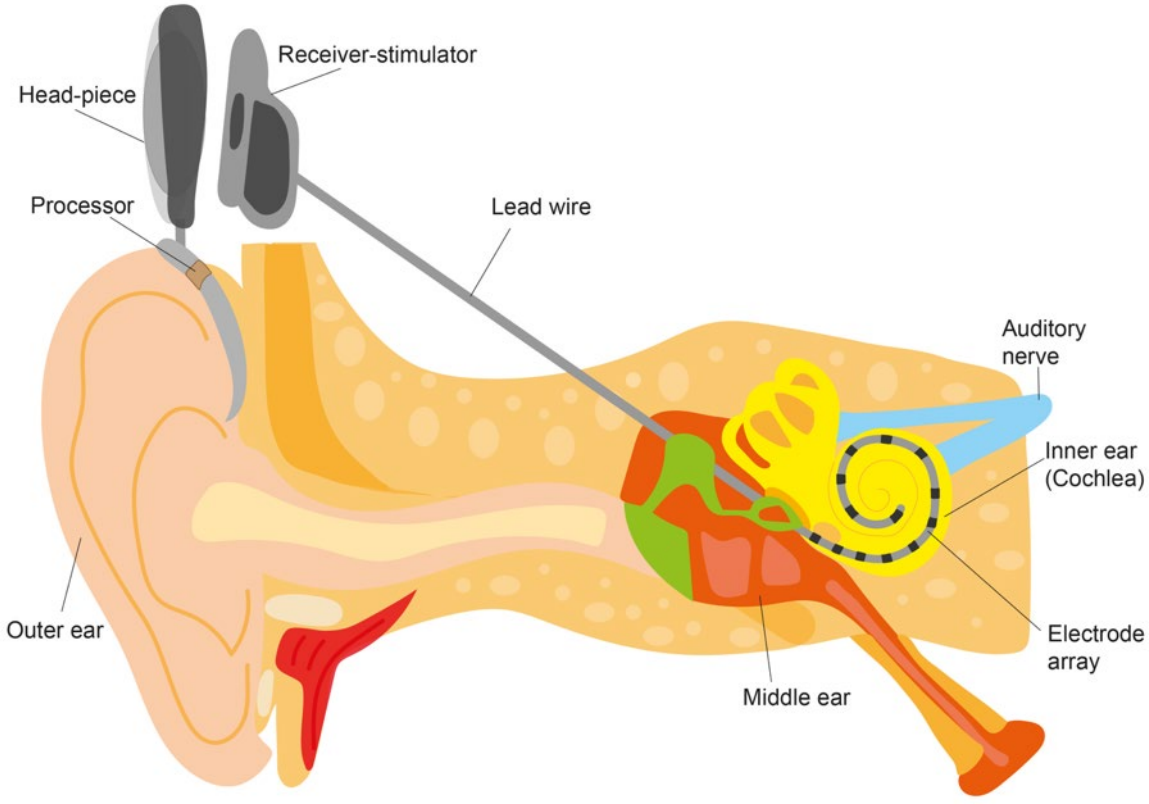


Figure 1.7 **Diagram to show the components of a cochlear implant**

This figure shows the components of a cochlear implant, including the externally worn sound processor with a microphone(s), and the receiver coil and stimulator which are surgically placed under the skin. The electrode array is surgically placed

into the cochlea and directly stimulates neurons of the auditory nerve sending sound information to the brain.

1.5 Neurobiological substrates of hearing loss

In order to understand the pathophysiology of hearing loss, it is necessary to determine the neurobiological substrates of hearing loss, e.g., key cellular players and cochlear regions involved in hearing loss. Sensorineural hearing loss (SNHL) is frequently due to the acute dysfunction and death of hair cells after insults such as noise exposure or, more chronically, with increasing age. Humans differ from other species as their hair cells are terminally differentiated (Mittal *et al.*, 2017; Shu *et al.*, 2019). Despite focused efforts to induce hair cell regeneration in humans, the field has not yet yielded a safe, effective way to achieve this. In addition to hair cells, other neurobiological substrates of hearing loss include SGNs, their axons and the synapses that form between the hair cells and the auditory nerve, all of which can become damaged and degenerate. Damage to the stria vascularis leads to increased permeability and leakage of the intrastrial fluid-blood barrier which disrupts the ionic composition in the cochlea. Figure 1.8 illustrates the different regions of the cochlea and central auditory axons which can become damaged, contributing to hearing loss. Macrophages are likely to have a role in the pathology associated with each region, which will be discussed further in Chapter 2**.**

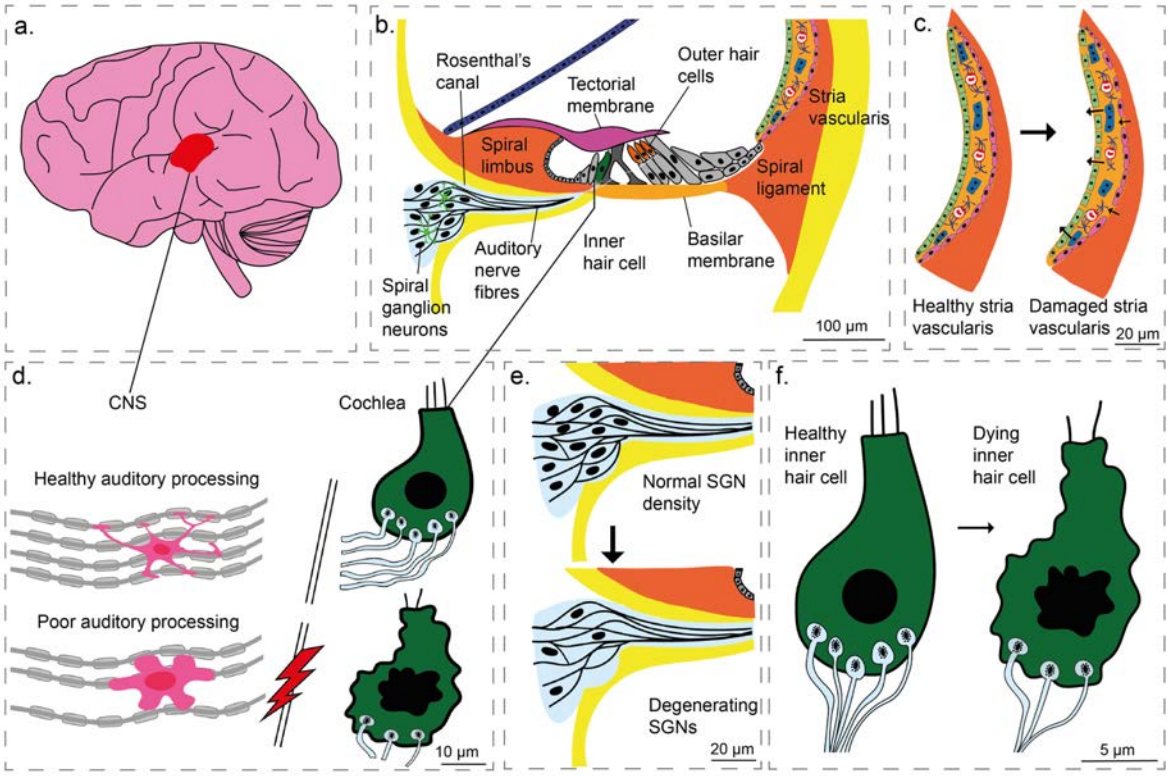


Figure 1.8 **Schematic illustrating the neurobiological substrates of hearing loss**

Key structures involved in auditory function and the pathology associated with these structures in hearing loss are highlighted.

a. Human brain with the auditory cortex indicated in red.

b. Key structures and regions of the cochlea involved in auditory function.

c. The stria vascularis regulates the ionic composition of the cochlear fluid and controls the entry and exit of entities between the cochlea and the circulation. Damage or atrophy of the stria vascularis disrupts these processes and is detrimental to cochlear function.

d. Auditory processing indicating a region of the auditory nerve in the CNS, sending signals to the SGNs in the cochlea that synapse onto hair cells. Poor auditory processing is caused by decreased signalling between damaged/dying hair cells within the cochlea and axons of the auditory nerve.

e. SGNs make synaptic connections between the hair cells and the neurons of the cochlear nucleus. They can become damaged and degenerate contributing to hearing loss.

f. Inner hair cells convert stereocilia movement into chemical signals that are transmitted along nerve fibres. Degenerating hair cells have reduced synaptic

connections and deafferentation of IHC terminals which contributes to elevated hearing thresholds.

Hair cells (including the stereocilia) were considered to be the primary site of injury that resulted in hearing loss (Bohne and Harding, 2000). Secondary damage or further loss of function occurs due to loss of spiral ganglion neurons (SGNs) and degeneration of cochlear synapses. This could have been because hair cell damage can be seen and measured within minutes after noise exposure, however cochlear synaptopathy and the death of SGNs takes months to years (Johnsson, 1974). Recent evidence in the mouse, however, has identified that the earliest site of injury may be the synapses between IHCs and SGNs (ribbon synapses) (Kujawa and Liberman, 2009). Noise exposure causing moderate but reversible threshold shifts results in deafferentation of IHC terminals (Bullen *et al.*, 2019) and loss of synaptic connections (Kujawa and Liberman, 2009). Loss of these structures occur within hours of noise exposure and before identifiable hair cell death in both noise-induced and age-related hearing loss (Kujawa and Liberman, 2009; Sergeyenko *et al.*, 2013; Fernandez *et al.*, 2015) and precedes the loss and degeneration of the peripheral axons of the SGNs which occurs over weeks after injury (Liberman and Liberman, 2015).

The cell body and central axons of SGNs survive for years in mice (Kujawa and Liberman, 2006) and humans after injury (W. Liu *et al.*, 2015), a finding that is supported by outcomes after cochlear implantation, where functional hearing can be achieved even after a considerable period of deafness (Beyea *et al.*, 2016). Contrastingly, in birds, both hair cells and auditory neurons can regenerate following acoustic or ototoxic injury (Pirvola *et al.*, 1997). In aged mice the loss of ribbon synapses occur at a similar rate to the loss of SGNs but following a delay of several months (Sergeyenko *et al.*, 2013). In aged human temporal bones, the number of ribbons per IHC (Viana *et al.*, 2015) and the number of SGNs (Makary *et al.*, 2011) decreased with increasing age. Importantly, degeneration of cochlear synapses and SGNs is not revealed by standard audiometric tests and is therefore considered 'hidden hearing loss' (Liberman, 2017; Liberman and Kujawa, 2017). This type of damage/dysfunction is responsible for hearing difficulties experienced in a noisy environment. The mechanisms resulting in synaptopathy are not fully understood. There is evidence of a local excitotoxic response (Kujawa and Liberman, 2009, 2015; Liberman and Kujawa, 2017) and macrophage recruitment has been shown after noise injury. Whether the mechanisms differ in response to different insults is unclear.

Strial dysfunction and degeneration in both animal and human cochleae has been reported after noise exposure and with age. Noise exposure activates PVM/Ms causing changes in their morphology, a detachment of these cells from capillary walls and a reduction in the expression of

PEDF. This results in a down-regulation of the expression of tight and adhesive junction proteins, therefore increasing the permeability of the intrastrial fluid-blood barrier (Zhang *et al.*, 2013). A trans-tympanic injection of lipopolysaccharide (LPS) into a mouse middle ear caused a breakdown in the integrity of the strial barrier and morphological changes in the PVM/Ms (Zhang *et al.*, 2015; Jiang *et al.*, 2019). These changes were associated with elevated hearing threshold resulting in a 30 dB SPL hearing loss in high frequencies compared to controls (Zhang *et al.*, 2015). Analysis of human temporal bones has identified strial atrophy and vascular changes in the stria from older cases (Suzuki *et al.*, 2006; Kurata *et al.*, 2016). These may alter the function of the blood-labyrinth barrier and regulation of the endocochlear potential and contribute to ARHL. The functional consequences of this age-related change have been seen in invasive studies in older mice that identified a drop in endocochlear potential, relative to younger animals (Ohlemiller *et al.*, 2009).

The interaction between ARHL and NIHL was investigated (Kujawa and Liberman, 2006). Young mice were exposed to a noise that caused a moderate but permanent threshold shift then were aged for 2 years to determine whether the noise exposure caused damage to the cochlea over time. They found that aged, noise-exposed mice had widespread loss of spiral ganglion cells despite no loss of hair cells, supporting the view that synaptopathy can occur without hair cell loss and develop over time (Kujawa and Liberman, 2006). Pre-exposing mice to noise exposure early in life had deleterious effects on the nature and progression of an ARHL, highlighting the link between early inflammatory insults and overall auditory health. Further research is needed to elucidate the potential mechanisms for the interaction with ARHL and NIHL, however initial evidence suggests that an interaction between pathologies can accelerate hearing loss.

1.6 Role of the immune system in the cochlea and in sensorineural hearing loss (SNHL)

1.6.1 Inflammatory response in the cochlea

Due to the tight blood-labyrinth barrier (BLB), it was long thought that the cochlea was an immunologically privileged organ as the barrier prevented entry of immune cells, antibodies and inner ear antigens (Juhn and Rybak, 1981). However, there is now evidence showing the induction of an inflammatory response and the influx of inflammatory markers such as pro-inflammatory cytokines/chemokines into the cochlea in response to cochlear insults such as noise exposure (Hirose *et al.*, 2005; Keithley, Wang and Barkdull, 2008), cochlear implantation (Bas *et al.*, 2015), ototoxicity (Sato *et al.*, 2010), and hair cell ablation (Kaur, Hirose, *et al.*, 2015). Multiple studies indicate a role for inflammation and the immune system in the physiology and pathophysiology of

hearing and hearing loss (Ma *et al.*, 2000; Wang *et al.*, 2003; Fujioka *et al.*, 2006; Keithley, Wang and Barkdull, 2008).

Cells within the cochlea mount an inflammatory response to both pathogens (Woo *et al.*, 2010) and cochlear insults such as noise (Hirose *et al.*, 2005), drugs (So *et al.*, 2007) or immune challenges (Hirose *et al.*, 2014; Jiang *et al.*, 2019). Cochlear insults lead to an activation of macrophages and fibrocytes resulting in an increased production of inflammatory mediators and subsequent tissue damage. Damage to cochlear structures with a limited ability to repair contributes to a progressive loss of function, potentially resulting in hearing loss.

Acute insults such as noise exposure causes the production of proinflammatory cytokines, tumour necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β) and interleukin 6 (IL-6) within the cochlea (Fujioka *et al.*, 2006). Production of these cytokines in the early stages after noise exposure causes an infiltration of inflammatory cells resulting in tissue damage leading to hearing loss (Fujioka *et al.*, 2006; Keithley, Wang and Barkdull, 2008). Treatment with Etanercept, a TNF-α inhibitor, reduced the infiltration of inflammatory cells, resulting in a reduction in hearing loss in an animal model of immune mediated labyrinthitis (Wang *et al.*, 2003). Similarly, inhibition of IL-6 using a rat anti-mouse IL-6 receptor antibody (MR16-1) had protective effects on the noise damaged cochlea through a reduction in the activation of macrophages amongst SGNs (Wakabayashi *et al.*, 2010). Evidence from these studies indicate an immune role of cytokines, produced by macrophages, following insults to the cochlea.

1.6.2 Macrophages in the cochlea

Macrophages are immune cells that have roles in both homeostasis and inflammation (Ginhoux and Guilliams, 2016; Watanabe *et al.*, 2019). They detect, phagocytose and clear senescent cells and harmful organisms around the body. Macrophages produce and release pro-inflammatory cytokines to initiate inflammation and anti-inflammatory cytokines to return the tissue to a homeostatic environment. In response to an insult and tissue injury, bone marrow-derived monocytes are recruited to the injured tissue and differentiate into macrophages and carry out a robust inflammatory response.

The cochlea has two resident macrophage populations, cochlear macrophages and perivascular macrophage-like melanocytes (PVM/Ms), that have been described in both human (O'Malley, Nadol and Mckenna, 2016; Liu *et al.*, 2018, 2019) and animal studies (Okano *et al.*, 2008; Sato *et al.*, 2010). In a healthy mouse cochlea, cochlear macrophages reside in the spiral ligament, spiral limbus, amongst SGNs and below the basilar membrane (Hirose *et al.*, 2005; Lang *et al.*, 2006; Okano *et al.*, 2008; Sato *et al.*, 2008). The presence of cochlear macrophages in the organ of Corti

has been described in human tissue analysis (Liu *et al.*, 2018). PVM/Ms reside in the central region of the stria vascularis, positioned around blood vessels, and play a role in controlling the permeability of the intrastrial fluid-blood barrier (Zhang *et al.*, 2012). Macrophages are either tissue-resident macrophages, primarily carrying out homeostatic roles in healthy conditions or infiltrated macrophages. Following a cochlear insult, such as a noise injury (Hirose *et al.*, 2005; Sautter *et al.*, 2006; Tornabene *et al.*, 2006; Yang *et al.*, 2015) or cochlear implantation (Bas *et al.*, 2015), circulating bone marrow-derived monocytes infiltrate the cochlea and differentiate into monocyte-derived/infiltrated macrophages. These cells exhibit a robust inflammatory response, in some cases leading to tissue damage and functional damage. The role of macrophages in the auditory system under homeostatic conditions and following different cochlear insults will be discussed in detail in Chapter 2.

1.6.3 Cochlear inflammation

Cochlear inflammation is a major pathogenic factor in conditions that cause SNHL including otitis media (Papp, Rezes and Jokay, 2014; Subramaniam, Ashkar and Rai, 2020), meningitis, autoimmune inner ear disease (Mijovic, Zeitouni and Colmegna, 2013) and ototoxicity (Tan, Thorne and Vlajkovic, 2016). The cochlear inflammatory response involves an acute stage followed by a chronic stage involving fibrosis and ossification. The acute stage includes the production and release of pro-inflammatory cytokines and chemokines, the recruitment and infiltration of immune cells and the breakdown of the intrastrial fluid-blood barrier. The acute stage of cochlear inflammation lasts approximately 3 to 7 days depending on the type of insult (Tan, 2013). The chronic stage includes the formation of a fibrous tissue growth in the perilymphatic spaces that undergoes ossification to become a bony occlusion known as labyrinthitis ossificans (Xu, Joglekar and Paparella, 2009). This chronic stage occurs rapidly in post-meningitis cases; this can cause complications when it comes to cochlear implantation in the patients who have profound hearing loss or deafness due to the meningitis, as this will cause difficulty inserting the electrode into the cochlea.

The use of anti-inflammatory drugs (glucocorticoids) are a beneficial treatment option for sudden SNHL, highlighting the role cochlear inflammation plays in this form of hearing loss (Kalinec *et al.*, 2017). Glucocorticoids bind to and activate soluble cytoplasmic glucocorticoid receptors that translocate to the nucleus and bind to specific DNA sites, downregulating the production of proinflammatory cytokines and adhesion molecules (Vandevyver *et al.*, 2013). Dexamethasone, a glucocorticoid, inhibited TNF-α secretion from spiral ligament fibrocytes in vitro, suppressing the inflammatory response on spiral ligament fibrocytes and therefore reducing cochlear malfunction (Maeda *et al.*, 2005). Additionally, dexamethasone treatment reduced trauma-induced hearing

loss by protecting hair cells from apoptosis induced by TNF-α in vitro, as dexamethasone down-regulated the production of the pro-inflammatory cytokine TNF-α (Haake *et al.*, 2009).

Glucocorticoids are given to treat SSNHL. Systemic glucocorticoids can reverse sudden SNHL (Chen, Halpin and Rauch, 2003; Slattery *et al.*, 2005) however several studies have identified adverse effects associated with this treatment, such as suppression of the adrenal response (Henzen *et al.*, 2000). In a study of 116 patients after 1 month of prednisone treatment, 57% of the patients showed improved hearing outcomes. However, 14% of the patients experienced adverse events; the most common being hyperglycaemia (Alexander *et al.*, 2009). Intratympanic administration of corticosteroids can be used in the management of SSNHL. This method of administration into the middle ear provides a direct route for absorption through the round window membrane, reducing the opportunity for off-target effects. The procedure can be carried out using local (topical) anaesthesia, whereby lidocaine is applied to the tympanic membrane prior to piercing it. This administration route results in the delivery of a high concentration of steroid to the affected ear. Intratympanic delivery can be used in those who cannot tolerate systemic steroid therapy (i.e., immunocompromised patients or diabetics). Intratympanic injections may lead to complications such as tympanic infection or perforation, pain, vertigo and further hearing loss (Gundogan *et al.*, 2013; Liu *et al.*, 2016). Out of a total of 128 patients who suffered from SSNHL, 88 patients (68.8%) responded to the treatment (5mg/ml of intratympanic-Dexamethasone) while 40 patients (31.3%) were non-responsive (Tsai *et al.*, 2011). Patients who received the treatment within seven days of disease onset (compared to later than seven days) had a significantly better response indicating that the timing of treatment and early administration is important. Initial studies suggest that intratympanic steroid application is a safe and effective treatment for sudden SNHL (Tsai *et al.*, 2011; Kanotra *et al.*, 2021; Li *et al.*, 2021) however due to the reporting of complications associated with intratympanic injection (Liu *et al.*, 2016), further high-quality studies that evaluate the risk and efficacy of intratympanic steroids for treating SSNHL are required.

The use of therapeutic interventions aims to target the acute stages of the inflammatory response, preventing a chronic inflammatory response that could lead to more severe and permanent complications. After cochlear implantation, glucocorticoid treatment may be given to reduce inflammation and prevent a chronic inflammatory response that causes fibrosis and ossification in the cochlea (Y. Liu *et al.*, 2015; Bas *et al.*, 2016; Honeder *et al.*, 2016). Severe intracochlear fibrosis can reduce CI performance and may lead to device failure, explantation and re-implantation. Some surgeons in CI centres across the UK give glucocorticoids after surgery as there has been some promising evidence from clinical and animals studies (Chang *et al.*, 2017). However, stronger evidence is required to make this a widespread, routine treatment.

1.7 Immune response to CIs and its likely impact on performance

1.7.1 Inflammatory response to cochlear implants

Surgical insertion of an electrode array into the cochlea causes physical trauma and an associated inflammatory response, which should resolve in the normal wound healing process. However, some individuals may experience a heightened, prolonged inflammatory response leading to poorer hearing outcomes. Damage to the cochlea from surgical implantation can cause immediate or delayed effects, as summarised in Figure 1.9. The acute inflammatory response includes the production and release of pro- and anti-inflammatory cytokines and chemokines, the recruitment and activation of immune cells, the breakdown of the intrastrial fluid-blood barrier and tissue damage. The acute inflammatory response can become chronic which, in some cases, leads to tissue damage and poor performance. A delayed, chronic effect includes a foreign body reaction (FBR), a pathological complication that can lead to recurrent wound infection and device failure whereby explantation of the device is necessary (Lim *et al.*, 2011). A chronic inflammatory response initiated by the body to the electrode can lead to fibrosis formation around the electrode array. In some individuals, an exaggerated inflammatory response and excessive fibrosis can lead to physical migration of the electrode array out of the cochlea, increased impedance, poor sound quality and speech perception leading to device failure. See Chapter 4 for further detail regarding the inflammatory response to cochlear implantation.

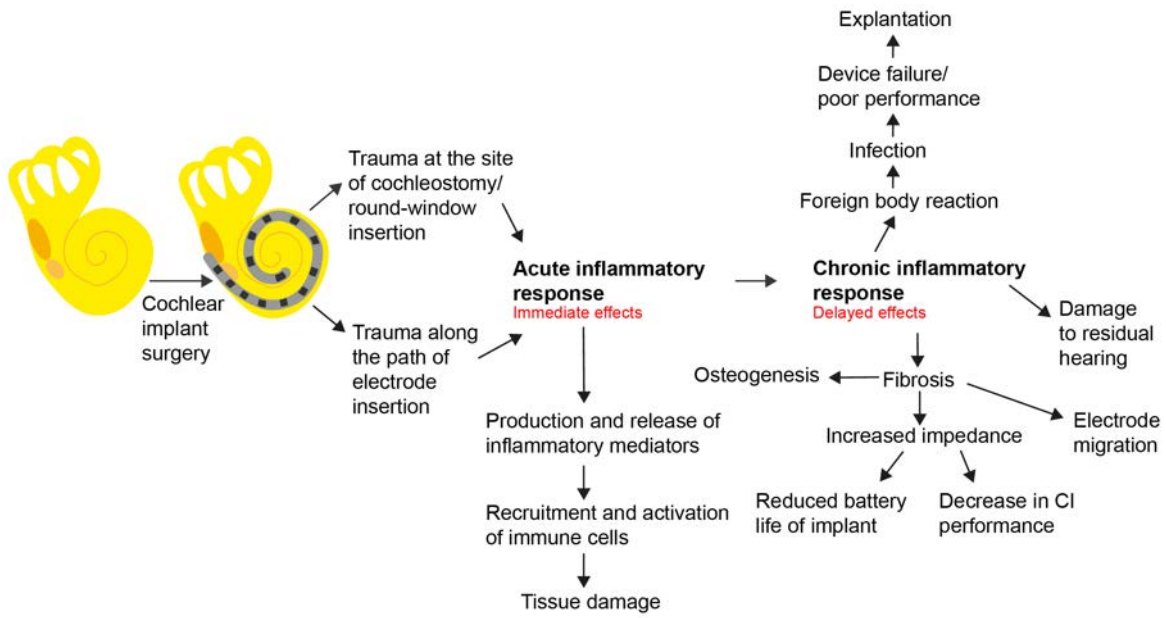


Figure 1.9 **Schematic of the inflammatory response to cochlear implantation involving an acute and chronic inflammatory response**

The events that occur in response to cochlear implantation including the immediate effects in the acute inflammatory response and more delayed effected as part of the chronic inflammatory response.

1.8 Sub-optimal performance with cochlear implants

Of the over 700,000 cochlear implant (CI) users worldwide (NIH, 2021), there is a small but significant group of individuals who experience less favourable hearing outcomes with their implants. Understanding the biological mechanisms responsible for these poorer outcomes is a focus of this research.

Severe cases of poor performance often lead to explantation/reimplantation (revision) surgery. The main reasons for explantation include device failure (hard and soft failure) and medical or surgical failure. Table 1.1 includes a description of each type of failure. The most common reason for revision surgery is device failure, including hard and soft failures (Wang *et al.*, 2014; Patnaik *et al.*, 2016; Batuk *et al.*, 2019; Karamert *et al.*, 2019; Stevens *et al.*, 2019; Wijaya *et al.*, 2019; Kim *et al.*, 2020; Kimura *et al.*, 2020; Layfield *et al.*, 2020; Rayamajhi *et al.*, 2020). A considerable improvement in electrode design, signal processing technology and surgical technique has occurred over recent years, evidenced by the decreasing incidence of hard failures (Stevens *et al.*, 2019). However, the number of soft failures is increasing. Causon et al carried out a retrospective meta-analysis study, analysing reasons for device failures resulting in the device being explanted over a 10-year period (Causon, Verschuur and Newman, 2013). Data reported to the U.S. Food

and Drug Administration (FDA) and recorded on the Manufacturer and User Facility Device Experience (MAUDE) database was analysed, informing the proportion of failures caused by hardware or surgical factors. The proportion of these that were not caused by either hardware or surgical factors were therefore considered a soft failure (Balkany *et al.*, 2005). The proportion of soft failures increased from 7% to 27%, while the proportion of hardware or surgical failures decreased from 93% to 73% (Figure 1.10). Therefore, despite continuing improvement in electrode design, signal processing technology and surgical technique, there is still performance variability between individuals and soft failures.

Table 1.1 **Descriptions of reasons for explantation**

Reason for explantation	**Description**
Hard failure	Intermittent or no communication between the speech processor and internal device or failed device integrity testing (Stevens *et al.*, 2019).
Soft failure	Poor post-implant audiologic performance or subjective adverse symptoms in the setting of a normal device integrity testing and radiographic studies (Balkany *et al.*, 2005; Kimura *et al.*, 2020). Resolution of adverse symptoms following reimplantation (Balkany *et al.*, 2005).
Medical or surgical failure	Infection, incision breakdown and an allergic reaction (Kimura *et al.*, 2020).
Presumed soft failure	Meets soft failure criteria but following explantation, the individual with a CI continues to experience symptoms/ no improvement (Kimura *et al.*, 2020).

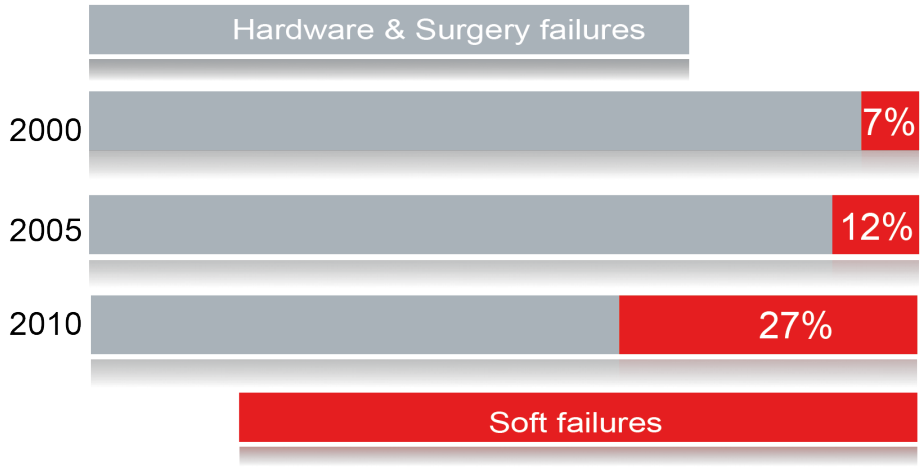


Figure 1.10 **Data obtained from a retrospective meta-analysis study to investigate the reasons for failures which lead to the cochlear implant being explanted over a 10-year period**

This figure shows the proportion of cochlear implant failures that were hardware and surgical failures and the proportion which were considered soft failures (Causon, Verschuur and Newman, 2013).

As hardware and surgical factors are ruled out in soft failure cases, it is plausible that the interaction at the biological interface between the tissue and CI, and the resultant tissue response, is responsible. The biological mechanisms driving these failures are unclear. However, several cases of soft failures illustrate a decline in performance, electrode extrusion and explantation (Nadol, Eddington and Burgess, 2008; Neilan *et al.*, 2012; Hough, Sanderson, *et al.*, 2021). Analysis of the tissue response associated with the array in these cases indicated a heightened inflammatory and fibrotic response to the electrode array, suggesting a role for the tissue response at the tissue-electrode interface in the performance decline and resulting soft failure. A retrospective review of CI revision surgeries identified that out of 14 soft failure cases, nearly one-half of these patients had an inflammatory condition (asthma, meningitis or inner ear malformation) (Chung *et al.*, 2010). Whereas, in the same study less than 10% of the patients with hardware or surgical failures had either of these inflammatory conditions. This evidence suggests that the pre-existing inflammatory status of the individual has an effect on the tissue response to the implant leading to the soft failures. Levels of inflammatory markers in the cochlear fluid of patients after CI surgery was measured and it was found that those expressing higher levels of interferon-γ six months post-surgery had less favourable outcomes with their CI compared to patients with lower levels of this inflammatory marker (Causon, 2015). These studies highlight a link between inflammatory status and CI outcomes.

Often soft failure cases described in studies are the worst-case scenarios, where the failure resulted in explantation. Cases of poor performance, not leading to explantation, were not accounted for therefore underestimating the influence of biological factors to underperformance. Little data exists which explains the mechanisms that drive the tissue response to CIs that cause adverse events.

Cochlear implant performance often refers to how well an individual does in a speech discrimination test such as the Bamford-Kowal-Bench (BKB) sentence test, which measures the ability to repeat sentences and spoken words. Unexplained performance variability and individual differences between CI users in speech discrimination outcomes are common amongst the adult population. Factors include aetiology of deafness (acquired or congenital), age of onset of deafness and duration of deafness all contribute to an individual's performance (Lazard *et al.*, 2012; Blamey and Artieres, 2013; Holden *et al.*, 2013; James *et al.*, 2019). Higher levels of residual hearing pre-implantation and previous hearing aid use predict better speech discrimination outcomes, whilst inner ear malformations, partial insertion of the electrode array and a longer duration of moderate-to-profound hearing loss negatively impacts performance (Buchman, Copeland, *et al.*, 2004; Lazard *et al.*, 2012; Moberly *et al.*, 2016).

Very little data are available regarding patients who are considered poor performers despite the high proportion of adults with a CI who may fall into this category (Lenarz *et al.*, 2012; Moberly *et al.*, 2016). Depending on how poor performance is defined, 10 – 50% of adults fall into this category (Lenarz *et al.*, 2012). Long-term hearing performance after implantation in post-lingually deafened adults found that 13% of the adults in their study were poor performers (Lenarz *et al.*, 2012). The reasons for poor performance are not well understood, making it difficult for clinicians to predict patients who are not as likely to do well and to intervene appropriately (Moberly *et al.*, 2016). People with a CI who are not performing well will undergo diagnostic procedures to investigate the source of the problem. These include a general remapping of the device to ensure the most appropriate stimulation parameters are used, computerised tomography (CT) to check electrode array position and finally a hardware integrity test carried out by the manufacturer. However, in many cases these tests do not identify the source of poor performance. Better tests, indicating the reason for underperformance, are necessary. Better understanding of the causes of variability and underperformance will enable strategies to improve speech and language outcomes.

1.8.1 Local inflammation, electrode impedance (EI) telemetry and hearing outcomes

Electrode impedance (EI) telemetry is a routine non-invasive clinical measure whereby fluctuations in EI can be an indicator of performance decline, a rampant tissue response or migration. EI describes the resistance with which electrical current flows through and between implanted electrodes. A current pulse is delivered by the CI that flows through the electrode of the implant and into the cochlear tissue. The pulse must deliver sufficient amount of charge to the spiral ganglion neurons (SGNs) to initiate signal transduction. Too much charge will damage the tissues and high electrode impedance means a higher voltage will be required to deliver enough charge. Therefore, a consistent high EI is not favourable for two reasons: the battery is drained at a faster rate due to increased energy consumption and more importantly, the higher voltage is spread across more SGNs reducing the dynamic range of stimulation and therefore sound quality (Wilk *et al.*, 2016). Lower electrode impedances are likely to lead to better hearing outcomes. EI is measured as part of the integrity test and is a useful way to quickly inform of any open or short circuit faults which can result in an electrode being de-activated.

Regular measures of EI could be used as an early indicator of performance decline and a way to detect a heightened inflammatory response at the tissue-electrode interface. It provides insight into the biological environment around the electrode array as the volume and cellular composition of the tissue, formed around the array during the inflammatory response, will affect the resistance of charge to flow across electrodes. EI also gives an indication of the dynamic changes in the cochlear fluid environment adjacent to the electrodes, due to the tissue response (Duan, Clark and Cowan, 2004).

Fibrosis within the cochlea and around the electrode array is associated with post-operative increases in impedance (Clark *et al.*, 1995; Wilk *et al.*, 2016; Ishai *et al.*, 2017). Several animal models have shown increases in impedance and fibrosis following cochlear implantation, which was reduced following local application or release of dexamethasone that was incorporated into the electrode arrays (Bas *et al.*, 2016; Wilk *et al.*, 2016). This evidence is supported by a recent human study whereby impedance measures were significantly lowered over a two year period in people with a dexamethasone-eluting CI, compared to a non-eluting CI (Briggs *et al.*, 2020). See 5.1.4 for further detail of these studies. These findings indicate that inflammation is a mechanism underpinning impedance changes and subsequently affects CI performance.

Clinical studies measuring impedance levels post-implantation have shown that electrode impedance fluctuations and spikes are associated with inner ear pathology following cochlear implantation (Choi *et al.*, 2017; Shaul *et al.*, 2019). A retrospective review of CI impedances measured at a CI clinic between 2010 – 2015 (770 patients), found that the percentage of patients

experiencing clinical events such as hearing loss, vestibular problems and tinnitus, was higher in the patients experiencing impedance spikes compared to the controls (Shaul *et al.*, 2019). Observations made in this study suggest that the impedance spikes are related to the tissue response and cochlear inflammation including the temporal relationship between impedance spikes and inner ear dysfunction (Shaul *et al.*, 2019). Detection of impedance fluctuations provides an opportunity for early intervention, such as the use of anti-inflammatory drugs, to reduce the cochlear inflammation that may responsible for the impedance spikes.

Changes in electrode impedance can be associated with performance where higher impedance is associated with poorer hearing outcomes such as lower average hearing threshold and comfortable listening levels (Busby, Plant and Whitford, 2013). The use of electrode impedance (EI) was explored to provide insight into the immune-mediated response to the CI and how this affected hearing performance (Sanderson *et al.*, 2019). A retrospective investigation was carried out using patient data from the University of Southampton Auditory Implant Service (USAIS) over a 5-year period (Sanderson *et al.*, 2019). The aims were to gain an understanding in trends of impedance and to identify how many individuals, amongst the dataset, exhibited significantly raised levels of impedance which were below the manufacturer's warning level of high impedance, and whether this could be used to identify the beginning of decline in performance. 8% of adults and 5% children were detected as having statistically raised electrode impedance (Sanderson *et al.*, 2019), which could have resulted in poorer hearing function and sound quality; highlighting the individual as vulnerable. This analysis of impedance is also beneficial as an objective method to determine electrode deactivations. In this study, subjective measures through patient reporting were the main method to determine whether an electrode should be deactivated (Sanderson *et al.*, 2019). Using EI as a biomarker of decreasing CI performance and inner ear pathology, combined with subjective measures from patient consultations, provides powerful tools to identify poor performers to either monitor more closely or intervene earlier on. This should improve outcomes for the individual and prevent a negative decline in hearing performance.

1.8.2 Systemic inflammation in relation to hearing outcomes

For a CI to function, the electrodes stimulate SGNs across the cochlea that bundle to form the auditory nerve transmitting the information to the brain. For the sound to be perceived by the user, there needs to be adequate stimulation of the central auditory processing pathways. Anything that interrupts the message transfer along the auditory pathway, and the ability of the cells in these pathways to respond, are likely to result in poorer hearing outcomes. The mechanisms that influence this message transfer and the factors that contribute to the health of

the auditory system are not well understood. However, many of the risk factors associated with the gradual decline in hearing function that occurs with aging are lifestyle factors such as repeated noise exposure, co-morbidities (such as cardiovascular disease), diet, smoking and chronic infections. All of these factors have an inflammatory component.

Chronic conditions (such as type 2 diabetes and cardiovascular disease) and aging are associated with changes in the immune system and with increased levels of circulating markers of inflammation (Shaw *et al.*, 2010; Franceschi and Campisi, 2014). These co-morbidities, alongside aging, are associated with an increased risk of hearing loss (Nash *et al.*, 2011; Gupta *et al.*, 2019). The link between inflammation and ARHL has been evidenced in several studies (Bainbridge, Cheng and Cowie, 2010; Verschuur *et al.*, 2012; Verschuur, Agyemang-Prempeh and Newman, 2014; Lassale *et al.*, 2020). Increased levels of inflammatory markers such as white blood cells (WBCs), neutrophils, IL-6 and C-reactive protein (CRP) were associated with overall lower hearing threshold in a cohort of aging individuals (Verschuur *et al.*, 2012). In a different cohort of aging people, an association between WBC count and hearing threshold was identified; this became greater in individuals over 75-years-old suggesting that inflammatory status continues to increase with age (inflammaging) (Verschuur, Agyemang-Prempeh and Newman, 2014). Similarly, WBC count was positively associated with hearing impairment in older adults (Lassale *et al.*, 2020). This data suggests that inflammation and the age-associated increase in inflammatory markers is a causal factor for ARHL.

Lifestyle factors, such as smoking and alcohol consumption, influence auditory performance throughout a lifetime (Fransen *et al.*, 2008; Verschuur *et al.*, 2012; Dawes *et al.*, 2014). The association between hearing loss and smoking and alcohol consumption were evaluated in a study with 164,770 participants (Dawes *et al.*, 2014). Smokers were more likely to have a hearing loss compared to non-smokers and non-smokers more regularly exposed to passive tobacco smoke were more likely to have a hearing loss compared to those not exposed (Dawes *et al.*, 2014). Conversely, moderate alcohol consumption meant a lower chance of having a hearing loss compared to individuals who had never consumed alcohol (Dawes *et al.*, 2014). A multi-centre study involving 4,083 subjects found that both noise exposure and smoking significantly increased high-frequency hearing loss and a high body mass index (BMI) resulted in a hearing loss across the frequency range (Fransen *et al.*, 2008). Whereas, moderate alcohol consumption had a protective effect against hearing loss (Fransen *et al.*, 2008). These studies highlight key drivers of progressive hearing loss which are also associated with the inflammatory status of an individual. If overall peripheral and systemic inflammation is affecting the health of the auditory system, could lifestyle factors and insults that affect our inflammatory status be playing a role in variable CI outcomes?

1.9 The innate immune response and microglial priming in the central nervous system

The innate immune response is implicated in the cellular changes, cell loss and tissue remodelling following a cochlear insult. Between individuals there is variability in the severity of damage from cochlear insults such as noise injury, cochlear implantation and aging. This results in variable hearing trajectories, depending on how their immune system responds to the insults. The accumulation of cochlear insults an individual is exposed to throughout their lifetime, alongside age-associated changes, influence the function of the auditory system and therefore trajectory of hearing loss (naturally and aided). As previously stated, there is a small group of people with a CI who do not experience favourable outcomes with their implants (Causon, Verschuur and Newman, 2015). Understanding the biological mechanisms that underpin the variable, inflammatory response in these individuals will enable pharmacological and clinical interventions to be established with the aim to improve hearing performance.

We hypothesise that one source of variability is the behaviour of innate immune cells, macrophages and microglial cells, in the auditory system in raising an inflammatory response to a cochlear insult. Macrophages and microglia can become 'primed', a state describing a change in their phenotype whereby the cells exhibit an exaggerated inflammatory response to a second insult after being exposed to an initial stimulus (Perry and Holmes, 2014). In the auditory system, we propose that macrophages become primed to an inflammatory insult, resulting in an exaggerated response when the macrophages are exposed to additional insults over time. Characteristics of a primed microglia profile has been defined. This includes 1) higher baseline expression of markers of inflammation and inflammatory mediators 2) a lower threshold to switch into a pro-inflammatory state and 3) an exaggerated inflammatory response to an immune activation (Lull and Block, 2010; Norden, Muccigrosso and Godbout, 2015). In the auditory system, an exaggerated response would include increased production of inflammatory meditators resulting in progressive cochlear damage. The primed microglia phenotype has been described in other progressive CNS conditions in models for aging (Godbout *et al.*, 2005), neurodegenerative disease (Cunningham *et al.*, 2005) and traumatic brain injury (Sandhir, Onyszchuk and Berman, 2008).

Priming of microglial cells was demonstrated by producing an exaggerated inflammatory response in the ME7 prion mouse model upon the administration of lipopolysaccharide (LPS) (*Salmonella equine abortus*, 2 μg), as shown in Figure 1.11 (Cunningham *et al.*, 2005). Both peripheral and systemic challenges of LPS resulted in an increased expression of IL-1β by microglia in the pre-symptomatic prion mice compared to the normal mice. The pre-existing neuroinflammation,

associated with the prion mouse, switches the microglial cells to a primed phenotype resulting in an exaggerated inflammatory response when exposed to the inflammatory stimulus LPS. Similarly in aged mice, systemic injection of LPS caused an exaggerated inflammatory response and sickness behaviour due to the elevation in inflammatory cytokines (Godbout *et al.*, 2005).

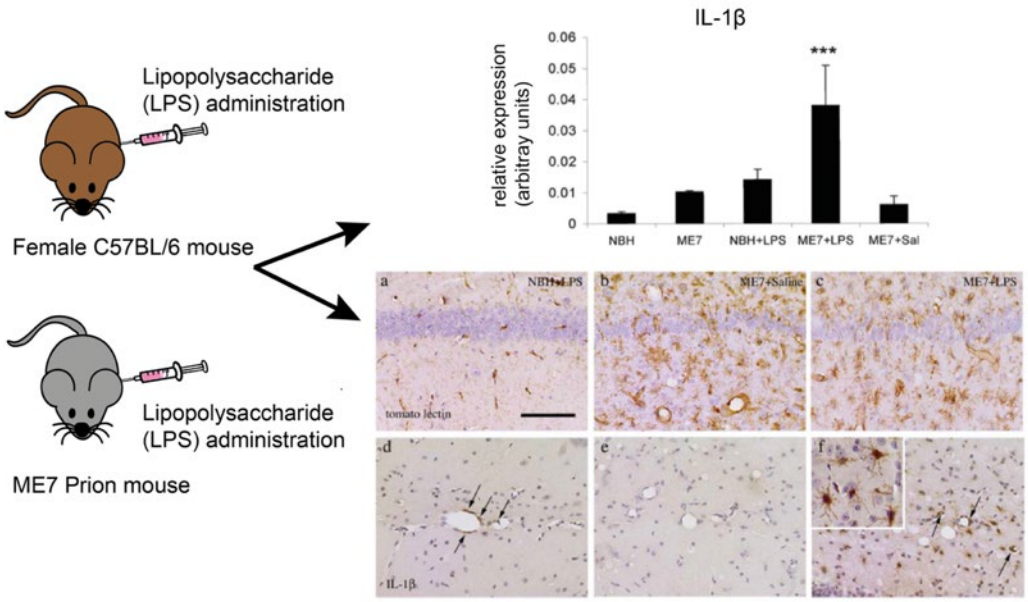


Figure 1.11 **Experimental design and outcome in the study by Cunningham et al**

Two mouse models used in the study were administered with LPS, both centrally and peripherally. Following LPS administration, ME7 prion mice with pre-existing neuroinflammation exhibited significantly increased microglial activation and expression of Il-1β compared to the control mice. Therefore, indicating an exaggerated inflammatory response in these mice through microglial priming (Cunningham et al., 2005).

CNS trauma models have provided similar evidence for microglial priming. An optic nerve crush model was used to investigate whether Wallerian degeneration of a neuronal tract, the optic nerve (Palin *et al.*, 2008), triggered priming in microglia associated with the tract. Optic nerve crush injury caused an increased expression of CD68+ and F4/80+ microglial cells which persisted for 28 days (Figure 1.12). When injured mice were injected peripherally with LPS (*Salmonella equine abortus*, 0.5 μg/g body weight) 28 days after injury, there was an exaggerated inflammatory response indicated by the increased expression of IL-1β, TNF-α and IL-6 compared to the controls (Palin *et al.*, 2008). Axonal degeneration of the optic nerve after injury 'primes' the microglia, making the CNS more susceptible to further damage through peripheral immune insults. In the cochlea and associated central auditory pathways, we hypothesise that previous cochlear insults and/or inflammatory challenges would prime the macrophages and microglial

cells leading to exacerbation of damage upon subsequent insults such as cochlear implantation. Microglia and macrophage priming could provide an explanation for the variability in hearing trajectories throughout life, and in outcomes between people with a CI.

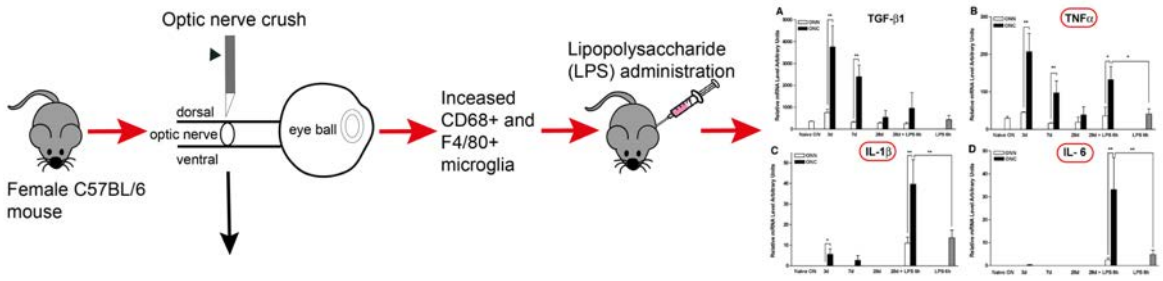


Figure 1.12 **Experimental design and outcome in the study by Palin and colleagues**

The use of the optic nerve crush model to investigate the level of pro-inflammatory cytokines in injured mice after the administration of LPS. Increased expression of TNFα, IL-1β and IL-6 cytokines was measured in injured mice that were administered with LPS compared to controls (Palin *et al.*, 2008).

Trained immunity refers to the ability of innate immune cells, such as microglia, to display a memory to an inflammatory challenge similar to the way the peripheral adaptive immune system develops immunological memory (Haley *et al.*, 2018). The exact molecular pathways responsible for microglial priming are not known, however it has been suggested that the mechanisms behind trained immunity are the mechanisms behind microglial priming. The characteristics of a primed microglial profile could be used to describe the changes that occur in trained immunity. It is likely that these terms are interchangeable and are referring to the same phenomenon.

An integrated nomenclature for innate immune memory suggests innate immune cells may be (i) 'primed' by an initial stimulus, resulting in innate immune memory in the form of a 'trained' or exaggerated response to subsequent stimuli. (ii) Innate immune cells may be desensitised by an initial stimulus resulting in a 'tolerant' or suppressed secondary response; immune tolerance (Neher and Cunningham, 2019). Additionally, innate immune cells may become fully activated but no memory responses occur resulting in a return to homeostatic levels. Therefore, 'microglial memory' therefore refers to all microglial responses that are dependent on prior exposures, either trained or tolerant with respect to the response of a naive microglial cell (Neher and Cunningham, 2019).

Immune tolerance describes a state where an initial stimulus may lead to innate immune cells switching to an alternative state and exhibiting tolerant responses, whereby there is a decrease in the responsiveness of an innate immune cell (compared to its naïve equivalent), following pre-

activation or 'priming' by a prior stimulus (Neher and Cunningham, 2019). Immune tolerance was first described in a study in 1946 which provided evidence that repeated daily injection of typhoid vaccine in rabbits resulted in a progressive decrease in fever induced by the vaccine (Beeson, 1946). In a later study in mice, prior injection with a sublethal dose of LPS protected the mice from LPS-induced lethality upon a subsequent dose of LPS (Watson and Kim, 1963). A key role of macrophages in the induction of immune tolerance has been demonstrated. LPS pre-treatment was shown to induce immune tolerance in a range of mouse strains but not in C3H/HeJ mice (Freudenberg and Galanos, 1988), which are LPS-resistant due to a mutation in the toll-like receptor 4 (TLR4) gene (Qureshi *et al.*, 1999). When CH3/HeJ mice were administered LPS-responsive macrophages before being treated with lethal LPS-D-galactosamine, the mice were sensitive to the lethal activity of LPS. When the mice were pre-treated with LPS before administration of LPS-responsive macrophages and lethal LPS-D-galactosamine, the LPS did not induce tolerance and protect the mice from the challenge of lethal LPS. Whereas if LPS-responsive macrophages were administered at the time of LPS pre-treatment, the mice were protected. These findings highlight the role of macrophages in the induction of immune tolerance (Freudenberg and Galanos, 1988). Many studies have shown that a main characteristic of immune/endotoxin tolerant monocytes and macrophages is the down regulation of genes for inflammatory cytokines and chemokines such as TNFα, IL-1β, IL-6, CCL3, CCL4 and CX3CL10 (Medvedev, Kopydlowski and Vogel, 2000; Foster, Hargreaves and Medzhitov, 2007; Biswas and Lopez-Collazo, 2009). Immune tolerant monocytes display increased phagocytic activity (del Fresno *et al.*, 2009).

Innate immune cells develop memory through epigenetic reprogramming which are changes to the rate of transcription through histone post-translational modifications (PTMs) (Haley *et al.*, 2018). Histone PTMS can either increase or decrease the rate transcription through altering the structure of chromatin to either an open (activating) or closed (repressing) state. Examples include acetylation, methylation, phosphorylation and ubiquitylation (Dawson and Kouzarides, 2012). Acetylation is the addition of an acetyl group to the N-terminal tail of histones, leading to more of an open conformation of chromatin structure and increasing transcription. Histone deacetylases (HDACs) are enzymes which remove acetyl groups making the DNA less accessible therefore decreasing the rate of transcription. There is some evidence suggesting that HDACs inhibitors have anti-inflammatory actions in CNS pathologies through preventing the inhibition of inflammatory genes (Kaminska, Mota and Pizzi, 2016). On the other hand, HDAC inhibiters have pro-inflammatory actions through the promotion of microglial ramification changing the microglia to a non-activated phenotype (Huang *et al.*, 2017). Despite the contrasting evidence of the role of

histone modifications in inflammatory actions, there is some evidence for the role of histone modifications in microglial priming in disease.

Transcriptional single-cell sorting was used to generate an epigenetic map of the immune cells in wild-type and Alzheimer's disease (AD) transgenic mice (in a mouse model of AD that expresses five human familial AD gene mutations - 5XFAD line Tg6799). The distribution of histone 3 lysine 4 dimethylation (H3K4me2), a methylation PTM that marks the promoter and enhancer regions, was measured (Keren-Shaul *et al.*, 2017). A novel type of protective microglia, the disease associated microglia (DAM) which accumulates around plaques was described. There was no difference in the distribution of H3K4me2 between the non-DAM and DAM in the transgenic mice, however significant differences were found between the WT and AD-transgenic mice (Keren-Shaul *et al.*, 2017). These results suggest that epigenetic changes have occurred in the progression of the disease, priming the microglia to later transition into DAM (Keren-Shaul *et al.*, 2017). Epigenetic modifications play a role in changing phenotype of microglia. This mirrors what is seen in trained immunity, when monocytes retain an 'immunological scar' from a previous infectious episode leading them to a state of increased responsiveness upon the next infectious episode. Epigenetic modifications occur at genomic regions called latent enhancers. These are unbound by transcription factors and have few histone modifications in unstimulated macrophages, acting as the 'immunological scar' leading to increased responsiveness upon the next infectious episode (Ostuni *et al.*, 2013; S. Saeed *et al.*, 2014).

There is evidence for epigenetic regulation in IL-1 cytokine family, key pro-inflammatory mediators. A feature of microglial priming is the increased expression of IL-1 upon repeated exposure to inflammatory stimuli, thought to be controlled by epigenetic modifications. C57BL/6 mice challenged with LPS (*Escherichia coli*, 0.33 mg/kg body weight), and aged mice, had decreased expression of DNA methylating enzymes and decreased DNA methylation in the Il-1β gene promoter in microglia. This increased Il-1β mRNA and protein production and increased sickness behaviour in these mice (Figure 1.13) (Matt, Lawson and Johnson, 2016), demonstrating that epigenetic modifications occur during microglia priming. Additionally, in human blood samples from elderly subjects and subjects with neurodegenerative diseases at the proximal promoter of IL-1β, encoding Il-1β, hypomethylation of two CpGs resulted in an increased expression of Il-1β suggesting that transcript levels are regulated by methylation. Together, these studies provide evidence for the epigenetic regulation of the phenotype and expression of microglia and the inflammatory mediators they express.

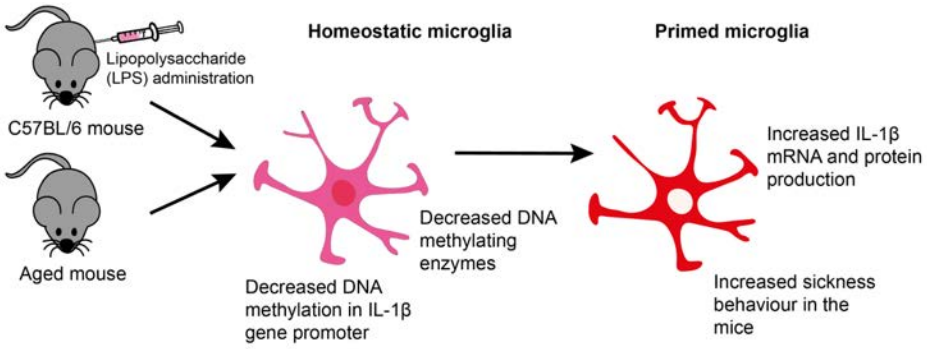


Figure 1.13 **Experimental design and outcome in the study by Matt and colleagues**

> Results from the study demonstrating that in mice challenged with LPS and in aged mice, the microglia had decreased DNA methylating enzymes and decreased DNA methylation in the IL-1β gene promoter which led to increased IL-1β mRNA and protein production and increased sickness behaviour in these mice. These results indicate that epigenetic modifications had occurred in microglial priming (Matt, Lawson and Johnson, 2016).

The epigenetic modifications of innate immune cells (macrophages and microglia) that occur in immune priming and tolerance modify the phenotype and function of the cells. These changes influence the inflammatory response and subsequent tissue response. There is not enough evidence to state that immune priming, leading to an exaggerated or 'trained' response, will always have damaging effects on the tissue. However in many studies, including the studies described above, microglial priming results in a heightened inflammatory response with increased production and release of pro-inflammatory mediators such as IL-1β which is damaging to surrounding tissue (Cunningham *et al.*, 2005; Palin *et al.*, 2008; Skelly *et al.*, 2019). Contrastingly, there is some evidence to suggest that immune tolerant responses in microglia can be beneficial in mouse models of brain pathology (Wendeln *et al.*, 2018). Further research is required to determine whether tolerant responses are always beneficial or whether in some cases, can be detrimental.

Based on the available evidence, we are hypothesising that innate immune priming (rather than immune tolerance) in the auditory system is a potential mechanism contributing to progressive hearing loss. Immune tolerance results in a decreased response upon a secondary stimulus and a reduction in the expression of pro-inflammatory cytokines likely to lead to minimal additional local tissue damage. Whereas, we are proposing that when microglia and macrophages in the auditory system are exposed to insults, this causes a change in phenotype resulting in an exaggerated inflammatory response when exposed to an additional insult. The exaggerated response is characterised by increased production of inflammatory mediators such as IL-1β

leading to local tissue damage and progressive loss of function to key structures required for auditory function; thus, contributing to progressive hearing loss.

The concept of microglial priming has been explored in detail in the CNS in the last ten years. However, there is little data investigating the role of macrophages and microglial cells in response to injury to the cochlea by insults such as noise exposure and cochlear implantation. As the concept that the cochlea is immune privileged no longer holds, it is plausible to suggest that the role of the immune cells in the cochlea, and the mechanisms behind the innate immune response, could be similar to the mechanisms that exist in the periphery and the brain and therefore correlates can be made.

1.9.1 The development of animal models to investigate the role of macrophages in the auditory system

There is a need for the development of models to enable the investigation of the role of macrophages in the auditory system following acute and chronic insults, particularly investigating the effect of cumulative insults and the immunological mechanisms responsible for this. The use of mouse models for TBI, neurodegeneration and aging have enabled detailed investigation into the mechanisms of microglial priming and the consequence of this on the surrounding tissue environment. Several studies (animal and human) have investigated the distribution, morphology and function of macrophages in the cochlea and how this changes following acute and chronic cochlear insults. This will be discussed in detail in Chapter 2. Very few studies have investigated the role of macrophages in the interaction of cochlear insults, what factors exacerbate cochlear inflammation following cochlear insults, and whether pre-existing inflammation in the auditory system results in an exaggerated inflammatory response when there is an additional insult. Using a combination of mouse models and human tissue samples (Figure 1.14), the aim of this research was to investigate the macrophage populations of the cochlea and auditory system and investigate whether cochlear macrophages influence the trajectory of hearing loss naturally and with a cochlear implant.

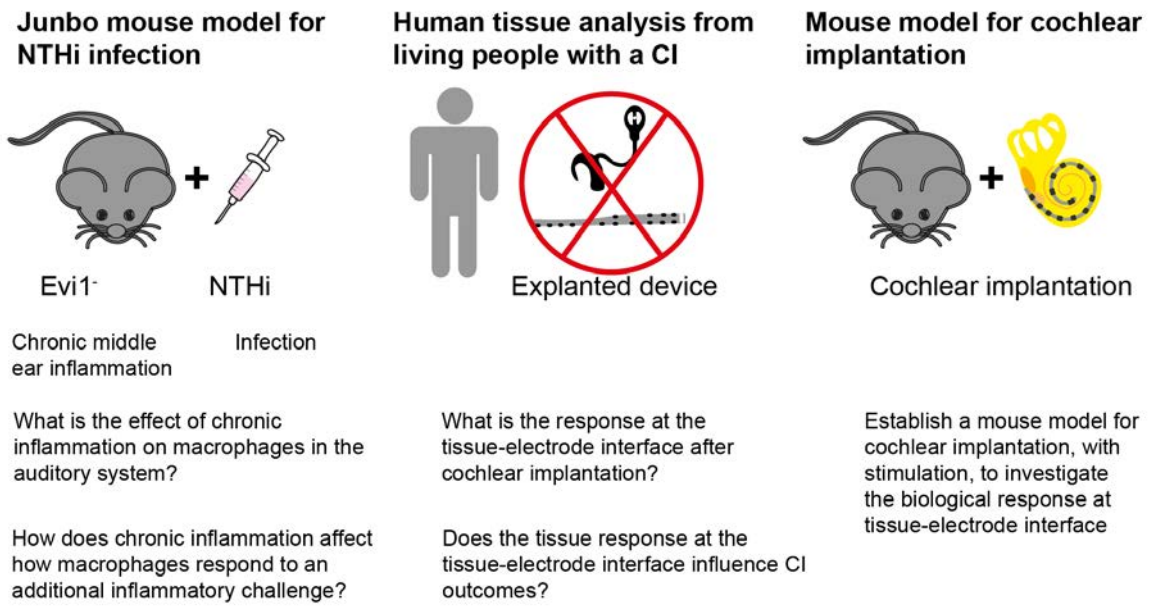


Figure 1.14 **Methods utilised in this project to investigate the role of macrophages in the auditory system following acute and chronic insults**

The different approaches used in this project including the Junbo mouse model for NTHi infection, human tissue analysis from living people with a cochlear implant and a mouse model for cochlear implantation. The main objectives are included.

1.10 Overarching aims and questions

Translational questions:

- What factors contribute to the variability seen in an individual's hearing loss trajectory?
- What factors contribute to performance outcomes following cochlear implantation?
- Why do some individuals experience sub-optimal performance with their cochlear implant?
- What is the tissue response to cochlear implantation?
- Does the tissue response at the tissue-electrode interface influence CI outcomes?

Macrophage biology questions:

- What is the macrophage population in the cochlea?
- What is the role of macrophages following acute and chronic insults to the auditory system?
- Do macrophages influence the trajectory of hearing loss?
- Do macrophages influence cochlear implant performance/hearing outcomes?

- Do macrophages in the auditory system become primed following acute and chronic insults, leading to an exaggerated inflammatory response when exposed to a secondary insult resulting in further tissue damage and a reduction in function?

1.11 Overview of methods

A range of techniques were used to investigate macrophage populations in the cochlea and auditory system and the role of these cells following acute and chronic insults including middle ear inflammation and cochlear implantation.

Literature search - A systematic approach was taken for critical evaluation of the current literature in the field. A meta-analysis style data extraction and distilling method was utilised when evaluating the current literature described in each chapter.

Mouse tissue - Mouse models including the Junbo mouse model and a CI mouse model were used to obtain tissue for detailed investigation of the macrophage populations and the tissue response in the cochlea and auditory system.

Human tissue - An explanted array with a fibrotic sheath attached was collected at explantation to enable analysis of the tissue on an explanted electrode array from a living cochlear implant user.

Histology - Histological stains including Haematoxylin and Eosin (H&E), Trichrome and Tetrachrome were used to investigate the tissue composition in both the mouse and human tissue.

Immunohistochemistry - An array of markers were used for investigation of the cellular composition in mouse and human tissue. Multiple macrophage markers and inflammatory and proliferative cell markers were included.

Imaging - Multiple imaging modalities were used to analyse and acquire images of human and mouse tissue sections stained histologically and immunohistochemically.

- Colour imaging microscopy
- Fluorescence microscopy
- Confocal microscopy

Micro-Computerised Tomography (μCT) - Micro-CT scanning and acquisition was carried out on a whole mouse cochlea and a mouse cochlea implanted with an electrode array.

Clinical data - Clinical measures including electrode impedance, speech perception tests (BKB sentence test) and electrode status were measured and recorded from an individual who was implanted then experienced a decrease in performance leading to explantation and reimplantation.

Chapter 2 Macrophages in the cochlea; an immunological link between risk factors and progressive hearing loss

2.1 Introduction

Innate immunity and inflammation have roles in both the physiology and pathophysiology of hearing loss. Hearing loss is an inability to hear sounds within what is considered the normal audible range. The most prevalent type of hearing loss (sensorineural) is due to damage or dysfunction in the cochlea and/or auditory nerve. Progression of hearing loss is principally attributed to a gradual loss of function in hair cells, the sensory cell, and their post-synaptic contacts in the cochlea. The time of onset and rate of progression of hearing loss varies. Age, a history of smoking, injury to the auditory system through e.g., noise exposure and infection, and common age-associated comorbidities such as cardiovascular disease and type 2 diabetes are risk factors for progressive hearing loss. The mechanistic links between these factors and the cellular changes within the auditory system that cause progressive hearing loss are not well understood. Findings from other neurodegenerative conditions have identified that tissue-resident macrophages contribute to tissue injury in the nervous system.

Currently, no effective medical or pharmacological treatments for SNHL are available, although acoustic hearing aids and cochlear implants (CIs) are able to reduce and manage the effects of hearing loss. CIs are neuro-prostheses which consist of an electrode array that has been surgically inserted into the cochlea to directly stimulate the auditory nerve (see 1.4.1). For effective CI function, there is a need to reserve residual structures within the auditory system. There is variability in the hearing trajectory of individuals with natural hearing, in addition to the performance variability observed in people with a CI. Better understanding of the cellular changes in the impaired auditory system and how these differ between people with hearing loss is required.

The auditory system comprises distinct anatomical regions and is broadly subdivided into the central auditory system (the regions from the cochlear nucleus in the brainstem to the auditory cortex) and the peripheral auditory system (the outer, middle and inner part of the ear). Refer to 1.2 and Figure 1.1 for an overview of the anatomical regions which comprise the central and peripheral auditory system. The cochlea is organised with a series of specialised soft-tissue layers located within the otic capsule in the temporal bone of the skull. The organ of Corti, stria

vascularis and Rosenthal's canal, housing the spiral ganglion neuron (SGNs) cell bodies, have distinct microenvironments important for auditory function (described in detail in 1.2).

Each anatomical region in the auditory system can become damaged, with resulting impairment of hearing function. The neurobiological substrates of hearing loss e.g., the key players and cochlear regions involved in hearing loss have been described in detail in 1.5 and illustrated in Figure 1.7. Neurobiological substrates of hearing loss include hair cells and their synapses, SGNs and their axons, and the stria vascularis. Each cell or region can become damaged and degenerate following a cochlear insult, contributing to a progressive loss of function and subsequent hearing loss. Macrophages are distributed across the auditory system, often in close proximity to these key cells and tissues.

As discussed in 1.6, insults to the cochlea such as noise exposure, cochlear implantation and aging cause an inflammatory response involving the recruitment and infiltration of immune cells and the increased production of inflammatory mediators. Macrophages are the primary and most abundant immune cell within the cochlea. Several studies have investigated the distribution, morphology and abundance of macrophages under homeostatic conditions and how this changes following cochlear insults (Hirose *et al.*, 2005; Lang *et al.*, 2006; Okano *et al.*, 2008; O'Malley, Nadol and Mckenna, 2016; Liu *et al.*, 2018; Noonan *et al.*, 2020; Okayasu *et al.*, 2020). It is unclear whether and when these changes are protective or detrimental to cochlear function. Following inflammatory insults to the cochlea (such as CI, noise exposure or infection), it is plausible that macrophages could play a role in accelerating damage to cochlear regions involved in hearing function leading to progressive hearing loss. For people with a CI, damage to cochlear structures following implantation may lead to increased inflammation, associated with increased fibrosis and poorer performance with the implant (Clark *et al.*, 1995; Wilk *et al.*, 2016; Ishai *et al.*, 2017).

Several questions regarding to the role of macrophages in auditory function remain, including: what are the roles of cochlear macrophages in injury/repair responses? What are the molecular mediators that control macrophage recruitment, signalling and turnover? Do macrophages in the cochlea contribute to hearing loss as a consequence of entering a primed, or altered activation, state? In order to improve hearing outcomes for people with hearing loss, we need to understand how best to modulate the auditory system at a cellular level. This requires a major advance in our understanding of the biology of macrophages in the auditory system. Figure 2.1 demonstrates the neurobiological substrates of hearing loss and indicates the distribution and role of macrophages amongst the auditory structures, which will be described within this chapter.

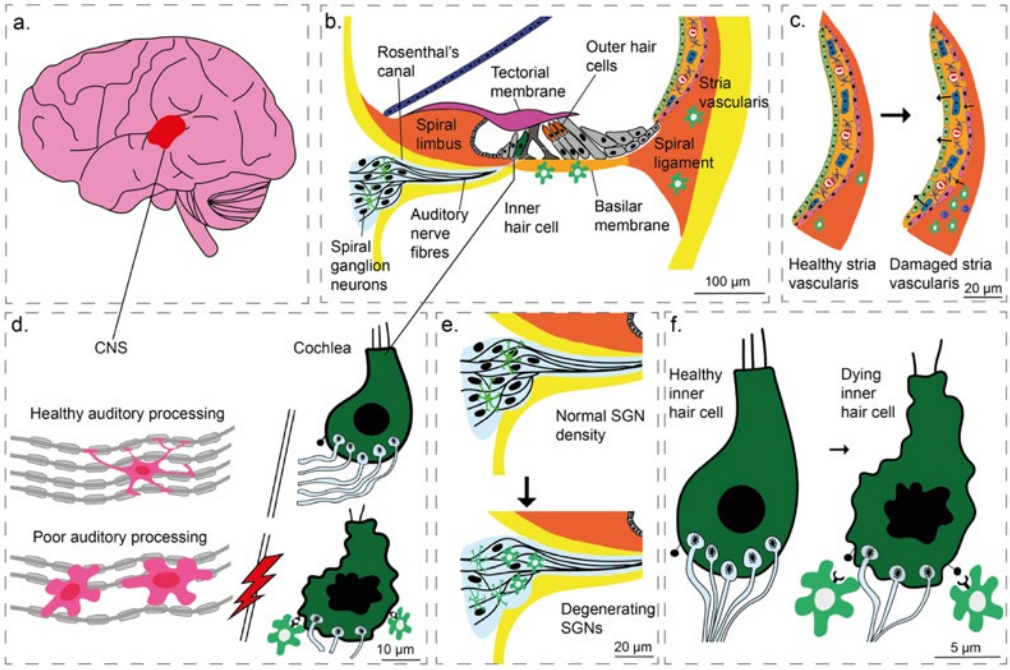


Figure 2.1 **Schematic illustrating the neurobiological substrates of hearing loss demonstrating the pathology within these structures and the distribution of macrophages**

See figure legend (Figure 1.8) for detail of the pathology within each region. The distribution, morphology and function of cochlear macrophages, associated with these structures, change following cochlear insults.

a. Human brain with the auditory cortex indicated in red.

b. Key structures and regions of the cochlea involved in auditory function.

c. PVM/Ms in the stria change morphology and display a physical detachment from capillary walls following cochlear insults.

d. Decreased signalling between damaged/dying hair cells within the cochlea and axons of the auditory nerve results in microglia in the CNS auditory nerve becoming activated and increasing in number.

e. With aging, SGNs degenerate and reduce in number. The number and morphology of macrophages amongst SGNs changes.

f. Following cochlear damage, macrophages are recruited to damaged SGNs and sensory cells through fractalkine signalling.

An extensive review of the literature was carried out to extract and highlight the available evidence for the distribution, morphology and abundance of macrophages in the auditory system and to determine how these change following acute and chronic insults to the cochlea. We propose that cochlear macrophages influence the trajectory of hearing loss across the lifespan and may play a role in the variability of CI performance, by contributing to decreased cochlear health and the progressive loss of function following an inflammatory insult. We hypothesise that

cochlear insults train or prime macrophages in the auditory system similar to what is reported in other progressive CNS conditions (Haley *et al.*, 2018; Neher and Cunningham, 2019). This priming response would then result in an exaggerated inflammatory response when the macrophages are exposed to subsequent cumulative insults. These exaggerated inflammatory responses are likely to cause progressive (albeit unintentional) damage to the cochlea and associated structures, manifesting as greater loss of hearing function. Priming of macrophages in the auditory system may play a role in the variability in hearing function that presents throughout individuals across their lifespan, whereby previous lifestyle choices or inflammatory events influence and alter the hearing trajectory.

2.2 Aims and objectives

- Review the evidence for a role of macrophages in progressive hearing loss
- Extract and distil the available evidence of the distribution, abundance and morphology of macrophages in the peripheral auditory system
- Propose our hypothesis suggesting a role of macrophages in the trajectory of hearing loss through macrophage priming

2.3 Methods

Description of the search strategy

A search was carried out using PubMed using the search terms ‘cochlear macrophages’ and ‘cochlear inflammation’ (04-04-20). The pdf of the paper was saved to Mendeley into a folder according to the search terms. Key information and data were extracted and added to a table. Any additional papers that were found during secondary data extraction, that were not part of the initial search, were added to a separate table and folder of Mendeley. Papers were organised into categories based on type of cochlear insult/ hearing loss e.g., NIHL (18), ARHL (11), ototoxicity (4), cochlear implantation (9). Data was extracted and a timeline was drafted for each of the groups capturing information on the insult, the measurements taken, the species, age, timepoints of the experimental design. The purpose of the search and extraction of secondary data was to put forward the evidence base for investigating macrophages, to illustrate what is known and where there are gaps in the literature regarding the role of macrophages following acute and chronic insults.

2.4 Results

2.4.1 Tissue-resident vs infiltrating macrophages in the auditory system

Distinct tissue-resident macrophage populations exist in different organs including microglia in the CNS (Li and Barres, 2018) and CC chemokine receptor 2- (CCR2-) cells in the heart (Z. Wang *et al.*, 2019). Microglia have essential roles in development, in homeostasis and diseases of the adult auditory CNS (Figure 2.2) (Li and Barres, 2018). They arise from embryonic yolk sac precursors (Ginhoux *et al.*, 2010) and maintain their population by self-renewal (Lawson, Perry and Gordon, 1992; Ajami *et al.*, 2007). The ability to identify the origin and kinetics of these cells in homeostatic or pathological tissue is an important step in understanding how other signalling pathways might influence their behaviour. Perivascular macrophages (PVMs), a second resident population in the brain, are located at the BBB between the systemic circulation and CNS parenchyma (Figure 2.2) (Galea *et al.*, 2008; Varatharaj and Galea, 2017; Galea and Perry, 2018; Lapenna, De Palma and Lewis, 2018). PVMs originate from yolk sac-derived, erythromyeloid progenitors that are seeded in the brain during early development (Goldmann *et al.*, 2016; Faraco *et al.*, 2017; Lapenna, De Palma and Lewis, 2018). PVMs have homeostatic and pathological roles in regulating the movement of solutes, pathogens and immune cells from blood to brain. Importantly both microglia and perivascular macrophages, resident populations in the brain, are long-lived and sensitive to epigenetic modification (Keren-Shaul *et al.*, 2017; Haley *et al.*, 2018) giving them the ability to develop a memory of previous insults to the tissue they reside in. Whereas, infiltrating monocytes enter the brain and cochlea from the blood stream following an insult/inflammation. Once at the site of injury, infiltrated monocytes differentiate into macrophages. These cells are therefore a different population to the resident macrophage population.

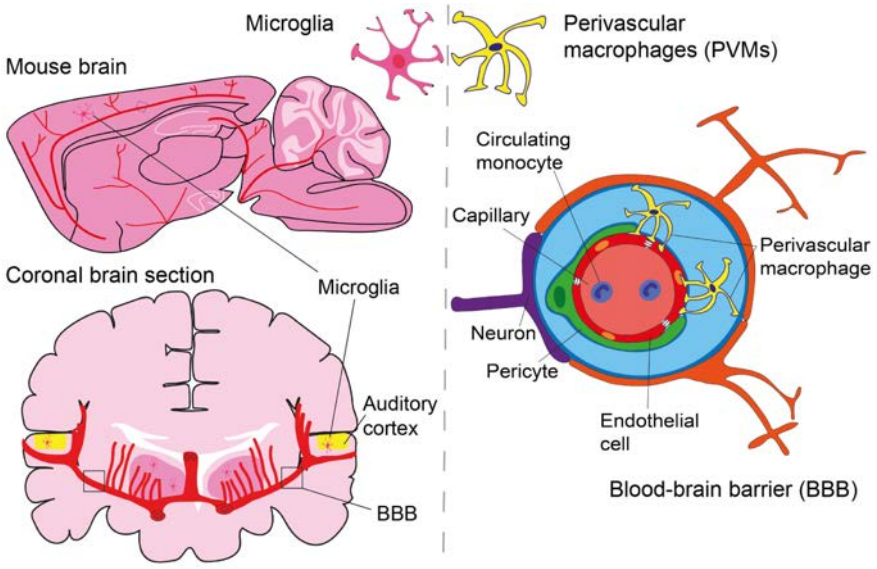


Figure 2.2 **Schematic illustrating two resident macrophage populations in the brain: microglia and perivascular macrophages**

Microglia and perivascular macrophages (PVMs) are two resident macrophage populations in the brain. Both are derived from yolk sac erythromyeloid precursors and maintain their population by self-renewal. Microglia carry out multiple homeostatic and pathological roles. Brain PVMs regulate the movement of solutes and immune cells from blood to brain.

2.4.2 Development and origin of cochlear macrophages

The origin and changes in the population of cochlear macrophages from embryogenesis to the development of the mature cochlea have been studied in the mouse (Okano *et al.*, 2008; Chen *et al.*, 2018; Dong *et al.*, 2018; Kishimoto *et al.*, 2019). Colony stimulating factor 1 (Csf1) signalling regulates the survival, proliferation and differentiation of brain-resident macrophages including microglia (Ryan *et al.*, 2001) and also controls seeding of macrophages in the cochlea during development (Kishimoto *et al.*, 2019). Two subtypes of resident macrophages exist in the embryonic mouse cochlea. A population of Csf1r-dependent macrophages that originate from the yolk sac, and Csf1r-independent macrophages that migrate from the fetal liver via the systemic circulation (Kishimoto *et al.*, 2019) have been identified (Figure 2.3). Csf1r-dependent macrophages make up the larger proportion of resident macrophages and express Iba1, CD68 or F4/80. They reside in the mesenchyme surrounding the otocyst at E10.5 and at E17.5 these macrophages appear in the spiral ganglion and spiral ligament. Csf1r-independent macrophages express CD11b at E10.5. At E17.5, these macrophages are restricted to the mesenchyme of the cochlear modiolus and the intraluminal surface of the perilymphatic space (Kishimoto *et al.*, 2019).

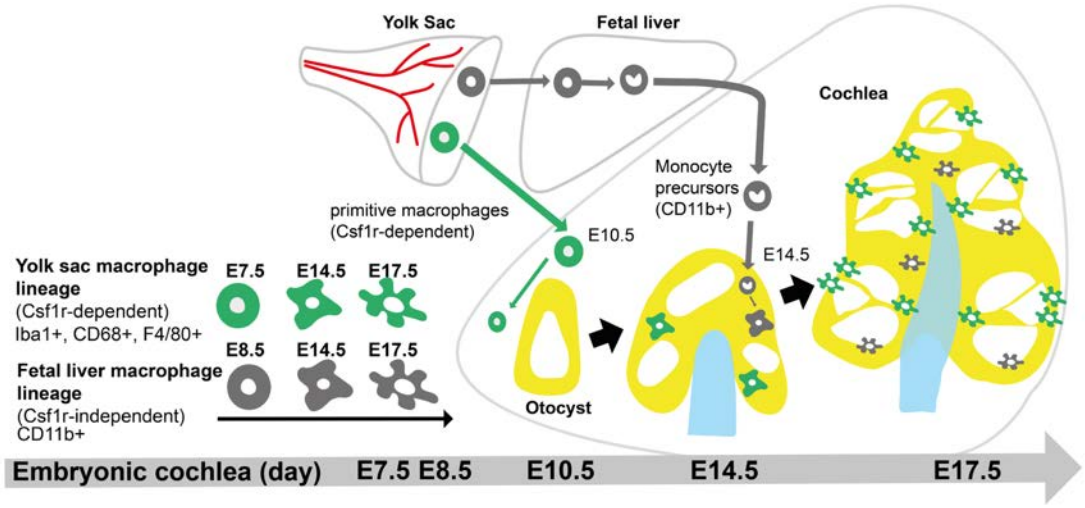


Figure 2.3 **The distribution and morphology of macrophages in the embryonic cochlea under homeostatic conditions**

Csf1r-dependent and csf1r-independent resident macrophages are found in the cochleae of the embryo. These subtypes vary in their origin, the markers they express, their abundance and distribution. Figure adapted from (Kishimoto *et al.*, 2019).

Changes in the macrophage population early in postnatal development have been studied in (C57BL/6J) mice (Figure 2.4) (Chen *et al.*, 2018; Dong *et al.*, 2018). Iba1-positive cells (Iba1+) were distributed in the modiolus, spiral lamina, spiral ganglion, spiral ligament (SL) and organ of Corti (OC) in P0 to P14 mouse cochleae (Chen *et al.*, 2018). The number of Iba1+ cells increased in number to P5 then decreased to P14 (Figure 2.4a) (Chen *et al.*, 2018). The population of macrophages in the SL changed throughout the postnatal period, indicating a potential role of the macrophages in extracellular matrix remodelling. The cells were located mainly in the peripheral and central regions at P0. Between P0 and P14 they migrated towards the subcentral and marginal region of the SL (Chen *et al.*, 2018). The morphology of the Iba1+ cells in the SL changed from amoeboid (P0) to ramified, a phenotype associated with homeostasis (Chen *et al.*, 2018). Two distinct macrophage populations have been identified in the organ of Corti (OC) and basilar membrane (BM) during postnatal development (Figure 2.4b) (Dong *et al.*, 2018). OC macrophages were short-lived and underwent developmental death as the epithelium matured. In contrast, BM macrophages differentiated from a monocyte morphology to mature resident macrophages (Dong *et al.*, 2018). The population of macrophages during postnatal development is dynamic and implies a role in the remodelling of the cochlea through development.

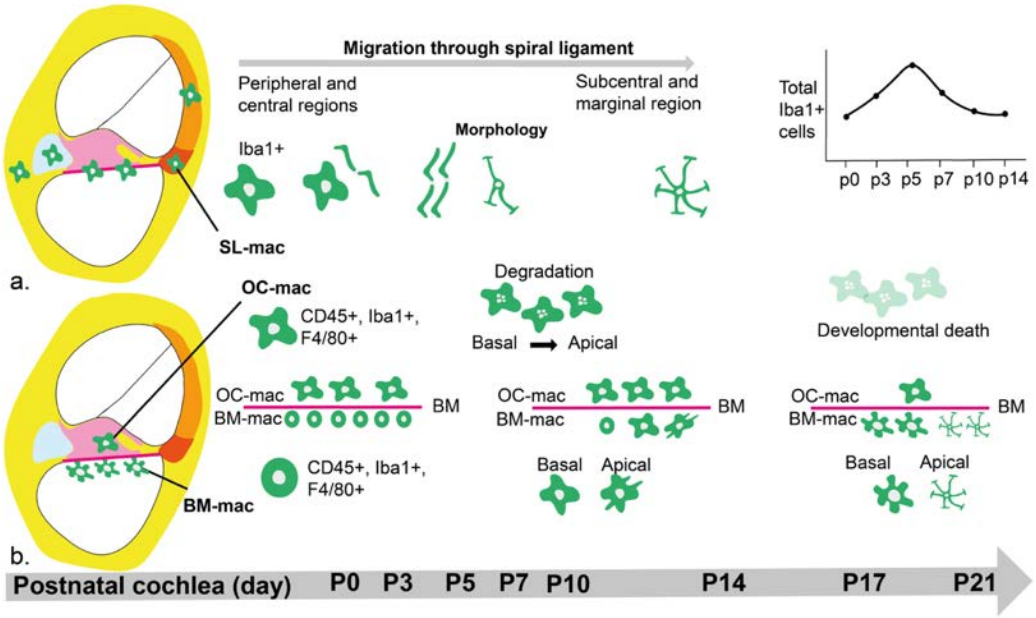


Figure 2.4 **The distribution and morphology of macrophages in the postnatal cochlea under homeostatic conditions**

The distribution and morphology of macrophages changes during postnatal development of the cochlea, this is most marked in the spiral ligament (SL) (**a**) (Chen *et al.*, 2018) and organ of Corti (OC) and (**b**) basilar membrane (BM) macrophages (Dong *et al.*, 2018).

In the adult cochlea, two resident populations of macrophages are necessary for homeostatic function; cochlear macrophages and perivascular macrophage-like melanocytes (PVM/Ms), (Figure 2.5). A mid-modiolar view of a mouse cochlea is shown in Figure 2.5, illustrating key anatomical regions and the distribution of cochlear macrophages. Resident cochlear macrophages are recruited from bone marrow precursors and renew their population through infiltration of circulating monocytes (Figure 2.5) (Hirose *et al.*, 2005; Lang *et al.*, 2006; Okano *et al.*, 2008; Tan, Lee and Ruan, 2008; Sato *et al.*, 2010; Shi, 2010). Yolk sac, fetal liver and bone marrow-derived resident macrophages have been identified in the adult cochlea (Okano *et al.*, 2008; Ginhoux and Guilliams, 2016; Kishimoto *et al.*, 2019). Cochlear macrophages display an amoeboid or ramified morphology and have both protective and degenerative roles under homeostatic and pathological conditions.

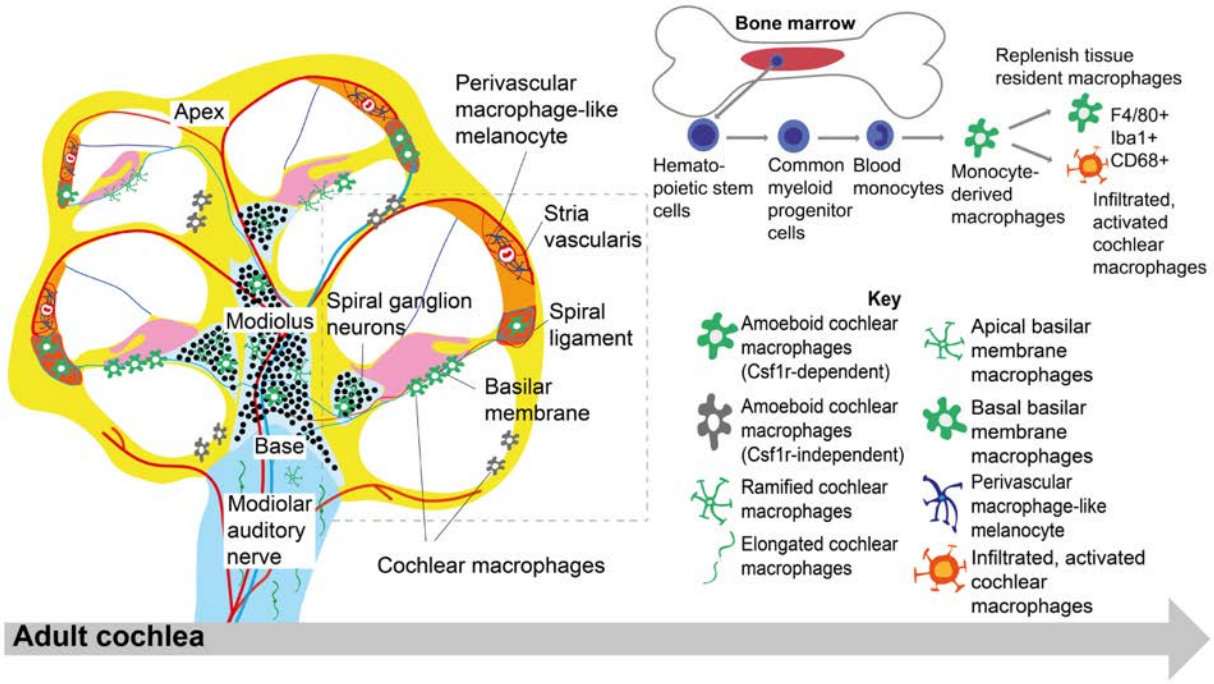


Figure 2.5 **The distribution and morphology of macrophages in the adult cochlea under homeostatic conditions**

The distribution, morphology and abundance of macrophages vary depending on the specific site within the tissue. The lineage of the macrophages is demonstrated, either yolk sac (Csf1r-dependent) or liver (Csf1r-independent). All of these factors determine the function of the cells under homeostatic conditions and following a cochlear insult. The resident macrophage population is replenished by bone marrow-derived cells.

PVM/Ms are positioned in close proximity to vascular structures of the inner ear (Shi, 2010; Zhang *et al.*, 2012). PVM/Ms have an estimated turnover time of 10 months in mice and are maintained through migration of monocytes into the cochlea (Shi, 2010). They have essential roles in maintaining the integrity of the intrastrial fluid-blood barrier and maintaining the endocochlear potential (Zhang *et al.*, 2012, 2013). Depletion of PVM/Ms in mice has been shown to cause hearing loss and a significant reduction in endocochlear potential (Zhang *et al.*, 2012).

Each of the macrophage populations in the auditory system reside in an anatomical niche, adopts a characteristic morphology and undertakes specific functions. Additionally, each population expresses unique and overlapping cellular markers, used experimentally to identify these cells across the brain and cochlea, as shown in Figure 2.6. The origins of the macrophage populations are reflected in the array of cellular markers. This chapter has drawn together the published evidence base with a focus on the cochlea to identify the markers that have been used in studies, summarised in Figure 2.6. Compared to CNS macrophages, the expression signature of cochlear

macrophages and the markers which can be used to identify these cells is less well documented. This needs to be considered when drawing conclusions in the field. Many of the earlier studies used CD45, a pan leukocyte marker. More recent studies used markers such as Iba1 or F4/80 for cochlear macrophage identification. Some reports have relied on cellular morphology to distinguish between macrophages and other leukocytes. CD11b is expressed by all of the resident macrophage populations. Markers listed in red in Figure 2.6 could be used to identify specific sub-populations of macrophages. A definitive list of specific reliable and experimentally tractable markers for cochlear macrophages is still needed and would be beneficial for advancing the field.

	Expression signature	
Resident cochlear macrophages	Iba1,CD45,CX_3CR1, CD68, MHC Class II, F4/80, CD163, CD11b	
Perivascular macrophage-like melanocytes (PVM/Ms)	F4/80, CD68, CD163, CD11b	
Infiltrating monocytes	$CD45^+Iba1^+CX_3CR1^+$, Ly6C, CCR2	
Microglial cells	MHC Class II^{low}, CX3CR1, $CD45^{low}$, CD11b, SALL1, P2RY12, TMEM119	
Perivascular macrophages (PVMs)	CX_3CR1, $CD45^{hi}$, CD163, MHC Class II^{hi}, CD11b, MERTK, CD206	

Figure 2.6 **Expression signature for macrophages and microglia of the peripheral and central auditory system**

There are several specific markers that identify different CNS glial populations; however, this is lacking in the peripheral auditory system macrophages. Clarification on the appropriate markers which can be used to identify cochlear macrophages and PVM/Ms is needed to improve our understanding of the role of these cells in homeostasis and hearing loss. CD11b, highlighted in blue text, is expressed by all of the resident macrophage populations described. Markers that are unique to sub-populations and that could be used to differentiate populations are highlighted in red text. See appendix B.1 for the citations associated with each cell marker.

The detailed expression profiles of microglia and macrophages of the brain have been well characterised using comparative transcriptomic studies (Crotti and Ransohoff, 2016; Li and Barres, 2018). Gene expression profiles consistent with macrophages have been identified in recent single-cell RNA sequencing of cochlear tissue (Rai *et al.*, 2020) and transcriptomic analysis of the C57 mouse cochlea has identified genes associated with macrophage activation (Su *et al.*, 2020). Extending this work to identify and establish the set of markers expressed by cochlear macrophages using multiplex and comparative transcriptomic studies will enable more reliable

identification of sub-populations and roles and comparison with other organ systems. Approaches need to enable region (cochlear sub-structure) specific macrophage information to be captured.

Research to identify the expression profile of macrophage sub-populations, or between cells in different regions in other tissues, has advanced faster than macrophages in the cochlea and has identified markers that indicate cell function. In the brain, microglia and infiltrating monocytes have been shown to display functional differences and contribute to disease pathologies differently such as after ischemic stroke or in multiple sclerosis (Yamasaki *et al.*, 2014; Ritzel *et al.*, 2015). Markers such as TMEM119, which appears to be specific for microglia in both human and mouse, can be used to distinguish resident microglia from blood-derived macrophages (Bennett *et al.*, 2016; Satoh *et al.*, 2016). Being able to distinguish resident and infiltrating macrophages in the cochlea may help elucidate the precise function of each sub-population and increase understanding in the mechanisms responsible for progressive loss of function.

2.4.3 Distribution and morphology under homeostatic conditions

The distribution, abundance and morphology of cochlear macrophages across different regions of the adult cochlea under homeostatic conditions has been investigated in both human and animal studies using immunohistochemistry. The principal findings from many of these studies are summarised in Table 2.1. Macrophages broadly adopt morphologies that are indicative of their function. Homeostatic macrophages have longer branched processes (Li and Barres, 2018). In contrast, macrophages migrating to an injury site or which are activated and phagocytic have fewer processes and appear amoeboid. The current understanding of the distribution and morphology of the cochlear macrophages is mapped in Figure 2.5 and described in Table 2.1.

Table 2.1 **The distribution of macrophages across the sub-structures of the cochlea and auditory nerve**

This table summarises key findings by anatomical location from studies of macrophage distribution and morphology in the cochlea and auditory nerve under homeostatic conditions. The data are from studies in mouse, rat and humans. The morphological characterisation of macrophages is indicative, though not definitive, of cellular function. More detailed characterisation of the cellular expression patterns and mediator release from the cells is needed to understand macrophage function and heterogeneity. Key: (H) Human primary study, (M) Mouse primary study, (R) Rat primary study.

Anatomical region	Auditory nerve (AN)	Rosenthal's canal (RC) - Spiral ganglion neurons (SGNs)	Osseous spiral lamina (OSL)	Organ of Corti (OC)	Basilar membrane (BM)	Scala tympani (ST)	Spiral ligament (SL)	Stria vascularis (SV)
Under homeostatic conditions	There are elongated Iba1+ cells with filamentous processes with terminal podosomes that attach to adjacent neurons in the AN (Liu *et al.*, 2019) ***(H).***	Macrophages in RC have a small cell body with ramified long processes (Fuentes-Santamaria *et al.*, 2017) ***(R).*** There are multiple macrophages among the SGNs. There are Iba1+ cells with pseudopodia and cell projections are observed between the satellite glial cell layer and nerve soma (Liu *et al.*, 2018) ***(H).*** The density of macrophages is positively correlated with the density of spiral ganglion cells (Okayasu *et al.*, 2020) ***(H).*** Iba1+ cells with a ramified, branching morphology with spider-like extensions (Noonan *et al.*, 2020) ***(H)***.	CD163+ and Iba1+ cells are present along OSL (O'Malley, Nadol and Mckenna, 2016) ***(H).*** Iba1+ and CD68+ present in the OSL (Noonan *et al.*, 2020) ***(H).***	Distinct macrophage populations are present during the postnatal developmental period ***(Dong et al., 2018) (M).*** Macrophages are rarely observed in the mouse OC under homeostatic conditions (Hirose *et al.*, 2005; Yang *et al.*, 2015) ***(M).*** The human OC contains some active, dendritic, lysosome containing macrophages (O'Malley, Nadol and Mckenna, 2016; Liu *et al.*, 2018, 2019) ***(H).***	Distinct macrophage populations are present during the postnatal developmental period ***(Dong et al., 2018) (M).*** *There* are apical vs basal differences in macrophage morphology (Frye *et al.*, 2017) ***(M).*** Macrophages are located on the perilymphatic side of the BM. Some processes are seen to penetrate through BM (Okayasu *et al.*, 2020) ***(H).*** Macrophages are located below inner hair cells and at the base of the Hensen cells (O'Malley, Nadol and Mckenna, 2016) ***(H).*** *F*ew cells were present in the basilar membrane of the OC (Noonan *et al.*, 2020) ***(H)***.	Macrophages (some PVM/Ms) are observed along the ST wall, usually amoeboid morphology. The cells are CD163+, Iba1+ and CD68***+*** (O'Malley, Nadol and Mckenna, 2016) ***(H).***	There are few macrophages observed in the SL. Those observed are often around blood vessels (Liu *et al.*, 2018) ***(H).*** CD163+ and Iba1+ cells are observed among various types of fibrocytes (O'Malley, Nadol and Mckenna, 2016) ***(H).***	Perivascular macrophages (PVM/Ms) have branched morphology (Zhang *et al.*, 2012, 2013) ***(M).*** PVM/Ms are tightly associated with endothelial cells (Neng *et al.*, 2015) ***(M).*** PVM/Ms are located near and around blood vessels and intermediate cells (Liu *et al.*, 2018) ***(H).*** Iba1+ and CD68+ cells present in SV (Noonan *et al.*, 2020) ***(H).***

2.4.3.1 Organ of Corti macrophages

Whether macrophages reside in the organ of Corti under homeostatic conditions is debated. The organ of Corti in the mouse is reported not to contain macrophages (Hirose *et al.*, 2005; Yang *et al.*, 2015). In contrast, analysis of human temporal bones identified Iba1+ macrophages with differing morphologies in the organ of Corti (O'Malley, Nadol and Mckenna, 2016; Liu *et al.*, 2018, 2019). This uncertainty is likely due to variability in species, age and differences in microscopy methods. Super-resolution structured illumination microscopy (SR-SIM) and transmission electron microscopy were used in the analysis of human tissue (Liu *et al.*, 2018, 2019). These techniques have not been reported in the mouse.

2.4.3.2 Basilar membrane macrophages

Macrophages in the organ of Corti are positioned along the perilymphatic side of the basilar membrane (BM) at the basal region of the tunnel of Corti or supporting cells and could be described as BM macrophages (O'Malley, Nadol and Mckenna, 2016; Liu *et al.*, 2018, 2019). BM macrophages reside on the perilymphatic side of the sensory epithelium, in the scala tympani. These macrophages have been shown to have processes which penetrate through the BM (Okayasu *et al.*, 2020). Morphological differences of mouse BM macrophages exist between the apical and basal regions of the cochlea. Macrophages in the apical region of the sensory epithelium have ramified morphology with long processes and a small cell body, which transition through to a typical amoeboid morphology in the basal regions (Figure 2.5) (Yang *et al.*, 2015; Frye *et al.*, 2017; Frye, Zhang and Hu, 2018).

2.4.3.3 Lateral wall macrophages

Perivascular resident macrophage-like melanocytes (PVM/Ms) are located near and around blood vessels in the healthy adult stria. They have a branched morphology and ensheath vessel walls with end-feet like processes (Zhang *et al.*, 2012, 2013). PVM/Ms are often found associated with endothelial cells (Neng *et al.*, 2015). Few cochlear macrophages are located in the spiral ligament, often around blood vessels (Liu *et al.*, 2018) and also found amongst various types of fibrocytes (O'Malley, Nadol and Mckenna, 2016).

2.4.3.4 Spiral ganglion neuron macrophages

A morphologically distinct population of cochlear macrophages is distributed amongst the SGN cell bodies. These macrophages have a morphology characteristic of homeostatic microglia (Fuentes-Santamaria *et al.*, 2017; Liu *et al.*, 2018). The rat and human modiolar auditory nerve is

populated with elongated macrophages with filamentous processes that contact adjacent neurons (Fuentes-Santamaria *et al.*, 2017; Liu *et al.*, 2019).

2.4.4 Immune/inflammatory response to insults to the cochlea

Insults to the cochlea lead to cells within the cochlea becoming damaged and mounting an inflammatory response. Model systems, predominantly with mice, have been used to investigate the distribution, morphology and potential function of macrophages and the role of inflammation after cochlear insults including noise exposure, aging and cochlear implantation. Reviewing the published evidence revealed a role for cochlear macrophages in response to tissue damage caused by noise exposure. Heterogeneity in experimental design between studies was demonstrated, even when considering the consequences of a single type of insult for example noise. This is important to note when drawing conclusions about the role of macrophages following an insult.

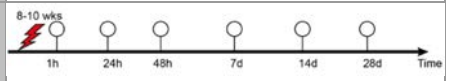
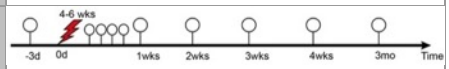
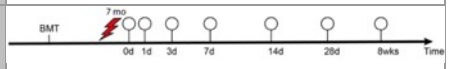
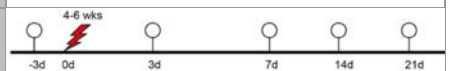
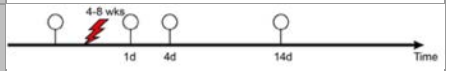
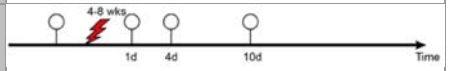
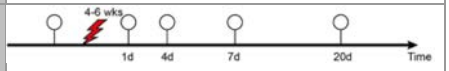
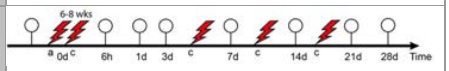
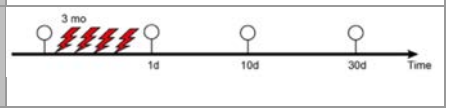
2.4.5 Variation in noise exposure study design

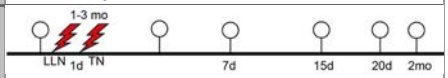
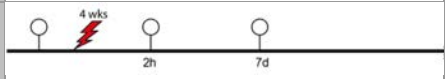
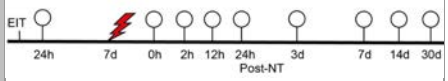
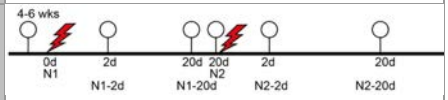
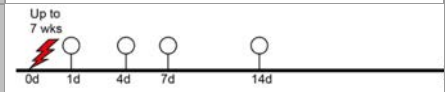
Differences in experimental design include intensity and duration of noise exposure, age and strain of mouse, whether the noise exposure caused temporary or reversible threshold shifts and frequency and type of measurements taken. There are notable differences in measurement approach. Table 2.2 highlights the noise exposure studies and experimental design variability.

Many of the studies have used relatively young mice (4 - 6 weeks). The immune/macrophage response is likely to be different if older mice were used as regional, age-dependent differences have been identified in microglial phenotypes between young (4 months) and aged (21 months) C57/BL6 mice (Hart *et al.*, 2012). This may have translational relevance. The level of noise exposure, both intensity and duration, is variable. A significant exposure such as 118 dB, 4 hours for 4 consecutive days designed to induce long-lasting auditory damage and a permanent threshold shift (Fuentes-Santamaria *et al.*, 2017) is likely to elicit a different inflammatory response compared to lower level noise exposure that only produces temporary threshold shifts (Frye, Zhang and Hu, 2018). The higher intensity exposure may provide insight into the damage caused by a single event e.g., a blast, whereas the lower level noise is more representative of daily noise exposure associated with certain industries. While both studies give insight, it is important to highlight the differences in experimental design as this makes integration, or extrapolation, of findings difficult.

Table 2.2 **Summary of study designs investigating the effect of a single type of insult on macrophages in the auditory system**

This table summarises key methodological variables in published studies, of noise damage to the auditory system, in which the effect on macrophage populations was assessed. Variables are: age, strain and gender of animal and also the intensity and duration of noise exposure. The timeline indicates the age at which the animals were exposed to noise and the number of measurements taken across the timeline of the experiment.

	Citation	Animal	Strain	Gender	Age	Intensity	Readout/measurement
1	(Hirose *et al.*, 2005)	Mouse	CBA/CaJ	M	8 – 10 weeks	0 dB, 106 dB, 112 dB, 120 dB	
2	(Sautter *et al.*, 2006)	Mouse	Tg CCL2$^{-/-}$ CX3CR1$^{+/GFP}$ & CCR2$^{-/-}$ CX3CR1$^{+/GFP}$	M and F	8 – 10 weeks	0 dB, 106 dB, 112 dB	
3	(Fujioka *et al.*, 2006)	Rat	Sprague Dawley	M	4 – 6 weeks	124 dB, 4 kHz, 2 hr	
4	(Tan, Lee and Ruan, 2008)	Mouse	C57BL/6J (M) GFP Tg (F)	M and F	7 months	120 dB, 2 hr	
5	(Sato *et al.*, 2008)	Mouse	C57BL/6 & CX3CR1 mutant		10 weeks	112 dB 8 – 16 kHz, 2 hr	
6	(Wakabayashi *et al.*, 2010)	Mouse	C57BL/6J	M	4 – 6 weeks	124 dB, 4 kHz, 2 hr	
7	(Zhang *et al.*, 2013)	Mouse	C57BL/6J	M	6 – 8 weeks	120 dB, 3 hr on day 1 and day 2	
8	(Cai *et al.*, 2014)	Mouse	C57BL/6 J, CBA/CaJ & B6.B10ScN-Tlr4lps-del/JthJ	M	4 – 8 weeks	120 dB, 1 – 7 kHz, 1 hr	
9	(Yang *et al.*, 2015)	Mouse	C57BL/6J	M and F	4 – 8 weeks	120 dB, 1 - 7 kHz, 1 hr	
10	(Vethanayagam *et al.*, 2016)	Mouse	C57BL/6 J & B6.B10ScN-Tlr4lps-del/JthJ	M and F	4 – 6 weeks	120 dB, 1 - 7 kHz, 1 hr	
11	(Tan, Thorne and Vlajkovic, 2016)	Mouse	C57BL/6	M	6 – 8 weeks	55 – 65 dB, Acute: 100 dB 24 hr Chronic: 90 dB, 2 hr per day	
12	(Mizushima *et al.*, 2017)	Mouse	C57BL/6	F	6 – 10 weeks	121 dB, 4 kHz, 4 hr	
13	(Fuentes-Santamaria *et al.*, 2017)	Rat	Wistar	F	3 months	118 dB, 4 hr, 4 consecutive days	

	Citation	Animal	Strain	Gender	Age	Intensity	Readout/measurement
14	(Frye, Zhang and Hu, 2018)	Mouse	CBA/CaJ	M and F	1 – 3 months	LLN: 95 dB, 8 – 16 kHz, 8 hr on 16 hr off. TN: 120 dB, 1 - 7 kHz, 1 hr	
15	(Kaur, Ohlemiller and Warchol, 2018)	Mouse	CX3CR1$^{1/1}$ CX3CR1$^{GFP/1}$, CX3CR1$^{GFP/GFP}$	M and F	6 -8 weeks	120 dB, 2 hr	
16	(Okano and Kishimoto, 2019)	Mouse	Csf $^{op/op}$ w/ B6C3F1	M and F	4 weeks	120 dB, 8 kHz, 1 hr	
17	(Kaur *et al.*, 2019)	Mouse	C57BL/6 (B6) & CX3CR1$^{+/+}$ CX3CR1$^{GFP/+}$ CX3CR1$^{GFP/GFP}$	M and F	6 weeks	90 dB, 8 – 16 kHz, 2 hr	
18	(Eshraghi *et al.*, 2019)	Guinea pig		M and F		120 dB 6-10 kHz centred at 8 kHz, 2 hr	
19	(Zhang *et al.*, 2020)	Mouse	C57BL/6J & B6.129P2(Cg) Cx3Cr1^{tm1Litt}/J (aka Cx3cr1$^{GFP/GFP}$)	M and F	4 – 6 weeks	120 dB SPL 1-7 kHz, 1 hr (N2 - Repeated 20d after first NE)	
20	(Rai *et al.*, 2020)	Mouse	C57BL/6J	M and F	Before 7 weeks	120 dB SPL, 8-16 kHz, 2 hr	

2.4.6 Changes in the distribution and morphology of macrophages after insults to the cochlea

Insults to the cochlea lead to the activation of resident cochlear macrophages with a switch to a pro-inflammatory phenotype across regions. This change in expression pattern and morphology is associated with changes in the distribution and number of cochlear macrophages, as summarised in Table 2.3. The resulting inflammatory response is associated with damage and degeneration within cochlear structures including sensory cells (Bohne and Harding, 2000), SGNs (Wang, Hirose and Liberman, 2002) and cochlear atrophy.

Following cochlear insults, PVM/Ms in the stria become activated and change their morphology, inducing strial dysfunction and atrophy (Zhang *et al.*, 2013; Neng *et al.*, 2015; Noble *et al.*, 2019). Morphological changes associated with aging were observed in PVM/Ms in mouse (Neng *et al.*, 2015) and human (Noble *et al.*, 2019) tissue. Exposure of mice to sound at 120 dB SPL (an exposure similar to sound produced by a jet aircraft) for 3 hours each day for 2 days resulted in a physical detachment of PVM/Ms from capillary walls, downregulated PEDF production,

breakdown of the intrastrial barrier, a lower endocochlear potential and reduced hearing function (Zhang *et al.*, 2013). Persistent activation of PVM/Ms in response to an inflammatory event is likely detrimental to the cochlea.

Table 2.3 **Changes in the distribution and morphology of macrophages in the cochlea and auditory pathway following cochlear insults including noise exposure, aging and cochlear implantation**

This table summarises the changes in distribution, morphology and abundance of macrophages following cochlear insults. Inflammation following cochlear insults is often associated with an increase in number and activation status of cochlear macrophages. These changes reflect the role of these cells in the tissue injury and repair response. Evidence from other pathologies suggests that dysregulated macrophage responses are associated with a change or loss of function. Key: † No reporting on this to be found, (H) Human primary study, (M) Mouse primary study, (R) Rat primary study.

Anatomical region	Modiolar auditory nerve (AN)	Rosenthal's canal (RC) - Spiral ganglion neurons (SGNs)	Osseous spiral lamina (OSL)	Organ of Corti (OC)	Basilar membrane (BM)	Scala tympani (ST)	Spiral ligament (SL)	Stria vascularis (SV)
After noise exposure	There is an increase in Iba1 staining. The cells resemble activated microglia (Fuentes-Santamaria *et al.*, 2017) ***(R).***	Macrophages become activated and display an amoeboid morphology (Fuentes-Santamaria *et al.*, 2017) ***(R).*** There is an increase in the number of macrophages (Kaur, Ohlemiller and Warchol, 2018) ***(M).***	There is an increase in the number of macrophages (Zhang *et al.*, 2020) ***(M).***	†	This is a site of migrating macrophages (Hirose *et al.*, 2005) ***(M).*** After acute noise exposure, there is an infiltration of monocytes (Yang *et al.*, 2015) ***(M).*** Low level noise causes macrophage activation to amoeboid morphology in the middle and basal portions of the BM and an increase in the macrophage population in regions adjacent to sensory cells (Frye, Zhang and Hu, 2018) ***(M).*** A marked increase in the number of macrophages with monocyte-like morphology 2d post-exposure. 20d post-exposure, the macrophages acquire amoeboid activated morphology. Following the second noise	There is an increase in the number of macrophages on the luminal surface of ST (Zhang *et al.*, 2020) ***(M).***	There is an increase in the number of macrophages and the number of activated macrophages (Fuentes-Santamaria *et al.*, 2017) ***(R).*** There is significant monocyte infiltration (Hirose *et al.*, 2005; Sautter *et al.*, 2006; Tornabene *et al.*, 2006) ***(M).***	Some PVM/Ms observed are smaller with shorter processes. Some have a flat, amoeboid morphology. There is a physical detachment of PVM/Ms from capillary walls (Zhang *et al.*, 2013) ***(M).***

Anatomical region	Modiolar auditory nerve (AN)	Rosenthal's canal (RC) - Spiral ganglion neurons (SGNs)	Osseous spiral lamina (OSL)	Organ of Corti (OC)	Basilar membrane (BM)	Scala tympani (ST)	Spiral ligament (SL)	Stria vascularis (SV)
					exposure, the number of macrophages and the number of activated macrophages is significantly higher compared to the first exposure (Zhang *et al.*, 2020) ***(M).***			
With aging	The population of macrophages increases with age and the macrophages have variable morphologies. There is an increase in the number of activated macrophages (Noble *et al.*, 2019) ***(H).***	The macrophages have a worm-like cytoarchitecture located near neuronal soma. There are increases in interactions between macrophages and glial cell-associated myelinated axonal projections of type I spiral ganglion cell bodies (Noble *et al.*, 2019) ***(H).***	Macrophages have a more bipolar architecture and flat encroaching filopodia-like structures suggesting interactions with peripheral neural projections (Noble *et al.*, 2019) ***(H).***	†	Mature tissue macrophages are involved in the pathology rather than the infiltrating macrophages. The number of apical macrophages increases whereas the number of basal macrophages decreases (Frye *et al.*, 2017; Hu, Zhang and Frye, 2018) ***(M).***	†	There is an increase in the number of macrophages in the middle and basal turns. There is also an increase in the number of activated macrophages with amoeboid morphology (Noble *et al.*, 2019)***(H).***	PVM/Ms are smaller with shorter processes (Neng *et al.*, 2015) ***(M).*** PVM/Ms are highly ramified in aged human temporal bones (Noble *et al.*, 2019) ***(H).***
	There is an increase in the infiltration of macrophages following implantation (Bas *et al.*, 2015) ***(M).***	Monocyte/macrophage/microglia invasion increases rapidly after implantation (Bas *et al.*, 2015) ***(M).*** The density of macrophages is	There is a higher density of macrophages in implanted compared to unimplanted ears. Macrophages in the implanted ears	There are increased IL-1β levels on day 14 which then remained stable until day 30 suggesting a pro-	There are a few macrophages observed beneath the OC in both implanted and implanted, with processes extending into the OC (Noonan *et al.*, 2020; Okayasu *et al.*, 2020) ***(H).***	Iba1+ macrophages within areas of fibrosis in the scala tympani (Noonan *et al.*, 2020) ***(H).***	There is no difference observed in the prevalence of macrophages between implanted and unimplanted	PVM/Ms are observed near blood vessels. There is an increase in the infiltration of monocytes /macrophages

Anatomical region	Modiolar auditory nerve (AN)	Rosenthal's canal (RC) - Spiral ganglion neurons (SGNs)	Osseous spiral lamina (OSL)	Organ of Corti (OC)	Basilar membrane (BM)	Scala tympani (ST)	Spiral ligament (SL)	Stria vascularis (SV)
After cochlear implantation		significantly greater in the basal RC in implanted compared to unimplanted. The macrophages have a more transitional, ramified morphology with long processes. The number of spiral ganglion cells is significantly less in implanted compared to unimplanted ears. The number of macrophages 'wrapping' spiral ganglion cells increases (Okayasu *et al.*, 2020) ***(H).***	have a more amoeboid morphology compared to unimplanted (Okayasu *et al.*, 2020) ***(H).***	inflammatory environment (Bas *et al.*, 2015) ***(M).*** There is no difference in the distribution of macrophages in the OC between implanted and unimplanted ears after 12 to 210 months after implantation (Okayasu *et al.*, 2020) ***(H).***			ears (Okayasu *et al.*, 2020) ***(H).***	over time. Maximum levels are observed on day 14 and day 30 post-implantation (Bas *et al.*, 2015) ***(M).*** There is no difference in the prevalence of macrophages between implanted and unimplanted ears (Okayasu *et al.*, 2020) ***(H).***

Following trauma to the cochlea, the number and activation status of macrophages is increased in the spiral ligament (Fuentes-Santamaria *et al.*, 2017; Noble *et al.*, 2019), with significant monocyte infiltration after a noise-induced injury (Hirose *et al.*, 2005; Sautter *et al.*, 2006; Tornabene *et al.*, 2006). The macrophages amongst the SGNs become activated; increasing in number and with an amoeboid appearance indicating a switch to a pro-inflammatory phenotype (Fuentes-Santamaria *et al.*, 2017; Kaur, Ohlemiller and Warchol, 2018). Similarly, macrophages in the modiolar auditory nerve increase in number and resemble an activated morphology following acoustic trauma (Fuentes-Santamaria *et al.*, 2017). They also display a shift to more variable morphologies with increased age (Noble *et al.*, 2019). Acute noise exposure results in the infiltration of monocytes to the basilar membrane (Yang *et al.*, 2015) and the activation of macrophage populations in regions adjacent to sensory cells (Frye, Zhang and Hu, 2018). Whereas, the chronic insult of aging causes the activation of mature tissue macrophages, with little monocyte infiltration (Frye *et al.*, 2017).

Damaged SGNs and surrounding resident cells produce pro-inflammatory cytokines that activate and recruit macrophages (Bas *et al.*, 2015; Fuentes-Santamaria *et al.*, 2017). Cochlear injury including selective hair cell ablation, aminoglycoside ototoxicity and noise trauma is associated with the recruitment of macrophages to the sensory epithelium and spiral ganglion of the cochlea. This is mediated through fractalkine signalling, promoting the survival of the SGNs and highlighting that macrophages can be neuroprotective in the injured cochlea (Kaur, Zamani, *et al.*, 2015; Kaur, Ohlemiller and Warchol, 2018; Kaur *et al.*, 2019). These findings identify a role for macrophages and fractalkine signalling in both cochlear degeneration and repair following an insult. Further investigation into the mechanisms that recruit the macrophages to the synaptic region is necessary, as well as establishing the origin and phenotype of the cells.

As discussed in 2.1 and in more detail in Figure 1.5, the neurobiological substrates of hearing loss are the key players and cochlear regions involved in hearing loss. Macrophages are distributed across the auditory system in close proximity to these regions under homeostatic conditions (Table 2.1). Following a cochlear insult, as discussed above, the distribution, morphology and abundance of macrophages within these regions change suggesting a role in the inflammatory events following an insult that contribute to progressive hearing loss.

Collating information from multiple studies, as displayed in Table 2.1 and Table 2.3, to begin to build a consensus understanding of macrophage phenotype and function across different regions of the cochlea after cochlear insults, will enable better understanding of how these cells respond to insults and subsequently contribute to an individual's cochlear health. An understanding of the precise molecular mediators that influence the macrophage phenotype after a cochlear insult is

lacking. Further investigation into other immune insults that could occur throughout a lifespan, such as peripheral and systemic infection and inflammation, which may influence the function and behaviour of cochlear macrophages is necessary.

2.4.7 Immune events following an acute insult

Acute insults to the cochlea, where the injury stimulus is short-lived, including noise exposure (Hirose *et al.*, 2005; Sato *et al.*, 2010; Frye, Zhang and Hu, 2018), ototoxicity (Sato *et al.*, 2010; Kaur, Ohlemiller and Warchol, 2018), selective hair cell ablation (Kaur, Zamani, *et al.*, 2015) and CI surgery (Seyyedi and Nadol, 2014; Bas *et al.*, 2015) lead to the rapid onset of sensory cell injury and tissue pathogenesis. This signals to resident cochlear macrophages, switching them to an activated, pro-inflammatory state. Pro-inflammatory macrophages release cytokines such as TNF-α (Keithley, Wang and Barkdull, 2008) and chemokines including CC chemokine ligand 2 (CCL2) (Sautter *et al.*, 2006; Bas *et al.*, 2015; Zhang *et al.*, 2020) which attract and recruit inflammatory, circulating monocytes into the cochlea (Hirose *et al.*, 2005; Tan, Lee and Ruan, 2008; Yang *et al.*, 2015; Vethanayagam *et al.*, 2016; Frye *et al.*, 2017; Okayasu *et al.*, 2020). These infiltrating monocytes differentiate into resident cochlear macrophages which carry out characteristic immunological functions including phagocytosis (Fredelius and Rask-Andersen, 1990), inflammatory mediator production and antigen presentation (Yang *et al.*, 2015).

The time course of monocyte infiltration following acute injury is variable, depending on the type and duration of injury. For example, following noise exposure, infiltration begins between 12 - 24 hours, peaks at 3 - 7 days then decreases gradually as the inflammation resolves (Hirose *et al.*, 2005; Tornabene *et al.*, 2006; Wakabayashi *et al.*, 2010). In contrast, a continuous presence of monocyte-like macrophages ($CD45^{high}$, $F4/80^{low}$, $Iba1^{low}$) in the cochlea at 20 days after noise exposure has been reported (Zhang *et al.*, 2020). Whereas, following cochlear implantation, monocyte infiltration to the wound-site increases rapidly at 1 day post-implantation with peak numbers detected at 7 days post-implantation (Bas *et al.*, 2015). The precise role and turnover rate of the newly infiltrated and now resident macrophages is not well understood.

Following an insult, the tissue reparative response is an acute wave of inflammatory activity that is closely followed by resolving, anti-inflammatory signalling. Anti-inflammatory macrophages (GFP^{high} and $Ly6C^{low}$) have been identified in the cochlea 20 days post-noise injury (Zhang *et al.*, 2020). Although cochlear implantation is a single event, the signalling and tissue response may vary. Surgical insertion of an electrode array inside the cochlea causes physical trauma. The inflammatory response to this should be as described above and should resolve as part of a normal wound healing response. However, in some individuals, there may be a chronic,

exaggerated inflammatory response promoting the excessive deposition of fibrotic tissue around the array, which in some cases leads to reduced CI performance (Nadol, Eddington and Burgess, 2008; Nadol *et al.*, 2014; Bas *et al.*, 2015; Hough, Sanderson, *et al.*, 2021).

2.4.8 Immune events associated with a chronic insult

Chronic damage/deterioration of the auditory system (for example, due to aging, chronic noise exposure or the accumulation of multiple insults) triggers a different immune response. The effects of cumulative low-grade cochlear insults, such as aging, have been investigated in a small number of studies (Frye *et al.*, 2017; Zhang *et al.*, 2017; Su *et al.*, 2020). Cumulative, progressive, sensory cell degeneration and death leads to the activation of resident mature macrophages, with little infiltration of circulating monocytes (Frye *et al.*, 2017). This parallels the innate immune response seen in chronic compared to acute CNS disease. Activation of resident macrophages is associated with a morphological change to cells with a larger cell body and amoeboid shape. The activated, mature macrophages adopt either a pro- or anti-inflammatory profile culminating in a return to homeostasis. Pro-inflammatory macrophages produce and release pro-inflammatory mediators including IL-1β, TNF-α, IL-6 which signal to surrounding cells resulting in further inflammation and cellular damage (Fujioka *et al.*, 2006; Tan, Thorne and Vlajkovic, 2016; W. Wang *et al.*, 2019). Anti-inflammatory macrophages produce tissue reparative mediators such as IL-10 and arginase.

2.5 Discussion

The current evidence for the role of cochlear macrophages under homeostatic conditions, and how this changes following acute and chronic insults, has been described and summarised in this chapter (Table 2.1 and Table 2.3). Several studies using both human temporal bones and animal models, have investigated the distribution and morphology of macrophages across different regions of the cochlea. Increases in abundance and activation status following variable insults to the cochlea has been measured. These studies indicate a role for macrophages in mediating an immune response to multiple types of insults. What is not clear is the mechanisms responsible for the tissue damage, loss of function and progressive hearing loss following the acute and chronic insults. Based on our understanding of macrophages in the cochlea and the mechanisms involved in other progressive CNS conditions, we are proposing that tissue-resident cochlear macrophages can become primed. Therefore, when exposed to additional insults, the macrophages display an exaggerated inflammatory response that leads to further inflammation and mediator release, tissue damage and subsequent loss of auditory function. Damage to cochlear structures results in progressive hearing loss which contributes to CI performance.

2.5.1 Priming of tissue-resident macrophages

Microglial cells can become activated and remain in a hyper-responsive, pro-inflammatory state making them more susceptible to a secondary inflammatory stimulus, which can trigger an exaggerated inflammatory response (Perry and Holmes, 2014). The nomenclature of the various activation states of microglia and macrophages is the subject of ongoing discussion in the literature (Perry and Teeling, 2013; Haley *et al.*, 2018; Neher and Cunningham, 2019). Primed is the term applied to microglia with an immunophenotype associated with memory of previous insults. There is overlap between *priming* and the term *trained* that is used to describe this state in macrophages in other organ systems. Primed microglia typically express higher baseline markers of inflammation, a lower threshold to switch to a pro-inflammatory state and an exaggerated inflammatory response to immune activation (with associated local tissue damage) (Lull and Block, 2010; Norden, Muccigrosso and Godbout, 2015). Primed microglia have been described in models of aging, neurodegenerative disease and traumatic brain injury (TBI) (Cunningham *et al.*, 2005; Kokiko-Cochran and Godbout, 2018; Neher and Cunningham, 2019; Lopez-Rodriguez *et al.*, 2021). Refer to 1.9 for detail of the key studies evidencing microglial priming. Priming of microglia/macrophages in the auditory system has not been described in the literature, despite the similarities in neurodegeneration within the auditory system.

2.5.2 Our hypothesis

Figure 2.7 integrates the findings from key studies and the sequence of immune events that take place in the cochlea during acute and chronic pathogenesis. The presence of primed macrophages has been added based on the hypothesis we are proposing. The timeline emphasises the differences in time-course between acute (across days) and chronic (across years/lifetime) pathogenesis. This allows for current and future studies to be put into context of where an experimental design sits in relation to the relative contribution of acute and chronic insults, the interaction between both, and the consequences of these insults on immune cell population and tissue damage.

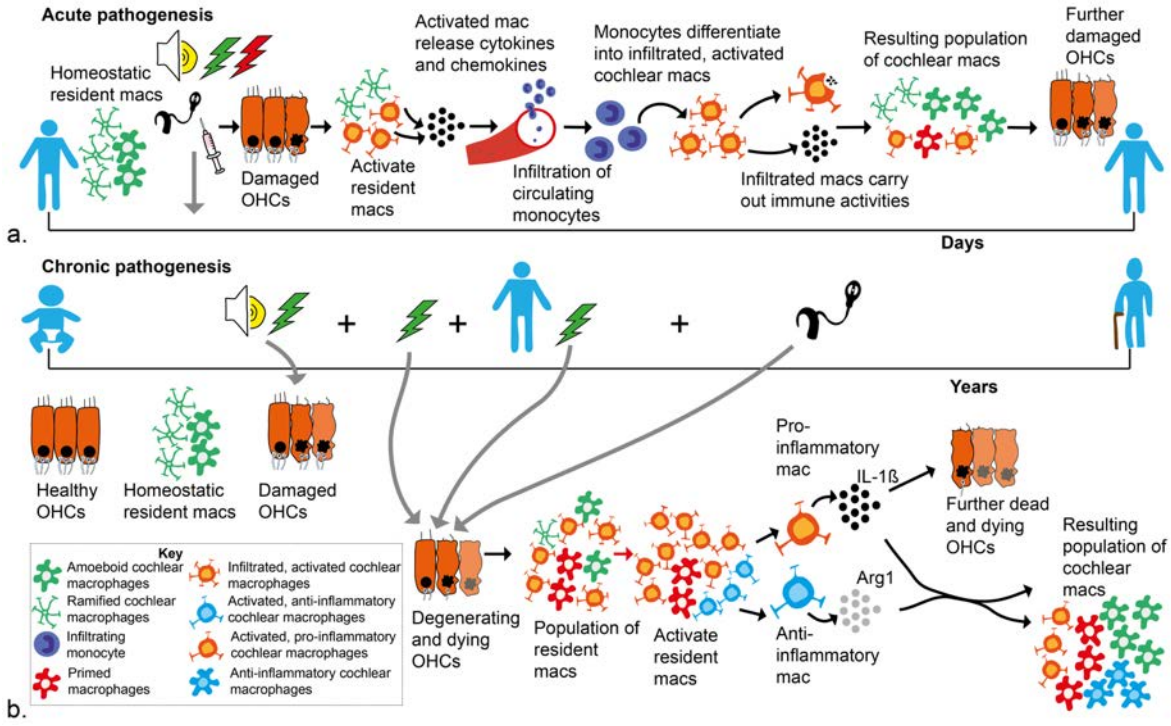


Figure 2.7 **A role for macrophage-mediated responses to acute and chronic insults in the cochlea**

A summary of the sequence of immune events that occur in the cochlea following (**a**) an acute insult and (**b**) how this may be altered in the context of chronic insults if activated cochlear macrophages can become primed.

a. An acute insult, with sensory cell and tissue injury, signals to resident cochlear macrophages resulting in their activation. Activated, pro-inflammatory cochlear macrophages release cytokines and chemokines which recruit circulating bone marrow-derived monocytes. On entry to the cochlea, the monocytes differentiate into activated macrophages. Further tissue damage may occur due to local release of mediators from these macrophages resulting in cellular dysfunction and hearing loss. Typically, a wave of inflammation is followed by induction of an anti-inflammatory

response that results in resolution of the inflammatory response. However, some macrophages may adopt an altered or primed phenotype in response to the initial activating insult.

b. Chronic, cumulative, progressive insults cause gradual sensory cell denervation and degeneration. This signals to activate resident cochlear macrophages. The activated macrophages adopt a pro-inflammatory profile and secrete pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. If cochlear macrophages can become primed by cochlear insults, activated macrophages which have responded to previous insults may now have a lower threshold to mount a pro-inflammatory response to subsequent insults or changes in the local micro-environment, i.e., are primed. We propose that some cochlear macrophages may remain in a pro-inflammatory, hyper-responsive state (primed) and respond to further insults with an exaggerated immune response. Tissue damage in the auditory system is likely to manifest as a progressive loss of hearing function, with the precise function that is impaired reflecting the site within the system that has been injured.

The timeline depicted in Figure 2.7b demonstrates that a single, acute insult (such as noise exposure) causes damage to OHCs but running in parallel, the accumulation of multiple insults over time lead to the development of chronic pathology. This involves the slow, progressive degeneration of the sensory cells and the activation of the long-lived resident cochlear macrophages. Based on the understanding of glial biology from other systems; we are hypothesising that some of the macrophages involved in this response are likely to have already been exposed to an immunological insult, or been trained, and are in a primed, or more responsive state. Upon activation through further cochlear insults or through chronic inflammation associated with aging and age-associated morbidities (risk factors for hearing loss), the number of activated macrophages within some regions of the cochlea increases making these regions more of an inflamed environment. Inflammation contributes to neuronal, axonal and tissue injury and degeneration. This damage will manifest as progressive hearing loss (Figure 2.8). For people with a CI, the exacerbated cochlear damage may manifest as variable or even poor performance outcomes.

2.5.3 Cumulative effect of cochlear insults

The cumulative effect of cochlear insults is not well understood. Investigation of the interactions between noise-induced hearing loss (NIHL) and age-related hearing loss (ARHL) found that early life noise exposure made the cochlea more vulnerable to changes commonly associated with aging (Kujawa and Liberman, 2006). There was widespread loss of spiral ganglion cells (not

associated with hair cell death) in older mice that had been noise-exposed that was not evident in unexposed age-matched controls (Kujawa and Liberman, 2006). Evidence from a repeated acoustic overstimulation model, highlighted the effect of prior macrophage activation on the cochlear response to subsequent acoustic trauma (Zhang *et al.*, 2020). In mice exposed to 120 dB SPL noise for 1 hour then re-exposed 20 days later, a greater increase in macrophage populations in the osseous spiral lamina, luminal surface of the scala tympani and underneath the basilar membrane was measured compared to cell numbers in those regions following the first noise exposure. Additionally, after the second insult (noise exposure), there was a significant increase in the number of macrophages with a mature morphology; suggestive of a transition to a more pro-inflammatory phenotype following an initial exposure. This is the first study to closely examine the macrophage distribution, morphology and phenotype following repeated insults and provides evidence for an exaggerated inflammatory response following a prior immune activation in the cochlea (Zhang *et al.*, 2020). Mechanistic links which may exist between NIHL and ARHL are not yet understood but macrophages and their ability to retain immunological memory are a putative link. This is supported by the evidence from the studies that identify the distribution and morphology of macrophages (Table 2.1 and Table 2.3) and the(ir) immune response to acute and chronic tissue changes seen in NIHL, ARHL and following cochlear implantation (Figure 2.7).

2.5.4 Do macrophages influence the trajectory of hearing loss?

2.5.4.1 Inflammaging and progressive hearing loss

Chronic inflammation or 'inflammaging' describes low-grade inflammation that can occur in aging tissues and worsens with age (Franceschi *et al.*, 2006, 2017; Gruver, Hudson and Sempowski, 2007; Watson *et al.*, 2017). It is related to age-related changes in immune function. The decreased ability to control the production of pro-inflammatory proteins that occur with immunosenescence results in an increased inflammatory state in the majority of tissues. Data from cross-sectional aging cohort studies identified that higher levels of circulating markers of inflammation are associated with a more marked ARHL (Bainbridge, Cheng and Cowie, 2010; Verschuur *et al.*, 2012; Simpson, Matthews and Dubno, 2013; Nash *et al.*, 2014; Verschuur, Agyemang-Prempeh and Newman, 2014). Similarly, data from a longitudinal aging study identified that WBC count was positively correlated with hearing impairment in older adults (Lassale *et al.*, 2020). In a small longitudinal population study from our own group (unpublished 57 adults aged 65-75 at time of recruitment) the greater the number of times across a 12-month period an excreted metabolite of activated macrophages (neopterin) was elevated above the basal rate for adults, the greater the likelihood of more marked low frequency hearing loss 2-3 years later. In common with other organs, the immune system within the auditory system is exposed to multiple insults, such as the

effects of co-morbidities across the lifespan. The combination of type, intensity and temporal sequence of insults, or immunobiography, is unique for each individual (Franceschi *et al.*, 2017; Haley *et al.*, 2018; Netea *et al.*, 2020).

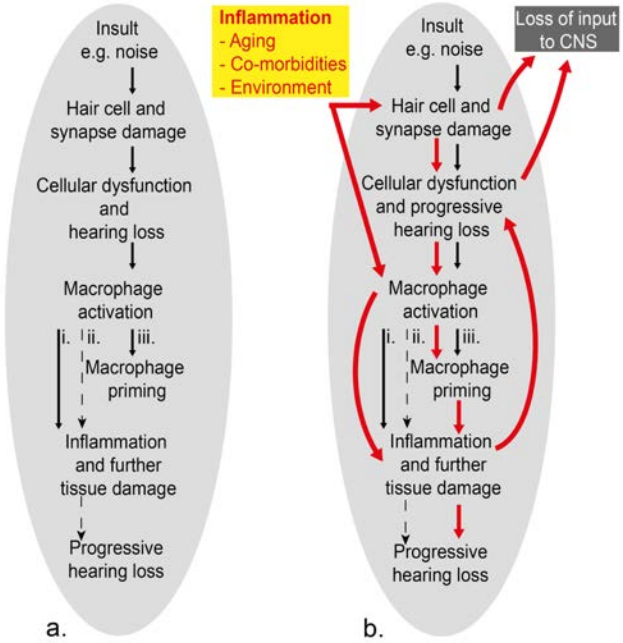


Figure 2.8 **Schematic of the hypothesis proposing the role for tissue-resident cochlear macrophages in contributing to the pathology of hearing loss**

a. An insult to the auditory system such as noise exposure, causes hair cell and synapse damage in the cochlea resulting in cellular dysfunction and hearing loss. Damaged sensory cells release mediators which recruit and activate resident cochlear macrophages (i). Activated macrophages will adopt a pro-inflammatory or anti-inflammatory phenotype. Pro-inflammatory macrophages produce pro-inflammatory cytokines and chemokines, resulting in inflammation and cellular damage or apoptosis. (ii). Some macrophages may already be in a pro-inflammatory state from a previous insult (primed), therefore upon activation display an exaggerated inflammatory response leading to further inflammation and damage to tissues in the local area. This will contribute to progressive hearing loss. (iii). Some macrophages may switch to a primed phenotype as a result of this insult.
b. Additional inflammation due to aging, co-morbidities or environmental factors results in further hair cell and synapse damage (and subsequent progressive hearing loss) and further macrophage activation. (iii). Macrophages in an active, pro-inflammatory state as a result of the previous noise exposure may be primed and therefore display an exaggerated inflammatory response to the additional inflammation, causing further tissue damage and hearing loss. Sensory cell and tissue

damage and the resulting loss of function of essential cells in the cochlea, causes loss of input to the central auditory pathways. Decreased activation of the ascending and descending central auditory pathway leads to dysfunctional auditory processing.

2.5.4.2 Do macrophages contribute to the trajectory of hearing loss and CI performance through priming?

It is evident from the literature that macrophage phenotype including morphology, expression of markers and the signals they produce and release, the way in which macrophages behave and how they respond to inflammatory insults, play a role in both normal and pathological auditory function across a lifetime. Cochlear insults such as noise exposure, the chronic deterioration of the auditory system that occurs with aging and conditions causing systemic inflammation are associated with a greater degree of hearing loss and the evidence is that the accumulation of insults may exacerbate this hearing loss (Kujawa and Liberman, 2006; Zhang *et al.*, 2020). Despite this, the mechanisms responsible for the pathology causing the hearing loss are not well understood although inflammation and cochlear macrophages are involved.

Based on the understanding from the fundamental studies discussed in this chapter and our wider knowledge of how macrophages respond to inflammatory insults in chronic conditions such as traumatic brain injury (TBI), Alzheimer's disease (AD) and age-associated changes, we hypothesise that innate immune memory and the priming of cochlear macrophages and microglia in the auditory pathway influence the trajectory of hearing loss. This would affect an individual's hearing ability through life and could affect how well the individual does with a cochlear implant.

Priming was first observed in peritoneal macrophages exposed to LPS. Isolated macrophages produced increased IL-1 and inducible nitric oxide synthase (iNOS) (relative to non-primed macrophages) if the cells were pre-exposed to or 'primed by' interferon gamma (Pace *et al.*, 1983). A study by Cunningham et al. demonstrated this priming effect in microglial cells when an enhanced immune response in the ME7 prion mouse model was measured after administration of LPS. Both direct challenge of the CNS and a systemic challenge with LPS, resulted in increased microglial activation and a greater inflammatory response in the prion mice compared to wild-type animals (Cunningham *et al.*, 2005). Similar observations have been described in models of aging (Godbout *et al.*, 2005), Wallerian (axonal) degeneration (Palin *et al.*, 2008) and Alzheimer's disease (Holtman *et al.*, 2015; Lopez-Rodriguez *et al.*, 2021).

Similarities can be drawn between how macrophages/ the immune system respond to insults across different organ systems. Traumatic brain injury (TBI) is defined as the functional disruption of the brain from an impact or penetrating injury (Centers for Disease Control and Prevention

(CDC), 2013). Typical pathological features of TBI include BBB disruption, neuronal and axonal injury, oedema, focal contusion and a widespread inflammatory response including microglial and astrocyte activation, infiltration of peripheral cells and increased production and release of inflammatory molecules (Puntambekar *et al.*, 2018). Many of these pathological features are similar to that following an insult to the cochlea or elsewhere in the auditory pathway. Similar to our hypothesis in the auditory system, the inflammatory response to TBI is influenced by previous and subsequent inflammatory challenges (Kokiko-Cochran and Godbout, 2018), potentially through the mechanism of microglial priming. To date, there are no effective interventions to reliably improve outcomes after TBI. A variety of TBI models are being used to investigate the mechanisms and pathology, however due to the different types and the complexity of TBI, and also the array of primary and secondary damage which occurs in human TBI, no single model fully recapitulates human TBI. This makes it difficult to understand the mechanisms. In all models however, there is a consistent microglia/macrophage inflammatory response highlighting these cells as critical mediators in the outcome.

In essence, noise injury is a traumatic injury to the cochlea and auditory system. Using a frequency-specific noise to create an injury in the cochlea, it is possible to reliably map the injury site, due to tonotopy of the cochlea. Tonotopicity describes the characteristic region within the cochlea that responds according to the frequency of the sound used. While with noise injury there is a small frequency shift, this shift is reproducible (Quraishe, Newman and Anderson, 2019). The resulting tissue damage and the local cellular response from the insult, in and around the area of damage, can be investigated. Functional readouts following the injury can be obtained to determine the severity of damage to the auditory system and any subsequent additional (or progressive) hearing loss. There is also the option to study the effect of a loss and re-establishment of function in the auditory system through the use of hearing aids or cochlear implants. Utilising the auditory system to investigate macrophage/microglial priming may be beneficial to inform how these cells behave in other pathologies.

The evidence is that inflammatory insults induce innate immune memory (trained immunity) and that exposure to insults over time form an individuals' immunobiography, influencing how they respond to immune insults across their lifespan. In terms of the auditory system, the integration of immunobiography, trained immunity and inflammaging explains why individuals exhibit heterogeneity in their hearing loss trajectory as they go through life, including both their natural hearing alone or with the addition of a hearing aid or cochlear implant. Macrophage priming/training may occur if the cell has been previously activated and returned to a quiescent state but retained a 'memory' of the event so that it produces exaggerated acute responses to subsequent challenges. On the other hand, persistent cochlear inflammation may result in a

chronic state of activation for the macrophages resulting in a persisting phenotype that is primed. A better understanding of the mechanisms behind how trained immunity may contribute to chronic inflammation with aging and what factors may exacerbate cochlear inflammaging may enable lifestyle or pharmaceutical interventions to slow down the decline in hearing function with age. Protecting key auditory structures from tissue damage will also be beneficial for people with a CI to improve performance.

Further studies are needed to investigate the role of macrophages following different cochlear insults and to investigate the effect of the accumulation of insults on the macrophage response, to look for evidence of macrophage priming. Animal models for acute and chronic inflammation are valuable for investigating the macrophage response and this approach allows for the investigation of the effect of multiple insults.

2.6 Conclusion

This chapter and the published review article (Hough, Verschuur, *et al.*, 2021) has synthesised the current knowledge of the distribution, morphology and function of macrophages under both homeostatic and pathological conditions in the cochlea. Following an acute insult, which can lead to sensory cell and synaptic damage, cochlear macrophages become activated and initiate a typical immune response involving both pro-inflammatory and anti-inflammatory signalling. The macrophage response, and molecular mediators involved in their function, following progressive accumulative insults over time need further investigation. We hypothesise, based on the evidence from other areas of the body, that macrophages contribute to the progressive loss of hearing experienced by many people worldwide and to the variability in hearing function that exists between individuals both with natural and aided hearing. Out of necessity, and reflecting the field, much of the evidence is from studies in mice though we have included human data where available. Further studies are to be carried out in animal models of inflammation to investigate macrophage priming in the auditory system. A better understanding of macrophage biology is needed as this may identify ways to limit the damage to essential structures within the cochlea that contribute to a loss of auditory function.

Chapter 3 The middle ear histopathology of the Junbo mouse, a model for the study of the macrophage in the consequences of otitis media

3.1 Introduction

3.1.1 Otitis media

Otitis media (OM) denotes all types of middle ear inflammation. Broadly, the different types of otitis media include acute otitis media (AOM), chronic otitis media (COM) and otitis media with effusion (OME). There is heterogeneity in the clinical presentation of OM. Acute otitis media (AOM) is short-term inflammation of the middle ear caused by either bacterial or viral infections, or both. Rapid onset of acute inflammation is associated with earache, fever and middle ear effusion (Lieberthal *et al.*, 2013). The principal bacteria involved is non-typeable *Haemophilus influenzae* (NTHi). AOM is a global health burden with an estimated 709 million cases each year with 51% of these occurring in children under five years (Monasta *et al.*, 2012). Studies in low and middle-income countries (LMIC) found that by the age of three, 80% of children will have experienced at least one episode of AOM (Teele, David W, Klein Jerome O, Rosner, 1989; Simões *et al.*, 2010) and 40% will have six or more occurrences by the age of seven (Casselbrant *et al.*, 2003). Otitis media with effusion (OME), also known as 'glue ear', is the accumulation of fluid within the middle ear space without signs of acute inflammation (Rosenfeld *et al.*, 2016). OME is asymptomatic and therefore can go undetected, making it difficult to establish accurate data on the incidence and prevalence. Epidemiology data from large cohort studies (Marchant *et al.*, 1984; Williamson *et al.*, 1994; Paradise *et al.*, 1997) show a point prevalence of OME on screening tests is 20% (Casselbrant *et al.*, 2003) and, by the age of three, almost all children have experienced at least one episode of OME (Schilder *et al.*, 2016).

Chronic otitis media (COM) can be separated into two distinct types: squamous and mucosal. Both types have two clinical subsets - active and inactive. Squamous COM can be congenital or acquired, however most cases are acquired. In congenital disease, there is no perforation or retraction of the tympanic membrane and it is seen in patients with no history of ear surgery. Acquired inactive squamous COM is characterised by a retraction pocket in the tympanic membrane which is a pocket of space whereby the tympanic membrane gets pulled towards the middle of the middle ear, thought to result from chronic negative pressure from Eustachian tube

dysfunction (Urík, Tedla and Hurník, 2021). Retraction pockets can narrow and trap debris leading to the formation of a larger cyst-like structure which may form into a cholesteatoma (Louw, 2010). Active squamous COM is characterised by the retraction of the tympanic membrane as well as the presence of a cholesteatoma in the tympanic membrane. Cholesteatomas are formed due to the accumulation of tissue debris and keratin, and can cause local osteitis resulting in a significant inflammatory response and local destruction of adjacent temporal bone (Louw, 2010). Destruction of bone in the middle (ossicles) and inner ear (cochlea and semi-circular canals) can cause conductive and sensorineural hearing loss, as well as vertigo. In severe cases, the inflammatory reaction and erosion of bone can spread to the intracranial cavity resulting in a significant risk of meningitis, brain abscesses and mastoiditis (Prasad *et al.*, 2013).

Mucosal COM is characterised by the perforation in the pars tensa and subsequent inflammation of the middle ear mucosa. In inactive mucosal COM, there is no active inflammation and suppuration (discharge) in the middle ear. Whereas in active mucosal COM, due to the perforated tympanic membrane, there is subsequent inflammation and associated discharge from the perforation (also known as chronic suppurative OM). An estimated 31 million people with AOM go on to develop active mucosal COM (or CSOM) each year, 22.6% are in children under five (Monasta *et al.*, 2012).

Conductive hearing loss is a common complication of OM and occurs due to the fluid accumulation in the middle ear space impairing the transmission of sound waves (Klein *et al.*, 2017). A frequent treatment for conductive hearing loss due to the 'glue ear' is grommets, the surgical insertion of ventilation tubes into the ear drum to enable air flow and improve hearing function (Browning *et al.*, 2010). Grommets can have a beneficial effect on hearing levels and time with effusion (Browning *et al.*, 2010). Although, as OME resolves naturally in many children and the insertion of grommets requires surgical operation, it is often difficult to determine individuals with OME that is likely to persist and therefore would benefit from grommets. Less common, but severe complications of OM, such as meningitis and mastoiditis, are associated with 21,000 deaths per annum (Geyik *et al.*, 2002; Dubey, Larawin and Molumi, 2010; Monasta *et al.*, 2012) and, in rare cases, intracranial problems such as brain abscesses (Penido *et al.*, 2005).

The incidence of OM, particularly active mucosal COM, is considerably higher in LMICs. Reasons for this include reduced access to specialist doctors and equipment leading to under- and over-diagnosing of OM. This is associated with increases in complication and sequelae associated with OM, particularly hearing loss. In LMIC, OM constitutes a major chronic disease (Vos *et al.*, 2015). The incidence of OM in Sub-Saharan Africa, South Asia and Oceania is two- to eight-fold higher than in developed world regions; with India and Sub-Saharan Africa accounting for the majority of

OM-related deaths (Vos *et al.*, 2015; Myburgh *et al.*, 2016). Increasing the availability of accessible tools to aid more accurate diagnosis and better understanding of genetic and environmental factors increasing the risk of OM, is necessary to reduce the global incidence of this disease. The different types, pathology and occurrence of OM are summarised in Table 3.1.

Table 3.1 **The different types of otitis media and the corresponding pathology**

Type of otitis media	**Pathology**	**Incidence**	**Hearing loss**	**Treatment**
Acute otitis media (AOM)	Short term inflammation/ear infection. Middle ear effusion, signs of acute infection e.g., pain, fever. Intact, bulging tympanic membrane, conductive hearing loss (Lieberthal *et al.*, 2013).	709 million cases a year. 51% of these occurring annually are in under-fives (Monasta *et al.*, 2012).	Hearing loss can be conductive or sensorineural.	Analgesic to relieve pain (Lieberthal *et al.*, 2013). Antibiotics therapy (dependent on age of child and severity of AOM) (Lieberthal *et al.*, 2013).
Recurrent acute otitis media (RAOM)	Three episodes of AOM in 6 months or ≥ four episodes in 12 months. Multiple episodes of: middle ear effusion, signs of acute infection e.g., pain, fever. Intact tympanic membrane, conductive hearing loss (Kong and Coates, 2009).	By the age of 2 years, 30% will have 2 or more episodes, 10% 3 or more, and 4% 4 or more (Teele, David W, Klein Jerome O, Rosner, 1989).	Decrease in hearing compared to AOM.	Antibiotics therapy, Grommets (only if middle ear effusion present), adenoidectomy
Otitis media with effusion (OME) 'glue ear'	Serous or mucoid effusion in the middle ear without signs of infection and intact tympanic membrane (Rosenfeld *et al.*, 2016).	Affects 5-6% of children in high income countries in second year of life (Bhutta, 2014).	Typically, mild conductive hearing loss.	Watchful waiting for three months.

Type of otitis media	Pathology	Incidence	Hearing loss	Treatment
Chronic otitis media with effusion (COME)	Otitis media with effusion, intact tympanic membrane and no signs of acute infection present for at least 3 months (Bhutta, Thornton, *et al.*, 2017).	Lack of reporting.	Associated with significant hearing loss in at least 50% children (Rosenfeld *et al.*, 2016).	Grommets = surgical insertion of ventilation tubes into the ear drum, steroids (Bhutta, Thornton, *et al.*, 2017).
Active Squamous chronic otitis media	Retraction of the tympanic membrane as well as the presence of a cholesteatoma in the tympanic membrane.	Approximately 9 to 12.6 cases per 100,000 adults and from 3 to 15 cases per 100,000 children (Kuo *et al.*, 2015).	Conductive hearing loss, sensorineural hearing loss if any bone destruction to the cochlea.	Surgery to remove the cholesteatoma and clean out the mastoid.
Inactive squamous chronic otitis media	Retraction pocket in the tympanic membrane. May be asymptomatic.	Lack of reporting.	Conductive hearing loss or no hearing loss.	
Active mucosal chronic otitis media	Perforation in the tympanic membrane, subsequent inflammation of the middle ear mucosa and chronically discharging ear for more than 6 weeks (Bhutta, Thornton, *et al.*, 2017).	31 million cases each year. 22.6% of these occurring annually are in under-fives (Monasta *et al.*, 2012). Rare (<1%) in high-income countries (Bhutta, 2015), relatively common in (>2%) in many LMIC (Bhutta,	Associated with significant conductive hearing loss due to quantity of middle ear fluid.	Antibiotic therapy, aural toileting, myringoplasty (Bhutta, Thornton, *et al.*, 2017), tympanoplasty.

Type of otitis media	Pathology	Incidence	Hearing loss	Treatment
		2015). Prevalent in some indigenous groups (Bhutta, 2015).		
Inactive mucosal chronic otitis media	Perforation of the tympanic membrane with no active inflammation and suppuration (discharge) in the middle ear.	Lack of reporting.	Conductive hearing loss.	Most tympanic membrane perforation will heal spontaneously however large perforations may persist and require surgical management (myringoplasty or tympanoplasty).

The heterogeneity of the pathology, associated with the different types of OM, and treatment varies between countries. There is no consensus for the most effective treatment and considerable debate has taken place due to the demand and use of antibiotics which varies among countries, ranging from 56% in the Netherlands to 95% in the USA (Akkerman *et al.*, 2005). In the UK, where AOM is the most common childhood infection, antibiotics are prescribed for 87% of the episodes, despite evidence indicating that without antibiotic treatment AOM symptoms improve in 24 hours for 60% of children and for 80% children – symptoms resolve within 3 days (Venekamp, Damoiseaux and Schilder, 2014). Lack of antibiotic use has been associated with a higher incidence of complications (Akkerman *et al.*, 2005). A higher incidence of acute mastoiditis was found in the Netherlands, where antibiotic use is relatively low compared to countries with higher antibiotic use (Zuijlen Van *et al.*, 2001). Antibiotic resistance has led to the recommendation that antibiotics are only used in severe cases (Lieberthal *et al.*, 2013). As prescribing and treatment regimes in OM continue to be refined, focus needs to be on better understanding of the long-term consequences to hearing health of middle ear inflammation.

3.1.2 Otitis media and hearing loss

Otitis media (OM) is associated with hearing impairment, whereby the severity is associated with the type and duration of the disease. In children, hearing loss caused by OM can have an effect on speech and language development as the hearing loss occurs in the critical periods of language acquisition and learning (Menyuk, 1986; Winskel, 2006). Hearing tests are essential to assess the impact of OM on hearing function, particularly in cases of OME, COME and active mucosal COM. Children with OME persisting for three months should be given hearing assessments, routine tympanometry and access to language and speech development. The hearing assessment should include tonal audiometry with air and bone conduction, and age-appropriate vocal audiometry (Vanneste and Page, 2019).

Hearing loss caused by OM can be conductive, sensorineural or both (mixed hearing loss). Conductive hearing loss is a direct cause of OM and is associated with AOM or OME due to the increased stiffness and mass of the tympanum caused by middle ear effusion (Cai and McPherson, 2017). Conductive hearing loss due to OM is mostly transient and resolves when middle ear effusion clears. However, in cases of recurrent and chronic otitis media, sensorineural hearing loss (SNHL) can occur. SNHL occurs when there is damage or loss to the sensory components of the cochlea and/or dysfunction to the auditory nerve. It can be a result of either type of OM and the hearing loss is permanent. SNHL is an indirect cause of OM, whereby middle ear inflammation causes increased cochlear inflammation and subsequent tissue damage. The exact mechanisms that give rise to SNHL including the SNHL associated with OM are not well understood.

As discussed in Chapter 2, inflammatory insults to the auditory system such as noise exposure, infections and cochlear implantation influence auditory function. In addition, more chronic events that occur over time, such as inflammation due to specific lifestyle factors and an increased inflammatory state associated with aging and co-morbidities, are also associated with an individual's hearing trajectory. We have proposed that macrophages in the auditory system contribute to the progression of hearing loss through the mechanism of innate immune priming. Otitis media is an example of an early life inflammatory condition of the middle ear, which in many cases leads to chronic middle ear inflammation that is associated with hearing loss later in life (Jensen, Koch and Homøe, 2013; Aarhus *et al.*, 2015; Aarhus, Homøe and Engdahl, 2020), as shown in Figure 3.1. Part of the mechanism by which OM causes damage to the auditory system may be mediated through macrophage responses.

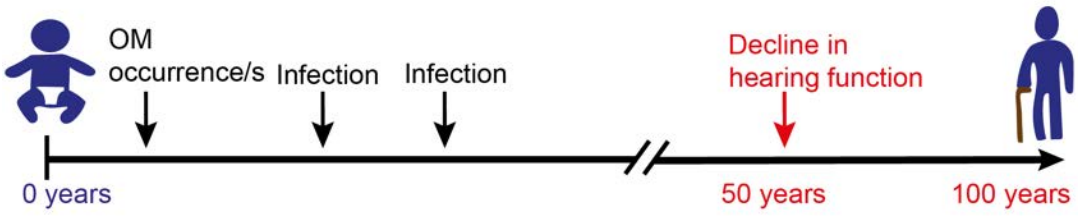


Figure 3.1 **A timeline for an individual who has experienced chronic and recurrent middle ear inflammation in childhood and goes on to develop hearing loss**

Otitis media, an example of an early life inflammatory condition, is associated with hearing loss later in life.

To investigate the role of macrophages in the auditory system and how macrophages respond to chronic inflammation associated with OM, we have used the Junbo mutant mouse as an experimental model for NTHi middle ear infection (Hood *et al.*, 2016). The Junbo mouse (Parkinson *et al.*, 2006) carries a mutation in the Evi1 transcription factor. Evi1 protein expression has been confirmed in middle ear basal epithelial cells, and in fibroblasts and neutrophils. Evi1 is implicated in mucin gene regulation (Parkinson *et al.*, 2006) and as a negative regulator of NFkβ (Xu *et al.*, 2012). Junbo mice spontaneously develop middle ear inflammation after 4-5 weeks. Hood and colleagues characterised the response to NTHi infection in the Junbo mouse (Hood *et al.*, 2016). Studies of the mouse have shown that this model recapitulates the pre-inflamed, fluid-filled middle ear environment seen in people who have experienced long-term and recurrent AOM and who then develop an NTHi infection resulting in more chronic inflammation (rAOM and COM – see Table 3.1). This makes the model relevant to human OM and valid as an animal model to investigate how macrophages in the auditory system respond to chronic inflammation, and how chronic inflammation affects the response of macrophages to an infection.

3.1.3 Middle ear anatomy and cell populations

The healthy middle ear is an air-filled cavity containing the three middle ear ossicles: the malleus, incus and the stapes (Figure 3.2). The ossicles form a chain between the tympanic membrane and oval window (Anthwal and Thompson, 2016) and transmit and amplify sound vibrations from the outer to the inner ear.

The tympanic cavity and it's sub-compartments (illustrated in Figure 3.2b) form the major part of the middle ear cavity (Luers and Hüttenbrink, 2016). The cavity is lined with a thin mucosal membrane that covers all structures and is continuous with the mastoid antrum, Eustachian tube and nasopharynx. The populations of epithelial cells, making up the mucosal membrane, change between the regions facilitating the function of the mucociliary transportation system (Massa *et*

al., 2015). The airway epithelium is the first line of defence against viruses and bacteria through different mechanisms, including mechanical (mucociliary transport), innate (inflammatory mediators and defensins) and adaptative (antigen specific and immune memory) immunity (Murphy *et al.*, 2013).

The anatomy and histology of the middle ear has been studied in animal and human tissue (Bluestone and Doyle, 1988; Park, Ueno and Lim, 1992; Ars *et al.*, 2012). Early studies (Lim, 1976, 1979) describe the morphology and cellular composition of the middle ear mucosa and Eustachian tube referring to data from human tissue and tissue from laboratory animals (guinea pigs and squirrel monkeys). Many papers have continued to include data from both human and animal studies (Massa *et al.*, 2015) which is valuable, however it is not always explicit what species are being referred to.

The human and mouse epitympanum is vascularised and consists primarily of squamous epithelial cells with islands of ciliated columnar cells. The mesotympanum is composed equally of squamous and ciliated columnar cells. The number of ciliated columnar cells increase through the middle ear cavity from the mesotympanum, through the hypotympanum and into the Eustachian tube (Massa *et al.*, 2015). The mucosal membrane at the entrance of the Eustachian tube is a pseudostratified epithelium rich in goblet and ciliated cells, which secrete mucus to trap pathogens and debris (Bluestone and Doyle, 1988; Park, Ueno and Lim, 1992; Ars *et al.*, 2012). Efficient function of the mucosal membrane clears secretions from the middle ear and Eustachian tube to minimise inappropriate microbial colonisation and infection.

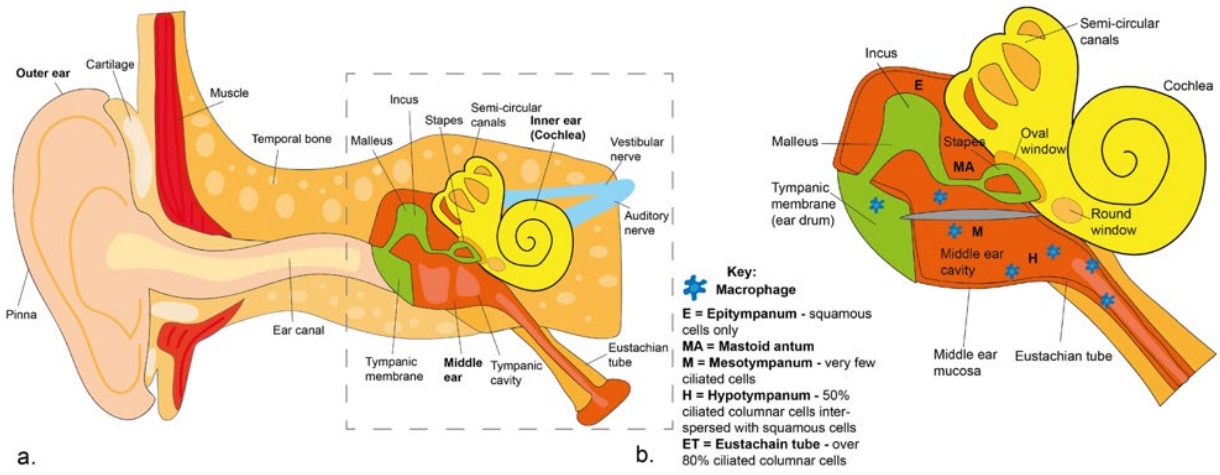


Figure 3.2 **Middle ear anatomy showing the structures of the middle ear and position in relation to the cochlea and an overview of the distribution of the macrophages across the sub-compartments**

a. Schematic highlighting the anatomy of the ear including the outer, middle and inner ear.

b. More detailed view of the middle ear positioned between the tympanic membrane and the stapes, which is inserted in the oval window of the cochlea. The middle ear mucosal membrane, consisting of different populations of cells, is separated into regions including epitympanum (E), mastoid antrum (MA), mesotympanum (M), hypotympanum (H) and the Eustachian tube (ET). Macrophages are distributed across the middle ear and Eustachian tube under homeostatic conditions, as indicated by the blue cells.

Following infection, the inflamed mucosa of the middle ear transforms into a respiratory-type inflamed epithelium with mucosal hyperplasia made up of pseudostratified, columnar epithelium with an increased population of ciliated, goblet and secretory cells (Tos, 1980; Bluestone and Doyle, 1988; Massa *et al.*, 2015).

As well as epithelial cells, populations of stromal cells, vascular cells, leukocytes including monocytes and macrophages, pericytes, mast cells and fibroblasts, and a rich vascular bed make up the middle ear mucosa (Mittal *et al.*, 2014; Ryan *et al.*, 2020). These populations were first characterised in the mouse on the basis of their ultrastructure (Lim and Hussl, 1969), and then by cellular expression profiles using immunohistochemistry (Takahashi, Peppard and Harris, 1989). Macrophages and granulocytes (Mac-1+ cells) were identified in the subepithelial stroma (localisation of macrophages highlighted in Figure 3.2b) in the middle ear of healthy BALB/c mice (Takahashi, Peppard and Harris, 1989).

Transcriptome studies have provided further insight into the cell types and genes expressed in the healthy and infected mouse middle ear (MacArthur, Hausman, Kempton, Choi, *et al.*, 2013; Hernandez *et al.*, 2015). Following NTHi middle ear infection, an innate immune response is rapidly activated. Gene clusters associated with neutrophil and macrophage recruitment and activation, leading to resolution of infection, were identified (Hernandez *et al.*, 2015). Information about the sub-regions of the middle ear, and different cell-type responses, were lost in these studies as middle ear tissue was bulk homogenised to extract RNA. Spatial information has been captured through single-cell transcriptomics, used to investigate gene expression of individual middle ear cells and elucidate the function of the individual cell types distributed in a healthy middle ear cavity prior to OM (Ryan *et al.*, 2020). Genes associated with innate immunity have been identified, highlighting the need to understand the contribution of different middle ear cell types to innate immunity. Analysis found a large number of negative regulators of immunity and inflammation in the transcriptomes of many different cell types, compared to the number of pro-inflammatory genes (Ryan *et al.*, 2020), indicating a homeostatic function of the innate immune cells in the healthy middle ear.

Cellular changes in the middle ear epithelium in OME, compared to healthy epithelia, have been studied using RNA-sequencing (Stabenau *et al.*, 2021). Tissue was collected from paediatric patients with OME undergoing tympanostomy and healthy paediatric patients undergoing cochlear implantation (Stabenau *et al.*, 2021). 1,292 genes involved in mucin production, immune function and inflammation were differentially expressed in middle ear epithelium of OME patients compared to controls.

Macrophages in the middle ear play a role in innate immunity. Resident macrophages in healthy mouse mucosa express genes consistent with a homeostatic and anti-inflammatory role such as those encoding complement components C1qa, C1qb and Ciqc and the fractalkine receptor Cx3cr1 (Ryan *et al.*, 2020). Many of the innate immune genes expressed are involved in pathogen detection, complement activation and both positive and negative inflammatory regulation (Ryan *et al.*, 2020), as well as a readiness to respond to infection. Following middle ear infection and inflammation, macrophages are recruited into the middle ear cavity. They increase in number and adopt a pro-inflammatory phenotype to carry out innate immune defence functions such as phagocytosis and intracellular killing of bacteria. Characterisation of this response has been carried out using the Junbo mouse model which will be discussed in this chapter. During OM, macrophages are a major component of cellular effusions. As OM progresses, the macrophages adopt an anti-inflammatory phenotype and play a role in the resolution (Hernandez *et al.*, 2008; Hur *et al.*, 2021). The recruited macrophages will become part of the resident macrophage population (Fang *et al.*, 2020).

3.1.4 Pathophysiology of acute and chronic otitis media

Analyses of middle ear tissue from humans and animal models (chinchilla, murine and rat), have identified a myriad of pathological changes in the middle ear and cochlea associated with OM. Figure 3.3 summarises the pathogenesis of OM from the initiation to the resolution, including potential sequelae and complications. The schematic indicates the key role of inflammatory mediators and immune cells in the pathological changes that occur with middle ear infection and inflammation.

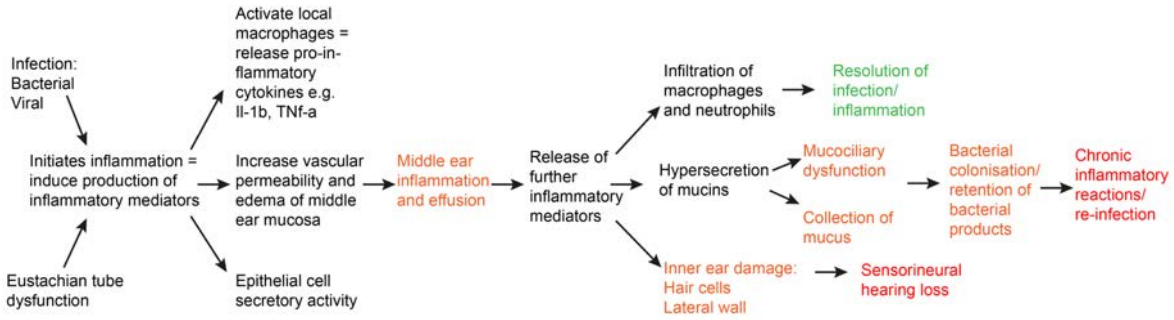


Figure 3.3 **A schematic describing the pathogenesis of otitis media, highlighting the complexity of the pathology involving inflammatory mediators and immune cells. Potential sequelae and complications are indicated**

This schematic describes the pathogenesis of OM beginning from an infection or dysfunction in the Eustachian tube. These factors initiate inflammation inducing the production of inflammatory mediators. These mediators signal to local cells to induce further inflammatory responses including activation of local macrophages, increasing vascular permeability and changes in epithelial cell secretory activity. This results in inflammation and effusion. Further inflammatory mediators are released, signalling to carry out functions including clearance of bacterial products by infiltrated macrophages and neutrophils, hypersecretion of mucins resulting in build of mucus and mucociliary dysfunction, and damage tissues of the inner ear. The consequence of mucus accumulation and mucociliary dysfunction is reduced bacterial clearance/bacterial colonisation leading to chronic inflammatory reactions. Green indicates resolution of inflammation, orange indicates damage or dysfunction and red indicates serious damage.

Pathological changes in the middle ear include mucosal hyperplasia, effusion, infiltration and proliferation of immune cells in the inflamed middle ear mucosa and cavity, upregulation of pro-inflammatory cytokines in the middle ear mucosa and hearing loss. Figure 3.4 indicates the studies which have identified these changes.

Early histopathology studies have provided insights into the mucosal cellular and overall tissue changes associated with different types of OM. In 60 patients with persistent OME, characteristic mucosal changes were identified including a thickening of the pseudostratified ciliated epithelium and an increase in goblet cell density (Tos, 1980). Analysis of temporal bones from 123 adult patients with COM revealed granulation tissue, epithelial metaplasia and osteitic changes of the ossicles, mastoid bone and otic capsule (Meyerhoff, Kim and Paparella, 1978). Histochemical analyses of acute and chronic animal models of OM have identified infiltration of immune cells into the middle ear cavity and increased thickness of the mucosal membrane (Keithley *et al.*, 1990; Melhus and Ryan, 2003; Kurabi *et al.*, 2015; Huang *et al.*, 2016).

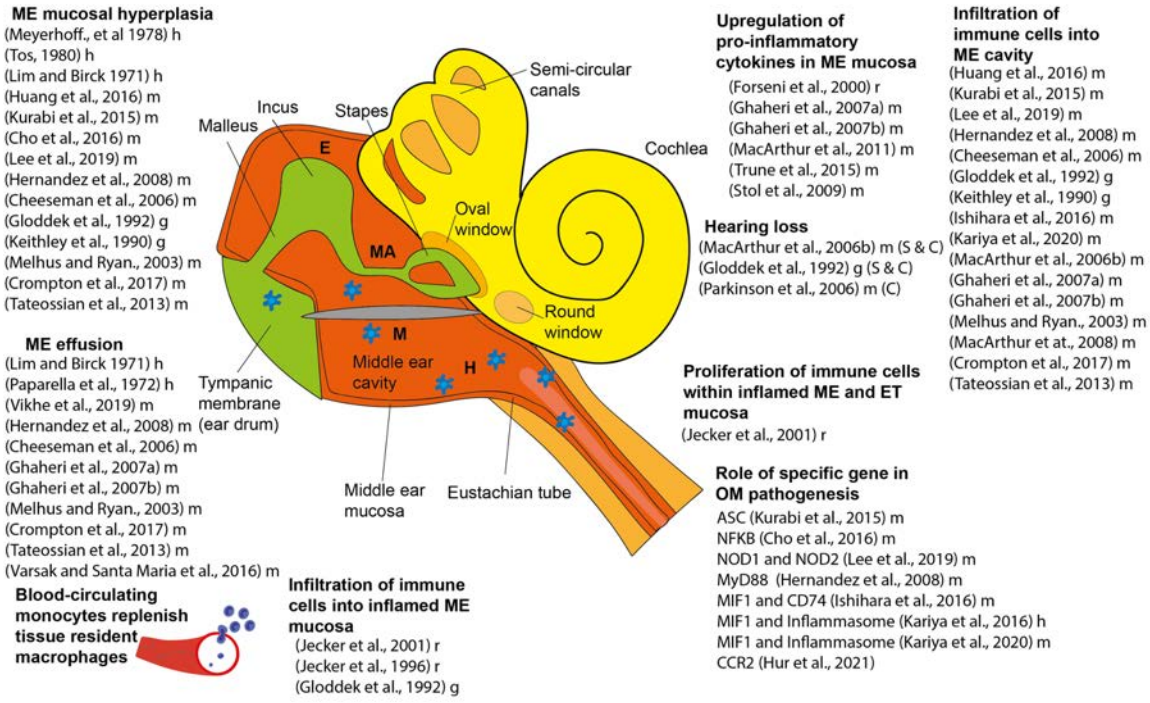


Figure 3.4 **Pathophysiology of otitis media in the middle ear**

Middle ear inflammation causes pathology within key regions of the middle ear. The pathology has been investigated in both acute and chronic models of OM (references listed). Key: human primary study h, mouse primary study m, rat primary study r, guinea pig primary study g.

Mucosal inflammation, reduced ability to clear NTHi infection and changes in immune cell infiltration (Hernandez *et al.*, 2008; Kurabi *et al.*, 2015; Hur *et al.*, 2021) have been described in genetic models of OM. These studies indicate a role of the innate immune response in the pathogenesis and resolution of OM.

Biofilms are communities of microorganisms embedded in a self-produced extracellular polymeric matrix that are attached to a surface (Donlan, 2002; Silva and Sillankorva, 2019). The role of

biofilms formed by otopathogens, and the way in which the biofilms contribute to the recurrent and chronic nature of OM has been investigated (Post, 2001; Kaya *et al.*, 2013; Novotny *et al.*, 2019; Barron *et al.*, 2020). The hypothesis for the role of biofilms in OM came from several repeated observations. For many years patient samples of middle ear effusion were culture negative and considered sterile. When the samples were analysed using polymerase chain reaction (PCR) methods, bacterial DNA was detected in culturally negative/sterile samples (Post *et al.*, 1995, 1996). Microscopy analysis of middle ear mucosa revealed the formation of biofilms in both animal (Post, 2001) and human tissue (Hall-Stoodley *et al.*, 2006; Silva and Sillankorva, 2019). NTHi, a key pathogen involved in OM, has the capacity to form biofilms (Murphy and Kirkham, 2002; Moriyama *et al.*, 2009; Puig *et al.*, 2014). NTHi clinical isolates from paediatric AOM patients showed that a high percentage (84.3%) were biofilm forming strains (Moriyama *et al.*, 2009). In addition, NTHi clinical isolates from AOM cases, not improved by amoxycillin treatment, had considerably higher level of biofilm formation than that of AOM cases who were improved by amoxicillin. This indicates a role of biofilm formation in the persistent and, in some cases, untreatable nature of AOM (Moriyama *et al.*, 2009). In contrast, a similar study found no association of biofilm production and AOM treatment failure or recurrence (Mizrahi *et al.*, 2014).

Bacterial biofilms have been demonstrated from patients with COM and cholesteatoma (Lee *et al.*, 2009; Saunders, Murray and Alleman, 2011; Lampikoski *et al.*, 2012; Kaya *et al.*, 2013; Gu *et al.*, 2014), using scanning electron microscopy (SEM). Analysis of 102 middle ear, mastoid tissue and ossicle samples collected during surgery from 34 patients revealed the presence of biofilms in 7/10 (70%) active mucosal COM patients, 6/11 (54.5%) inactive mucosal COM patients and 8/13 (61.5%) patients with cholesteatoma (active squamous COM) (Kaya *et al.*, 2013). Higher rates were found in hypertrophic and granulated tissue samples compared to normal middle ear mucosa (Kaya *et al.*, 2013). Similar rates of biofilm presence were reported in active mucosal COM patients 6/10 (60%) (Lee *et al.*, 2009) and 19/29 (66%) active mucosal COM patients in the mastoid mucosa (Lampikoski *et al.*, 2012). Based on the evidence from these and further studies, it has been proposed that biofilms may attach to inflamed and damaged tissue in patients with active mucosal COM, resulting in persistent infections and resistance to antibiotic therapy. There has been considerable debate whether biofilms exist in the middle ear and play a role in the pathogenesis of OM. However, the evidence suggesting that biofilms do play a role in both acute recurrent OM and COM is becoming more robust.

Neutrophils, the first line responders to invading pathogens, are associated with the increased persistence of bacterial infection seen in OM. Neutrophils can form neutrophil extracellular traps (NETs) in response to NTHi infection (Juneau *et al.*, 2011; Arazna, Pruchniak and Demkow, 2013; Schachern *et al.*, 2017). NETs are characterised by a double-stranded DNA lattice decorated with

histones and elastase (Brinkmann *et al.*, 2004). NTHi survives within NET structures and when entrapped within NET structures is resistant to extracellular killing and phagocytic killing by infiltrating neutrophils (Hong *et al.*, 2009). This provides a mechanism of bacterial persistence as bacteria can survive within the middle ear chamber within these structures.

The precise mechanisms involved in the pathophysiology of OM, including the exact role of macrophages and the effect of the infection on macrophages, are not fully understood. However, these studies highlight a role of the innate immune response and macrophages in the initiation and resolution of OM.

3.1.5 Cochlear pathology and otitis media

Different mechanisms have been proposed for how middle ear inflammation during OM causes cochlear pathology and subsequent SNHL. Figure 3.5 summarises the two main theories in the literature, a) an anatomical mechanism whereby cytokines and bacterial components from the middle ear cavity, move through the semi-permeable round window membrane into the basal region of the cochlea (Figure 3.5a) and b) via inflammatory cytokine induction (Figure 3.5 b). The round window membrane (RWM) is permeable to various chemical mediators (Goycoolea *et al.*, 1980; Harris and Ryan, 1985; Juhn, 1997). An increase in keyhole-limpet hemocyanin (KLH) was observed in the cochlea after the middle ear was challenged with KLH (Harris and Ryan, 1985). Passage of inflammatory mediators and bacterial components, found in increased quantities during OM, into the basal region of the cochlea can cause tissue damage to cochlear structures (Cureoglu *et al.*, 2004; Papp, Rezes and Jokay, 2014).

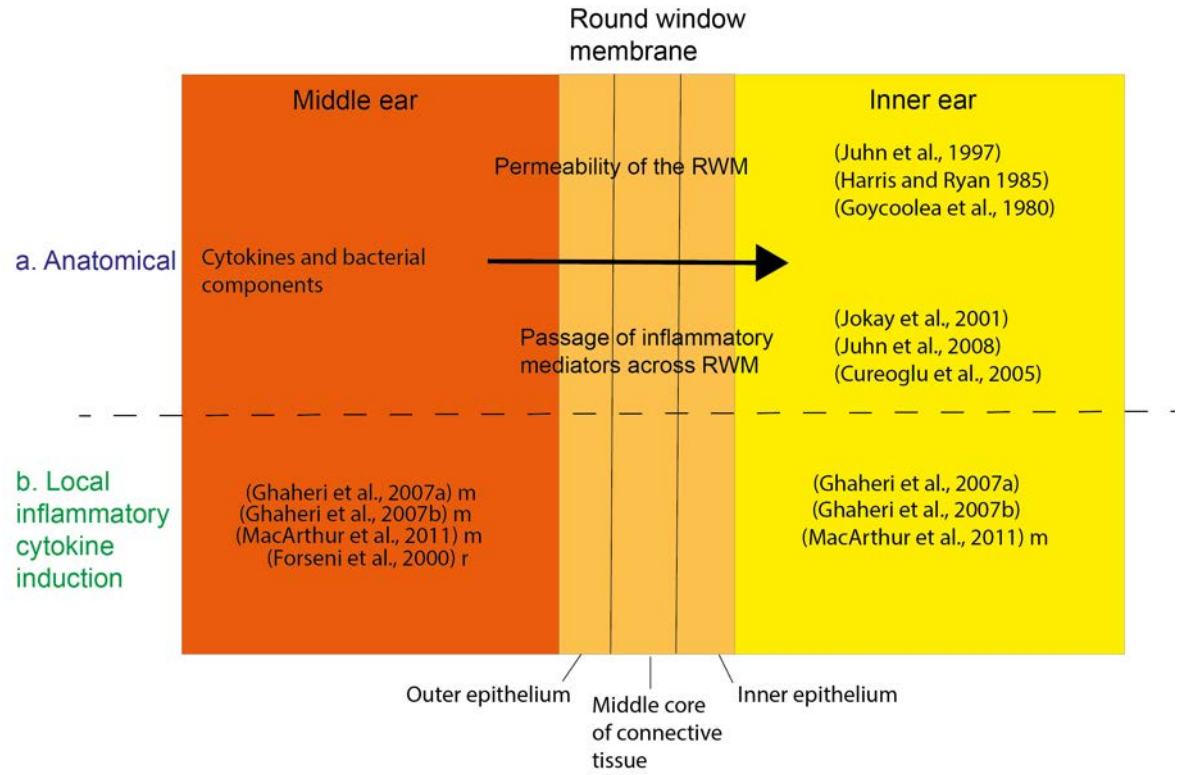


Figure 3.5 **Mechanisms proposing how middle ear inflammation causes damage to the cochlea and subsequent SNHL**

a. Anatomical mechanism via round window membrane. Inflammatory mediators and molecules such as pro-inflammatory cytokines and bacterium pass through the semi-permeable round window membrane into the basal region of the cochlea. This causes local tissue damage.

b. Via local inflammatory cytokine induction. Middle ear inflammation causes an increase in the production and expression of pro-inflammatory mediators in middle and inner ear tissue. An increase in the expression of pro-inflammatory mediators in the cochlea will cause local tissue damage and activation of cochlear macrophages which can cause further damage.

Studies using different animal models for OM have shown that middle ear inflammation causes an increase in the production and expression of pro-inflammatory cytokines in both middle ear and cochlear tissues (Forséni, Bagger-Sjobach and Huhcrantz, 2001; Ghaheri *et al.*, 2007a, 2007b; MacArthur *et al.*, 2011). Increased expression of cytokines (mRNA) and protein have been reported in a model of acute (Ghaheri *et al.*, 2007a) and spontaneous chronic OM (Ghaheri *et al.*, 2007b), indicating that cytokine expression in the cochlea occurs in response to the middle ear inflammation. Altered, overexpression of middle and inner ear cytokines related to inflammation, tissue remodelling and angiogenesis in models for acute and chronic otitis media have been shown (MacArthur *et al.*, 2011; Trune *et al.*, 2015). Therefore, it is possible that middle ear

inflammation can (indirectly) signal to the cochlea. Increased expression of pro-inflammatory cytokines in the cochlea can cause damage to key structures with limited capacity for repair such as the stria vascularis and hair cells and decrease function. Middle ear inflammation can signal both directly and indirectly to the cochlea to switch on inflammation-related genes.

There is increasing evidence of pathological changes that occur in the cochlea, following middle ear inflammation, in both human temporal bone and animal studies. Figure 3.6 summarises these changes in the cochlea and the studies where the evidence has come from. Damage to structures within the cochlea leads to dysfunction causing SNHL. A loss of inner and outer hair cells, and a decrease in stria and spiral ligament area in the basal turn of the cochlea, was measured in temporal bones from 15 patients with COM (with an age range of 8-88 years) compared to control ears (Cureoglu *et al.*, 2004). Similar cochlear changes were found in a larger study (614 temporal bones with OM, 47 (30 cases) with COM and 35 (21 cases) with purulent otitis media were selected) (Joglekar *et al.*, 2010). Morphological changes in the basal turn of the cochlea in COM results in sensorineural often high-frequency hearing loss in these patients. No hearing measures were available in these studies meaning associations could not be made between the morphological changes, degree of these changes and hearing impairment.

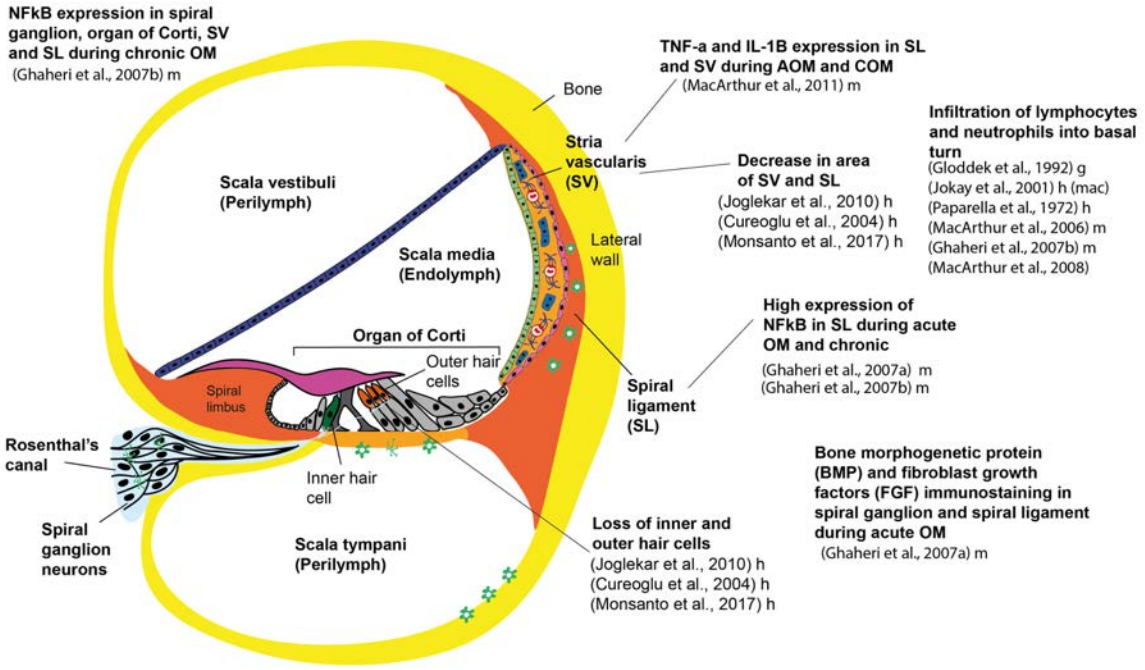


Figure 3.6 **Evidence for the effects of otitis media on the cochlea**

Middle ear inflammation causes pathology within key regions and tissues in the cochlea. The pathology has been investigated in both acute and chronic animal models of OM and in human temporal bone studies (references listed). Key: human

primary study h, mouse primary study m, rat primary study r, guinea pig primary study g.

Increased expression of innate immune genes and pro-inflammatory mediators in cochlear tissues, in mice with middle ear inflammation has been shown (MacArthur, Hefeneider, Kempton and Trune, 2006; Ghaheri *et al.*, 2007a, 2007b; MacArthur *et al.*, 2011; Trune *et al.*, 2015). Infiltration of inflammatory cells in the basal turn of the cochlea of mice has also been identified (MacArthur, Hefeneider, Kempton and Trune, 2006; Ghaheri *et al.*, 2007b; MacArthur *et al.*, 2008). The presence of inflammatory cells, which release inflammatory mediators, in the basal turn may signal to activate resident macrophages or cause damage to the local tissue environment. In a model for COM, caused by a mutation in the TLR4 gene, histological analysis showed inner ear inflammation which paralleled elevations in hearing thresholds suggestive of SNHL (MacArthur, Hefeneider, Kempton and Trune, 2006). It is clear from these data that middle ear inflammation causes cochlear pathology, particularly in the basal region. It is widely evidenced that basal cochlear regions are more susceptible to damage in humans and animals, resulting in high frequency hearing loss.

Clinical studies have investigated associations between the sub-types of OM and SNHL. Audiometric changes in the early stages of AOM in 27 patients have been reported (Kasemodel *et al.*, 2020). Air and bone conduction thresholds identified hearing loss in 90% of the ears with AOM, 46.67% conductive and 43.33% mixed hearing loss; suggesting SNHL is present in patients with AOM (Kasemodel *et al.*, 2020). A review of data from 121 patients with unilateral active mucosal COM identified an association with SNHL (Papp, Rezes and Jokay, 2014). Bone conduction threshold averages were significantly higher in the patients with active mucosal COM compared to controls (Papp, Rezes and Jokay, 2014). A retrospective cohort study, in which 10,248 patients with newly diagnosed COM were compared with 30,744 controls, identified the incidence of sudden SNHL was 3 times higher in patients with COM compared to the control group (Yen *et al.*, 2015). The stated definition for COM was a minimum of three outpatient service claims or any single hospitalisation with a corresponding diagnosis (Yen *et al.*, 2015). Similarly, the incidence of SNHL was measured in a population of 137 patients with active mucosal COM compared to a control group (137 people). Active mucosal COM involves recurrent episodes of ear discharge through a perforation in the tympanic membrane. SNHL was found in 71.4% of COM cases with an ear discharge duration of more than 5 years (Subramaniam, Ashkar and Rai, 2020). Patients with a longer duration of disease and larger size of tympanic membrane perforation were more likely to develop SNHL.

Long-term epidemiological follow up studies have investigated associations between childhood OM and hearing loss later in life. The Norwegian HUNT study is a population-based cohort study which measured participants pure-tone audiometry in primary school then again at ages later in life (Aarhus *et al.*, 2015; Aarhus, Homøe and Engdahl, 2020). 30 and 40-year follow up studies found a clear association between childhood COM and adult hearing loss. Similarly, a study of two Greenlandic population-based paediatric cohorts identified that active mucosal COM was associated with permanent hearing loss in adolescents (Jensen, Koch and Homøe, 2013). These clinical studies identify a clear link between OM and SNHL including between childhood OM and hearing loss in adulthood.

The clinical and experimental evidence above describes the link between OM and cochlear pathology and suggests some mechanisms which are likely to be responsible for the hearing impairment. However, there are still gaps in knowledge. OM is a multifactorial disease where the pathogenesis is affected by immune status of the individual, pathogen virulence and strain, genetic susceptibility and Eustachian tube structure and function (Trune and Zheng, 2009); making it a complicated disease to model and understand. The varying severity and outcomes for people with OM, particularly in terms of hearing impairment, adds a further complexity when finding appropriate therapeutic measures.

Cochlear implants (CIs) are an appropriate therapy for severe to profound SSNHL. CIs can restore a level of hearing function in individuals, however there is a population of people with a CI who experience poorer outcomes. The reasons for the poor outcomes are not well understood. OM, at the time of implantation, can be associated with increased CI complications and infection. It is not known whether prior OM influences CI outcomes later in life. Little is understood about how factors an individual is exposed to throughout their lifetime influence innate immune cells and how this impacts auditory function.

In order to investigate whether childhood OM influences the function of the auditory system later in life and therefore CI performance, adequate recording of middle ear inflammation history is necessary during the pre-implant assessment. Analysis of patient clinical records will provide insight into factors associated with poorer hearing outcomes later in life. As well as using clinical data to investigate the links between early life inflammation and hearing loss, genetic mouse models for diseases such as OM, enable detailed investigation of the cellular mechanisms involved in the pathogenesis.

3.1.6 Genetics of otitis media

The genetic aetiology for OM is poorly understood. Very few underlying genes have been identified despite genome wide association studies (GWAS) being carried out. The first GWAS investigated genetic determinants of OM susceptibility in childhood. This study identified CAPN14 and GALNT14 on chromosome 2p23.1 and the BPIFA gene cluster on chromosome 20q11.21 as novel candidate genes (Rye *et al.*, 2012). These findings failed to be replicated in an independent population of chronic otitis media/recurrent otitis media. A later GWAS was carried out to investigate COME and recurrent OM and identified a novel susceptibility locus on chromosome 2q31.1 (Allen *et al.*, 2013). In both studies, the sample size was small and a limited number of genetic variants were examined which meant that signals did not break the threshold of genome-wide significance ($P< 5 \times 10^{-8}$). These GWAS to identify genetic determinants of OM were underpowered to detect alleles of mild and moderate disease risk. In a GWAS, a fibronectin type III domain containing 1 (FNDC1) gene, associated with AOM in children (van Ingen *et al.*, 2016), was identified. The mouse homologue of this gene, Fndc1, was expressed in middle ear tissue and upregulated by LPS suggesting an immune role in OM. FUT2, encoding a human secretion gene alpha-(1,2)-fucosyltransferase which controls the expression of specific antigens on the mucosal epithelia, has been associated with paediatric ear infections (Tian *et al.*, 2017) and to confer susceptibility to recurrent/chronic familial OM (Santos-Cortez *et al.*, 2018). Further investigation of the pathways involving these genes is required.

Candidate gene studies overcome some of the limitations of GWAS. Candidate gene studies of innate immunity and inflammation have been carried out on different cohorts from across the world. A recent review (Giese *et al.*, 2020) has identified 100 alleles that confer susceptibility to OM (Giese *et al.*, 2020). A candidate gene study was carried out on a cohort of children with COME to test for associations in four candidate loci identified in mouse models for chronic otitis media (Bhutta, Lambie, *et al.*, 2017). The study identified an association with the loci TGIF1 and FBX011 but not Evi1 or NISCH (Bhutta, Lambie, *et al.*, 2017). Both FBX011 and TGIF1 are involved in TGF-β signalling and may be critical for the development of persistent middle ear inflammation. Further GWAS and candidate gene studies are required to establish pivotal gene loci involved in the complex mechanisms behind the pathologies in OM. Human gene studies alongside genetic mouse models will complement findings and accelerate the field in understanding the genetic basis of OM.

3.1.7 Animal models to investigate otitis media

Chinchillas were the first animal model system for the study of AOM which involved challenge with S. *pneumoniae* (Giebink *et al.*, 1976). Chinchillas have a large middle ear and Eustachian tube, closely resembling the anatomy of humans. These studies provided insight into the molecular mechanisms and immune response to pneumococcal infection (Giebink *et al.*, 1976). The first dual infection model of OM was established using chinchillas as a host (Giebink *et al.*, 1980). Increased incidence of OM (67%) was seen in chinchillas infected with S. *pneumoniae* and co-inoculated with influenza A virus, compared to those infected with influenza A alone (4%) and S. *pneumoniae* (21%) alone. These studies were pivotal in understanding how respiratory viral infection contribute to the pathogenesis of acute OM. Chinchillas are still frequently used for NTHi infection and biofilm studies, and vaccine development (Bakaletz, 2009; Novotny *et al.*, 2019; Bailey *et al.*, 2020).

Murine models to investigate OM have been developed and are widely used due to the availability of reagents, a well characterised immune response and well-defined microbiological status (Bhutta, 2012). A benefit of using mice is that the completion of the mouse genome sequence enables annotation of mouse genes to investigate the effects of these gene mutations (Consortium, 2002).

Multiple animal models for both acute and chronic OM have been established and investigated (see appendix B.2 and B.3, summarising the different types of models for OM). Methods used to induce the acute or chronic OM include inoculation with an antigen or bacteria (Figure 3.7), surgical manipulation (Figure 3.8) and genetic mutation (Figure 3.9). Direct inoculation, to induce AOM, can either be through transtympanic, transbullar or via intranasal inoculation of bacteria, which mimics naturally ascending Eustachian tube infection and produces varying levels of middle ear infection. Inflammation is short-lived and the infection resolves within a couple of days. Figure 3.7 summarises the experimental design for models of AOM induced by bacterial inoculation. COM can be induced in mice through surgical manipulation such as tympanic membrane perforation or obstruction of the Eustachian tube (Varsak and Santa Maria, 2016) or by genetic mutation (Zheng, 2006). Figure 3.8 summarises the experimental design.

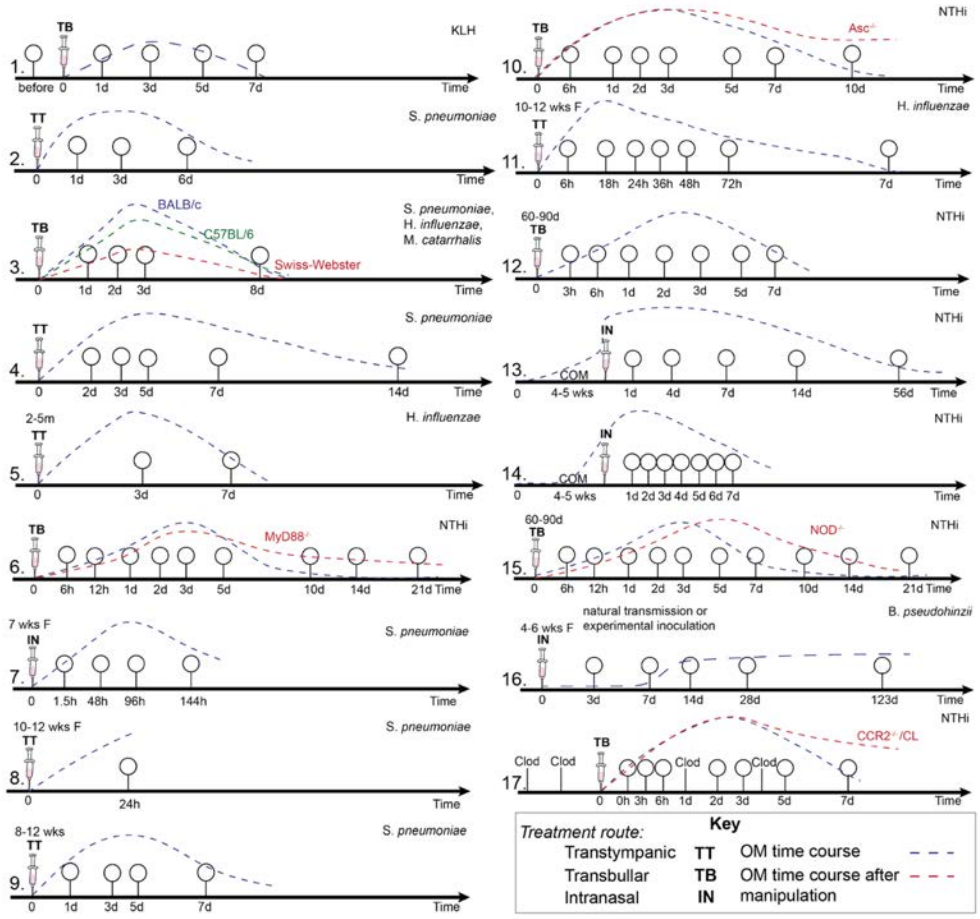


Figure 3.7 **Study design timelines for models of acute otitis media induced by bacterial (or bacteria-derived antigen) inoculation**

The timeline includes age or time at which animals were exposed to the treatment to induce AOM and the number of measurements taken across the study. The dashed line (blue) indicates the time course of the middle ear inflammation. A red dashed line indicates the change in the time course of the middle ear inflammation in that

model of AOM in a mouse with a specific gene knocked-out. Treatment route (transtympanic, transbullar or intranasal) and the type of bacteria used is included.

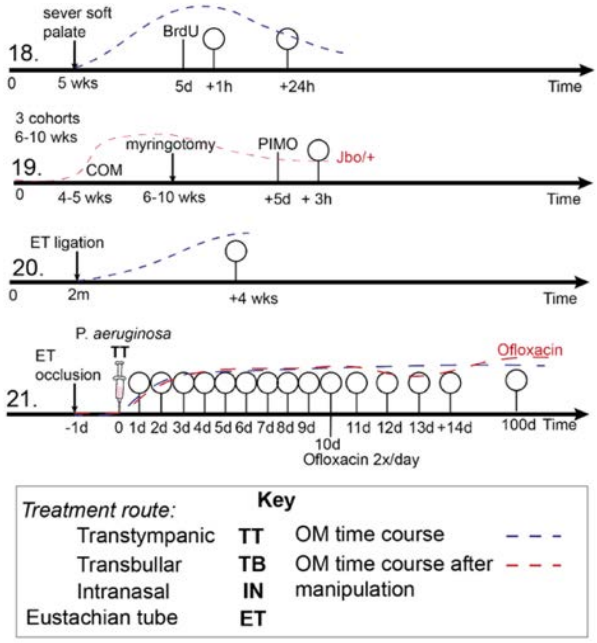


Figure 3.8 **Study design timelines for models of acute and chronic otitis media induced by surgical manipulation**

The timeline includes the age/time at which the animals were exposed to the surgical manipulation and the number of measurements taken across the experiment. The dashed line (blue) indicates the time course of the middle ear inflammation. A red dashed line indicates the change in the time course of the middle ear inflammation in that model of OM in a mouse with a specific gene mutated (Jbo/+).

Many studies have investigated the role of innate immune receptors and effectors in the middle ear inflammatory response and resolution of OM using either gene-driven (MacArthur, Hefeneider, Kempton and Trune, 2006; Hernandez *et al.*, 2008; Kurabi *et al.*, 2015; Hur *et al.*, 2021) or phenotype-driven (Hardisty *et al.*, 2003; Parkinson *et al.*, 2006; Crompton *et al.*, 2017) genetic approaches. Figure 3.9 summarises the experimental design of several genetic models for OM. In gene-driven approaches, specific knock-in or knock-out mutations are carried out with the aim of investigating the resulting phenotype. C3H/HeJ mice have mutation in toll-like receptor 4 (TLR4) meaning they are unable to clear Gram-negative bacteria (MacArthur, Hefeneider, Kempton and Trune, 2006). C3H/HeJ mice spontaneously develop OM without the need for bacterial inoculation. Other genetic models for OM involve both a gene mutation and bacterial inoculation. Inflammatory and innate immune response genes in OM, including myeloid differentiation primary response gene (MyD88) (Hernandez *et al.*, 2008), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (Kurabi *et al.*, 2015) and

chemokine ligand 2 (CCL2) (Hur *et al.*, 2021) were knocked down in mice before inducing AOM by middle ear injection of NTHi. These studies indicate a role of the innate immune receptors and signalling molecules in the pathogenesis and resolution of OM.

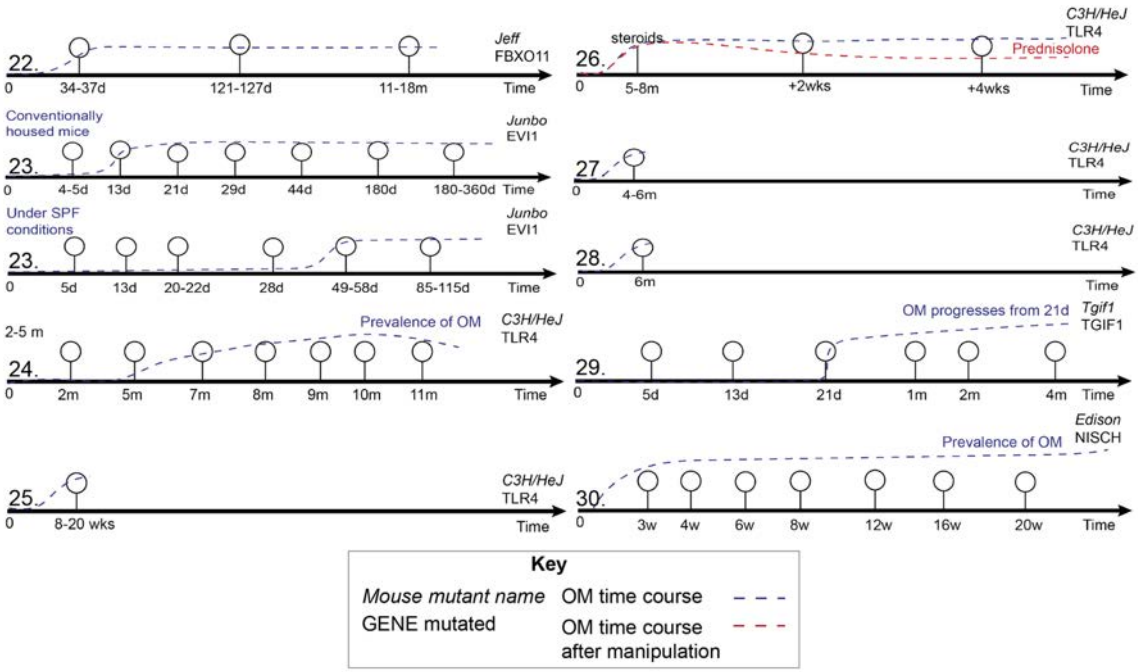


Figure 3.9 **Study design timelines for models of chronic otitis media induced by genetic mutation**

The timelines include the number of measurements taken across the timeline of the experiment in models of COM. The dashed line (blue) indicates the time course of the middle ear inflammation. The blue dashed line indicates the change in prevalence of the middle ear inflammation across time. The name of the mouse mutant and the gene mutated is included.

Phenotype-driven approaches involve screening of large numbers of mutant mice, produced by chemical N-ethyl-N-nitrosourea (ENU) mutagenesis, for disease phenotypes (Trune and Zheng, 2009). This is a powerful way to investigate the link between the mutated gene and disease phenotype and is advantageous for OM as the genetic aetiology is poor. The MRC Harwell mutagenesis program in the UK (Brown and Hardisty, 2003) has identified Junbo (Jbo) (Parkinson *et al.*, 2006), Jeff (Jf) (Hardisty *et al.*, 2003; Hardisty-Hughes *et al.*, 2006) and Edsin (Crompton *et al.*, 2017) mouse mutants with a conductive deafness phenotype that display pathological signs comparable to OM in humans.

Figure 3.10 compares genetic models for OM, whereby NTHi infection is overlaid. In (Hernndez et al., 2008; Hur et al., 2021; Kurabi et al., 2015), specific genes involved in the innate immune signalling and response were knocked down before inducing AOM with bacterial inoculation of NTHi. In comparison, in Junbo mice a mutation in the Evi1 gene induces spontaneous active

mucosal COM (aka CSOM) (Parkinson *et al.*, 2006). A model for middle ear infection was established by inoculation with NTHi (Hood *et al.*, 2016).

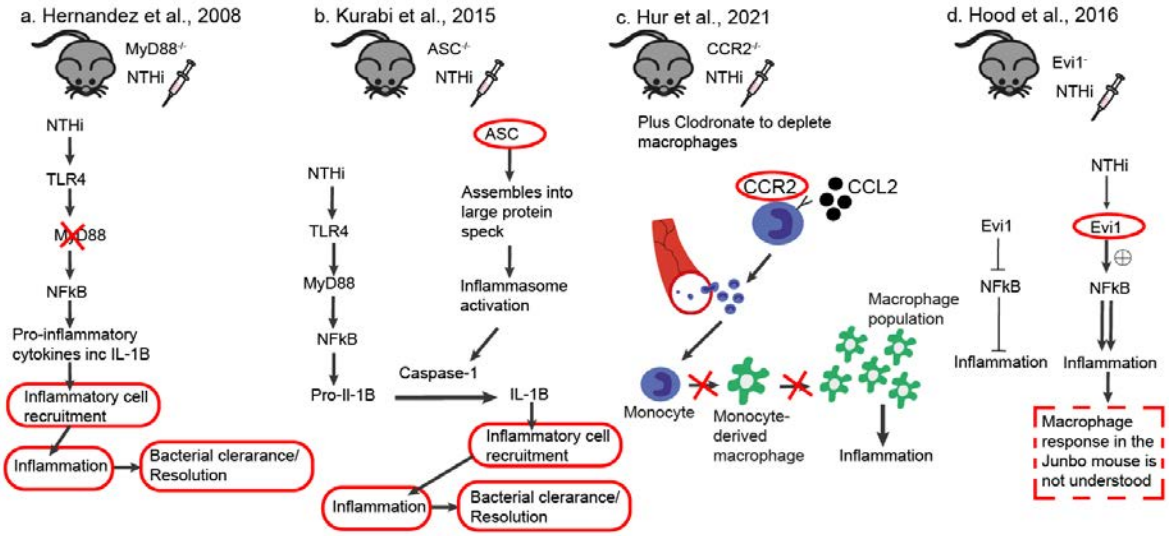


Figure 3.10 **Investigation of the role of the innate immune system in otitis media using genetically modified mouse models**

a. MyD88 is an adaptor for toll-like receptors to induce NFkβ gene expression. Following NTHi infection, MyD88$^{-/-}$ mice exhibited prolonged middle ear mucosal thickening, delayed recruitment of neutrophils and macrophages to the middle ear, and delayed clearance of NTHi from the middle ear following NTHi infection compared to WT mice.

b. ASC is a scaffolding protein and a key component of the inflammasome complex, involved in the innate immune response to infections by regulating the maturation of Il-1β. Asc$^{-/-}$ mice showed an increased degree and duration of mucosal epithelial hyperplasia and a reduced ability to clear bacteria from the middle ear compared to WT mice. A reduction in leukocyte recruitment and infiltration into the middle ear cavity and reduced macrophage NTHi phagocytosis was measured.

c. CCR2 is a protein expressed on the surface of monocytes involved in the recruitment and differentiation of monocytes into macrophages through the action of the chemokine, CCL2. CCR2-/- mice were exposed to clodronate to deplete to macrophage population. These mice exhibited enhanced middle ear inflammation, prolonged and increased infiltration of neutrophils into infected middle ear cavities, and increased persistence of infection compared to WT mice.

d. A mutation in the Evi1 gene was found to induce CSOM in Junbo mice. Evi1 is an inducible negative regulator of NFkβ, a transcription factor that modulates

inflammatory responses by regulating the expression of pro-inflammatory mediators. Mutation in Evi1 leads to increased NTHi induced inflammation through NFkβ upregulation. Junbo mice, with pre-inflamed middle ears, have increased susceptibility to NTHi infection.

These studies indicate a role for innate immune signalling pathways involving TLRs, NFkβ and Il-1β, in addition to macrophages in the pathogenesis and persistence of OM.

All chronic OM mouse mutants spontaneously develop middle ear inflammation but not all mutants have bulla fluid which is culture positive (Bhutta, Thornton, *et al.*, 2017).

There are no animal models that fully replicate the development or progression of COM in humans. The chronic OM mouse mutant, Junbo, has spontaneously inflamed middle ears and develops high rates of bulla infection when receiving an intranasal challenge of NTHi (Hood *et al.*, 2016). This is a promising model for studies of the pathology of COM and the consequences of a pre-inflamed middle ear environment when a bacterial infection is overlaid.

3.1.8 The Junbo mouse model

The Junbo mouse model is a well-established mouse model of OM. Junbo mice (Jbo/+) have a single nucleotide polymorphism mutation (Asn763Ile) in the gene encoding the transcription factor Evi1 (also known as Mecom) (Parkinson *et al.*, 2006). This mutation causes AOM to develop spontaneously in the postnatal period, developing into active mucosal COM with otorrhea. Evi1 is an inducible negative regulator of NFkβ, a transcription factor that modulates inflammatory responses by regulating the expression of pro-inflammatory mediators (Xu *et al.*, 2012). Evi1 negatively regulates NTHi-induced NFkβ-dependent inflammation in the lung (Xu *et al.*, 2012). Therefore, loss of function mutation in Evi1 will lead to increased NTHi-induced inflammation. Evi1 represses the TGF-β signalling pathway, resulting in negative regulation of cell growth and differentiation (Kurokawa *et al.*, 1998). The inflammatory response in the Junbo mice is restricted to the middle ear (Parkinson *et al.*, 2006). A mutation at the Evi1 locus causes susceptibility to OM. Using this model, detailed investigation of the mechanisms involved in the COM and how the increased middle ear inflammation affects susceptibility for infection can be carried out.

3.1.9 The use of the Junbo mutant mouse for the study of NTHi middle ear infection

Junbo, the mutant mouse that was identified in the large scale mutagenesis program at MRC Harwell UK (Brown and Hardisty, 2003), was used to develop a novel model for the study of NTHi middle ear infection (Hood *et al.*, 2016). Junbo mice spontaneously develop middle ear

inflammation and effusion in specific pathogen-free conditions at around 4-5 weeks. The mice demonstrated a hearing loss, in a click-box test, at ~40 days after birth, likely to be a conductive hearing loss (Parkinson *et al.*, 2006). Hood et al hypothesised that the middle ear inflammation would provide an environment where a single inoculation of NTHi would establish infection following spread along the Eustachian tube (Hood *et al.*, 2016). They found that a single intranasal inoculation of NTHi infected the middle ear of Junbo mice, persisting at high rates for 56 days (Hood *et al.*, 2016). This is a novel genetic model for COM where the mice spontaneously develop middle ear inflammation and show increased susceptibility to NTHi infection, whereby after a single NTHi challenge 80% of the Junbo middle ears were infected with NTHi.

Extensive studies have been carried out to determine the microbiological and immunological characteristics of this model. The dynamics and host response to nasopharynx colonisation and OM were characterised using distinct strains of NTHi (Hood *et al.*, 2016). Histological analysis of the middle ear of NTHi-infected Junbo mice at 7 days post-inoculation was carried out. The bulla fluid had a necrotic caseous core of neutrophils, surrounded by viable and apoptotic neutrophils, and an outer thick band of foamy macrophages (Hood *et al.*, 2016). Neutrophils were observed in the Eustachian tube lumen, adjacent to the bulla opening and at the nasopharynx junction. This histopathological data highlights the large NTHi populations that 'pool' in the middle ear, causing repeated reinfections through the descending spread from the middle ear to the nasopharynx with efflux of exudate (Hood *et al.*, 2016). Through inoculating age-matched wild-type mice, it was evident that pre-existing inflammation and bulla fluid is required for successful colonisation of NTHi and efficient infection. In NTHi-inoculated wild-type mice, 71 of 73 had healthy OM-free ears with a clear tympanic membrane and no bulla fluid. These middle ears were NTHi culture negative (Hood *et al.*, 2016). An activated, inflammatory immune environment prior to infection is an important factor in how OM progresses. This mouse model is an important model for the study of disease progression of OM with NTHi infection (Hood *et al.*, 2016).

In AOM, the presence of pathogenic or virulent material in the middle ear initiates an inflammatory response, involving immune cells such as macrophages and neutrophils. The accumulation of an immune-cell rich middle ear fluid increases the risk of recurrent AOM. Therefore, the composition of cells in the bulla fluid is likely to play a role in clearance or persistence of this material. As around 80% of the middle ears with fluid were positive for NTHi (Hood *et al.*, 2016), the cellular content of the middle ear fluid was analysed to see whether it played a role in the ability of NTHi to infect the Junbo mice (Vikhe *et al.*, 2018). A grading system of the Junbo bulla fluid from 1 to 5 was generated to account for the differences in middle ear fluid. Grade 1 was serous/ clear fluid and grade 5 was viscous/opaque fluid. Bulla fluid type was measured across 153 ears, highlighting variation in type from serous to more dense/viscous fluids

(Vikhe *et al.*, 2018). Higher bacterial titre was positively correlated with higher grade of fluid. Total cell count indicated a higher total cell count in the middle ear fluid of the inoculated mice compared to non-inoculated, suggesting the inoculation with NTHi causes an infiltration of cells into the middle ear.

The viability of the middle ear fluid cells was measured using flow cytometry to compare the proportion of necrotic and apoptotic cells in the fluid. A significant increase in the number of apoptotic cells was seen in higher grade fluid in Junbo mice inoculated with NTHi suggesting that NTHi infection stimulates apoptosis in the middle ear (Vikhe *et al.*, 2018). Lower grade fluids had a higher proportion of live cells compared to higher grade fluids. NTHi inoculation significantly increased live cell number in the higher-grade fluids. A higher number of necrotic cells was measured in higher grade fluid compared to lower grade fluid (Vikhe *et al.*, 2018). Increased numbers of live and necrotic cells in high grade NTHi positive fluids suggest the infiltration of cells into the middle ear cavity upon NTHi infection. These data show associations between the nature of the cells in the fluid, the properties of fluid and the presence of NTHi indicating a role of inflammation and fluid accumulation in NTHi infection.

Flow cytometry was used to identify the inflammatory cells infiltrating the middle ear (Vikhe *et al.*, 2018). Neutrophils were found in all grade fluids, with significantly higher numbers in higher grade fluids. NTHi inoculation did not alter neutrophil levels in lower grade fluids but caused a significant increase in the higher grade fluids (Vikhe *et al.*, 2018), indicating an infiltration of neutrophils following infection. Macrophage (CD11b+veF4/80+veLy6G-veLy6C-ve) number did not vary across the grades of fluid but were generally higher in lower grade fluids. Monocyte (CD11b+veLy6G-veLy6C-veF4/80±ve) number was constant across the grades of fluid in non-inoculated middle ears but increased in the inoculated mice (Vikhe *et al.*, 2018). Lymphocyte number (CD11b-veCD5+ve) was constant across grades of fluid for non-inoculated mice but significantly increased in number following NTHi inoculation. These results suggest a dynamic relationship between the infiltration of immune cells in response to NTHi, playing a role in the infection of the Junbo middle ear.

Cytokine expression was measured using immunoblotting to gain insight into the function of the innate immune cells within the fluid. An increase in the expression of cytokines and chemokines in the fluid with NTHi infection including IL-17A, IFN-gamma, TNF-α, VEGF-A, CCL2, MCP-5 and RANTES was measured compared to without NTHi infection (Vikhe *et al.*, 2018). Many of these cytokines and chemokines play a crucial role in inflammatory processes. Tumour necrosis factor-alpha (TNF-α) and vascular endothelial growth factor (VEGF-A) signal to recruit neutrophils. CCL2

is a chemokine that regulates the recruitment and differentiation of monocytes to the site of inflammation (Baba and Mukaida, 2014).

The cellular dynamics and the cytokine/chemokine response to NTHi in the higher grade, viscous fluids that support NTHi infection, were investigated to better understand the immune response to infection over time in the Junbo mice (Vikhe *et al.*, 2019). A gradual increase in fluid cell number was measured across a 7 day period following NTHi infection. The middle ear fluid of a non-inoculated Junbo mouse had a high number of neutrophils and necrotic cells. Sustained NTHi infection further increased the number of neutrophils and resulted in increased cellular necrosis in the fluid. In Junbo mice, NTHi infection first increased the number of neutrophils followed by the T-reg cell population in the fluid. An increase in T-reg cells results in an immune suppressive response by reducing the number of infiltrating immune cells to clear the infection, leading to longer-term infection (Vikhe *et al.*, 2019).

Cytokine and chemokine levels were measured to investigate the function of the infiltrated immune cells in high grade fluid following NTHi infection. The most significant changes in cytokine/chemokine levels across the 7 days of NTHi infection were for IL-12p40/p70, IL-4, and TGF-β (Vikhe *et al.*, 2019) which are involved in the Th2 and Th3 T-cell adaptive immune response. The changes in expression of these pro- and anti-inflammatory cytokines and chemokines in response to NTHi, alongside changes in cell count of different immune cells, provide insight to the pathology of the middle ear to better understand how the infection manifests and reoccurs in OM.

3.1.10 Conclusions

The Junbo mouse model, including the middle ear fluid dynamics, has been characterised thoroughly using microbiological and immunological techniques (Parkinson *et al.*, 2006; Hood *et al.*, 2016; Vikhe *et al.*, 2018, 2019). The Junbo mouse model for middle ear NTHi infection is a good animal model which recapitulates many aspects of the human disease including middle ear inflammation, effusion, infection and hearing loss. Understanding key aspects of the pathology in these mice is valuable to be able to utilise the model, to investigate the role of middle ear and cochlear macrophages in OM and inner ear pathology and begin to investigate the role of macrophages in the progression of hearing loss.

3.2 Aims and objectives

3.2.1 Overarching questions

The overarching question of this work is whether early life inflammatory events, like otitis media, predispose us to be at a greater risk of hearing loss later in life. Using the Junbo mouse model, we are investigating how macrophages in the auditory system respond to chronic inflammation and how chronic inflammation affects how these cells respond to an additional inflammatory challenge? To answer these questions, the following objectives will be addressed.

3.2.2 Objectives

- To characterise the middle ear inflammation/pathology in the Junbo mouse model for OM
- To characterise the population of macrophages in the middle ear mucosa and exudate in Junbo mice with and without NTHi infection overlaid – how do middle ear macrophages respond to chronic inflammation?
- To investigate the effect of middle ear inflammation on the cochlea
- To investigate the evidence of inflammation in the cochlea
- To characterise the population of macrophages in the cochlea in Junbo mice with and without NTHi overlaid – how do cochlear macrophages respond to chronic middle ear inflammation? Is there greater macrophage activation in the Junbo mice plus NTHi?
- What are the macrophage populations in the cochlea?
- Is there evidence of increased activation of macrophages in the cochlea?

3.2.3 Hypothesis

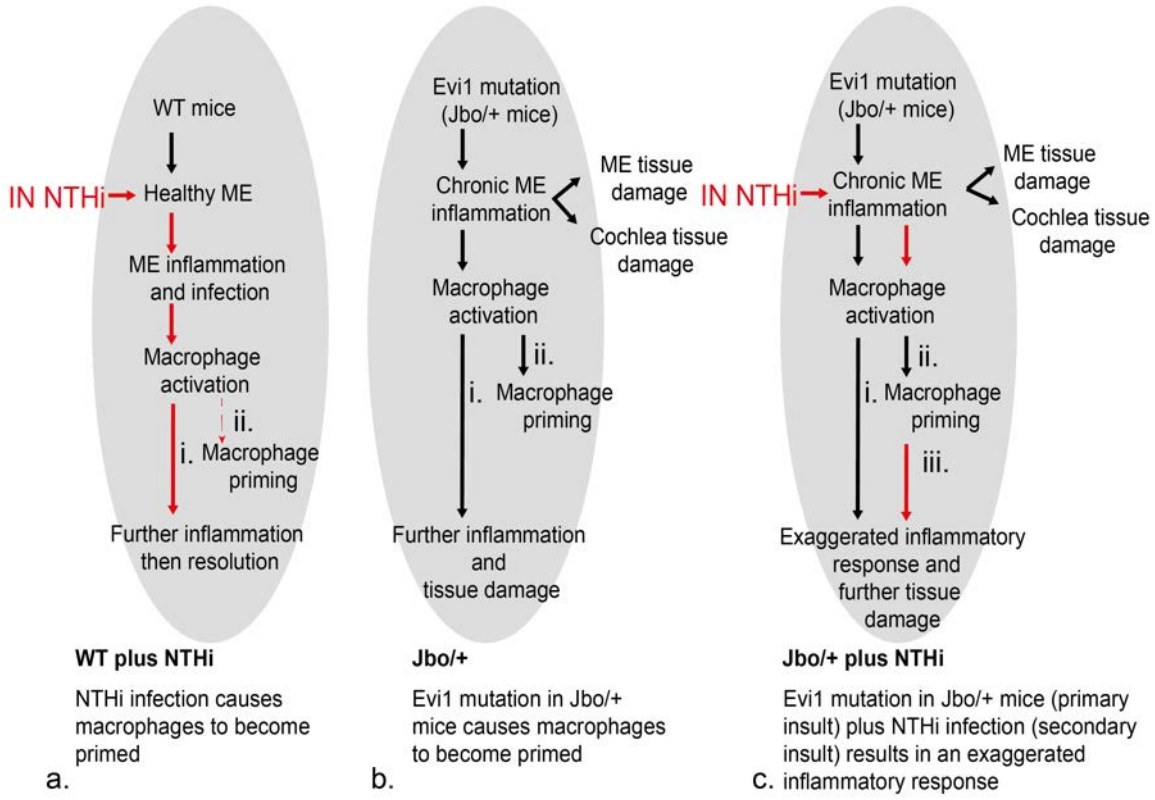


Figure 3.11 **The hypothesis proposing the role for macrophage activation and priming in middle ear and cochlea, in the model for NTHi middle ear infection in the Junbo mouse**

a. In WT mice with a healthy middle ear, a single inoculation of NTHi results in increased middle ear inflammation and infection (although in few mice (Hood *et al.*, 2016)). (i) This results in macrophage activation leading to further inflammation then resolution of the infection. (ii) Some activated macrophages may switch to a primed phenotype as a result of the infection with NTHi. If these primed macrophages are exposed to an additional insult (later in life), they will display an exaggerated inflammatory response leading to further tissue damage and loss of function.

b. Junbo mice develop chronic middle ear inflammation from 4-5 weeks of age. Cells within the inflamed middle ear cavity and cellular exudate will release inflammatory mediators, signalling to activate resident macrophages (i). Activated macrophages release pro-inflammatory mediators that will increase inflammation and cause further tissue damage. (ii) Some macrophages may switch to a primed phenotype as a result of the chronic middle ear inflammation.

c. In addition to this, following a single intranasal inoculation of NTHi, the macrophages in the primed phenotype (due to the middle ear inflammation) in the Junbo mice will display an exaggerated inflammatory response, releasing

increased levels of pro-inflammatory mediators such as Il-1β. This would cause further tissue damage and decreased function and inhibit the macrophages from resolving infection in OM.

3.3 Methods

The aim of the first phase of the study was to gather qualitative evidence to establish key validity of the model and likelihood of core hypotheses being correct. We used an opportunistic sampling method and obtained tissue from ongoing studies at MRC Harwell. Due to the low numbers of mice in this pilot study, quantitative analysis to allow for statistical analysis was not possible. However, to demonstrate the more detailed study plan including likely methods for quantification and statistical analysis that would be used for this study (if there were greater numbers of mice) some quantification and analysis has been carried out.

Sample size

The NC3Rs Experimental Design Assistant (EDA) tool is a web application designed to help researchers improve the design of animal experiments (EDA, 2021). The EDA compliments the NC3Rs ARRIVE guidelines for reporting animal research (Kilkenny *et al.*, 2010). Due to the opportunistic sampling method that was used in this study, a planned minimum sample size was not attained prior to the pilot study. However, the EDA tool was used for detailed study design and to carry out the power calculation to determine the sample size for a powered study. The effect size could be inferred from the pilot quantitative analysis, carried out in this chapter, investigating macrophage density in this mouse model. The primary outcome measure is the number of positively labelled macrophages per unit area. The effect size, d = 0.0004 number of cells/μm^2. Variability (standard deviation), obtained from data collected from a preliminary experiment, s = 0.00029 number of cells/μm^2. The NC3Rs power calculation tool for an unpaired t-test was used. Sample size (n) = 13 per group. Significance level, α = 0.05, power (1-β) = 0.9.

The formula for the power calculation:

$$n = 1 + 2C\left(\frac{S}{d}\right)^2$$

C is dependent on values chosen for significance level (α) and power (1-β) (Van Sluyters and Obernier, 2003). S is standard deviation and d is effect size.

3.3.1 The Junbo model

Junbo mice that were congenic on a C3H/HeH genetic background were used in most experiments (Parkinson *et al.*, 2006) with Junbo mice congenic on a BALB/c background used for comparative analysis. Detail of husbandry and microbiological surveillance of SPF Junbo and NTHi strain used for inoculation (Hood *et al.*, 2016).

For intranasal challenge, mice were inoculated intranasally at 4 or 11 weeks with 10^6 CFU NTHi 162sr (Hood *et al.*, 2016).

All mice were genotyped. Genotyping is carried out on ear biopsies (carried out by MLC) from the mice at 2 to 4 weeks of age. Genotyping services extract the DNA from the biopsies and qPCR is carried out on an automated system. Completed genotyping data is then passed on to the relevant scientific teams and the MLC through the in-house mouse information handling system (AnonyMus).

Mice were culled by an intraperitoneal overdose of sodium pentobarbital (euthatal). After culling, the mouse heads were placed in 10% neutral buffered formalin for 48 hours to fix. To decalcify, the skulls were placed in a large volume of D.F.B decalcifying agent (H.K. Kristenson) for 3-4 days until the tissue was soft and easily cut with a scalpel. The skulls were cut with a single incision bilaterally resulting in two half skulls, in a sagittal orientation. A second incision was made to remove the eyes and snout. Each half skull was placed in a histology cassette and placed in the tissue processor for 48 hours, whereby the tissue was put through different % alcohols and xylenes followed by paraffin to embed the tissue in a wax block for sectioning. Prior to commencing sectioning, the wax blocks were chilled on ice for 15 minutes. Sequential (5 µm) sections were cut and collected onto electrostatically charged slides and dried overnight at 37°C.

Samples were blinded prior to the tissue being made available for histological analysis. Each mouse was given a unique ID code, the codes were not un-blinded until after analyses of the immunohistochemical labelled sections. As the pathology associated with some mice is very evident, 20% of samples were re-blinded to the researcher after the first quantification by another member of the group and re-analysed. The variation between analysis 1 and 2 was compared and shown to have less than 5% variation.

3.3.2 Histological staining

For each mouse, 40 mid-modiolar cochlea and middle ear sections were obtained. 1 slide for every 10 out of each set were used for histological staining with Haematoxylin and Eosin (H&E) to ensure tissue quality and that directly comparative anatomical regions of the tissue were being studied (Cheeseman *et al.*, 2011; Hood *et al.*, 2016).

3.3.3 Immunohistochemistry

A sagittal cut was made in the skull before embedding and sectioning. Forty sections were collected from each tissue block, ten of which included a section through the cochlea with the

correct orientation for subsequent staining and image analysis. This resulted in one slide from each mouse labelled using the selected antibodies.

Iba1, CD68, TMEM119, IL-1β and anti-NTHi were used to characterise the macrophage populations in the middle ear, auditory nerve and cochlea. The secondary antibody and Vectastain Elite ABC kit was purchased from Vector labs. Colourisation was achieved using 3,3'-Diaminobenzidine (DAB) before counter staining with haematoxylin (Harris').

See appendix A.1 for a table of the primary and secondary antibodies used with their type, working dilution, host, catalogue number, manufacturer and details of the protocol stated.

3.3.4 Imaging acquisition

Images of the labelled sections were captured using a Nikon Microscope. Images were obtained using the 4x, 10x and 20x objectives and the 40x and 100x oil immersion objectives. ImageJ software was used to view and analyse the images.

For initial qualitative analysis and identification of the anatomy and cell locations within it, multiple images were taken at several magnifications for each region of interest. These include: middle ear mucosa and fluid, auditory nerve, stria vascularis, spiral ganglion neurons in the modiolus and organ of Corti. An image for quantification was taken at 20x and 40x magnification in the position of the middle ear mucosa and auditory nerve indicated in Figure 3.22a and Figure 3.30a.

3.3.5 Qualitative and quantitative image analysis

Qualitative analysis of the middle ear and cochlear histopathology of this mouse model, including the distribution of innate immune cells within the middle and inner ear, was carried out. Quantitative analysis was carried out to determine the number of positively labelled cells in the middle region of the mucosa and auditory nerve. The mean thickness of the middle ear mucosa was measured to give an indication of middle ear inflammation. Images of sections labelled with Iba1 and CD68 were measured using ImageJ, three measurements of mucosal thickness (μm) were made per section and the mean was calculated.

An image was captured in the same anatomical region of the auditory nerve and middle ear mucosa for each slide and marker at 20x and 40x magnification. Staining and quantification was carried on one section per antibody per mouse. Quantification of macrophages/microglia was carried out by manually counting cells using ImageJ. Cells were counted if there was brown staining (DAB chromogen) clearly associated with a cell body, identified by a visible cross-section

through the nucleus. Regions of interest within sections were highlighted using the drawing tool in ImageJ. The area of the tissue was calculated after the removal of areas of bone or other clearly identifiable structures within the tissue such as blood vessels. All images were processed using ImageJ to determine cell density (number of cells/μm^2).

The number of positively labelled macrophages per unit area (μm^2) in the middle ear mucosa (Iba1+ and CD68+) were measured. The number of positively labelled microglia per unit area (μm^2) in the auditory nerve (Iba1+ and TMEM119+) were measured.

3.3.6 Statistical analysis

To demonstrate how inferential statistical tests might be applied to the data, the assumption has been made that the data were normally distributed. This has not been confirmed due to the small sample size. If an adequate sample size was achieved, a Shapiro-Wilks test would be used to test for normality. If the data were not normally distributed, non-parametric equivalent tests would be carried out e.g., Kruskall-Wallis instead of the one-way ANOVA and the Mann-Whitney U instead of the two-way ANOVA.

Initial analysis comparing the middle ear histopathology in the 4- and 12-week-old mice (WT plus NTHi and Junbo plus NTHi) suggested minimal differences between the WT and Junbo mice at 4-weeks-old. This is likely because the Junbo phenotype is only fully expressed around 4-5 weeks of age. Therefore, the data for the 4-week-old mice were excluded from the one-way ANOVA. The number of macrophages/microglia per unit area (μm^2) (in the middle ear mucosa or auditory nerve, using varying markers) and the average thickness of the middle mucosa (μm) for 12-week-old mice were analysed using a one-way ANOVAs and LSD multiple comparison tests for post hoc testing. The number of macrophages/microglia per unit area (in the middle ear mucosa or auditory nerve, using varying markers) for the 4- and 12-week-old WT plus NTHi and Junbo plus NTHi mice were analysed by a two-way ANOVA. Where post hoc comparisons were found to be significant, data are presented as mean±95% confidence intervals (CI). $P<0.05$ were considered statistically significant.

3.3.7 Groups of mice and study design

- 4-weeks-old – WT plus NTHi (3 days) (n = 2), Jbo plus NTHi (3 days) (n = 2)
- 12-weeks-old – WT plus NTHi (7 days) (n = 2), Jbo plus NTHi (7 days) (n = 2), Jbo no NTHi (n = 4)
- 15-weeks-old – Jbo plus NTHi (28 days) (n = 5), Jbo no NTHi (28 days) (n = 3), WT no NTHi (28 days) (n = 4) (future work)

Table 3.2 **Number of available mice for each experimental group. The 15-week-old samples will be investigated for future work**

Age	**WT**	**WT plus NTHi**	**Junbo**	**Junbo plus NTHi**
4-weeks-old	n = 0	n = 2	n = 0	n = 2
12-weeks-old	n = 0	n = 2	n = 4	n = 2
15-weeks-old (future)	n = 4	n = 0	n = 3	n = 5

Note: NTHi doesn't survive in WT mice so there would be little point in aging infected mice for 28 days.

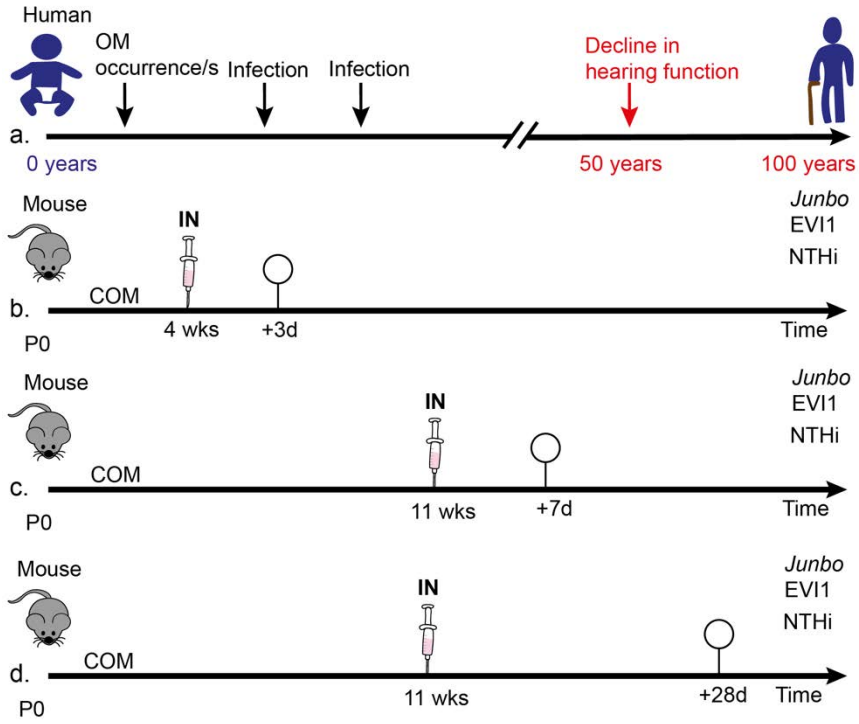


Figure 3.12 **Timeline of OM for humans (a) and the model for NTHi middle ear infection in the Junbo mutant mouse (b)**

a. Timeline for someone who had OM in early life, then were exposed to varying infections throughout their lifetime. This individual experienced a decline in hearing function later in life.

b – d. The experimental timelines in this study, whereby the Junbo mice spontaneously develop chronic middle ear inflammation by the age of 4-5 weeks.

b. The first group of mice (4-weeks-old) received a single intranasal inoculation of NTHi at 4-weeks-old and were culled 3 days later.

c. The second group (12-weeks-old) received a single inoculation of NTHi at 11-weeks-old and were culled 7 days later.

d. The third group (15-weeks-old) received a single inoculation of NTHi at 11-weeks-old and were culled 28 days later.

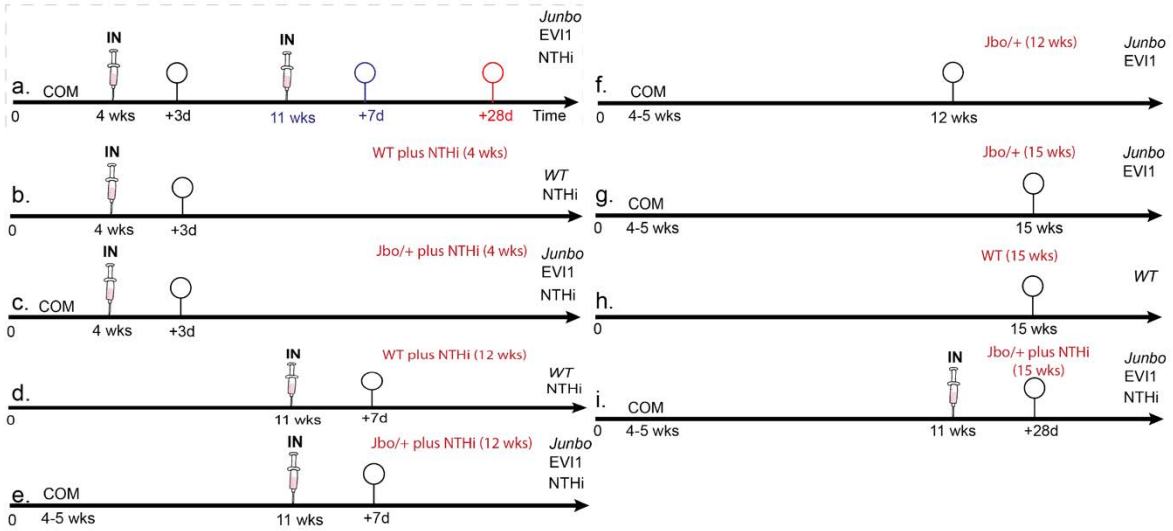


Figure 3.13 **Timelines of study design for the groups of mice used in this pilot study**

a. An overall timeline summarising the treatments involving the WT and Jbo/+ mice in this study.

b,c. A timeline for 4-weeks-old WT (b) and Jbo/+ (c) mice given intranasal inoculation of NTHi and culled 3 days later.

d,e. A timeline for 11-weeks-old WT (d) and Jbo/+ (e) mice given intranasal inoculation of NTHi and culled 7 days later.

f. A timeline for 12-weeks-old Jbo/+ mice.

g. A timeline for 15-weeks-old Jbo/+ mice.

h. A timeline for 15-weeks-old WT mice

i. A timeline for 11-weeks-old Jbo/+ mice given an intranasal inoculation of NTHi and culled 28 days later.

3.4 Results

3.4.1 Histological staining of mid-modiolar cochlea and middle ear sections to determine overall middle ear histopathology

Previous analysis of the middle ears of Junbo (Jbo/+) mice, using histochemistry, highlighted variability between the mice in the composition of the middle ear fluid ranging from serous/clear with no NTHi infection to viscous/cloudy with high NTHi infection rate (Hood *et al.*, 2016; Vikhe *et al.*, 2018). Haematoxylin & Eosin (H&E) stain was used to provide information of the gross structure of the tissue, to identify and establish any differences that exist between the WT and Jbo/+ mice and WT and Jbo/+ mice after NTHi inoculation; examining the middle ear histopathology. Figure 3.14 shows a typical mid-modiolar cochlea and middle ear section from a WT mouse, labelled to highlight key regions of the auditory nerve, cochlea and the middle ear cavity. A mid-modiolar sagittal section allows the visualisation of all cochlear turns and the organ of Corti, stria vascularis and spiral ganglion region. The air-filled middle ear cavity is on the right-hand side of the image.

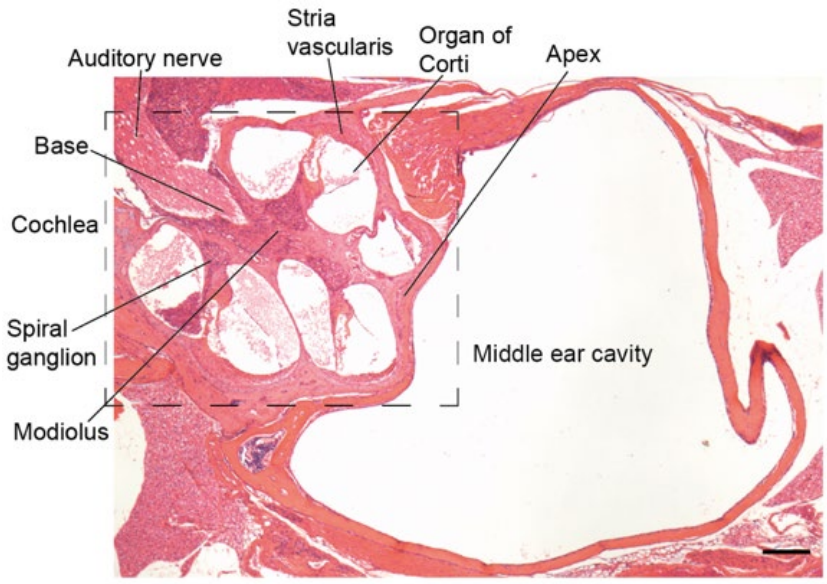


Figure 3.14 **Mid-modiolar cochlea and middle ear section of wild-type mouse**

A mid-modiolar cochlea and middle ear section histologically stained. Key regions labelled including cochlea, spiral ganglion, organ of Corti, stria vascularis, modiolus, middle ear cavity and auditory nerve. Histochemical stain = Haematoxylin and Eosin. Scale bar = 200 μm.

The panel of images in Figure 3.15 displays a representative, low magnification image of the groups of mice investigated in this study. Due to the pilot nature of this study and the way in the

tissue was attained through a collaboration with a group investigating the microbiology of this model, there are some groups of mice we were not able to analyse (indicated by the diagonal lines in Figure 3.15). However, by comparing different groups of mice we have been able to effectively characterise the middle ear histopathology of the Junbo mouse model for OM.

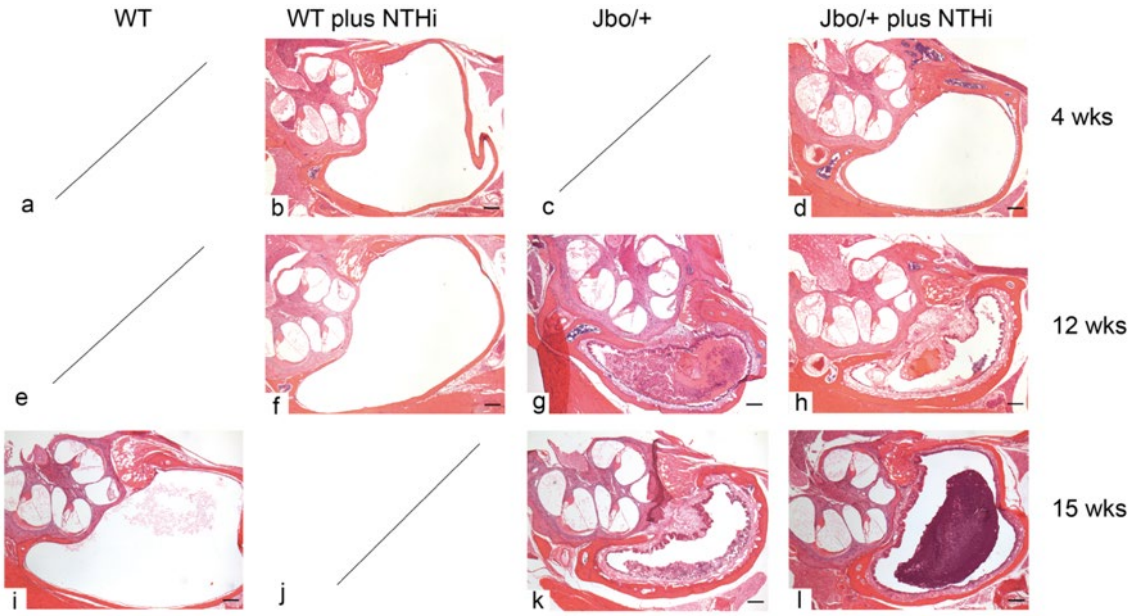


Figure 3.15 **Mid-modiolar cochlea and middle ear sections of WT and Jbo/+ mice; an example image of the groups of mice in this study**

Mid-modiolar cochlea and middle ear sections histologically stained to show the overall morphological features of three different age groups of WT (**i**), WT plus NTHi (**b, f**), Jbo/+ (**g, k**) and Jbo/+ plus NTHi (**d,h,j**) mice. The time points include: 4-weeks-old WT (n=2) (**b**) and Jbo/+ (n=2) (**d**) mice culled 3 days following NTHi inoculation, 12-weeks-old Jbo/+ mice (no NTHi) (n=4) (**g**) and 12-weeks-old WT (n=2) (**f**) and Jbo/+ (n=2) (**h**) mice culled 7 days following NTHi inoculation and 15-weeks-old WT (n=4) (**i**) and Jbo/+ (n=3) mice (**k**), 15-weeks-old Jbo/+ mice culled 28 days following NTHi inoculation (n=5) (**l**). The diagonal line represents no available tissue for this group. Histochemical stain = Haematoxylin and Eosin Scale bar = 200 μm.

There is heterogeneity in the morphology of the middle ear cavities in the Junbo mouse model. A grading system from 1-5 for the middle ear/fluid was generated in a similar manner to the grading system described previously (Vikhe *et al.*, 2018). Table 3.3 includes a description of the characteristics of middle ear cavities from 1-5. This grading system allows the categorisation of the Junbo mice middle ears based on the observed differences of the anatomy.

Table 3.3 **Description of the grading system (1-5) for the middle ear cavities of the Junbo mice**

Grade	Description
1	Clear cavity, no inflamed mucosa
2	Thin layer of inflamed mucosa with some fluid
3	Inflamed, fluid-filled cavity
4	Inflamed, fluid-filled cavity with lower volume of clear space
5	Highly inflamed, darkly stained, highly viscous, fluid-filled cavity

The images of H&E stained sections demonstrate differences in the middle ear cavities and fluid. The middle ear in Figure 3.16a is grade 1, as the middle ear cavity is clear with no inflamed mucosa (WT). The middle ear in Figure 3.16b is grade 2, as there is a thin layer of inflamed mucosa. The middle ear in Figure 3.16c is grade 3, as there is a layer of inflamed mucosa around the perimeter of the middle ear. The middle ear in Figure 3.16d is grade 4, as there is an inflamed fluid-filled middle ear cavity. The middle ear in Figure 3.16e is a grade 5 middle ear cavity, as the middle ear is an inflamed, viscous, and cellular fluid-filled cavity. There is heterogeneity between the viscosity, opacity and cellularity of the middle ear fluids of the Junbo mice, similar to what is observed in human OM.

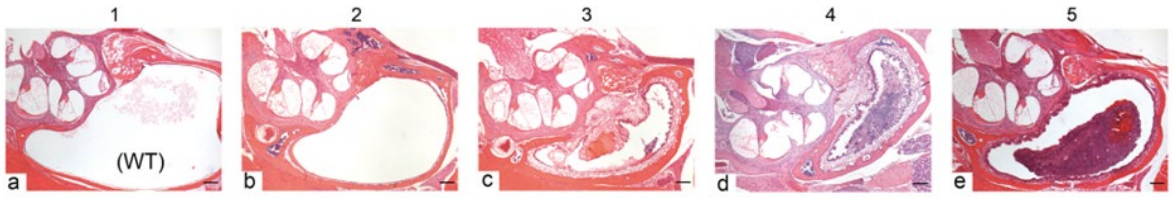


Figure 3.16 **Mid-modiolar cochlea and middle ear sections of Junbo mice showing an image of middle ear cavities graded from 1 to 5**

The morphology of middle ear cavities ranging from a clear cavity with no inflamed mucosa or fluid in a WT mouse (**a**) to middle ear cavities with increasing amounts of inflammation and fluid accumulation resulting in a middle ear cavity with a dense cellular exudate and inflamed mucosal membrane (**e**). Histochemical stain = Haematoxylin and Eosin. Scale bar = a-e 200 μm.

Histological staining was used to characterise and compare the morphology of the middle ear and cochlea of the WT and Jbo/+ mice used in this study. Figure 3.17a-c shows differences in the morphology of the middle ear cavities, and fluid that fill the cavities, between the WT and Jbo/+ mice. The Jbo/+ mice (with and without receiving NTHi inoculation) demonstrate inflamed middle

ears of varying degree, with differences in the cellularity and properties of fluid. WT mice have a clear, air-filled middle ear space with no visible inflammation (Figure 3.17a). The mucosa, lining the middle ear cavity, is thin and consists of ciliated epithelial cells in an organised distribution (indicated by the box in Figure 3.17g). Jbo/+ mice have an inflamed middle ear mucosa, which varies in cellularity between Jbo/+ mice and following NTHi inoculation. The mucosal membrane is biconcave, with many invaginations around the perimeter of the middle ear cavity. The ciliated epithelial cells appear disorganised in the chronically inflamed middle ears (Figure 3.17h and i). The middle ear fluid in the Jbo/+ mice has a dense population of neutrophils (Figure 3.17k and o) and foamy macrophages, observed in the dense cellular fluid (Figure 3.17l). Clusters of red blood cells (RBCs) are visible in the inflamed mucosa of Jbo/+ mice (Figure 3.17n).

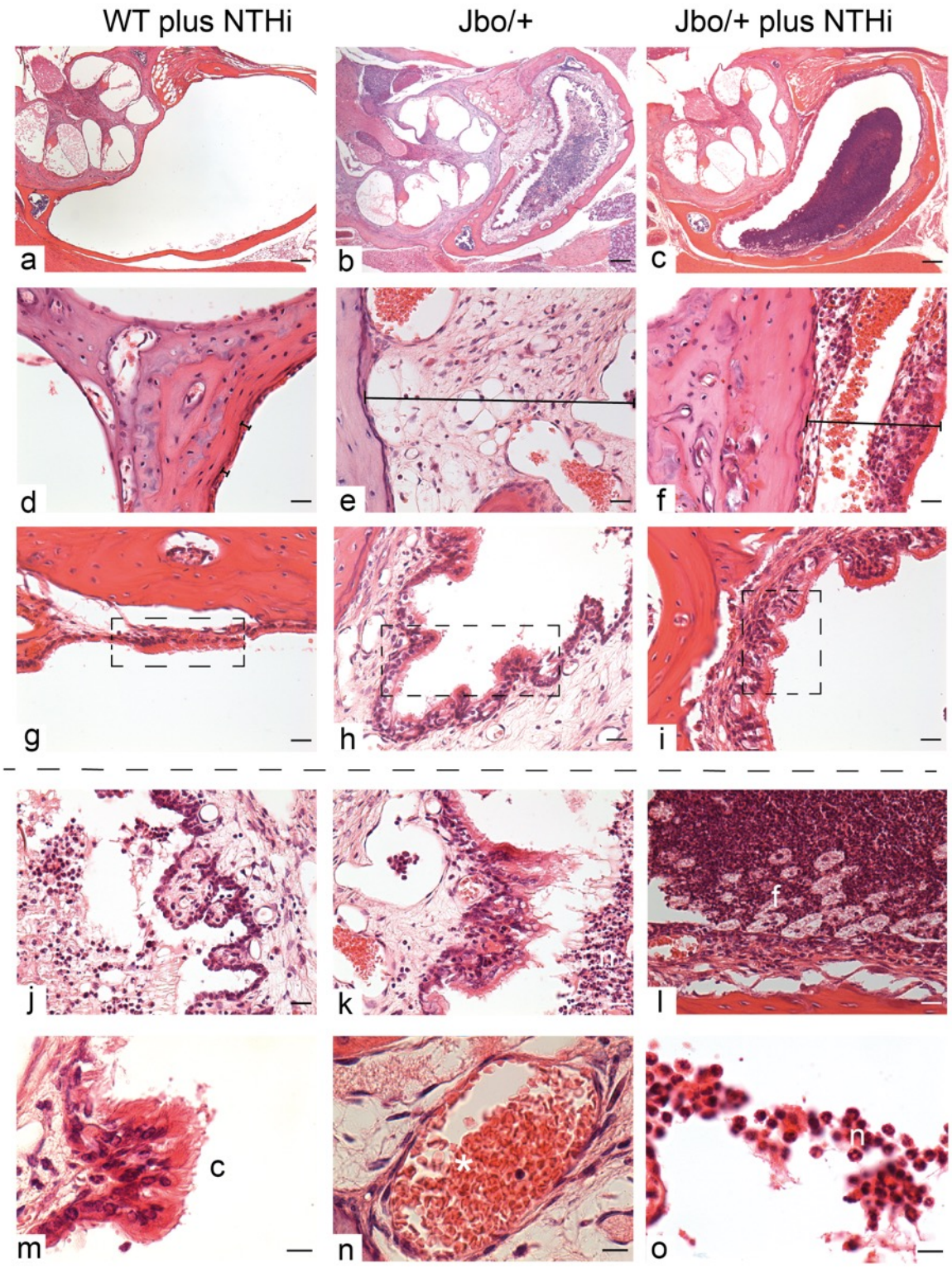


Figure 3.17 **Middle ear histopathology in Jbo/+ mice and WT and Jbo/+ mice after inoculation with NTHi**

Jbo/+ mice have heterogenous, inflamed middle ear cavities from 4-5 weeks old. Cochlea and middle ear sections of WT plus NTHi (**a**), Jbo/+ (**b**) and Jbo/+ plus NTHi mice (**c**). Vast heterogeneity is seen between the middle ear cavities in the Jbo/+ mice with levels of inflammation and cellular fluid. Mucosal hyperplasia is observed

in the middle ear cavities of the Jbo/+ mice (**e, f**) evidenced by a dense cellular mucosa, compared to the thin mucosa in WT mice (**d**). Ciliated epithelial cells line the middle ear cavity in a thin, organised distribution, as shown in (**g**) indicated by the box. Jbo/+ mice display an inflamed, densely cellular mucosa lining the middle ear cavity which has many invaginations. The ciliated epithelial cells appear disorganised (**e, f**) compared to that seen in WT mice (**g**). Characteristics of the inflamed middle ear cavities found in Jbo/+ mice include a dense, cellular mucosa lining the cavity (**j,k**) and a cellular fluid containing neutrophils and foamy macrophages (**l**). Higher magnification view of ciliated epithelial cells (**m**), RBCs in the inflamed middle ear mucosa indicated by the white asterisk (**n**) and neutrophils in the middle ear fluid (**o**). Histochemical stain = Haematoxylin and Eosin. Scale bar = a-c 200 μm, d-l 20 μm, m-o 10 μm.

3.4.2 Immunohistochemical staining to investigate middle ear inflammation and macrophage populations in the Junbo mouse model for otitis media

3.4.2.1 Middle ear inflammation in the Junbo mouse model for otitis media

Junbo mice spontaneously develop chronic middle ear inflammation from 4-5 weeks of age. We aimed to characterise this inflammation using various histological methods and immunohistochemical markers, to investigate the macrophage populations in the middle ear. Ionised calcium binding adaptor molecule 1 (Iba1) (Wako, Alpha labs, 1:1000 dilution) is a microglia and macrophage specific antibody that was used to investigate the macrophage populations in the middle ear and cochlea of the 4- and 12-week-old mice. Figure 3.18a-c shows morphological differences in the middle ear cavities. Figure 3.18d-l demonstrate inflammation across the middle ear mucosa and fluid. There is a high expression of Iba1-positive cells in the middle ear mucosa of Jbo/+ mice (Figure 3.18d-h). Iba1-positive foamy macrophages are distributed on the far side of the middle ear cavity, amongst the dense cellular exudate (Figure 3.18i). Large, amoeboid Iba1-positive cells are found in close proximity to the foamy macrophages. A high number of neutrophils can be observed in the fluid of both Jbo/+ (Figure 3.18j) and Jbo/+ plus NTHi mice (Figure 3.18k) middle ear fluids. In Junbo mice inoculated with NTHi, many of the neutrophils in the middle ear cavity are positively labelled with NTHi suggesting the neutrophils have engulfed and phagocytosed the NTHi. Following infection, there is a rapid infiltration of neutrophils. Alongside macrophages, their role is to clear bacteria and cellular debris to resolve the infection. A dense, cellular fluid results in a lack of bacterial clearance leading to an increased likelihood of re-emergence of infection. In Figure 3.18l, the tetrachrome stain highlights the rich blood supply in the inflamed mucosa of Jbo/+ mice as RBCs appear yellow.

During bacterial infection, endotoxin, a component of the bacterial cell wall, initiates inflammation by inducing an increase in the production of inflammatory mediators. These signal to increase vascular permeability and epithelial secretory activity, leading to middle ear inflammation and effusion.

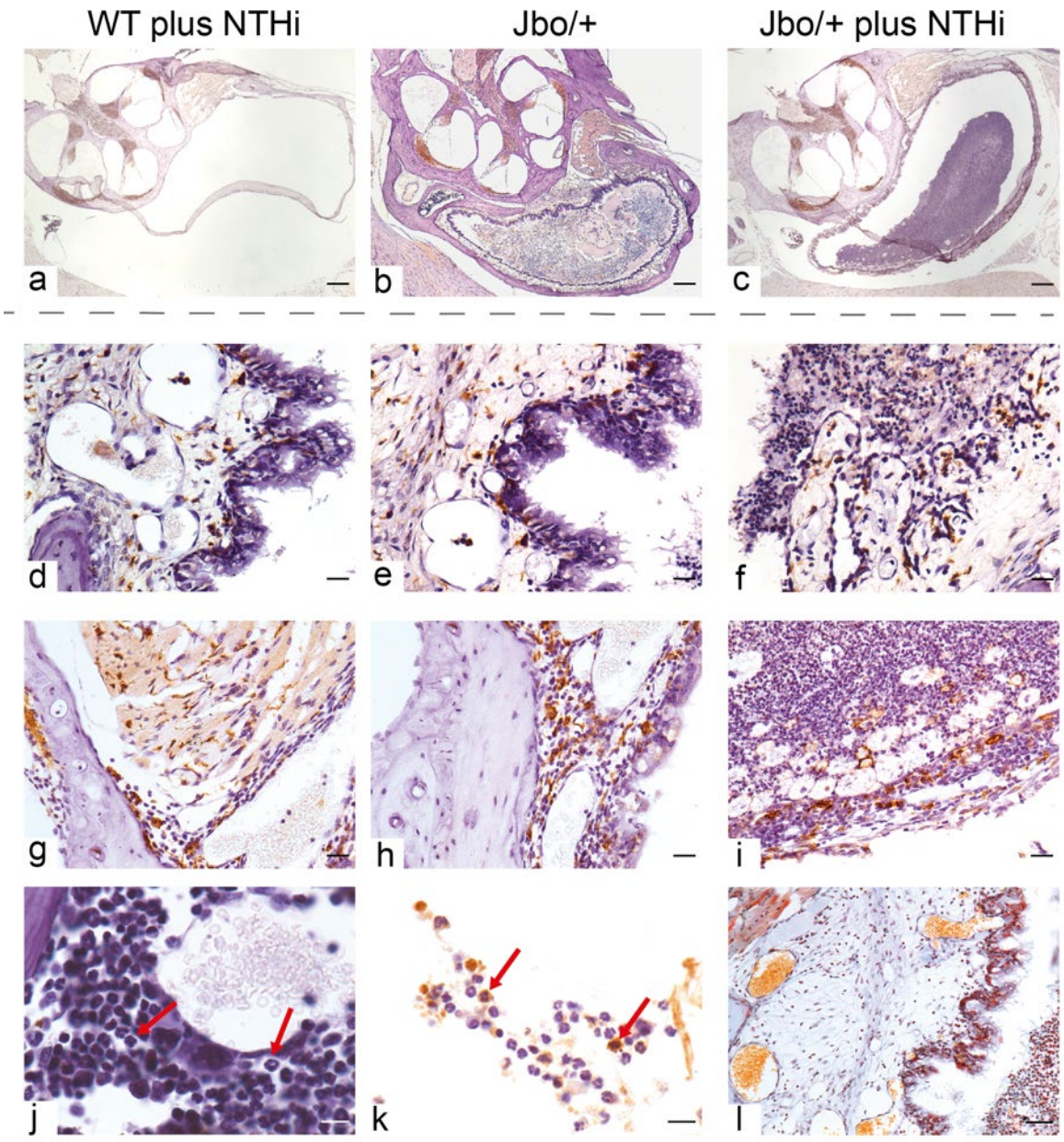


Figure 3.18 **Junbo mice have increased mucosal inflammation, cellular exudate and active immune cells within the middle ear cavity**

a-c. Iba1 stained mid-modiolar cochlea and middle ear sections from 12-week-old Jbo/+ mice and WT +/+ and Jbo/+ mice after NTHi inoculation. Jbo/+ mice (**b, c**) have

inflammation and fluid deposition in the middle ear cavities, compared to the air-filled space in WT mice (**a**).

d-l. Evidence of middle ear inflammation, dense cellular exudate and active immune cells in Jbo/+ mice. Highly inflamed mucosa and a viscous fluid containing many positively labelled macrophages (Iba1+) in an activated, amoeboid morphology (indicated by an arrowhead) (**d, e, f**). Layer of thickened mucosa close to the apex of the cochlea, with several Iba1-positive macrophages (**g, h**). Dense cellular exudate with foamy macrophages (**i**).

A high number of neutrophils are observed in the middle ear fluid of both Jbo/+ (**j**) and Jbo/+ mice following inoculation with NTHi (**k**), highlighted with red arrows. Some neutrophils in (**k**) are positively labelled with NTHi. Pools of RBCs (yellow) are identified in the inflamed middle ear mucosa. Immunohistochemical marker = Iba1 (a-i, l), NTHi (j, k). Counterstain = Haematoxylin. Scale bar = a-c 200 μm, d-i 20 μm, j-k 10 μm, l 50 μm.

3.4.2.2 NTHi expression in the Junbo mouse model for otitis media

Previous studies demonstrated that pre-existing middle ear inflammation and cellular fluid in Jbo/+ mice was required for successful colonisation and infection following intranasal inoculation with NTHi (Hood *et al.*, 2016). Using an antibody specific to the strain of NTHi inoculated (Anti NTHi-162, MRC Harwell, 1:5000 dilution), we investigated the expression of NTHi in the middle ears of WT and Jbo/+ mice following intranasal inoculation with NTHi. Jbo/+ mice have higher NTHi expression in the middle ear mucosa and fluid compared to WT mice after NTHi inoculation. As shown in Figure 3.19a, NTHi expression is very low in the middle ear of the WT mouse. A NTHi-positive neutrophil is visible in the apical turn of the cochlea (Figure 3.19c) and a larger, rounder NTHi-positive cell, likely to be a macrophage, is highlighted in the mucosa (Figure 3.19d). As shown in Figure 3.19b, Jbo/+ mice have a high number of NTHi-positive cells in the middle ear mucosa (Figure 3.19e) and fluid (Figure 3.19f). These findings indicate that Junbo mice have increased susceptibility to middle ear infection following NTHi inoculation.

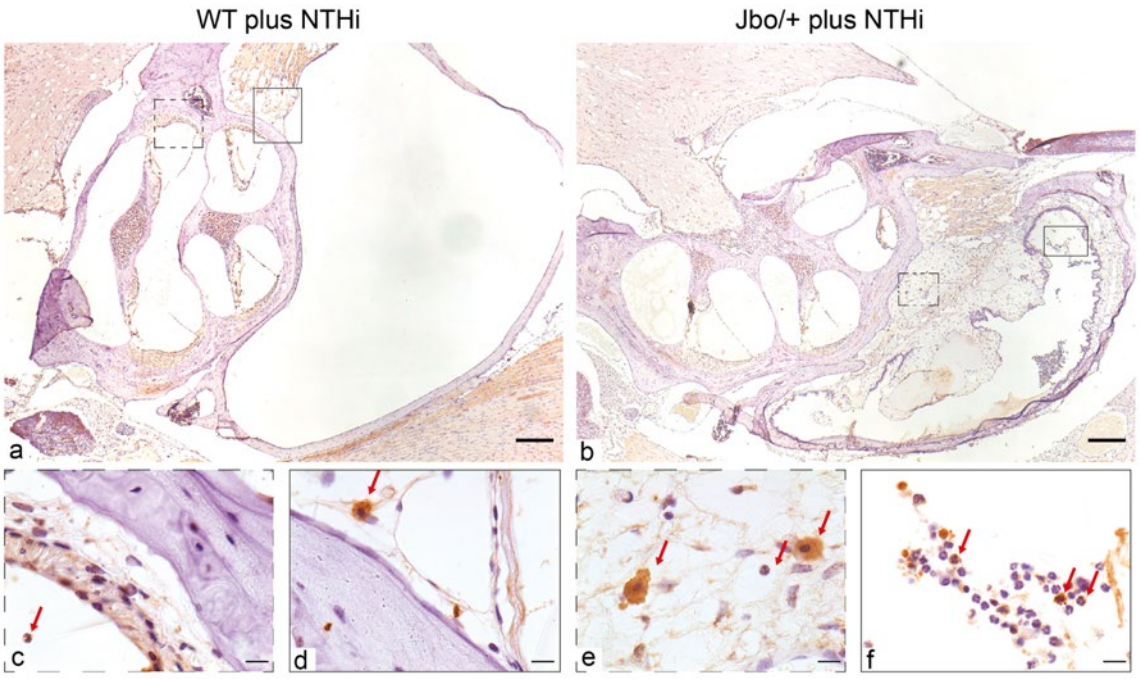


Figure 3.19 **Jbo/+ mice have increased susceptibility to middle ear NTHi infection following intranasal inoculation with NTHi**

NTHi stained mid-modiolar cochlea and middle ear sections from a 12-week-old WT (**a**) and Jbo/+ mouse (**b**) following inoculation with NTHi (culled 7 days later). Following inoculation, Jbo/+ mice with inflamed middle ear cavities filled with thick cellular exudate, had higher expression of NTHi-positive cells within the middle ear fluid and mucosa compared to WT mice. WT mice with air-filled cavities showed no NTHi infection, with only a small number of identifiable NTHi-positive cells in the mucosa lining the cavity.

c-d. A NTHi-positive cell in the WT mouse following NTHi inoculation. A NTHi-positively labelled neutrophil was observed in the apical turn of the cochlea (**c**), higher magnification view as an insert in (**c**). A NTHi-positive cell in the mucosa of the middle ear cavity, close to the apex of the cochlea (**d**).

e-f. NTHi-positively labelled cells in a Jbo/+ mouse following NTHi inoculation. There were many positively labelled cells in the mucosa and fluid. The arrows indicate amoeboid cells, likely to be macrophages, which are NTHi-positive (**e**). The arrows in (**f**) indicate NTHi-positively labelled neutrophils in the middle ear fluid.

Immunohistochemical marker = NTHi. Counterstain = Haematoxylin. Scale bar = a-b 200 μm, c-f 10 μm.

3.4.2.3 Macrophage populations in the middle ear mucosa and fluid

Iba1 (Wako, Alpha labs, 1:1000 dilution) is a macrophage and microglia specific marker. Iba1 is a calcium binding protein involved in membrane ruffling and phagocytosis of activated microglia and macrophages. The morphology of Iba1-positive cells gives an indication of the function and phenotype of the cell. Activated macrophages have a large, round amoeboid morphology, whereas homeostatic macrophages/microglia have a smaller cell body with a greater number of processes.

WT mice have a thin layer of mucosa lining the cavity of the middle ear. Few Iba1-positive cells are observed within this mucosa (Figure 3.20a). Jbo/+ mice have an inflamed mucosa with a population of large, amoeboid Iba1-positive macrophages (Figure 3.20b). Jbo/+ mice after NTHi inoculation have a densely cellular inflamed mucosa with a high number of Iba1-positive, activated macrophages (Figure 3.20c).

There is a high Iba1 expression in the middle ears (mucosa and fluid) of Jbo/+ mice and potentially even greater expression in the Jbo/+ mice with NTHi infection overlaid. The number of macrophages and number of activated macrophages, based on morphology, are increased in Jbo/+ mice compared to WT mice.

In Jbo/+ mice with a dense cellular exudate, a population of foamy macrophages is on the far side of the middle ear cavity (Figure 3.20h and i). Foamy macrophages are associated with necrotic changes in tissue and can be identified based on the circular morphology. The viscous middle ear fluid in Jbo/+ mice (with and without NTHi) consists of several populations of macrophages including activated Iba1-positive, CD68-positive and foamy macrophages. Neutrophils make up a large proportion of the middle ear fluid, identified by their distinctive morphology.

CD68 (Bio-Rad, FA-11, 1:250 dilution) is a lysosomal protein expressed in high levels in macrophages and activated microglia. CD68-positive cells are phagocytic macrophages involved in engulfing and clearing cellular debris. The cells have a large, round morphology. In WT mice, similar to the expression of Iba1-positive cells, there are few CD68-positive macrophages in the thin mucosal lining of the middle ear cavity (Figure 3.20d). In the inflamed middle ear cavity of Jbo/+ mice, CD68-positive cells are large and amoeboid shaped (Figure 3.20e) and are distributed mainly in the mucosa rather than the exudate. In Jbo/+ mice that have been inoculated with NTHi, CD68-positive macrophages are found in both the inflamed mucosa and the dense cellular exudate. However, overall, a higher population are distributed in the inflamed mucosa (compare

Figure 3.20f and Figure 3.20l). There appears to be greater expression of CD68-positive cells in Jbo/+ mice inoculated with NTHi compared to Jbo+/ mice (non-inoculated). Compared with Iba1 expression, there is lower overall expression of CD68-positive macrophages in the inflamed Jbo/+ middle ears.

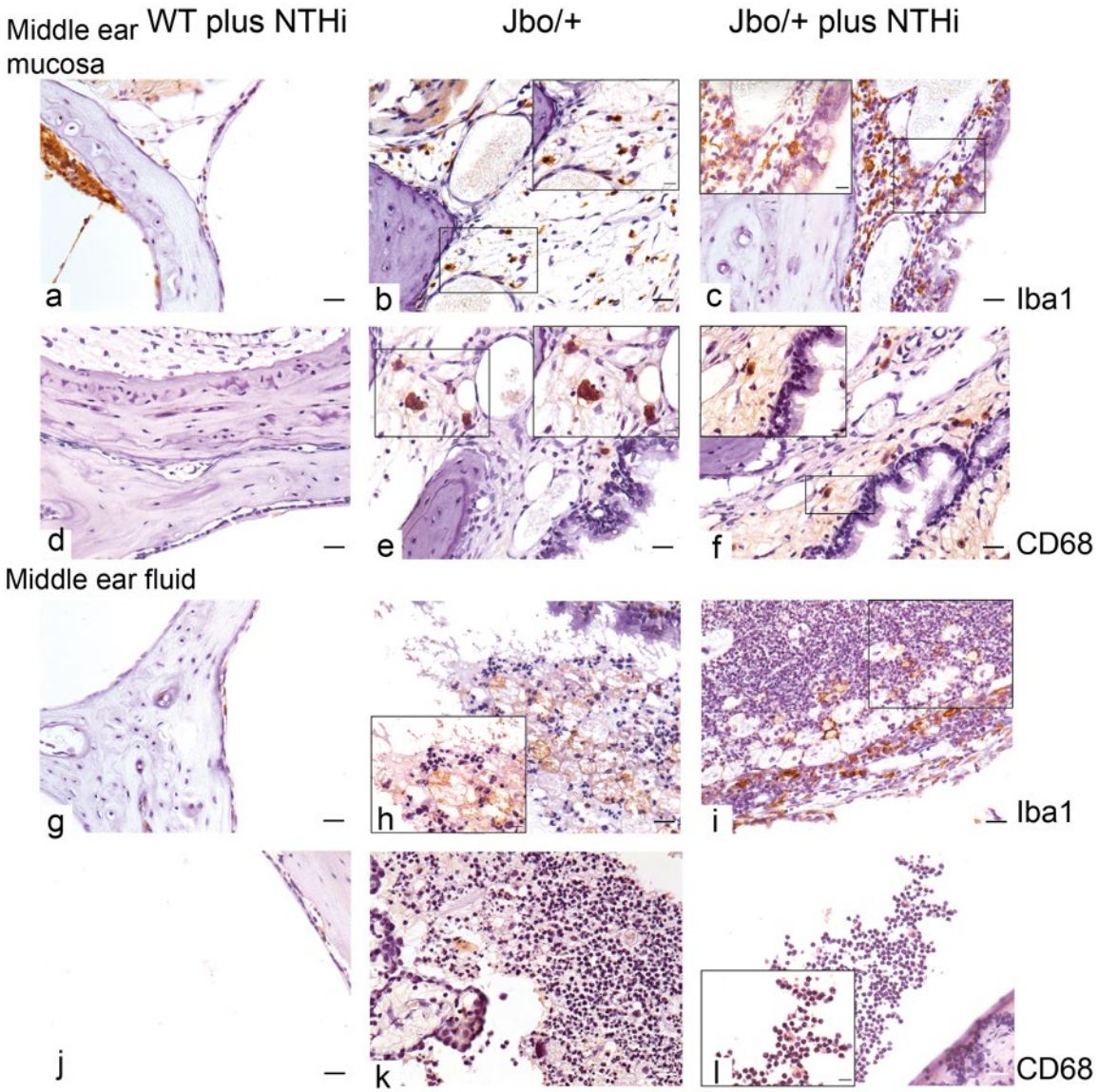


Figure 3.20 **Macrophage populations in the middle ear of Jbo/+ mice and WT and Jbo/+ mice after NTHi inoculation**

Two different macrophage markers, Iba1 and CD68, have been used to characterise the populations of macrophages in the middle ear mucosa lining the cavity and in the middle ear fluid of mice with inflamed middle ears.

a-c. Iba1-positive cells in the mucosa of WT plus NTHi (**a**), Jbo/+ (**b**) and Jbo/+ mice following NTHi inoculation (**c**).

d-f. CD68-positive cells in the mucosa of WT plus NTHi (**d**), Jbo/+ (**e**) and Jbo/+ mice following NTHi inoculation (**f**).

g-i. Iba1-positive cells in the middle ear fluid of Jbo/+ (**h**) and Jbo/+ after NTHi inoculation (**i**) (n.b. no fluid in WT plus NTHi mice (**g**)).

j-l. CD68-positive cells in the middle ear fluid of Jbo/+ (**k**) and Jbo/+ after NTHi inoculation (**l**) (n.b. no fluid in WT plus NTHi mice (**j**)). Immunohistochemical markers = Iba1 and CD68. Counterstain = Haematoxylin. Scale bar = 20 μm, inserts = 10 μm.

We have identified several macrophage populations within the middle ear of the WT and Jbo/+ mice based on morphology and positive staining with Iba1 and CD68. To investigate the function of these cells, a marker for the pro-inflammatory cytokine Il-1β (Peprotech, 500-P51, 1:50 dilution) was used. Macrophages in an activated and pro-inflammatory state produce Il-1β, an inflammatory mediator, which signals to surrounding cells to enhance the inflammatory response. Consecutive tissue sections were stained with Iba1 and Il-1β to investigate whether there was any co-expression between these two markers on the consecutive tissue sections. Iba1-positive macrophages on one slide were matched, based on positioning, to determine whether there was Il-1β expression on the consecutive slide in the same cell. Co-expression would indicate a macrophage in a pro-inflammatory state. Macrophages, previously exposed to an inflammatory insult may be in a primed state. Upon an additional inflammatory challenge, primed macrophages produce increased levels of Il-1β.

In WT mice (after NTHi inoculation), there are few pro-inflammatory macrophages in the middle ear mucosa (red arrow indicating a pro-inflammatory macrophage in Figure 3.21a and d). In Jbo/+ mice, there are several pro-inflammatory macrophages in the inflamed mucosa as indicated in the consecutive sections with a red arrow (Figure 3.21b and e). Similarly, in Jbo/+ mice following NTHi inoculation, there are several pro-inflammatory macrophages co-expressing Iba1 and Il-1β in the mucosa (Figure 3.21c and f).

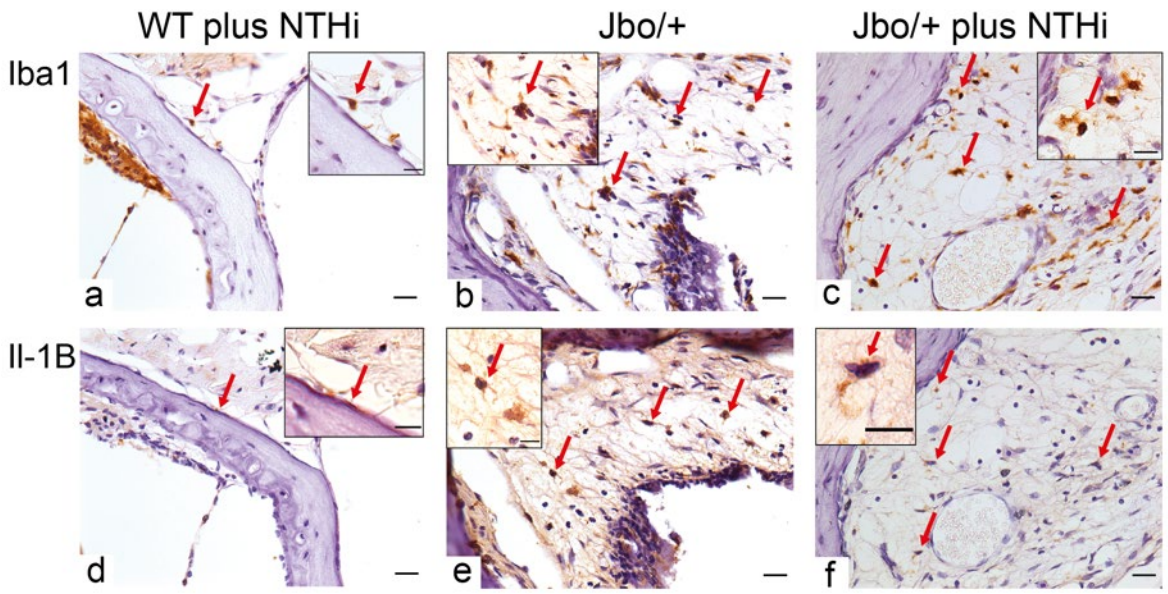


Figure 3.21 **Evidence of pro-inflammatory macrophages in the middle ear mucosa**

Consecutive slides were stained with the macrophage marker Iba1 and the pro-inflammatory cytokine marker Il-1β to investigate the function of the mucosal macrophages.

a-c. Iba1-positive macrophages in the middle ear mucosa of WT plus NTHi (**a**), Jbo/+ (**b**) and Jbo/+ plus NTHi mice (**c**).

d-f. Il-1β-positive macrophages in the middle ear mucosa of WT plus NTHi (**d**), Jbo/+ (**e**) and Jbo/+ plus NTHi mice (**f**). The red arrows indicate where there is co-expression between a macrophage and the pro-inflammatory cytokine Il-1β, indicating that these cells have a pro-inflammatory phenotype.

Immunohistochemical markers = Iba1 and Il-1β. Counterstain = Haematoxylin. Scale bar = 20 μm, inserts = 10 μm.

3.4.2.4 Quantification of middle ear histological changes, preliminary analysis

The data collected for the number of macrophages (Iba1+ or CD68+) per unit area in the middle ear mucosa and mucosal thickness are displayed in the box and whisker plots in Figure 3.22. The regions where measurements were obtained are highlighted in Figure 3.22a. Mucosal thickness (μm) was measured on Iba1 (Figure 3.22c) and CD68 (Figure 3.22d) stained sections. There is a large difference in mucosal thickness (μm) between WT plus NTHi and Junbo or Junbo plus NTHi mice. Mucosal thickness (μm) is greater in Junbo/Junbo plus NTHi mice compared to WT plus NTHi mice (Figure 3.22c and d) suggesting increased middle ear inflammation in the Junbo mice. The number of Iba1+ macrophages per unit area (μm^2) in the mucosa is highly variable between the groups of mice (Figure 3.22e). Whereas, the number of CD68+ macrophages per unit area

(μm^2) in the mucosa is higher in the 4- and 12-week-old Junbo and Junbo plus NTHi mice compared to WT plus NTHi mice. Figure 3.20 shows representative images of the middle ear mucosa with Iba1+ (a-c) and CD68+ (d-f) labelled macrophages in the groups of mice.

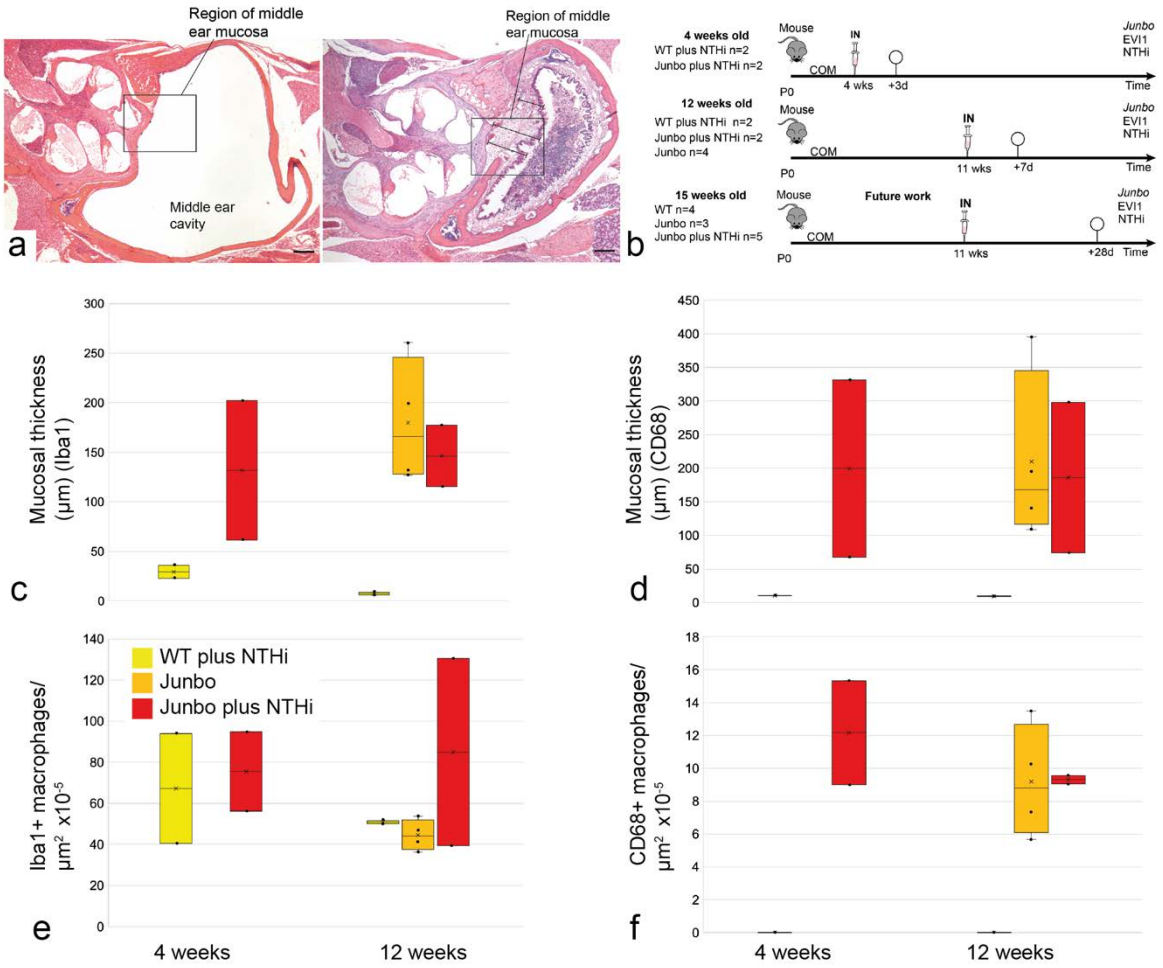


Figure 3.22 **Differences in mucosal thickness (μm) and the number of positively labelled macrophages/μm² in the middle ear mucosa between the groups of mice, presented on box and whisker plots**

Quantification of mucosal thickness (μm) (Iba1, CD68) and the number of positively labelled macrophages per unit area (μm²) in the middle ear mucosa (Iba1+, CD68+). The key and x-axis label (4 and 12 weeks) refers to all plots.

a. Sections of the cochlea and middle ear (WT plus NTHi, Junbo plus NTHi [left to right]) identifying the regions measured (box,|-|).

Histochemical stain = Haematoxylin and Eosin. Scale bar = 200 μm.

b. The timeline for each experimental group including the number of mice for each group. Quantification was carried out on 4- and 12-week-old mice. Future work will involve analysis of the 15-week-old mice.

c-d. Mucosal thickness (μm) of 4- and 12-week-old mice on Iba1 (c) and CD68 (d) stained sections. The mean of three measurements was calculated.

e-f. Number of positively labelled macrophages per unit area (μm²) of the middle ear mucosa: Iba1+ (e) and CD68+ (f) in 4- and 12-week-old mice.

Data displayed in (**c-f**) was collected from the following mice: 4-week-old WT plus NTHi (n=2) and Junbo plus NTHi mice (n=2); 12-week-old WT plus NTHi (n=2), Junbo (n=4), Junbo plus NTHi mice (n=2). Individual data points are indicated with a small black circle.

Mucosal thickness (μm) (Iba1) of 12-week-old Junbo plus NTHi, WT plus NTHi and Junbo mice was measured. The results identify a significant difference in the mucosal thickness between the groups as determined by a one-way ANOVA ($F(2,5) = 7.225$, $p < 0.05$). A least significant difference (LSD) test for multiple comparisons found that the mean mucosal thickness was significantly greater in the Junbo plus NTHi compared to WT plus NTHi mice (p=0.047, 95% C.I. = [2.844, 275.130]) and significantly greater in the Junbo mice compared to the WT plus NTHi mice (p=0.013, 95% C.I. = [54.58.8, 290.393]). There was no significant difference in the mucosal thickness between Junbo and Junbo plus NTHi mice (p= 4.98). Mucosal thickness (μm) (CD68) of 12-week-old Junbo plus NTHi, WT plus NTHi and Junbo mice was measured. There was no significant difference between the groups as determined by a one-way ANOVA ($F(2,5) = 1.887$, $p = 0.245$). A caveat to these findings is that the study is underpowered and requires larger group sizes to confirm these findings.

The number of positively labelled macrophages (Iba1+ or CD68+) per unit area (μm^2) in the middle ear mucosa of 12-week-old Junbo plus NTHi, WT plus NTHi and Junbo mice were measured. There was no significant difference in the number of Iba1+ macrophages per μm^2 between the groups as determined by a one-way ANOVA ($F(2,5) = 1.305$, $p = 0.350$). There was a significant difference in the number of CD68+ macrophages/μm^2 between the groups as determined by a one-way ANOVA ($F(2,5) = 9.031$, $p < 0.05$). An LSD test for multiple comparisons found that the number of CD68+ macrophages/μm^2 was significantly greater in the Junbo plus NTHi compared to WT plus NTHi mice (p=0.017, 95% C.I. = [0.0000, 0.0002]). An LSD test for multiple comparisons found that the number of CD68+ macrophages/μm^2 was significantly greater in the Junbo compared to WT plus NTHi mice (p= 0.010, 95% C.I. = [0.0000, 0.0002]). There was no significant difference in the number of CD68+ macrophages/μm^2 between Junbo and Junbo plus NTHi mice (p= 0.960).

To examine the effect of age (4- or 12-weeks-old) and group (Junbo plus NTHi and WT plus NTHi) on the number of positively labelled macrophages/μm^2 in the middle ear mucosa, a two-way ANOVA was conducted. There was no significant interaction between the effects of age and group ($F(1,4) = 0.212$, $p = 0.669$) on the number of Iba1+ macrophages/μm^2 in the middle ear mucosa. There was no significant interaction between the effects of age and group ($F(1,4) = 0.810$, $p = 0.419$) on the number of CD68+ macrophages per μm^2 in the middle ear mucosa.

3.4.3 Immunohistochemical staining to investigate cochlear inflammation and microglial and macrophage populations in the auditory nerve and cochlea in the Junbo mouse model

Using histological and immunohistochemical techniques, we have characterised the histopathology, inflammation and immune cell populations of the middle ear of this mouse model. Junbo mice have increased inflammation, cellular exudate and active immune cells in the middle ear cavity (Figure 3.18). Due to the chronic middle ear inflammation, Junbo mice have increased susceptibility to NTHi infection (Figure 3.19). We have characterised the populations of macrophages in the middle ear mucosa and fluid of the Junbo mice and investigated how the populations change following NTHi infection. We have identified activated (amoeboid Iba1), phagocytic (CD68) and pro-inflammatory (Iba1 and Il-1β) macrophages in the middle ear mucosa and cellular fluid in the Junbo mice.

Due to the close proximity of the middle ear cavity to the cochlea and the evidence of the different mechanisms for how middle ear inflammation causes cochlear inflammation, we investigated evidence of cochlear inflammation and characterised the macrophage populations in the cochlea and auditory nerve that may be activated as a result of local inflammation. Figure 3.23 is an image of cochlear section, stained with the marker Iba1, beside a labelled schematic of the cochlea. Key regions and structures in the cochlea have been highlighted, alongside the distribution of macrophages under homeostatic conditions (Figure 3.23b).

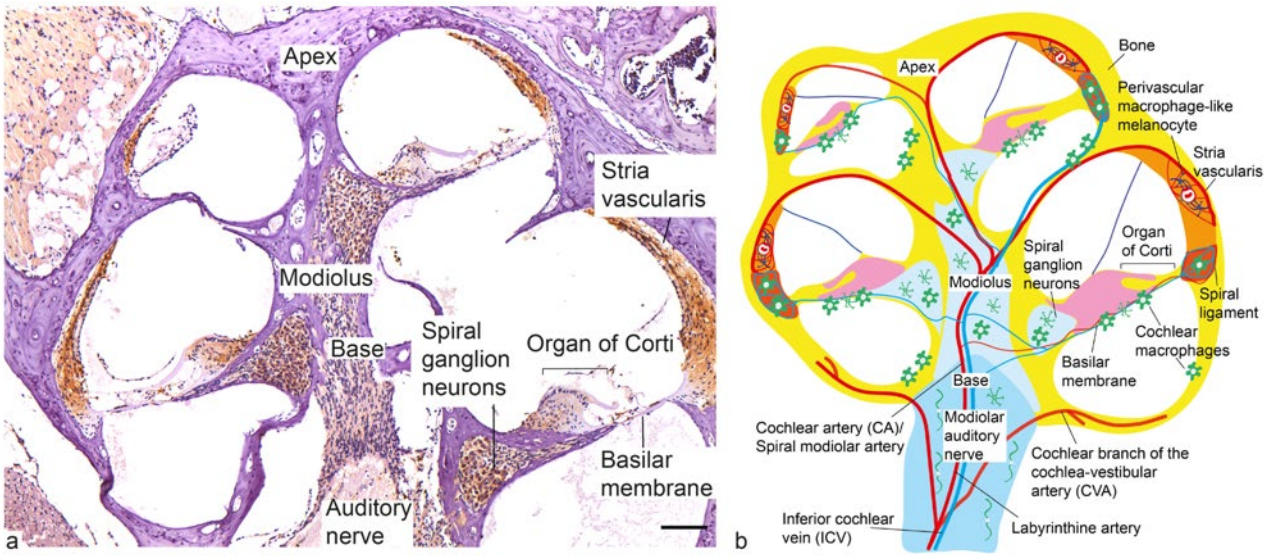


Figure 3.23 **Labelled mid-modiolar cochlear section**

a. Mid-modiolar cochlear section from a Jbo/+ mouse labelled with the marker Iba1. Key regions have been labelled. Immunohistochemical markers = Iba1. Counterstain = Haematoxylin. Scale bar = 100 μm.

b. A labelled schematic of the cochlea. The distribution of cochlear macrophages of different morphologies is shown in green.

3.4.3.1 Cochlear inflammation and damage

H&E stained sections were examined for evidence of tissue damage and inflammation. Evidence of pools of red blood cells (RBCs) were observed in the scala tympani of the basal turn of the cochlea in the Jbo/+ mice (Figure 3.24). The presence of an accumulation of RBCs within the cochlea basal turn was unexpected and may suggest damage or increased permeability of the round window membrane. As described in Figure 3.5, middle ear inflammation causes damage to the cochlea through the movement of inflammatory mediators across the round window membrane into the basal turn of the cochlea. Increased inflammation within the basal turn may cause damage to cochlear structures leading to movement of cells such as RBCs or pathogens into the cochlea. Inflammation causes increased permeability of the blood-labyrinth barrier (BLB) due to the down-regulation of tight and adherens junction proteins. Intratympanic instillation of LPS for 48 hours induced labyrinthitis in mice which resulted in morphological changes of PVM/Ms, ultrastructural damage to tight junctions and a loose intercellular connection between endothelial cells and perivascular cells in the BLB (Jiang et al., 2019). Tight junction protein, Zonula occludens-1 (ZO-1), was significantly down-regulated in the cochlea of LPS-treated mice thought to be associated with the increased expression of MMP-9 (Jiang et al., 2019) - a key member of MMP superfamily associated with extracellular matrix remodelling. Increased MMP-9 expression and

degradation of ZO-1 have also been shown to be responsible for the breakdown of the BBB in pathological conditions (Feng et al., 2011). Breakdown of BLB integrity resulting in the movement of cells and inflammatory mediators into the cochlea, may cause further damage to cochlear structures. Further histological stains will be used such as Perls Prussian blue to indicate the presence of non-heme iron in tissues such as hemosiderin. This will determine whether the RBCs have lysed and are releasing chemical signals, likely to cause damage to surrounding tissue. In addition to RBCs in the cochlea, few NTHi-positive neutrophils were observed within the cochlea of a WT mouse that had been inoculated with NTHi (Figure 3.19). It is not clear how the cells entered the cochlea, however the NTHi infection and/or middle ear inflammation may cause changes in the permeability of the cochlea barriers and the vasculature. Evidence of a breach of the cochlear barriers, including intrastrial fluid-blood barrier and the BLB, will be further investigated in the Junbo mice to determine whether middle ear inflammation alters the permeability of these barriers.

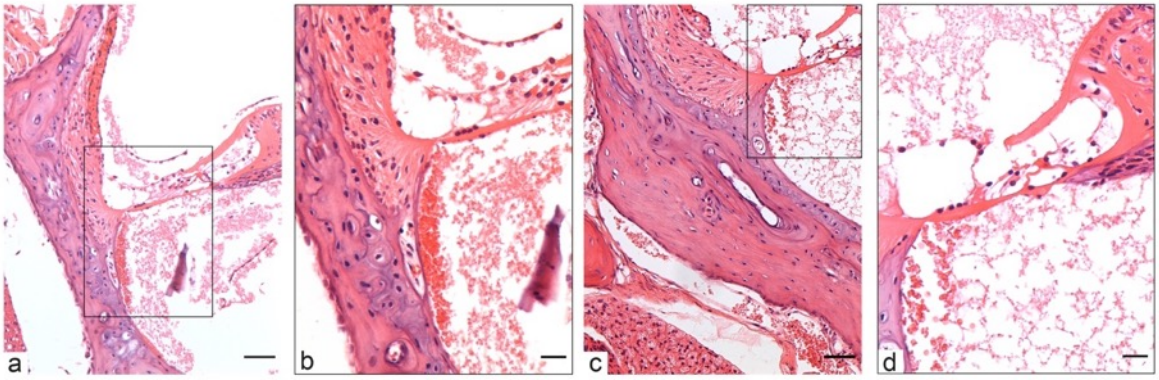


Figure 3.24 **Evidence of red blood cells (RBCs) in the basal turn of the cochlea in Junbo mice**

a, c. Left side cochlear basal turn from a Jbo/+ mouse showing the lateral wall, organ of Corti and an accumulation of RBCs in the scala tympani, below the basilar membrane.

b, d. Higher magnification view of the pool of RBCs below the basilar membrane in the scala tympani of Jbo/+ mice. Histochemical stain = Haematoxylin and Eosin. Scale bar = a, c 50 μm, b, d 20 μm.

3.4.3.2 Macrophage response to NTHi infection

To investigate whether middle ear inflammation and infection in Junbo mice resulted in cochlear inflammation, we aimed to characterise the population of macrophages in key regions of the cochlea and in the auditory nerve (AN), immediately adjacent to the modiolus. Iba1 was used to identify microglial cells in the AN and macrophages in the cochlea.

All mice have cochlear macrophages distributed amongst the SGNs in the osseus spiral lamina and spiral ligament. As discussed in Chapter 2, in the event of an inflammatory insult, these cochlear macrophages may become activated and release pro-inflammatory mediators which signal for the infiltration of monocytes. Iba1-positive macrophages are distributed in the basal turn of the cochlea of all mice. Figure 3.25 shows a high magnification image of a WT plus NTHi mouse organ of Corti which is representative of the distribution of Iba1-positive cells seen in all mice in this study. There is Iba1-positive staining at the base of the inner and outer pillar cells and amongst the Boettcher's cells. A population of Iba1-positive basilar membrane macrophages are seen in varying numbers between the mice.

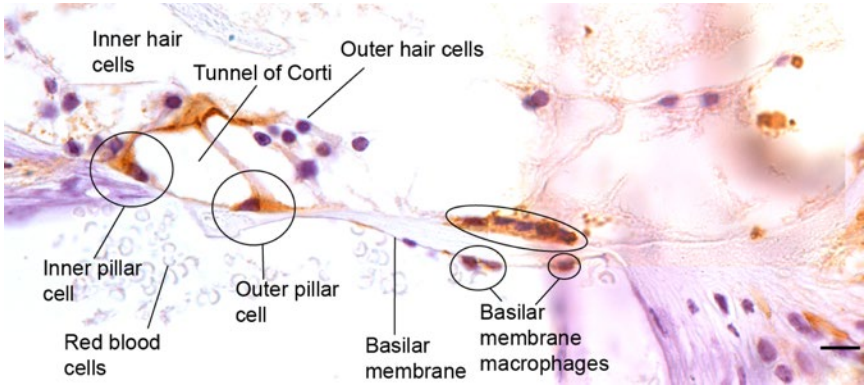


Figure 3.25 **High magnification view of the mouse organ of Corti with Iba1-positive cells circled**

Inner and outer hair cells and the supporting cells of the organ of Corti have been labelled. Iba1-positive cells are circled which includes inner and outer pillar cells, basilar membrane macrophages and Boettcher's cells. All mice in this study have similar Iba1-positive cell expression within the organ of Corti. Immunohistochemical markers = Iba1. Counterstain = Haematoxylin. Scale bar = 10 μm.

The overall distribution and expression of Iba1-positive cochlear macrophages change between WT plus NTHi, non-inoculated Jbo/+ and Jbo/+ inoculated with NTHi for 3 and 7 days (Figure 3.26). There is higher overall expression of Iba1-positive cells in the basal turn of the cochlea in the Jbo/+ and Jbo/+ plus NTHi mice. Figure 3.26c shows a Jbo/+ mouse inoculated with NTHi then culled 3 days later. 3 days after inoculation is the peak of macrophage activation which is followed by the infiltration of monocytes into the cochlea. Basilar membrane and scala tympani macrophages, likely to have infiltrated following inflammation, are indicated by an arrow in Figure 3.26c. Figure 3.26d shows a Jbo/+ mouse inoculated with NTHi then culled 7 days later. The acute inflammatory response to the NTHi is likely to have decreased by 7 days, which may explain the lower expression of basilar membrane and scala tympani macrophages in Figure 3.26d.

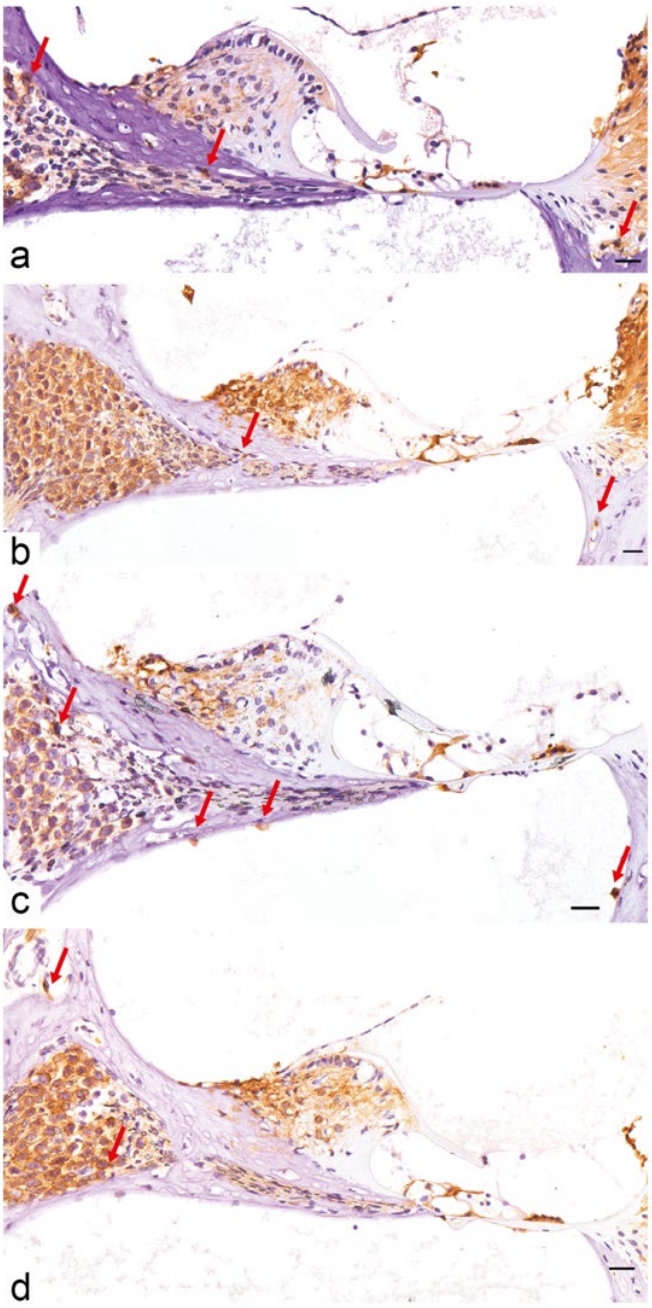


Figure 3.26 **Cochlear macrophage response to NTHi inoculation**

The expression of Iba1-positive macrophages changes across the time course of NTHi infection. **a-d.** Iba1-positive macrophages (indicated by the red arrow) in the basal turn of the cochlea in the following mice: non-inoculated Jbo/+ (**a**), WT plus NTHi (+7 days) (**b**), Jbo/+ plus NTHi (+3 days) (**c**) and Jbo/+ plus NTHi (+7 days) (**d**). Immunohistochemical markers = Iba1. Counterstain = Haematoxylin. Scale bar = 20 μm.

3.4.3.3 Microglial cell populations in the auditory nerve (AN)

Adjacent to the middle ear cavity on the tissue sections is the cochlea. The region of the auditory nerve (AN), which runs into the cochlea, is included on the tissue sections. This allows us to

investigate the population of microglial cells distributed in the AN. Iba1 was used to identify AN microglia. Iba1-positive cells are distributed across in the AN (in all groups of mice) and many have an elongated morphology with long processes running along the nerve tract. The morphology of the Iba1-positive cells from Jbo/+ plus NTHi mice (Figure 3.27c) display an activated morphology with a larger soma area and a greater number of processes that appear thicker compared the cells from non-inoculated Jbo/+ (Figure 3.27b) and WT plus NTHi mice (Figure 3.27a).

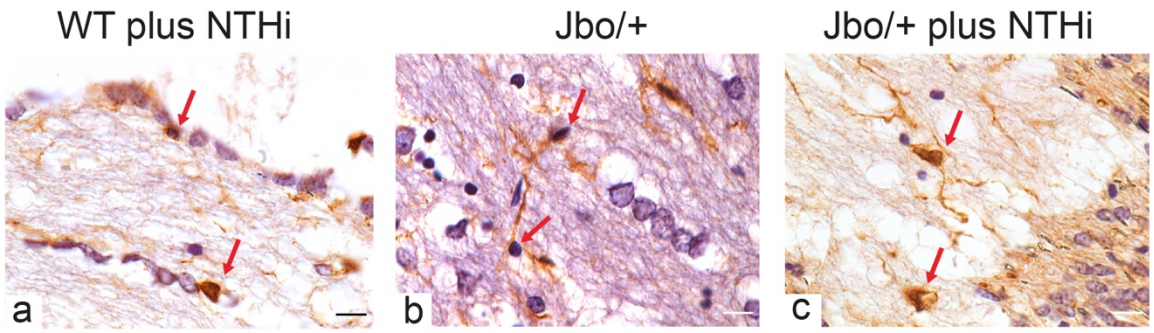


Figure 3.27 **High magnification images of Iba1-positive elongated microglia in the auditory nerve highlighting differences in cell morphology**

Iba1-positive microglia in the AN of WT plus NTHi (**a**), Jbo/+ (**b**) and Jbo/+ plus NTHi (**c**) mice. Immunohistochemical marker = Iba1. Counterstain = Haematoxylin. Scale bar = 10 μm.

To identify whether these cells were pro-inflammatory, consecutive tissue sections were stained with Iba1 and Il-1β and examined for co-expression. Figure 3.28 shows a panel of images from WT plus NTHi (a, d), Jbo/+ (b, e) and Jbo/+ plus NTHi (c, f) sections stained with Iba1 (a-c) and Il-1β (d-f). There is a greater number of Iba1-positive microglia in the AN of the Jbo/+ mice (with and without NTHi) compared to WT plus NTHi. The red arrows, in the same position on the Iba1 and Il-1β image, indicate positively labelled pro-inflammatory cells co-expressing Iba1 and Il-1β. There is no evidence of pro-inflammatory microglia in the AN of WT plus NTHi mice (Figure 3.28a and d), whereas the Jbo/+ and Jbo/+ plus NTHi mice have pro-inflammatory microglia distributed in the AN. The black arrows in Figure 3.28 indicate Iba1 positive microglial cells not co-expressing Il-1β, suggesting a homeostatic function.

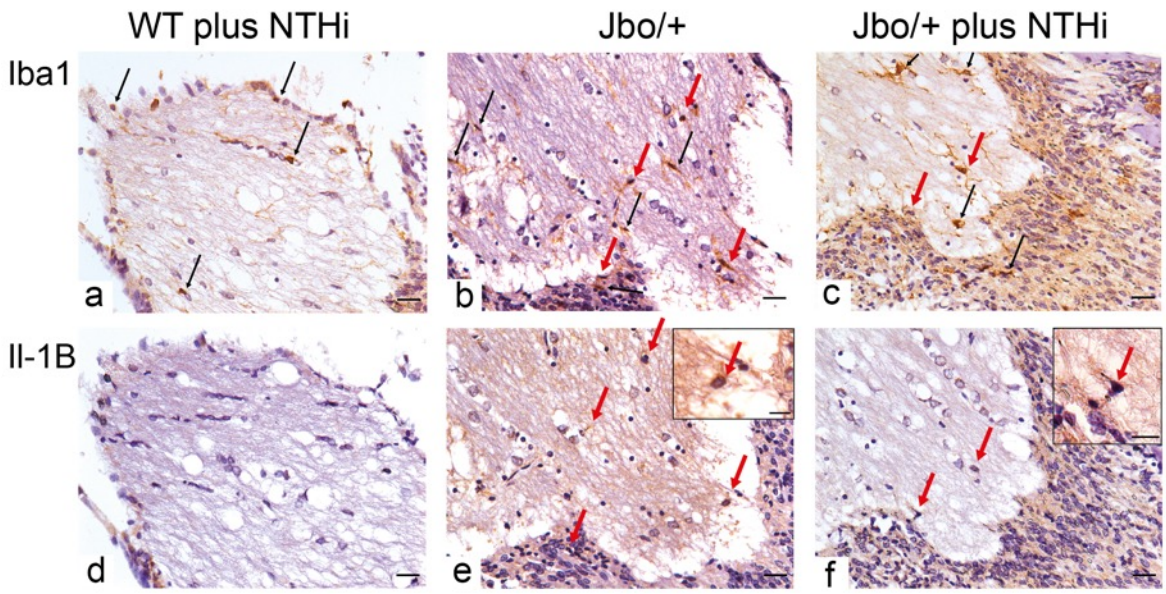


Figure 3.28 **Evidence of pro-inflammatory microglia in the auditory nerve**

Consecutive slides were stained with Iba1 and Il-1β to investigate the function of the microglial cells in the AN, proximal to the cochlea.

a-c. Iba1-positive microglia in the AN of WT plus NTHi (**a**), Jbo/+ (**b**) and Jbo/+ plus NTHi mice (**c**). Black arrows indicate Iba1-positive cells not co-expressing Il-1β.

d-f. Il-1β-positive microglia in the AN of WT plus NTHi (no positive cells) (**d**), Jbo/+ (**e**) and Jbo/+ plus NTHi mice (**f**). The red arrows indicate co-expression between a microglial cell and the pro-inflammatory cytokine Il-1β, indicating a pro-inflammatory phenotype. Immunohistochemical markers = Iba1 and Il-1β. Counterstain = Haematoxylin. Scale bar = 20 μm, inserts = 10 μm.

To further characterise the population of microglia in the AN, a marker for TMEM119 (Abcam, ab209064, 1:1500) was used. TMEM119 is a microglial specific transmembrane protein that has high expression in homeostatic microglia in the CNS. Figure 3.29 shows the expression of TMEM119-positive cells in the AN in WT plus NTHi mice (Figure 3.29a), Jbo/+ (Figure 3.29b) and Jbo/+ plus NTHi (Figure 3.29c) mice. Positively labelled cells are indicated by red arrows. Figure 3.29d-f show a higher magnification view of a region of the AN. There is higher expression of TMEM119-positive cells in the AN of the Jbo/+ mice compared to the Jbo/+ mice with NTHi infection overlaid. TMEM119 is homeostatic marker and it appears that NTHi infection in the Jbo/+ mice decreases the expression of this marker in microglia in the AN.

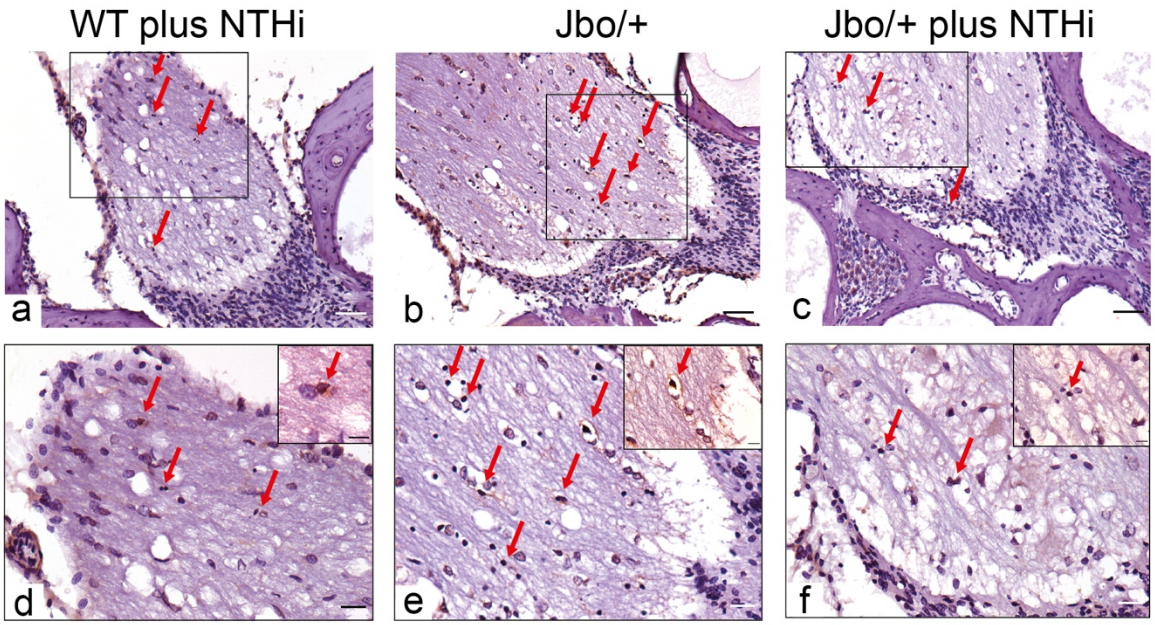


Figure 3.29 **A population of microglia in the auditory nerve identified by the marker TMEM119**

a-d. TMEM119-positive cells in the AN of WT plus NTHi (**a**), Jbo/+ (**b**) and Jbo/+ plus NTHi (**c**) mice.

d-f. Higher magnification images of the AN with the TMEM119-positive cells indicated by the red arrows. Immunohistochemical marker = TMEM119. Counterstain = Haematoxylin. Scale bar = a-c 50 μm, d-f 20 μm, inserts = 10 μm.

3.4.3.4 Quantification of the number of microglia/μm² in the auditory nerve

The data collected for the number of Iba1+ or TMEM119+ microglia per unit area in the auditory nerve is displayed in the box and whisker plots in Figure 3.30. The region where measurements were obtained are highlighted in Figure 3.30a. Representative images of Iba1+ and TMEM119+ labelled microglia in the auditory nerve, from each group, are shown in Figure 3.28a-c (Iba1+) and Figure 3.29 (TMEM119+). The number of Iba1+ and TMEM119+ microglia per unit area (μm²) in the auditory nerve is highly variable between the groups of mice. Due to the high variability in cell density across regions and groups of mice, the number of mice should be increased to determine any differences.

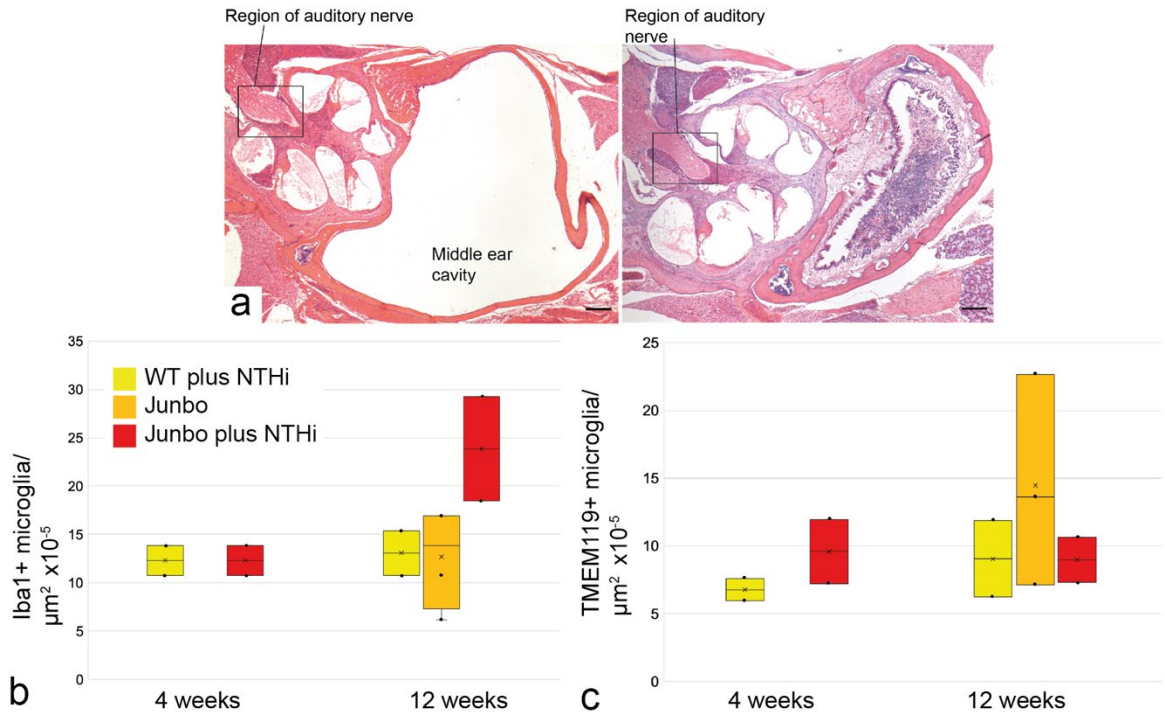


Figure 3.30 **Differences in the number of positively labelled microglia per µm² in the auditory nerve between the groups of mice, presented on box and whisker plots**

Quantification of the number of positively labelled microglia per unit area (µm²) in the auditory nerve (Iba1+, TMEM119+). The key refers to both plots.

a. Sections of the cochlea and middle ear (WT plus NTHi, Junbo plus NTHi [left to right]) identifying the region of auditory nerve measured (box,|-|).

Histochemical stain = Haematoxylin and Eosin. Scale bar = 200 µm.

b-c. Number of positively labelled microglia per unit area (µm²) of the auditory nerve: Iba1+ (**b**) and TMEM119+ (**c**) in 4- and 12-week-old mice.

Data displayed in (**b, c**) was collected from the following mice: 4-week-old WT plus NTHi (n=2) and Junbo plus NTHi mice (n=2); 12-week-old WT plus NTHi (n=2), Junbo (n=4), Junbo plus NTHi mice (n=2). Individual data points are indicated with a small black circle.

The number of positively labelled microglia (Iba1+ or TMEM119+) per unit area (µm²) in the auditory nerve of 12-week-old Junbo plus NTHi, WT plus NTHi and Junbo mice was measured. There was no significant difference in the number of Iba1+ microglia/µm² between the groups as determined by a one-way ANOVA ($F(2,5) = 3.024$, $p = 0.138$). There was no significant difference in the number of TMEM119+ microglia/µm² between the groups as determined by a one-way ANOVA ($F(2,5) = 0.713$, $p = 0.544$).

To examine the effect of age (4- or 12-weeks-old) and group (Junbo plus NTHi and WT plus NTHi) on the number of positively labelled microglia/μm^2 in the auditory nerve, a two-way ANOVA was conducted. There was no significant interaction between the effects of age and group ($F(1,4) = 2.970$, $p = 0.160$) on the number of Iba1+ microglia per μm^2 in the auditory nerve and no significant interaction between the effects of age and group ($F(1,4) = 0.488$, $p = 0.523$) on the number of TMEM119+ microglia per μm^2 in the auditory nerve.

3.4.3.5 Microglia/macrophage populations within key regions of the cochlea

Regions of the cochlea including organ of Corti, spiral ganglion neurons (SGNs) and the stria vascularis were examined for the presence of TMEM119-positive cells. There is TMEM119-specific staining amongst the SGN cell bodies in WT plus NTHi (Figure 3.31a), Jbo/+ (Figure 3.31b) and Jbo/+ plus NTHi (Figure 3.31c) mice. TMEM119-positive staining is observed in the stria vascularis in all mice (Figure 3.31d-f), indicating the distribution of resident PVM/Ms in the stria.

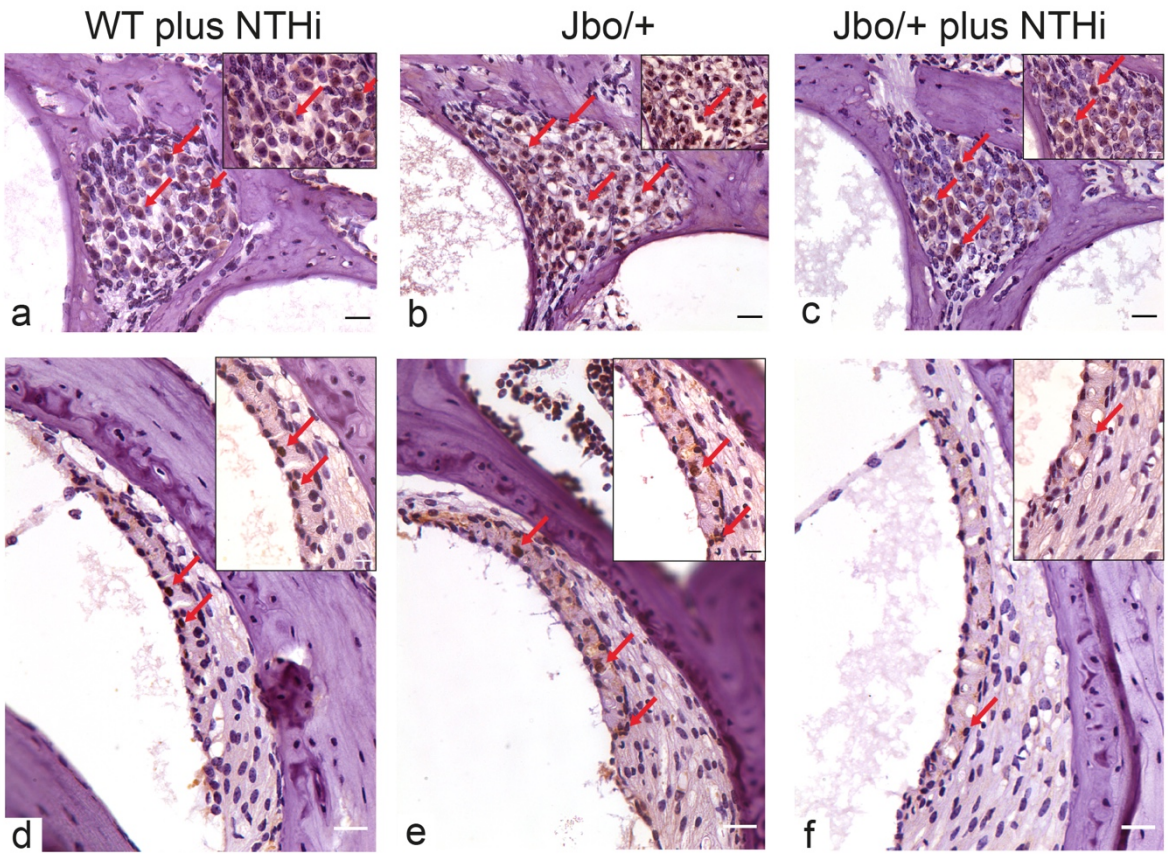


Figure 3.31 **Evidence of TMEM119 expression amongst the spiral ganglion neuron (SGN) cell bodies and in the stria vascularis**

a-c. TMEM119 expression amongst the SGN cell bodies of WT plus NTHi (**a**), Jbo/+ (**b**) and Jbo/+ plus NTHi mice.

d-f. TMEM119 expression in the stria vascularis of WT plus NTHi (**d**), Jbo/+ (**e**) and Jbo/+ plus NTHi (**f**) mice. Examples of positively labelled cells are indicated by a red arrow. Immunohistochemical markers = TMEM119. Counterstain = Haematoxylin. Scale bar = 20 μm, inserts = 10 μm.

The population of macrophages amongst the SGNs in the modiolus and osseus spiral lamina were investigated. Specific Iba1 staining is observed in the modiolus and regions close to the osseous spiral lamina, which houses the SGN cell bodies. Iba1 staining is often brown and diffuse amongst the SGNs, making it difficult to distinguish between specific and non-specific staining. In the images in Figure 3.32, only cell-specific Iba1-positive staining is highlighted. Figure 3.32a-c shows modiolar SGNs with Iba1-positive cells indicated by a black arrow in WT plus NTHi (Figure 3.32a), Jbo/+ (Figure 3.32b) and Jbo/+ plus NTHi (Figure 3.32c) mice. Iba1-positive macrophages within this region, co-expressing Il-1β on the consecutive slide, are labelled with a red arrow. WT mice following NTHi inoculation have very low levels/no expression of Il-1β. Non-inoculated and inoculated Jbo/+ mice have pro-inflammatory cochlear macrophages in the modiolar and spiral

ganglion regions, suggesting that mice with increased middle ear inflammation have increased macrophage activation within these regions.

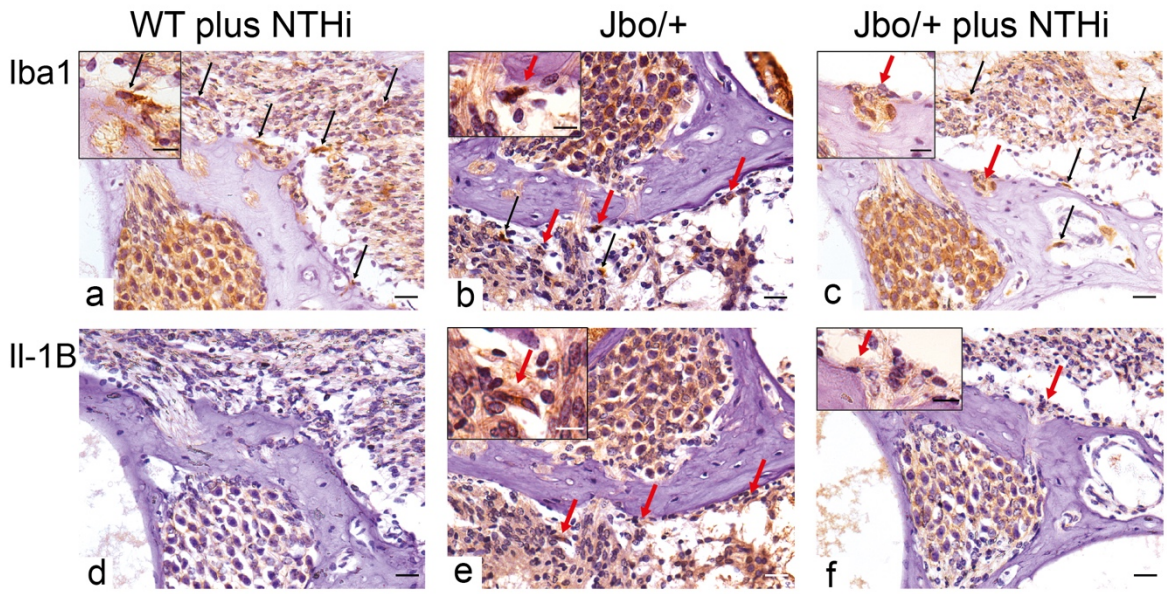


Figure 3.32 **Evidence of pro-inflammatory macrophages amongst the spiral ganglion neurons (SGNs) in the modiolus and osseus spiral lamina**

Consecutive slides were stained with the macrophage marker Iba1 and the pro-inflammatory cytokine marker Il-1β, to investigate the function of the cochlear macrophages in the modiolus and osseus spiral lamina.

a-c. Iba1-positive macrophages in the modiolus and osseus spiral lamina of WT plus NTHi (**a**), Jbo/+ (**b**) and Jbo/+ plus NTHi mice (**c**). The black arrows indicate Iba1-positive cells not co-expressing Il-1β.

d-f. Il-1β-positive macrophages in the modiolus and osseus spiral lamina of WT plus NTHi (**d**), Jbo/+ (**e**) and Jbo/+ plus NTHi mice (**f**). The red arrows indicate where there is co-expression between an Iba1-positive macrophage and the pro-inflammatory cytokine Il-1β, indicating a pro-inflammatory phenotype. Immunohistochemical markers = Iba1 and Il-1β. Counterstain = Haematoxylin. Scale bar = 20 μm, inserts = 10 μm.

To investigate the population of macrophages in the stria, the marker CD68 was used as it is an effective marker for labelling perivascular macrophage-like melanocytes (PVM/Ms). PVM/Ms are positioned in the middle region of the stria in close proximity to vascular structures. CD68-positive PVM/Ms are highlighted by a red arrow (Figure 3.33). Higher magnification view of the PVM/Ms is shown in Figure 3.33d-f. PVM/Ms become activated following inflammatory insults and change their morphology, causing strial dysfunction and atrophy. The CD68-positive PVM/M in the Figure 3.33e, from a Jbo/+ mouse with NTHi infection overlaid, appears to have a larger more ameboid

morphology compared to the smaller CD68-positive PVM/M indicated in the WT mouse following NTHi infection. Changes in the morphology of these cells following inflammation and infection may cause changes in the permeability of the intrastrial fluid-blood barrier resulting in changes in auditory function.

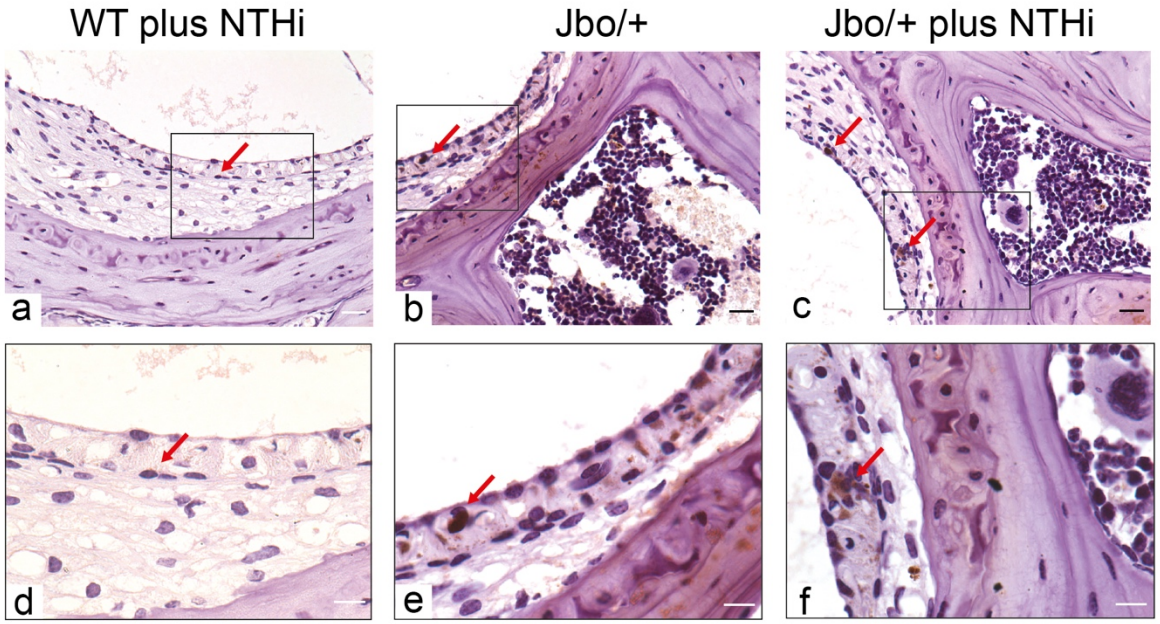


Figure 3.33 **Perivascular macrophage-like melanocytes (PVM/Ms) in the stria vascularis labelled with CD68**

a-c. CD68-positive PVM/Ms in the stria vascularis in the lateral wall of the cochlea in WT plus NTHi (**a**), Jbo/+ (**b**) and Jbo/+ plus NTHi (**c**) mice.
d-f. Higher magnification view of a region of the stria vascularis with CD68-positive macrophages indicated by a red arrow. Immunohistochemical markers = CD68. Counterstain = Haematoxylin. Scale bar = a-c 20 μm, d-f 10 μm.

3.5 Discussion

Otitis media (OM) is a significant global health problem affecting many children across the world. There is currently no effective vaccine and no consensus for the most effective treatment, resulting in OM being the leading cause of antimicrobial prescription to children worldwide. There is a need for good, robust mouse models to investigate the biological underpinnings of OM and to develop therapies for the prevention and treatment of OM.

3.5.1 The Junbo mouse model for middle ear NTHi infection is a good model to investigate the pathogenesis of otitis media

A model for NTHi middle ear infection in the Junbo mouse was established (Hood *et al.*, 2016). Junbo mice have a mutation in the Evi1 gene, encoding a transcription factor that is involved in host innate immune responses (Parkinson *et al.*, 2006). This mutation causes Junbo mice to spontaneously develop middle ear inflammation and effusion with hearing impairment after 4-5 weeks and when inoculated intranasally with NTHi, develops middle ear infection. Extensive microbiological studies of this mouse model were carried out to investigate the pathogenesis of acute and chronic otitis media. In the Junbo mice 80% of middle ears became infected after NTHi inoculation, whereas in the WT mice the middle ears were not efficiently infected by NTHi. These findings suggest that the pre-inflamed middle ear creates an environment that promotes bacterial infection and fluid accumulation upon the addition of an inflammatory challenge (Hood *et al.*, 2016). This model for NTHi infection in the Junbo mouse mimics several aspects of the disease course in human OM, in cases where recurrent AOM causes an inflamed middle ear environment which then may be exposed to the pathogens such as NTHi, to cause high levels of infection. This is a valuable model and tool to investigate the pathogenesis of acute OM leading to chronic OM.

3.5.2 The Junbo mouse model is a good model for investigating the effect of chronic inflammation in the auditory system on middle ear and cochlear macrophage populations

In addition to the value of better understanding the mechanisms underlying the complex pathology in OM, the Junbo mouse model is an excellent tool to investigate the way in which macrophages in the auditory system, respond to chronic inflammation. Chapter 2 discussed many studies which have investigated the changes in the morphology, distribution and function of macrophages in the auditory system following different acute insults. These studies demonstrate that macrophages, in the cochlea and auditory nerve, play a role in the inflammatory response to acute insults which can have both damaging and neuroprotective effects in different regions of

the cochlea. Junbo mice have a mutation resulting in a chronically inflamed middle ear environment, which perturbs their innate immune response to pathogens (Hood *et al.*, 2016). This creates a model to investigate the changes in the morphology, distribution and function of macrophages in the middle ear, auditory nerve and cochlea following a chronic inflammatory insult. Our aims were to investigate how macrophages respond to chronic inflammation in the Junbo mice and to investigate the effect of the pre-inflamed middle ear environment on how the macrophages respond to an additional inflammatory insult; NTHi inoculation. Examining the middle ear and cochlear macrophage populations and their response in the Junbo mouse model may enable a better understanding of the role of macrophages in progressive hearing loss.

3.5.3 Junbo mice have increased inflammation, cellular exudate and active innate immune cells in the middle ear

The first objective was to characterise the middle ear histopathology in the Junbo mouse model. Histological analysis was carried out to determine the morphology and level of inflammation in the middle ear cavities. Using H&E staining, morphological features of the middle ears of Junbo mice were described. These included an inflamed middle ear mucosa forming the perimeter of the cavity and a thick bulla fluid in the cavity (Hood *et al.*, 2016). Similarly, we identified an inflamed mucosa lining the middle ear cavity of Junbo mice (inoculated and non-inoculated) with a viscous, cellular fluid in the cavity. There was heterogeneity in the histopathology of the middle ear cavities similar to the pathology associated with human OM. The middle ears of WT mice (inoculated and non-inoculated) were air-filled spaces with no mucosal inflammation.

The middle ear cavities of Junbo mice displayed markers of inflammation characteristic of human OM including mucosal hyperplasia, damaged ciliated epithelial cells, immune cell infiltration and fluid accumulation, which were not observed in WT mice. The cellularity of the middle ear fluid was variable in the Junbo mice. The grading of middle ear fluid and cavity has been described (Vikhe *et al.*, 2018).

Junbo mice with a pre-inflamed middle ear environment have increased susceptibility to NTHi infection. Following NTHi inoculation, 80% of Junbo mice with fluid-filled middle ear cavities were positive for NTHi infection. Whereas, 71 of 73 WT mice following NTHi inoculation had no middle ear inflammation and fluid accumulation and were NTHi negative (Hood *et al.*, 2016). We identified increased NTHi expression in the middle ears of Junbo mice following NTHi inoculation, compared to the low expression in WT mice following NTHi inoculation. There are several reasons why a pre-inflamed middle ear environment in Junbo mice have increased susceptibility to NTHi infection and middle ear bacterial colonisation. Middle ear inflammation results in/is synonymous

with mucosal changes including thickening of the mucosal layer, invaginations of the membrane, epithelial injury and increased permeability of the mucosal barrier (Tos, 1980; Ars *et al.*, 2012; Massa *et al.*, 2015). These changes contribute to the accumulation of fluid in the middle ear cavity creating a new environmental niche for NTHi to colonise. In addition, the damaged and inflamed mucosa associated with OM is a site for biofilm formation in patients with COM (Kaya *et al.*, 2013), again creating an environment for NTHi to live resulting in persistent infections. The middle ear chemical environment may also influence growth of NTHi. NTHi is a microaerophilic bacterium, meaning they are unable to grow in normal atmospheric oxygen tensions and are adapted to low oxygen environments (Krieg and Hoffman, 1986). The middle ear fluid and mucosa in Junbo mice were analysed for evidence of hypoxia (Cheeseman *et al.*, 2011). Cellular hypoxia in foamy macrophages in the middle ear fluid and a hypoxic middle ear mucosa was identified. The hypoxic middle ear environment of Junbo mice may favour the growth of NTHi.

The cellular composition of the middle ear fluid in Junbo mice influences NTHi infection rate and bacterial load. There are direct correlations between the composition and grade of the bulla fluid and NTHi bacterial load in Junbo mice (Vikhe *et al.*, 2018). Following NTHi inoculation, high grade viscous middle ear fluids, showed increased infection rate and NTHi expression in the middle ear fluid. Whereas lower grade, serous fluid had lower infection rates. Cell necrosis was increased after NTHi inoculation and was observed across 7 days of sustained infection (Vikhe *et al.*, 2019). In NTHi-positive middle ears, there was a high number of necrotic and live cells indicating the infiltration of cells into the middle cavity following NTHi infection.

The first responders in the inflammatory response to NTHi infection are neutrophils. Persistent high numbers of neutrophils can be toxic and damaging for cells and tissue within that environment (Wilgus, Roy and McDaniel, 2013). Non-inoculated Junbo mice have a high number of neutrophils in the middle ear fluid that is further increased following inoculation with NTHi (Vikhe *et al.*, 2019), indicating neutrophil infiltration. The presence of NTHi in viscous fluid resulted in a continuous infiltration of neutrophils (Vikhe *et al.*, 2018). We found similar results with high numbers of neutrophils in the dense cellular fluid, which increased in mice inoculated with NTHi. Some of the neutrophils were NTHi-positive indicating that these cells had phagocytosed the NTHi as a mechanism of killing and removal. NTHi can initiate the formation of neutrophil extracellular traps (NETs) which trap NTHi and prevent bacterial killing (Juneau *et al.*, 2011). NTHi biofilms survive within NET structures therefore inhibiting bacterial clearance and potentially facilitating NTHi growth (Hong *et al.*, 2009; Juneau *et al.*, 2011). The increased number of apoptotic and necrotic cells within the middle ear of NTHi-infected Junbo mice suggest a similar process. Compiling the microbiological data from the Junbo mouse, it is likely that multiple forms

of NTHi exist in the middle ear cavity; in the biofilm associated form, planktonic and also host-cell associated forms (Vikhe *et al.*, 2018).

3.5.4 Junbo mice have distinct macrophage populations in the middle ear and cochlea, some of which show evidence of being in a pro-inflammatory phenotype

The chronic middle ear inflammation and high levels of NTHi infection in the Junbo mouse middle ears indicate a rampant innate immune response, likely to be signalling to immune cells within the middle and inner ear environment to cause tissue damage. The immune cell populations of the middle ear fluid have been characterised using flow cytometry. Macrophages are distributed across the middle ear and play a key role in maintaining immune homeostasis. During middle ear inflammation and infection, macrophages infiltrate the cavity and play a role in the initiation and resolution of OM by initiating the inflammation and clearing the bacteria by phagocytosis. Detailed understanding of the role and mechanisms involving macrophages in OM and how macrophages respond to chronic inflammation over time is limited.

Using several immunohistochemical macrophage and microglial cell markers (Iba1, CD68, TMEM119), we characterised macrophage populations in the middle ear cavity (fluid and mucosa), auditory nerve and cochlea. The morphology of Iba1-positive cells provides information about the phenotype. Amoeboid macrophages, a morphology suggestive of an activated phenotype, were observed in high numbers in the mucosa and fluid of Junbo mice which increased following NTHi infection. Foamy macrophages are fat-laden phagocytic cells, a hallmark of atherosclerosis (Z. Wang *et al.*, 2019). Iba1-positive foamy macrophages were observed in the middle ear cavities filled with a dense, cellular fluid; similar to what has been described (Hood *et al.*, 2016). CD68-positive macrophages were observed in the inflamed middle ear mucosa of Junbo mice, which increased following NTHi infection. These data indicate different populations of activated macrophages in the middle ear of Junbo mice as a result of the chronic inflammation and infection.

The function of the Iba1-positive macrophages was investigated by co-expression analysis with the marker, Il-1β. Evidence of numerous activated (determined by morphology) and pro-inflammatory (co-expression with Il-1β) macrophages was observed in the inflamed mucosa of Junbo mice (inoculated and non-inoculated). As discussed in Chapter 2, microglia and macrophages can become primed, whereby an inflammatory insult causes a change in the phenotype of the cells meaning when exposed to an additional inflammatory challenge, the cells display an exaggerated inflammatory response. This heightened response can be shown by an increased production of Il-1β. Following a direct challenge to the central and peripheral nervous

system with LPS, there was increased microglial activation and inflammatory response in the ME7 prion mice (with chronic neuroinflammation), compared to WT animals (Cunningham *et al.*, 2005). Thus, indicating that microglial cells in the mice with existing inflammation (ME7 mice) were in an activated state so when exposed to an LPS challenge, display an exaggerated inflammatory response. In the Junbo mouse, chronic middle ear inflammation leads to macrophage activation switching the macrophages to a pro-inflammatory state. Some of these macrophages may be primed therefore when exposed to NTHi infection, display an exaggerated inflammatory response (increased Il-1β expression). The increased release of inflammatory mediators, associated with an exaggerated inflammatory response, can lead to tissue damage.

In OM, chronic inflammation causes the activation of resident middle ear macrophages followed by the release of pro-inflammatory mediators. This leads to the infiltration of macrophages into the middle ear cavity, further inflammation and fluid accumulation. Infection with NTHi exacerbates this response by inducing further production of inflammatory mediators, activation of resident macrophages and the infiltration of monocytes which differentiate into macrophages. The role of macrophages in infected middle ear cavities is to engulf and phagocytose NTHi to clear the infection (Bakaletz, DeMaria and Lim, 1987). As OM progresses, macrophages switch to an anti-inflammatory phenotype (Ryan *et al.*, 2020) in an attempt to return the middle ear to a homeostatic environment. In Junbo mice the inflammation is chronic, contributing to the persistence of the infection; making this a good model to investigate the effects on chronic inflammation and infection on the macrophages in the middle ear.

Due to the close anatomical proximity of the middle ear and cochlea and the evidence indicating the association of OM and SNHL, the populations of macrophages and microglial cells were investigated in the cochlea and auditory nerve (AN). As discussed in Chapter 2, microglial cells are distributed in the distal central region of the AN and cochlear macrophages are distributed across regions of the cochlea. Acute (Frye, Zhang and Hu, 2018; Zhang *et al.*, 2020) and chronic (Neng *et al.*, 2015; Frye *et al.*, 2017; Noble *et al.*, 2019) insults activate these macrophages resulting in changes in morphology, distribution and function. Activated macrophages release inflammatory mediators as part of the inflammatory response and persistent release can lead to tissue damage.

We aimed to investigate and begin to characterise the macrophage populations in the AN and cochlea in this mouse model of chronic inflammation, to investigate whether chronic middle ear inflammation effects the phenotype and function of macrophages. Increased number of activated and pro-inflammatory macrophages in the cochlea may cause tissue damage to key structures leading to loss to function over time (SNHL).

Cochlear macrophages, positively labelled with Iba1, were observed across the cochlea in the stria, organ of Corti and amongst SGNs. Staining with Iba1 in the stria and amongst the SGNs in the cochlea appears diffuse, making it difficult to determine specific staining with this marker within these regions. Iba1-positive staining was observed in the organ of Corti with a similar distribution in all mice. Macrophages resided in the tunnel of Corti at the base of the inner and outer pillar cells, similar to what was observed in human cochlear sections (Liu *et al.*, 2018). The population of basilar membrane macrophages in the basal cochlear turn changed in number and distribution when comparing the WT plus NTHi and Junbo mice following infection. There was no basilar membrane or scala tympani macrophages in WT plus NTHi mice, whereas in Junbo mice plus NTHi (3 days) there was increased expression. This indicates an infiltration of these cells of following inoculation. Several studies have shown the basilar membrane (Hirose *et al.*, 2005; Yang *et al.*, 2015; Frye, Zhang and Hu, 2018) and scala tympani (Zhang *et al.*, 2020) to be a site of infiltrating monocytes and macrophage activation following an acute noise exposure. The number of macrophages decreased after 7 days of infection, indicating a decrease in the acute inflammatory response to infection.

Spiral ganglion regions were investigated for macrophage expression. The modiolus, the central region of the cochlea, and the osseus spiral lamina had Iba1-positive macrophages. Multiple macrophages are distributed amongst the SGNs in the human cochlea (Liu *et al.*, 2018; Okayasu *et al.*, 2020). In Junbo mice (inoculated and non-inoculated), there were several pro-inflammatory macrophages which were not evident in the WT mice. These findings suggest that middle ear inflammation, causes inflammation and increased activation of macrophage populations in the cochlea. Further studies will be carried out to determine whether increased macrophage activation in the cochlea causes damage to cochlear regions and therefore influence sensorineural hearing function.

The stria vascularis forms the structural barrier in the lateral wall of the cochlea. Perivascular macrophage-like melanocytes (PVM/Ms) are located in the middle region of the stria and play a key role in maintaining the integrity of the intrastrial fluid-blood barrier (Neng *et al.*, 2013). CD68 was used to label PVM/Ms in the stria. The morphology of the CD68-positive PVM/Ms varied between the WT and Junbo mice. The PVM/Ms in Junbo mice plus NTHi had an activated morphology. Changes in the morphology of these cells following inflammation and infection may cause changes in the permeability of the intrastrial fluid-blood barrier leading to altered auditory function. Further studies will be carried out to investigate changes in strial integrity and permeability by measuring the expression of tight- and adherens-junction proteins (Zhang *et al.*, 2013).

TMEM119 is a microglial specific marker (Bennett *et al.*, 2016). It was expressed exclusively on a subset of Iba1+CD68+ microglia in human Alzheimer's disease brains and not expressed on Iba1+CD68+ infiltrating macrophages in lesions of multiple sclerosis (Satoh *et al.*, 2016), indicating TMEM119 is a marker of resident macrophages in the brain (microglia). TMEM119 was used to investigate the expression of homeostatic microglial cells in the AN, as TMEM119 is expressed highly in homeostatic microglia (Bennett *et al.*, 2016). There was a higher distribution of TMEM119-positive microglial cells in Junbo mice compared to Junbo plus NTHi mice. This may be suggesting that the presence of infection is downregulating expression of TMEM119 in microglial cells in Junbo mice and that in the presence of NTHi infection, AN microglial cells are in an activated phenotype.

TMEM119-positive staining was observed amongst the SGN cell bodies and in the stria. These cells are not likely to be microglia due to the distribution in the cochlea, however this marker could be more indicative of whether these cells are infiltrated or resident macrophages. TMEM119-positive staining may indicate resident cochlear macrophages, however expression of TMEM119 in the cochlea has not been illustrated in many studies. No expression of TMEM119 was detected in the human cochlea (Liu *et al.*, 2019). Whereas, TMEM119 protein expression was detected in the stria in the postnatal and adult mouse cochlea (Bassiouni *et al.*, 2019). As discussed in Chapter 2, a group of markers to identify the specific cochlear macrophage populations are not yet established. TMEM119 may therefore be a useful marker to distinguish between resident and infiltrated cochlear macrophages.

Iba1-positive microglia with an elongated morphology and long processes were identified in the auditory nerve (AN) of all mice, similar to the cells described in the human AN (Liu *et al.*, 2019). In Junbo mice plus NTHi, the microglia switched to an activated morphology with a larger number of thicker processes and larger cell soma (Kettenmann *et al.*, 2011), indicating inflammation within this region. Several pro-inflammatory microglia were identified in the AN of Junbo and Junbo plus NTHi mice, whereas evidence of activated, inflammatory microglial cells were not observed in the AN of WT plus NTHi mice. These results indicate that middle ear inflammation is causing activation of microglial cells in the AN. Middle ear inflammation increases the expression of pro-inflammatory cytokines in the cochlea (Ghaheri *et al.*, 2007b, 2007a; MacArthur *et al.*, 2011), which may signal to activate resident cochlear macrophages or AN microglial cells.

3.5.5 Relevance of the model

Animal models are commonly used to investigate the complex pathology, microbiology and histopathology of OM. The use of mouse models of OM has increased over time (Bhutta, 2012).

An array of methods has been established to induce acute and chronic OM including inoculation of a human (or mouse) pathogen either via transtympanic, transbullar or intranasal route (acute), through surgical manipulation (acute or chronic) and by genetic mutation (chronic). (See Figure 3.7, Figure 3.8, Figure 3.9 for detail of experimental design). The multiple types of OM models including the advantages and disadvantages will be discussed, followed by why the Junbo mouse model is an effective model. The main reasons include; for the investigation of the pathogenesis of acute recurrent OM and active mucosal COM and for the investigation of the effect of chronic inflammation on macrophage populations in the middle ear and cochlea.

Advances in the establishment of effective mouse models of OM have been relatively slow due to the complexity and heterogeneity of the different types of OM, the models are trying to recapitulate. Many models for AOM are induced by directly inoculating high numbers of pathogens into the middle ear cavity. This induces rapid, acute inflammation and fluid accumulation in the middle ear which peaks at around 3 days and decreases by 7 days post-inoculation (MacArthur, Hefeneider, Kempton, Parrish, *et al.*, 2006; Sautter *et al.*, 2011; Cho *et al.*, 2016). This allows for mechanisms of AOM to be investigated, however limits the investigation of the pathogenesis of COM associated with increased pathology and complications. In addition, by inducing infection by direct inoculation into the middle ear this bypasses the natural progression and colonisation of bacteria through the nasopharynx and Eustachian tube. Therefore, it is not possible to investigate how the pathogen ascends, colonises and causes infection as it naturally occurs in human OM. A small number of models, including the Junbo mouse model (Stol *et al.*, 2009; Hood *et al.*, 2016; Dewan *et al.*, 2019), have used intranasal methods for inoculation which is advantageous and enables this investigation. Injecting bacterium into the middle ear is an invasive method and can cause additional tissue damage.

Most models of COM are induced by surgical manipulation (Bhutta, Cheeseman and Brown, 2014; Varsak and Santa Maria, 2016; Khomtchouk *et al.*, 2020) and genetic mutation ((Hardisty *et al.*, 2003; Parkinson *et al.*, 2006; Hood *et al.*, 2016). Surgical occlusion of a mouse Eustachian tube allowed for the investigation of Eustachian tube dysfunction-related conditions and how this causes OM (Varsak and Santa Maria, 2016). However, surgical manipulation has some disadvantages as it is invasive and can result in additional tissue damage and inflammation. Genetic mouse models have been established through inducing a mutation in a specific gene such as TLR4 (MacArthur, Hefeneider, Kempton and Trune, 2006) and TGIF1 (Tateossian *et al.*, 2013). These models enable the investigation of the pathways involved in OM, however there is often low penetrance of the phenotype. Several mouse genetic models for COME and COM were identified by a deafness screen as part of a larger scale N-ethyl-N-nitrosourea (ENU) mutagenesis program; namely Jeff (Hardisty *et al.*, 2003), Junbo (Parkinson *et al.*, 2006) and Edison (Crompton

et al., 2017). These mice have a single point mutation, responsible for the onset and progression of COM. An advantage of this method to induce COM, is that there is no need for surgery and the mice spontaneously develop COM without the need for further manipulation.

The main pathological features of OM include middle ear inflammation, fluid accumulation, infection and hearing loss; either conductive and/or sensorineural. It is therefore important that an animal model covers all or many aspects of this pathology in order to better understand the pathogenesis of the human disease. In the different models, ideally, we would want to be able to investigate the following: how the pathogen ascends and colonises in the Eustachian tube, the pathogenesis of OM in the middle ear including induction, progression and potentially resolution of OM, mechanisms of host immunity, treatments and interventions, and the level/type of hearing loss. The Junbo mouse model is one of many models established for the study of OM. Multiple aspects and characteristics of this model indicate that the Junbo mouse model for middle ear infection is a good system for investigating the pathogenesis of acute and chronic OM; with many advantages over other mouse models. Throughout this chapter, the microbiological, immunological and histopathological characteristics of the Junbo mouse model have been described. Colleagues at the MRC Harwell carried out extensive microbiological and immunological studies (Parkinson *et al.*, 2006; Hood *et al.*, 2016; Vikhe *et al.*, 2018, 2019). Data from histological and immunohistochemical studies to investigate middle ear inflammation and characterise the macrophage populations in the middle ear and cochlea were described in 3.4. This model exhibits the main pathological features of OM and enables detailed investigation of multiple aspects of the OM pathogenesis listed above.

The mutation in the Evi1 gene causes spontaneous middle ear inflammation and effusion, without the need for invasive surgery to induce the OM. NTHi is inoculated via intranasal route and the bacteria rapidly ascends the Eustachian tube and colonises in the middle ear (Hood *et al.*, 2016), mirroring the natural route in humans. This allows for investigation of how NTHi ascends and colonises in the middle ear. The pre-inflamed middle ear environment recapitulates the situation in human OM whereby cases of recurrent AOM led to an inflamed middle ear environment, then when exposed to a pathogen there is high level of infection and lack of clearance. This is an effective model to investigate the immunological mechanisms for how chronic middle ear inflammation causes increased, recurrent and persistent infection and the potential functional damage of this on the auditory system.

A mouse model for chronic, transmissible OM has been established which displays several pathological features of OM (Dewan *et al.*, 2019), similar to the Junbo mouse model. In this model low numbers of a mouse respiratory pathogen, *Bordetella pseudohinzi,* is intranasally inoculated

then colonises in middle ears. The bacterium is transmitted to cage mates and naturally ascends the Eustachian tube in mice to colonise the middle ear. This causes a progressive hearing loss and both acute and chronic histopathological changes. Similar to the Junbo model, investigation of the progression of colonisation, level of infection and the long-term effects of chronic inflammation and infection is possible with this model (Dewan *et al.*, 2019). A disadvantage of this model is the use of the mouse pathogen, compared to inducing infection with a human pathogen such as NTHi in the Junbo mouse model which is the principal bacterium in OM.

Junbo mice show heterogeneity in the middle ear inflammatory response displaying variable middle ear inflammation, bulla fluid properties and infection rates. This is similar to the heterogeneous human disease phenotype. There are some disadvantages of the model. The hallmark features of human active mucosal COM are tympanic membrane perforation and purulent otorrhea however, these features are not often both observed in genetic mouse models. Otorrhea occurs in Junbo mice in low-health-status housing over 6 months but not in high-health status specific pathogen-free conditions (Parkinson *et al.*, 2006). This highlights the difficulty for laboratory animals, which are not exposed to the environmental pathogens that humans are exposed to, to be exact models for human disease. If the Junbo mice were not raised in specific-pathogen-free conditions then the model may be a better model for human active mucosal COM and provide more information about the mechanisms behind the pathogenesis.

The heterozygote Jeff (Jf) mutant, also identified in the N-ethyl-N-nitrosourea (ENU) mutagenesis program, develops spontaneous active mucosal COM with conductive hearing loss in specific-pathogen-free conditions (Hardisty *et al.*, 2003). The Jeff mutation was mapped to chromosome 17 and was later found to be a mutation in the F-box gene, Fbxo11 (Hardisty-Hughes *et al.*, 2006). FBXO11 is a member of a large family of F-box proteins that is involved in ubiquitination. It is expressed in the middle ear epithelium in the period before the mice develop OM (Hardisty-Hughes *et al.*, 2006). Hardisty-Hughes et al speculated that ubiquitination could be playing a role in inflammatory signalling pathways involving NFkβ in the middle ear in response to NTHi (Hardisty-Hughes *et al.*, 2006). Unlike Junbo mice, Jeff heterozygotes display physical characteristics different to their wild-type littermates including smaller size and a mild craniofacial abnormality (Hardisty-Hughes *et al.*, 2006). Additionally, older Jeff mice have high hearing thresholds and low endocochlear potentials, suggestive of SNHL due to cochlear damage (Hardisty-Hughes *et al.*, 2006). Junbo mice do not present these characteristics which is an advantage of this model over Jeff mice.

Differences in middle ear pathology of Junbo and Jeff mice have been described. Both mice have hypoxic inflammatory cells in the bulla lumen, while Junbo mice also have a hypoxic mucosa

(Cheeseman *et al.*, 2011). Middle ear inflammation and bulla fluid cell numbers were higher in Junbo compared to Jeff mice. The inflammatory middle ear environment in Junbo mice is advantageous for investigating mechanisms of NTHi infection and clearance. In one of the largest human candidate gene association studies to date, FBXO11 was shown to be associated chronic otitis media (Bhutta, Lambie, *et al.*, 2017). The Jeff model is therefore an important model to investigate the mechanisms of COM and it useful to make comparisons with the Junbo mouse.

3.5.6 Limitations of this work

This work is being carried out in collaboration with Derek Hood and colleagues at the MRC Harwell, Oxford. The Junbo mice were bred, inoculated and housed at the Mary Lyon centre (MRC Harwell). Due to the slowing down of all in vivo experiments during the first year of COVID-19, the full planned experiments were not carried out as anticipated resulting in an incomplete set of mice. There was difficulty in obtaining the tissue we desired, in the correct quantity and time frame. Upon facilities opening again following COVID-19, there were delays in experimental plans and timelines as all animal breeding had to restart.

A limitation of this study is the low number of mice as the results described would be better validated with a more powered study. However, due to lack of previous evidence in this area, whereby the macrophage populations in the middle ear and cochlea of a mouse model of this type have not been characterised there was an initial need for reliable pilot data. In this initial pilot study, the middle ear histopathology and middle and inner ear macrophage populations have been characterised in the Junbo mouse model for NTHi infection. This work is valuable and is necessary for future work to be carried out.

3.5.7 Future work

The Junbo mouse model is an informative model for investigating the pathogenesis of OM. In addition, the model enables the investigation of the effect of chronic inflammation on middle ear and cochlear macrophage populations. In the same tissue that we have been investigating and with the addition of tissue of later timepoints, there are further questions to address using additional techniques.

- Determine the hearing function of the Junbo mice using ABR
- Investigate the function of the macrophages by measuring changes in gene expression
- Look for evidence of damage in the cochlea and middle ear cytoarchitecture due to the inflammation

- Investigate the effect of chronic inflammation and infection for a longer time period on the middle ear and cochlear macrophage populations
- Investigate the effect of additional insults overlaid onto the chronic inflammation on the middle ear and cochlear macrophage populations

Click-box tests were carried out in Junbo mice in initial experiments, revealing a conductive hearing loss. However, it will be useful to investigate the hearing function in greater detail using auditory brainstem response (ABR) tests, to better understand the sensorineural hearing function.

RNAscope is a technique providing information about the genes the cells are expressing. The gold standard method for measuring gene expression is quantitative polymerase chain reaction (qPCR). With this technique, RNA is extracted by a homogenisation approach which involves mixing the tissue together and destroying the tissue-specific context of gene expression. RNAscope is RNA in situ hybridisation, a method that allows the detection of target RNA within intact cells in the context of the tissue they are in. RNAscope uses labelled complementary RNA probes to localise specific RNA in tissue. It allows single molecule visualisation in individual cells achieved through a novel probe design strategy and a hybridisation-based signal amplification system, to simultaneously amplify signals and suppress background. A panel of genes will be used to provide insight into the origin of the cells (CD11b) and the phenotype i.e., whether they are pro- (Itgax) or anti-inflammatory (IL-10) and whether there is any evidence of priming (Il-1β).

Characteristics of the cochlea and middle ear cytoarchitecture will be investigated using techniques including tetrachrome and trichrome histological stains, additional IHC markers and label-free imaging techniques such as second-harmonic generation microscopy. The Junbo mouse middle ear cavity will be investigated for evidence of damage caused by the chronic inflammation such as epithelial remodelling, submucosal fibrosis and collagen deposition. In the trichrome stain, tissue of differing composition can be visualised with different colours indicating the tissue type. For example, cell nuclei are stained brown, collagen is stained blue, muscle is stained red and cytoplasm is pink. This stain will enable the investigation of differences, in tissue structure and composition in the cochlea and middle ear, between WT and Junbo mice. A tetrachrome stain provides information about the maturation and mineralisation of the bone. Osteoid tissue, the unmineralized organic portion of the bone matrix, is stained deep blue. The mineralised bone is stained red (Villanueva, Hattner and Frost, 1964). Changes in the bone composition in the apical regions of the cochlea (in direct contact with the inflamed middle ear in the Junbo mice) and the middle ear bone will be examined.

Second-harmonic generation microscopy is a label-free imaging technique, effective at visualising the supramolecular assembly of collagen tissues at a high level of detail to provide qualitative and

quantitative information (Campagnola and Dong, 2011; Chen *et al.*, 2012). This technique will give insight into the structure and composition of the middle ear and cochlear connective tissue in the Junbo mouse and determine any pathological changes associated with inflammation or infection. Changes in the tissue and bone composition of the middle ear may impact the transmission and amplification of sound waves to the cochlea.

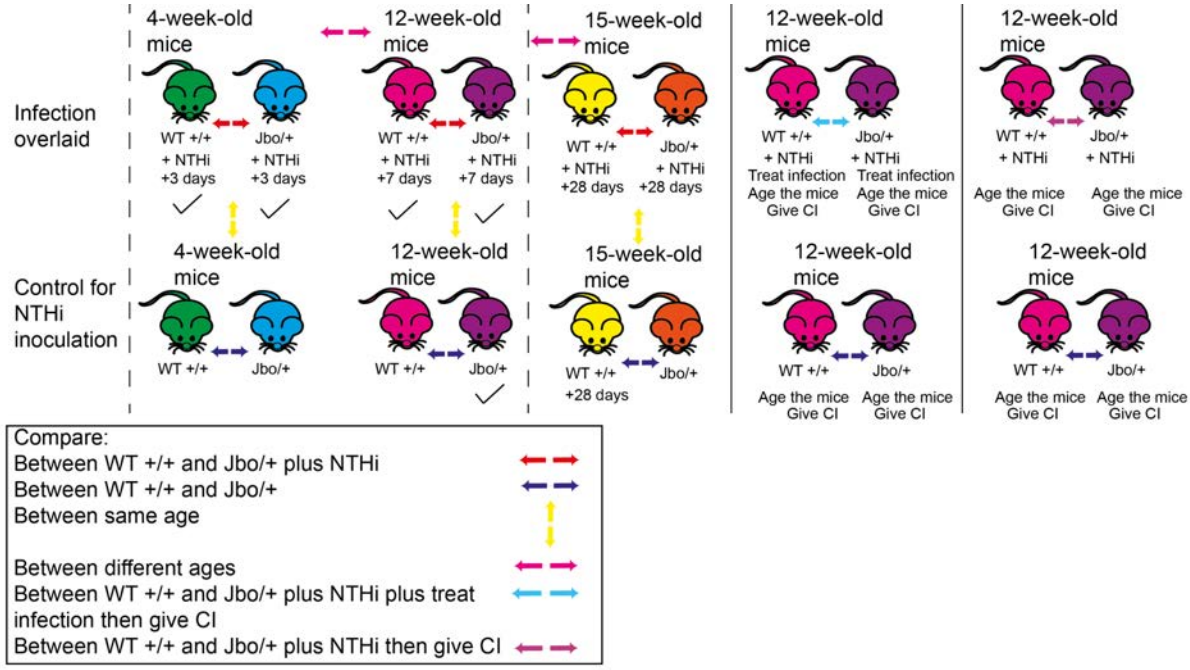


Figure 3.34 **The experimental design of the Junbo mouse model for middle ear infection to investigate the macrophage response in the auditory system**

The groups of mice and possible variables involved in this study. Between the two dashed lines, indicate the mice described in this chapter. Beyond the dashed line indicate the additional groups of mice with differing variables such as age, time with infection, treatment of infection and cochlear implantation to be investigated in future experiments.

To investigate the consequences of COM on the cochlea and the macrophage populations in the cochlea, a future experiment will be to age the Junbo mice (15-weeks-old) (Figure 3.34) and investigate differences in middle ear and cochlear histopathology and macrophage populations in older Junbo mice which have had COM for longer. Further to this, to investigate whether older Junbo mice respond differently to NTHi infection overlaid.

As we are interested in how early life inflammation affects the macrophage populations in the auditory system and whether this influences the trajectory for hearing loss, a future experiment would aim to mirror a human experience. Whereby an individual experiences early life middle ear inflammation and infection, then treatment and resolution of the infection followed by receiving

a CI later in life. We are interested in whether chronic middle ear inflammation and infection, causes changes in the auditory system influencing the response to and function of a CI.

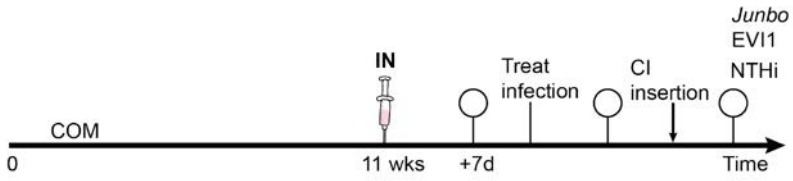


Figure 3.35 **A timeline for the experimental design of a future study whereby Junbo mice are inoculated with NTHi, the infection is treated and resolved then the mice are inserted with an electrode array into the cochlea**

This timeline mimics a human situation of early life middle ear inflammation followed by cochlear implantation later in life.

3.5.7.1 Relevance of this work to human health

We are interested in investigating whether early life inflammation/previous inflammatory insults influence the trajectory of hearing naturally, and with a CI later in life, and the potential mechanisms responsible. As discussed in Chapter 2, several risk factors for progressive hearing loss are associated with changes/increases in inflammation such as aging, noise exposure and lifestyle factors (smoking and diet). Co-morbidities with an inflammatory component such as type 2 diabetes and cardiovascular disease, are associated with increased levels of circulating inflammatory markers. Several studies in older people have found an association between the level of blood inflammatory markers and severity of hearing impairment (Masuda *et al.*, 2012; Verschuur *et al.*, 2012; Nash *et al.*, 2014; Verschuur, Agyemang-Prempeh and Newman, 2014). These studies show a clear association between inflammation and progressive hearing loss. We are hypothesising a link mediated by macrophages in the auditory system.

Using the Junbo mouse model, we have begun to investigate the effect of chronic inflammation on macrophages in the middle ear and cochlea and how chronic inflammation effects how the macrophage populations respond to an additional inflammatory challenge, NTHi infection. Evidence from other areas of the body suggest that macrophages and microglial cells can become primed by an inflammatory insult, resulting in an exaggerated inflammatory response when exposed to an additional inflammatory insult (Cunningham *et al.*, 2005; Godbout *et al.*, 2005; Lopez-Rodriguez *et al.*, 2021). This can lead to further tissue damage and loss of function.

In OM, chronic and recurrent middle ear inflammation early in life may cause macrophages in the auditory system to become primed. Exposure to additional inflammatory challenges through life such as further infections or cochlear implantation, may result in an exaggerated response leading

to tissue damage in the auditory system. This may contribute to progressive hearing loss and damage to auditory structures resulting in poorer performance with aided hearing including hearing aids and cochlear implants.

In order for us to understand whether early life inflammation (such as OM) influences the progression of hearing loss through life and whether this will affect CI performance, we need to determine whether this information is documented correctly in the clinical setting. A service evaluation to establish documenting practice of middle ear inflammatory histories, prior to cochlear implantation surgery at the University of Southampton Auditory Implant Service, was carried out. The results suggested an underreporting of historic episodes of middle ear inflammation across paediatric and adult cohorts. Additionally, this study identified how history taking could be improved so that the role of middle ear inflammation as a prognostic factor, and whether early life middle ear inflammation influences CI outcomes, could be determined. Further to this study, our group is utilising CI clinical records with machine learning approaches to establish relationships/associations between inflammatory conditions and CI performance in human cohort studies.

Translation of this work on the Junbo mouse model to human samples is clinically relevant and important for unpicking the immunological mechanisms of human OM. In a study, designed between our lab at the University of Southampton and University of Manchester, human middle ear fluid and mucosal samples will be collected from children with COME before having grommet surgery. For control tissue, the same samples will be collected from children with no OM who are having CI surgery. The innate immune environment of the middle ear fluid and mucosal samples will be analysed, similarly to the Junbo mouse model.

3.5.7.2 Implications of a history of OM for people with hearing loss who are given a cochlear implant

If early life inflammation causes changes in the auditory system resulting in damage to key structures and decreased function, this could impact an individual's hearing trajectory and also how well an individual does with a cochlear implant. Understanding the mechanism for how early life, chronic inflammation may affect auditory function and whether macrophages play a role in this will better inform future treatments and therapies for OM.

CI surgery is an acute inflammatory insult to the cochlea resulting in a wound healing response involving inflammation, proliferation and tissue remodelling. At implantation, if there is a population of macrophages in a primed state from a previous insult such as OM; this would result in an exaggerated inflammatory response. Increased inflammation may lead to an unresolved

wound healing response and fibrosis, which can cause a decline in hearing performance (Kamakura and Nadol, 2016).

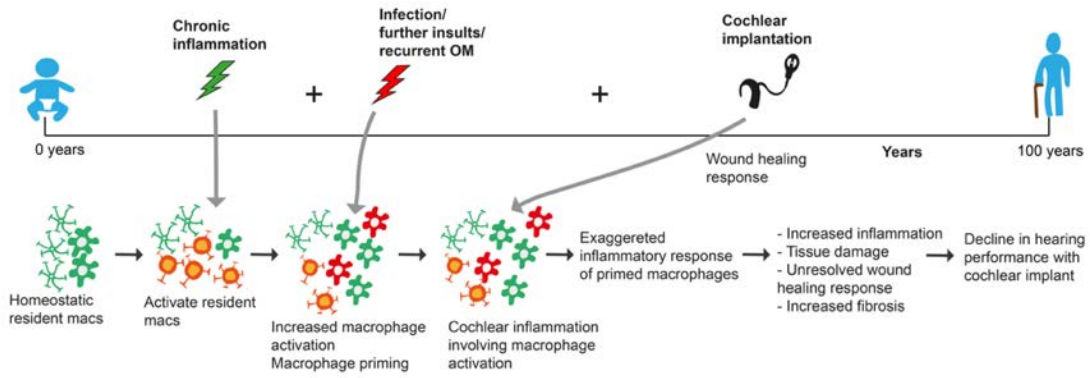


Figure 3.36 **Schematic demonstrating the effect of chronic inflammation and additional insults on resident macrophages in the cochlea and the implications for cochlear implantation**

This figure proposes that macrophages can become primed by chronic inflammation resulting in an exaggerated inflammatory response following the surgical insertion of a CI. An exaggerated inflammatory response involving increased production of pro-inflammatory mediators results in increased tissue damage, an unresolved tissue response and increased fibrosis. This leads to a decline in hearing performance and poorer CI outcomes.

Understanding an individual's history of OM, along with their history of other chronic inflammatory conditions, may be relevant as a predictor of CI performance. In individuals who experience a decline in hearing function through their lifetime and then receive a CI, a history of recurrent persistence or chronic OM may be a useful predictor and marker to identify this individual as someone who may not experience optimal hearing performance. In addition to this, reasons that lead to poor/underperformance with a CI are still not well understood.

Chapter 4 Investigating cochlear implant (CI) failure cases where a significant deterioration in hearing performance results in explantation – does the biological response at the tissue-electrode interface influence CI performance outcomes?

4.1 Introduction

4.1.1 An opportunity to investigate the biological response to cochlear implantation in a soft failure case

As discussed in 1.1, despite the success with cochlear implants (CIs) there is a small but significant group of individuals who fail to achieve the desired hearing outcome after implantation. Factors that contribute to CI performance include patient and intervention factors and the tissue response. Patient factors include medical history, cause of deafness, age of deafness and age at implantation. Intervention factors include surgical methods such as soft/atraumatic surgery, round window insertion vs cochleostomy, and properties of the electrode array such as length, thickness, flexibility and electrode material. The tissue response includes the type, distribution and function of the cells in the vicinity of the electrode array. The role of the tissue response in the variability in performance, and in some cases complete failure experienced by people with a CI, is poorly understood. CI failures are often caused by hardware or surgical failures. In cases where hardware and surgical failures can be ruled out, the failure is described as a soft failure. Soft failures can be caused by the tissue response to cochlear implantation occurring over time. Detailed understanding of the mechanisms of the inflammatory response at the tissue-electrode interface are lacking. We were presented with a unique opportunity to investigate the tissue response of the fibrotic sheath that formed around an electrode array in a case of migration and soft failure, in a living person with a CI who was undergoing explantation and reimplantation.

4.1.2 Cochlear implants

Cochlear implants are the most successful neuro-prosthesis, recommended for people with severe to profound deafness who do not receive adequate benefit from hearing aids. Around 19,000 people are living with cochlear implants in the UK (Cullington, 2021). Hearing loss occurs

as the cochlea loses its ability to convert mechanical energy from sound waves into neural impulses due to loss of or damage to the hair cells. The CI functions by directly stimulating neurons of the auditory nerve sending sound information to the brain. Therefore, for a CI to function, a functional nerve and implantable cochlea is required.

Cochlear implants are made up of two main components, a sound processor and an implant receiver, highlighted in Figure 4.1. The sound processor, worn on the back of the ear, captures the sound and transforms it into a digital signal. This is then transmitted to an implant receiver surgically placed under the skin. The receiver transforms the digital information to an electronic signal which goes to the electrodes in the cochlea. Each electrode on the array corresponds to a signal frequency. When the encoded signal is transmitted to the corresponding electrode, spiral ganglion neurons forming the auditory nerve are stimulated. The auditory nerve transmits the information to the brain via the central auditory pathway. For sound to be perceived by the user, adequate stimulation of the central auditory processing pathways is required. Cochlear implants are beneficial for people who have very little to no hearing function, giving them the ability to communicate, listen to music and succeed in a work environment.

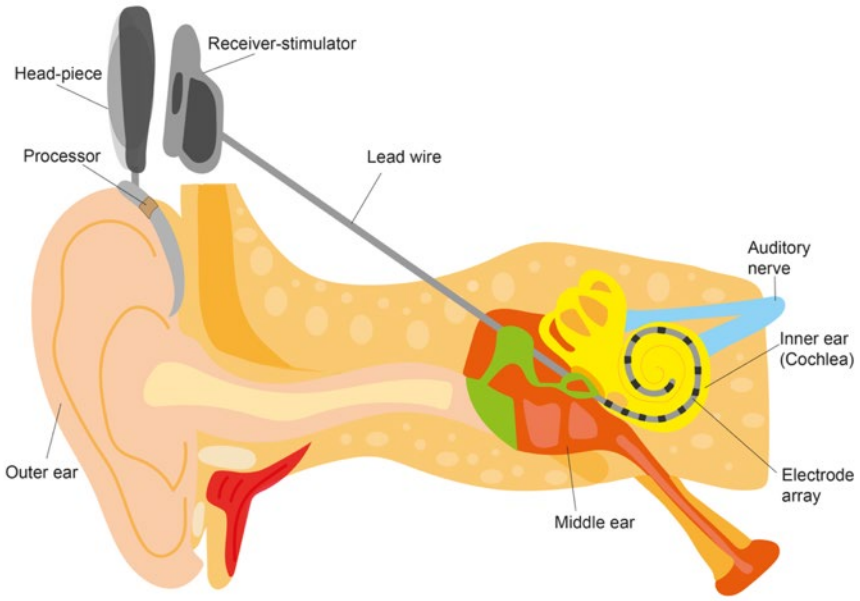


Figure 4.1 **Diagram to show the components of a cochlear implant**

The components of a CI including the externally worn sound processor with a microphone(s) and the receiver coil and stimulator, surgically placed under the skin. The electrode array is surgically placed into the cochlea.

4.1.3 Cochlear implant candidacy

The assessment for hearing function in the UK occurs soon after birth. Under the NHS new born hearing screening programme, all new born babies are screened for hearing difficulties in the first 26 days of birth. Those with hearing loss should receive hearing aids within 2 months of the diagnosis. Ongoing audiological assessment will assess whether a baby/child is suitable for a CI. If it is thought that the child is a potential candidate for a CI, they will be referred to one of the CI centres in the UK. For adults, hearing tests are available if they feel that their hearing has deteriorated and hearing aids may be offered to them. The function and benefit of the hearing aids will be reviewed every 4 years by audiology departments. If the audiologist deems the individual as a potential candidate, they will be referred to a CI centre in the UK.

In the UK, there is a specific assessment to determine CI candidacy under the National Institute of Health Care Excellence (NICE) guidelines. For adults, unilateral cochlear implantation is recommended for people with severe to profound deafness who do not receive adequate benefit from acoustic hearing aids. In January 2019, the eligibility criteria for cochlear implants in the NICE guidelines were updated and amended (NICE, 2019). Severe to profound deafness is defined as only hearing sounds that are louder than 80 dB at two or more frequencies (500 Hz, 1,000 Hz, 2,000 Hz, 3,000 Hz and 4,000 Hz) bilaterally without hearing aids. The classification for receiving adequate benefit from a hearing aid for adults, was a score of 50% or greater on Arthur

Boothroyd word test presented at 70 dB SPL. These updated recommendations are estimated to increase the number of people who receive cochlear implants by 70%. For children, the speech, language and listening skills are measured along with the developmental stage and cognitive ability using the hearing aid. Children and adults, who are blind or have other disabilities that increase their reliance on auditory stimuli, are recommended simultaneous bilateral CIs. There is an audiology assessment, medical and radiological evaluation and psychological assessment. If the individual is deemed suitable and that they would benefit from a CI, a multidisciplinary team decide the best route of action for the person to be implanted. The updated guidelines and increased eligibility will bring the UK into closer alignment with other European countries.

4.1.4 Wound healing response to cochlear implantation

At implantation, electrode arrays are inserted into the cochlea either by cochleostomy, where a hole is drilled into the bone of the cochlear wall, or by round window insertion (through the round window membrane). The biological response following surgical implantation of the electrode involves an acute and chronic inflammatory response. This should resolve as part of the normal wound healing response, consisting of three phases: inflammation, proliferation and remodelling (Figure 4.2) and results in the formation of a fibrotic sheath around the implanted device. The inflammatory phase occurs within hours and lasts for a small number of days. Damage to capillaries results in blood clots leading to proteins, such as fibrinogen and albumin, to be adsorbed onto the implanted electrode forming the provisional matrix. Platelets in the blood clot release chemokines which signal to recruit immune cells such as neutrophils, macrophages and fibroblasts to the site and adhere to the protein matrix. Inflammatory mediators are released from infiltrated immune cells as part of the inflammatory response. The proliferative phase involves active angiogenesis, required for the delivery of oxygen and nutrients to the wound site. Fibroblasts, key cells involved in extra-cellular matrix production and maintenance, proliferate into myofibroblasts which are collagen-producing cells. Collagen forms a large part of the fibrotic sheath. The remodelling phase involves tissue remodelling and formation of the mature scar tissue around the array.

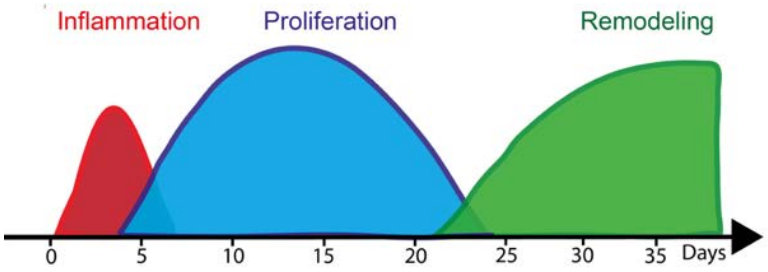


Figure 4.2 **Stages of the wound healing response to cochlear implantation**

Inflammation involves the recruitment of immune cells to the injury site and an increase in the production of inflammatory mediators. The proliferation stage involves the formation of new blood vessels and the differentiation of fibroblasts into myofibroblasts, collagen producing cells. The remodelling stage involves tissue remodelling where a mature scar forms around the implanted array.

4.1.5 Inflammatory response to cochlear implants

Most of our understanding of the tissue response at the electrode array following cochlear implantation comes from human post-mortem (Lee, Eddington and Nadol, 2011; Nadol *et al.*, 2014; Seyyedi and Nadol, 2014) and animal studies (Mistry *et al.*, 2014; Bas *et al.*, 2015). Tissue damage at the site of cochleostomy or round window membrane, and along the path of electrode insertion, causes an acute inflammatory response involving increased production of inflammatory cytokines and the recruitment of immune cells. A chronic inflammatory response follows, involving the formation of a fibrotic sheath around the electrode array and in some cases new bone formation, evidenced by human temporal bone studies (Li *et al.*, 2007; Fayad, Makarem and Linthicum, 2009; Kamakura and Nadol, 2016; Ishai *et al.*, 2017). Analysis of the cellular profile of the fibrotic sheath revealed a characteristic immune response involving B and T-cell lymphocytes, macrophages and foreign body giant cells (Nadol *et al.*, 2014; Seyyedi and Nadol, 2014). The analysis of the tissue response in these post-mortem studies have identified the cellular composition and tissue response around the electrode array at the end of life. These studies have been fundamental in building our understanding of the biological response to cochlear implantation. Further understanding of the wound healing response to cochlear implantation is necessary.

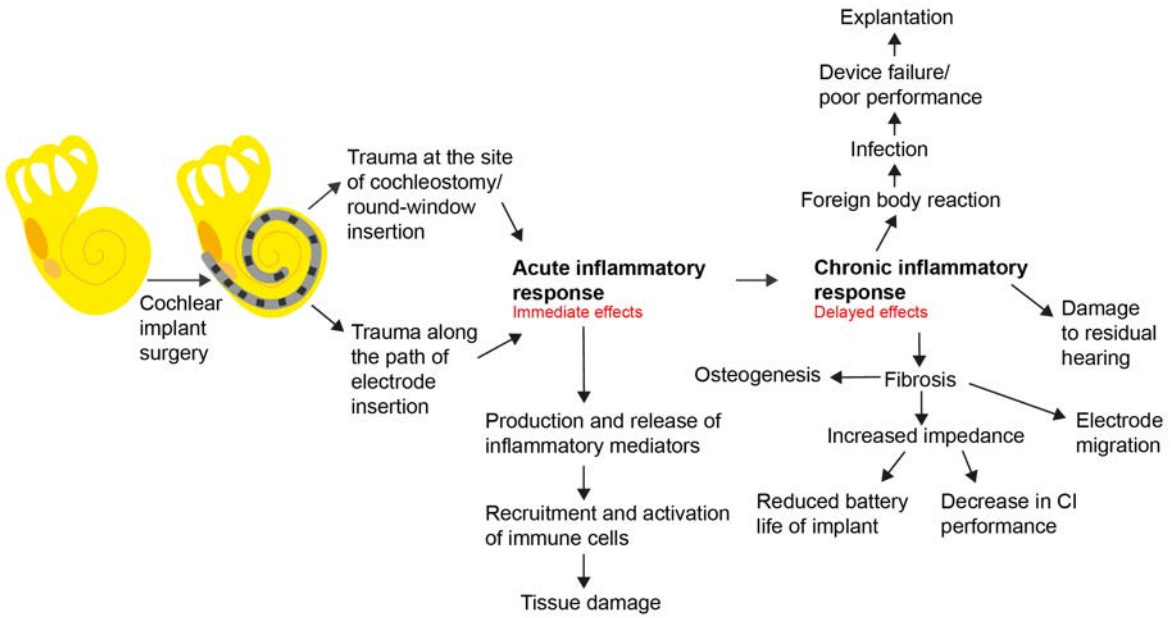


Figure 4.3 **Schematic of the inflammatory response to cochlear implantation involving an acute and chronic inflammatory response**

The events that occur in response to cochlear implantation including the immediate effects in the acute inflammatory response and the more delayed effects as part of the chronic inflammatory response.

4.1.6 Chronic inflammation and fibrosis

In some individuals, the normal wound healing response does not resolve leading to chronic inflammation and fibrosis. There is heterogeneity in this response between individuals. Chronic inflammation can result in a foreign body reaction (FBR) which is a pathological complication following implantation involving macrophages and foreign body giant cells. It is the end-stage response of the wound healing process following implantation of a medical prosthesis (Anderson, Rodriguez and Chang, 2008). FBR can lead to recurrent wound infection and device failure resulting in further surgery to remove the implant (Lim *et al.*, 2011). An exaggerated FBR can progress to neo-ossification (Foggia, Quevedo and Hansen, 2019). New bone growth can influence post-implant hearing performance (Kamakura and Nadol, 2016).

The immunological response to cochlear implantation involves the formation of a fibrotic sheath around the electrode array inside the cochlea which can be beneficial to secure the electrode in place. The size of fibrotic sheath varies and, in some individuals, progresses to a bony occlusion. The presence of new bone and fibrotic tissue throughout the cochlea and ensheathing the electrode has undesirable effects in terms of both overall device function and for the success of further surgeries (as an individual is likely to need multiple CIs throughout their lifetime).

Significant fibrosis and new bone growth around the electrode lead to post-operative increases in impedance, diminishing device performance. Impedance is the resistance of the electric circuit to the current flow when a voltage is applied. Higher impedance causes higher voltages generated across the tissue-electrode interface, which may lead to an excessive spread of current over an area and decrease the dynamic range of stimulation (Wilk *et al.*, 2016). An unnecessary increase in voltage leads to increased energy consumption reducing the durability of the implants batteries, a considerable problem for life-span of the implant (Wilk *et al.*, 2016). A positive correlation between fibrous tissue growth and increased impedance was shown in guinea pigs, which could be reduced with localised treatment of the anti-inflammatory agent, dexamethasone (Wilk *et al.*, 2016). Reducing local inflammation may be a way to reduce excessive fibrosis and poor performance. Impedance measurements are being investigated as a diagnostic tool to determine to the level, and temporal profile of fibrous tissue growth after cochlear implantation (see 1.8.1).

4.1.7 Cochlear duct length variability

Cochlear duct length is the distance from the natural entrance of the cochlea through to the helicotrema and varies between individuals. This variability is likely to contribute to the poorer outcomes following implantation. With such variation between individuals, a one size fits all approach is not appropriate and will not result in optimal outcomes. Evidence of a correlation between higher electrode insertion depth and improved hearing outcomes has been described (Hochmair *et al.*, 2003; O'Connell *et al.*, 2016; Büchner *et al.*, 2017). In a clinical study performed on 10 people with a MED-EL COMBI 40+ CI with an insertion depth of 30mm, stimulation was either distributed across the whole length of the cochlea or concentrated at the basal end to mimic an insertion depth of 20mm (Hochmair *et al.*, 2003). Improvement in speech perception in quiet and noise was measured when stimulating the entire length of the cochlea including the apical region, compared to just stimulating electrodes in the basal region (Hochmair *et al.*, 2003). A larger study involving 91 adults implanted with a FLEX^{20}, FLEX^{24} or FLEX^{28} electrode, measured speech perception in quiet and noise at 3- and 6-months of use with electric-stimulation (ES) or electric-acoustic-stimulation (EAS) (Büchner *et al.*, 2017). In solely electric-stimulation users, those with the longest array (FLEX^{28}) had the best speech comprehension scores after 3- and 6-months suggesting better hearing outcomes following deeper electrode insertion.

Deeper electrode insertion however may increase the risk of insertion trauma, intracochlear damage and inflammation (Adunka and Kiefer, 2006; Suhling *et al.*, 2016). Intracochlear trauma is likely to affect the preservation of residual hearing and the health of the spiral ganglion neurons. In a retrospective study of 120 adult patients with preoperative residual hearing, implanted with

different thin flexible electrode arrays of varying lengths (TFEA 20, 24 or 28), pure-tone audiometry scores were used to determine the degree of hearing preservation and how this related to electrode array length (Suhling *et al.*, 2016). Hearing preservation was possible in the majority of patients implanted with each array. Interestingly, shorter electrode arrays resulted in greater hearing preservation and lower levels of hearing loss compared to the longer arrays. There is continued debate in the field between the optimal insertion depth for good hearing performance outcomes and reduced insertion depth to minimise intracochlear trauma and damage to cochlear structures essential for the use of residual hearing.

A selection of arrays with different designs and length are now available, therefore accurate preoperative imaging methods to evaluate the patient's cochlear duct length and anatomy are required. Computed tomography (CT) and magnetic resonance imaging (MRI) prior to surgical implantation is commonly used for prospective CI patients (Widmann *et al.*, 2020). Preoperative CT imaging provides information about the cochlear bone morphology and can identify cochlear malformations. This information is used to inform surgical technique and indicate the need for a specific array design. MRI provides key soft tissue detail about the membranous labyrinth and anatomical information about the cochleovestibular nerve.

For intra- and post-operative imaging in cochlear implantation, cone-beam computed tomography (CBCT) is a newer technique that uses a cone-shaped x-ray beam directed through the target to produce accurate three-dimensional imaging. A full volume of image can be captured in a single rotation, whereby the sample does not need to rotate. The advantages of CBCT include lower radiation dose, higher resolution, shorter imaging time and less intense metallic artifacts than conventional CT (Dahmani-Causse *et al.*, 2011).The ability to estimate the position of electrode arrays using CBCT imaging was initially investigated by implanting eight fresh frozen temporal bones with either Cochlear Slim-Straight (n= 5) and Contour Advance electrode arrays (n= 3) (S. R. Saeed *et al.*, 2014). CBCT imaging and histological examination of the temporal bones was carried out to estimate the electrode array scalar location of each temporal bone. This study demonstrated that CBCT imaging produced clear images whereby electrode contacts and scalar position could be reliably determined. CBCT is now utilised during and following surgery for cochlear implantation. Intra-operative CBCT is a safe, rapid and reliable method to identify misplacement or fold over of the electrode array to initiate immediate correction (Jia *et al.*, 2018). Post-operative CBCT is used for the evaluation of electrode array position, whereby it is possible to determine the number of electrodes inserted and to measure insertion depth angle and length following implantation (Widmann *et al.*, 2020).

4.1.8 Variable/poor cochlear implant performance

CI performance is variable across people with a CI. In addition to this, the way to describe and define performance and poor performance has not been standardised. There is a population who are not achieving maximal benefit but who are also not yet identified as a failure case as they may not need revision surgery. Different clinical diagnostic tests, such as remapping of the device and a device integrity test, can be used to determine the function and attempt to identify the problem with the CI. Continuous and regular electrode impedance recordings is a useful measure of CI performance (Busby, Plant and Whitford, 2013; Sanderson *et al.*, 2019). Higher impedance was associated with poor performance including a lower average hearing threshold and comfortable listening skills (Busby, Plant and Whitford, 2013). Electrode impedance measures of CI users over a 5-year period were analysed and individuals with significantly raised impedance (which were below the manufacturers warning level of high impedance) were identified (Sanderson *et al.*, 2019). This is clinically relevant, if implemented routinely, as these patients could be monitored more closely to determine if the impedance continues to increase and whether this is an indicator of a decline in function. Currently there is no consensus for what is a failure or decrease in performance from a patient perspective. Therefore, it is difficult to determine and establish what is the threshold for failing from a patient's point of view. What is a failure and what meets a criterion for a failure? Being better informed of the factors that influence CI performance and having better methods and biomarkers to measure CI performance will result in earlier intervention and increased patient specific, personalised treatment to prevent poor performance outcomes.

4.1.9 Explantation and reimplantation/ revision surgery

With the increasing numbers of people receiving cochlear implants worldwide, there is an increase in the number of cases of explantation and reimplantation/revision surgery (Kimura *et al.*, 2020). Explantation is the removal of a device and reimplantation is the insertion of a new, sometimes different device. Revision surgery involves the removal or repositioning of the CI, either for medical reasons or because of device failure. Many studies have reviewed the rate of revision surgery and reimplantation in different cohorts and CI centres and determined the reasons for failures (Sorrentino *et al.*, 2009; Wang *et al.*, 2014; Ravikumar, Vishwakarma and Keshri, 2018; Karamert *et al.*, 2019; Lane *et al.*, 2019; Bourdoncle *et al.*, 2020; Gumus *et al.*, 2020; Layfield *et al.*, 2020). The revision surgery/reimplantation rate varies between 2.9 - 8.7% in these retrospective studies. Many factors relating to study design will influence this rate including follow-up duration of the study, the cohort size, paediatric and/or adult population in the cohort, and date of implantation of that cohort (later date will mean more technologically advanced

devices and some dates may include batch failures that may skew results for that period). Rates of reimplantation and CI revision are higher in children compared to adults, due to skull growth and the higher incidence of falls and physical contact in children resulting in head trauma (Weise *et al.*, 2005; Côté *et al.*, 2007; Wijaya *et al.*, 2019).

The main reasons for explantation include device failure (hard and soft failure) and medical or surgical failure. A hard failure is marked by intermittent or no communication between the speech processor and internal device or failed device integrity testing (Stevens *et al.*, 2019). A medical or surgical failure includes infection, incision breakdown and an allergic reaction (Kimura *et al.*, 2020). A soft failure occurs in cases with poor post-implant audiologic performance or subjective adverse symptoms in the setting of a normal device integrity testing and radiographic studies (Balkany *et al.*, 2005; Kimura *et al.*, 2020). Soft failures are adverse events that are not caused by hardware or surgical failures and therefore, it is likely that the interaction between the individual's biology and the CI is responsible. The biological mechanisms driving these failures are not well understood, however there is evidence to suggest that an aberrant immunological response may play a role (Hough, Sanderson, *et al.*, 2021). Following revision surgery, a return of function or resolution of adverse symptoms supports the diagnosis of soft failure (Balkany *et al.*, 2005). Some CI failure cases that initially fit the criteria for a soft failure, do not meet the criteria following reimplantation as they continue to experience adverse symptoms or do not experience an improvement in outcome. This group have been subcategorised as 'presumed soft failure' (Kimura *et al.*, 2020).

In cases of hard failures, the decision to undertake revision surgery is more straightforward and the time between diagnosis and reimplantation is often shorter (Buchman, Higgins, *et al.*, 2004; Kimura *et al.*, 2020). Whereas in cases of suspected soft failure, it is often more difficult for an audiologist to diagnose when presented with the array of symptoms that can occur with soft failures. This is especially true in young patients where it is harder to communicate poor CI function. Therefore, the time between the onset of symptoms and reimplantation is longer which could negatively impact the individual's speech and language development.

The most common reason for revision surgery is device failure including hard and soft failures (Wang *et al.*, 2014; Patnaik *et al.*, 2016; Batuk *et al.*, 2019; Karamert *et al.*, 2019; Stevens *et al.*, 2019; Wijaya *et al.*, 2019; Kim *et al.*, 2020; Kimura *et al.*, 2020; Layfield *et al.*, 2020; Rayamajhi *et al.*, 2020). In the last 10 years CI design and the accompanying technology has continued to advance, reflected in a decrease in the incidence of device (hard) failures and subsequent overall revision rate (Stevens *et al.*, 2019). Despite this, there have been instances of voluntary device recall when a device has a fault (recall of the CI500 series in 2011, Advanced Bionics HiRes 90K

device in 2010). These batch failures cause an increase/spike in the incidence of device failures and revision surgery in the following year or two.

The reported incidence of revision surgery for hard failures is variable. The number of revision surgeries for hard failures in an adult cohort was significantly lower in patients implanted after 2011, compared with those implanted before (following the recall of the CI500 device) (Stevens *et al.*, 2019). The overall revision surgery rate in the adult cohort of 432 patients was 7.4% and since 2011, a decline in the overall revision rate was measured. No known hard failures were recorded in patients implanted after 2011 (Stevens *et al.*, 2019).Whereas between 2005-2015 in the 81 revision cases for 64 patients, hard failures were the most common reason for revision surgery accounting for 53.1% (34/64) cases (Kimura *et al.*, 2020).

The incidence of soft failures appears to be increasing. Data from a retrospective meta-analysis study investigating reasons for failure between 2000 and 2010, revealed an increase in the proportion of soft failures from 7% to 27% while the proportion of hardware or surgical failures decreased from 93% to 73% (Causon, Verschuur and Newman, 2013). This data highlights that despite improvement in electrode design, signal processing technology and surgical technique; there is performance variability between individuals whereby the biology is responsible. Out of 81 revisions (64 patients), 29.7% (19/64) were soft failures (true and presumed), of which 68.4% (13/19) were true soft failures and 31.6% (6/19) were presumed soft failure (Kimura *et al.*, 2020) meaning this percentage failed to improve after surgery. Better understanding of the biological response to cochlear implantation and the factors that may affect this tissue response is fundamental for understanding the performance variability that can lead to soft failures.

Correlations between the reason for failure and reimplantation performance outcomes have been investigated. In most cases revision surgery was effective, particularly when the reason for explantation was device failure (Rivas *et al.*, 2008; Venail *et al.*, 2008; Stevens *et al.*, 2019; Kimura *et al.*, 2020). The number and outcomes of revision surgery performed during a 10-year period were reviewed, whereby out of 1469 procedures, 81 (5.5%) revisions were performed (Kimura *et al.*, 2020). 78.1% of the revision CI patients showed improvement postoperatively (Kimura *et al.*, 2020). Hard failures had the best outcomes, whereas the soft failure group had the lowest rate of performance improvement following revision surgery. This may be due an exaggerated/more chronic inflammatory response following the surgical insertion of the CI, in an already inflamed immune environment. Further investigation into the different causes and type of failures (and the biological mechanisms responsible) is necessary. In addition, better understanding of which types of failures resulted in better outcomes following reimplantation/revision surgery would be useful. This may inform more tailored, clinical decisions that are beneficial to the individual.

4.1.10 Electrode migration

Electrode migration is the movement of electrodes post-implantation resulting in extracochlear electrodes. It can be asymptomatic or present with a decrease in performance, gradual increase in impedance values in the basal electrodes, change in sound, pain sensation and facial nerve stimulation (Alenzi *et al.*, 2021). Electrode migration is a major complication that accounts for 1 - 15% of all revision surgery (Connell *et al.*, 2008; Brown *et al.*, 2009), and could be more common than previously thought (Dietz *et al.*, 2016). Audiological and electrophysiological tests and imaging techniques are required to confirm a suspected migration case. Often high-resolution radiological imaging such as CT or cone beam CT is necessary to determine the positioning of the electrode. Recording of electrode voltages (REVS) using surface electrodes is an effective method to detect electrode migration and extracochlear electrodes in adult CI users (Grasmeder *et al.*, 2021).

The reasons for electrode migration are not fully understood however some studies have suggested potential reasons for extrusion such as intracochlear factors (Rivas *et al.*, 2008; Zeitler, Budenz and Roland, 2009) including fibrosis, ossification or cochlear malformations and ossification after meningitis. Extracochlear factors include scar formation/fibrosis at the round window, middle ear and mastoid adhesions, cholesteatoma, skull growth (children) and extrinsic causes e.g. trauma and infections (Dietz *et al.*, 2016; Di Laora *et al.*, 2019). Electrode migration is more common in straight (lateral wall) electrodes compared to preformed (perimodiolar or modiolus hugging electrodes) (Mittmann *et al.*, 2015; Dietz *et al.*, 2016; von Mitzlaff *et al.*, 2020).

Electrode migration is thought to either occur rapidly in the early stages post-implantation or occur more delayed after several months. Rapid migration in the early stages has been proposed to be due to the lack of fibrotic sheath or ossification anchoring the array in position (Rivas *et al.*, 2008; Van Der Marel *et al.*, 2012). Three cases of migration occurred immediately after insertion, within 2 weeks (Green *et al.*, 2004). In cases of suspected delayed migration, silicone allergy is a potential cause. Three cases of delayed migration, occurring several months after implantation, were confirmed to have silicone allergy (Kunda *et al.*, 2006). Suspected electrode migration requires for prompt revision surgery to give the best chances for optimal reimplantation performance, as the migrated electrode may be causing tissue reactions such as fibrosis and ossification which may impair reinsertion (Rader *et al.*, 2016).

Many factors may be contributing to the mechanism of electrode migration including the mechanical (shape of electrode array) (Dietz *et al.*, 2016) and chemical (material coating electrode) (Kunda *et al.*, 2006) electrode factors and the biological factors (the cellular environment and how this may change following surgical insertion) (Kunda *et al.*, 2006; Di Laora

et al., 2019). The case study described in this chapter is a migration-related soft failure. Detailed investigation of the tissue composition and cellular environment of the fibrotic sheath, that formed around the migrating array over 10 months, will be carried out

4.1.11 Rationale for explant analysis

The two main ways to investigate the effects of cochlear implantation at a cellular and molecular level are by analysing tissue from animal models for cochlear implantation and from human temporal bone studies. Animal models are valuable as it is possible to manipulate the system whilst controlling all other aspects that may affect the response. A disadvantage of animal models is that often the materials used to implant into the cochlea are made of inert fluorocarbons and therefore do not mimic the materials used in human arrays. See Chapter 5 for further detail regarding animal models for cochlear implantation. Human temporal bone analysis allows the investigation of the bone that the CI has been anchored in for many years. These studies have provided the majority of the information that is currently known about the tissue response. A disadvantage of these studies is that the temporal bones are collected when the individual has died, meaning it is difficult to interpret when the biological response and damage has occurred, and how long it lasted for. There is often limited clinical and aetiological data available about the CI users.

A meta-analysis style approach was taken to compare the measures collected and results of multiple studies that have been carried out to investigate to the tissue response to cochlear implantation in human post-mortem tissue (see appendix B.4 for further detail of the studies). Details including; number of temporal bones analysed, measures collected, number of years of implantation and clinical details were extracted and compared. Each cross (X) on Figure 4.4 illustrates a single study. The number of temporal bones investigated and the average years of implantation of the temporal bones is shown. There are few studies that have investigated the tissue response at the early time points following cochlear implantation (red circle), likely due to lack of access to this tissue as in most cases the tissue is obtained at the end of life.

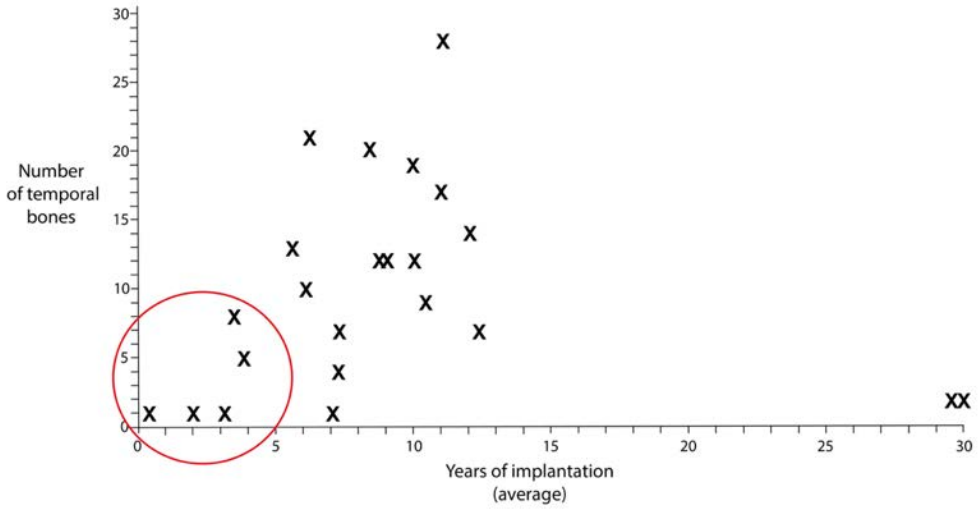


Figure 4.4 **Graph indicating the studies carried out to investigate the human tissue response to cochlear implantation**

The human temporal bone studies carried out to investigate the tissue response to cochlear implantation. Each cross represents one study. On the x-axis is average years of implantation of the temporal bones used in each study and on the y axis- the number of temporal bones in each study. The red circle indicates where further studies are needed.

Data richness of each study was calculated using a systematic approach whereby each individual method was scored e.g., histology and immunohistochemistry (3), clinical measures, CT imaging and 3D reconstruction methods (2) and bacterial assay, scanning electron microscopy (SEM) and fluorescent microscopy (1). The sum of the scores were calculated to given an overall data richness score (see appendix B.4). Figure 4.5 shows the variation in data richness between the studies, the studies with greater richness of data and the number of temporal bones investigated. Only three of the 23 studies used the cell-specific technique, immunohistochemistry, to investigate the tissue response to cochlear implantation. It is informative to have multiple measures collected in each study including histology and immunohistochemistry alongside clinical and imaging data. Having aetiological and clinical data alongside characterising the biological response would be beneficial in better understanding the mechanisms responsible for the tissue response and what factors may influence this. Clinical/hearing performance data gives an indication of how the tissue response may have affected hearing performance with the CI.

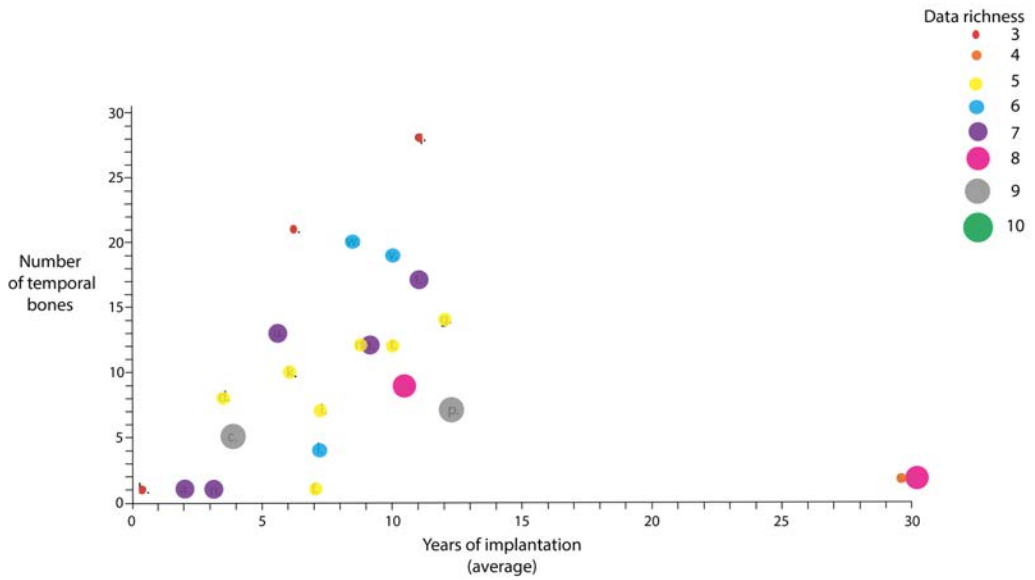


Figure 4.5 **Figure to highlight the variation in data richness across the human CI tissue response studies**

The data richness for each of the human temporal bone studies, based on the techniques used and the type of data collected. To assign a data richness score, each method was scored e.g., histology and IHC (3), clinical, CT imaging and 3D reconstruction (2) etc. For each study, the data richness score was calculated. This figure provides a clear visual to identify the type of data obtained from each study, and where this falls on the figure regarding the number of temporal bones and the average years of implantation.

Drawbacks of the human temporal bone studies include the relatively small number of studies, the small sample size, the large variability between the people with a CI (age, device, cause of deafness, surgical technique) in different samples and the difficulty in obtaining tissue and investigating the tissue response in living individuals. In addition, these studies are retrospective, and they often lack any aetiological or clinical data from the individuals. Investigating the response at the end of life, and with little clinical/hearing performance data, it is difficult to unpick the mechanisms involved in the tissue response and to determine whether the tissue response affects performance outcomes. More research should be carried out to better understand what mechanisms drive the tissue response to cochlear implantation that cause poor performance/soft failures. A way to investigate this is to characterise the tissue response at the tissue-electrode interface in a living person with a CI, who are undergoing explantation and reimplantation.

4.1.12 Case details

A female in her 50s was implanted with a Cochlear Nucleus CI522 device after developing a progressive SNHL over a decade. At the time of implantation, her hearing loss was profound for frequencies of 2 kHz and above and moderate for low frequencies (250 and 500 Hz). The device was inserted by round window insertion using a hearing preservation technique and the application of steroids. The device was activated after 6 weeks using the ACE processing strategy with a stimulation rate of 900 Hz, pulse width of 37ms, 10 maxima, and stimulation mode MP1+2. At activation, the two most basal electrodes gave open circuits on impedance telemetry so were deactivated. Over the course of 10 months, successive basal electrodes were deactivated due to open circuits and reports of non-auditory sensations and poor loudness growth. Rapid and dramatic changes in impedance were recorded over time. The clinical measures described, in addition to a CT scan 3 months post-activation, suggested electrode migration. At 10 months post-implantation, the decision was made to explant the device and reimplant with a new device. At explantation, the surgeon reported fibrosis, mechanical resistance to remove the array and five extracochlear electrodes. Routine manufacturer integrity testing confirmed the device was functional, diagnosing the case as a soft failure. Patient consent and ethical approval confirmed that full investigation of the fibrotic tissue associated with the array could be carried out.

4.2 Aims and objectives

Overarching question:

Does the tissue response at the tissue-electrode interface influence CI outcomes?

Objectives:

- To characterise the composition and organisation of the tissue surrounding the explanted array
- To characterise the cellular composition and organisation and determine what this tells us about the inflammatory status of the tissue at the tissue-electrode interface
- Correlate the biological findings of this migration-related soft failure with the documented clinical indicators to shed light on the potential explanations of the biological mechanism behind the soft failure
- Establish an effective protocol for further explant analysis

4.3 Methods

4.3.1 Tissue processing, embedding, sectioning

The electrode array and fibrotic tissue was placed in formalin solution for tissue fixation following explantation. The array was transferred to 70% ethanol and stored at 4°C. Figure 4.6a shows a photograph of the full length of the electrode array with the fibrotic tissue attached. The insert image in Figure 4.6a illustrates how the tissue has been split into four regions (R1-R4). Region one is the lead wire, region two is from the lead wire to the array, region three and four include electrode contacts. The black arrows in Figure 4.6a,b indicates the four most basal electrodes covered by the tissue. Figure 4.6b is a higher magnification micrograph showing the fibrotic tissue covering four electrode contacts. The asterisk indicates the suspected shear point of the tissue at explantation.

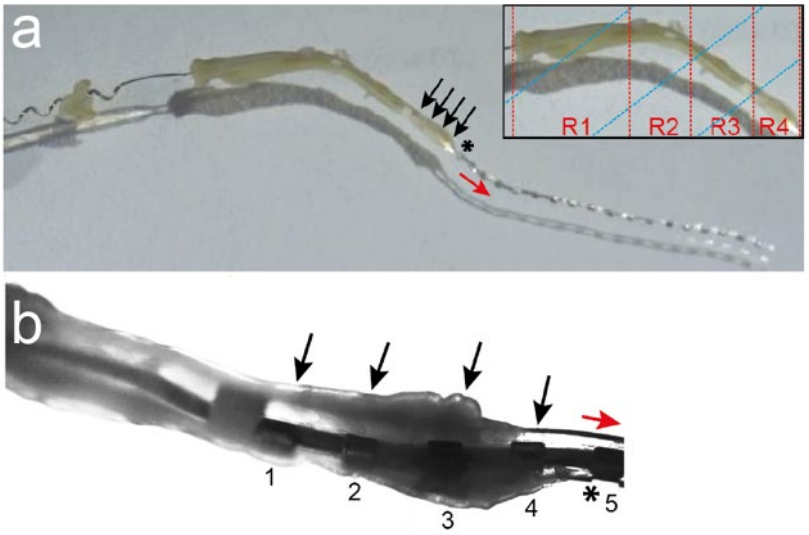


Figure 4.6 **Images of the explanted electrode array with the fibrotic sheath attached**

- **a.** Photograph of the full length of the explanted electrode array with the fibrotic tissue enveloping the basal portion of the array. The insert indicates the four regions of tissue that the tissue has been sectioned into.
- **b.** Micrograph showing the fibrotic sheath around the electrode array, covering at least four electrode contacts (labelled 1 - 4). The asterisk represents the suspected shear point of the tissue. The red arrow indicates the direction of the array tip.

To remove the fibrotic sheath, a single incision along the length of tissue was made. The tissue was embedded in optimum cutting temperature (OCT) cryomatrix and placed in isopentane, then cooled to -80°C to freeze. The tissue was sectioned (10 µm) using a cryostat and mounted on glass

3-Aminopropyltriethoxysilane (APES) coated slides. The sections were grouped into four separate regions (R1 - R4), from the lead wire (R1) through to the electrode contacts (R4), as shown in Figure 4.7. For the purpose of the tissue analysis, the mid-region (indicated in R1, Figure 4.7e) was divided into three layers as shown. The asterisk represents where the electrode array had been. Layer one is proximal to this point and layer three is distal from the array position.

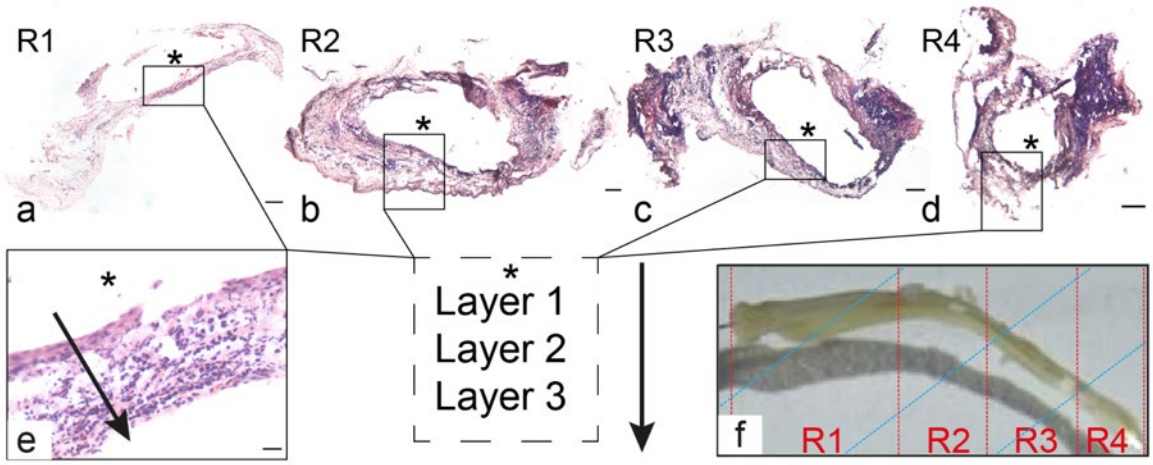


Figure 4.7 **Detail of how the fibrotic tissue was split into four regions and within each region there are three layers**

R1 is the lead wire, R2 is from the lead wire to the array, R3 and R4 include electrode contacts. R4 is closest to the round window niche. The blue dashed lines indicate the tissue sectioning plane. Within each region, the middle area of tissue, which would have been in contact the electrode array, is layer one moving further away from the electrode array is layer three. The asterisk represents where the electrode array would have been. Tissue composition and cellular organisation will be compared between the four regions and between layers.

4.3.2 Histological staining

Cryosections were stained with Haematoxylin & Eosin (H&E) and MSB Trichrome following manufacturer's instructions (Hough, Sanderson, *et al.*, 2021). Following staining, the tissue was briefly dehydrated through alcohols, cleared using clearene then cover-slipped with dibutylphthalate polystyrene xylene (DPX) mountant.

4.3.3 Immunohistochemical staining

For immunostaining, cryosections were air-dried for 30 minutes followed by 30 minutes in 37^{o}C incubator before rinsing in PBS. Endogenous peroxidase activity was quenched for 15 minutes using 1% hydrogen peroxide in methanol followed by 2 x PBS washes. Antigen retrieval was

optimised for each antibody (see A.1). The sections were incubated in blocking agent for 1 hour before 2 x PBS washes followed by overnight incubation in primary antibody, in a humid chamber at 4^{o}C (see A.1). After primary incubation, sections were rinsed in 2 washes of PBS. Secondary antibodies diluted 1:200 and appropriate, for the host species of primaries, were applied for 1 hour at RT then rinsed with 2 x PBS washes. Sections were incubated in Avidin-biotin horseradish peroxidase (standard ABC kit, Vector labs) for 30 minutes at RT and rinsed in PBS. Colourisation was achieved using diaminobenzidine (DAB) and hydrogen peroxide (see A.1). Sections were then rinsed in PBS, counter stained with haematoxylin, dehydrated in graded alcohols and then cover-slipped with dibutylphthalate polystyrene xylene (DPX) mountant.

4.3.4 Image acquisition

The stained tissue was viewed using bright-field light microscopy. Images were captured systematically across regions 1-4, using a Nikon Eclipse E400 microscope and Nikon HB-101004F light source. Images were captured using 4x, 10x, 20x, 40x (oil) and 100x (oil) objectives. ImageJ software was used to visualise the images and adjust brightness and contrast when necessary.

4.3.5 Clinical measures

Electrode status describes whether the electrode is active, de-activated or indicating an open circuit. Speech recognition scores were measured using BKB sentence test between 2 and 8 months of implantation. Impedance telemetry in common ground mode was measured from activation until shortly before re-implantation. X-ray micrograph was obtained at 1-day post-implantation.

4.3.6 Study workflow

The workflow of the study from cochlear implantation to explantation, tissue collection, processing and analysis is illustrated in Figure 4.8.

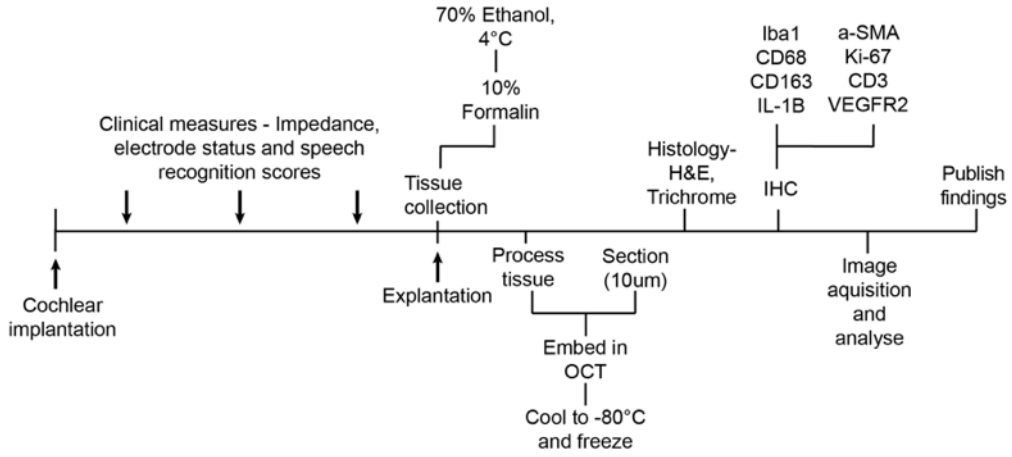


Figure 4.8 **Schematic of the experimental workflow for the novel explant tissue analysis from a living person with a CI**

This figure describes the workflow from cochlear implantation. Following activation, clinical measures including impedance, electrode status and speech recognition scores were measured across the period of implantation (10 months). At explantation, the tissue was collected, processed and sectioned (10 μm) using a cryostat. Histological and immunohistochemical analysis was carried out followed by image acquisition and analysis.

4.4 Results

4.4.1 Clinical evidence of migration, decreased performance and increased impedance

Over the course of 10 months of implantation the person was experiencing a decline in hearing performance, a gradual increase in impedance and a physical migration of the array out of the cochlea. Clinical data that was captured over the 10-month period is displayed in Figure 4.9. At device activation, the two most basal electrodes (E1 and E2) gave open circuits so were deactivated. Over the 10 months, successive basal electrodes within the cochlea were either open circuit or deactivated (Figure 4.9a), whereby 13 out of 22 electrodes were either deactivated or open circuit by the final appointment before explantation. BKB sentence test scores decreased significantly between 3 and 6 months for both hybrid (hearing aid plus CI) and CI alone (Figure 4.9). The score for CI alone went from ~60% at 3 months to ~38% at 6 months and the score for hybrid went from ~90% at 3 months to ~75% at 6 months and then ~50% at 7 months.

Electrode impedance measures for the 8 most basal electrodes are shown in Figure 4.9b. There is a shift in impedance across the period of implantation, where there is an initial peak in electrodes 1 and 2. As electrodes are deactivated, the impedance peak shifts across electrodes 3-8 over the time of implantation.

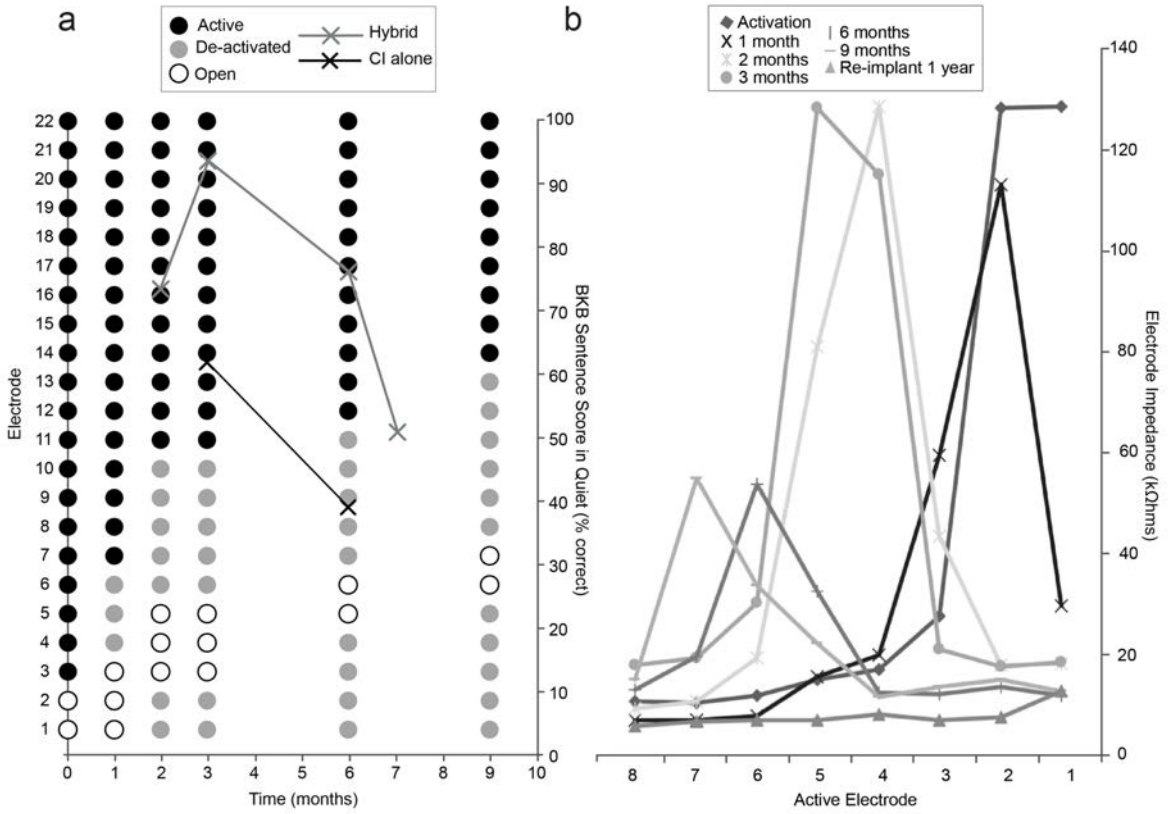


Figure 4.9 **Clinical measures including speech recognition score and electrode impedance, recorded over the 10-month period of implantation indicate a decrease in speech recognition scores and rapid increases in electrode impedance**

a. Electrode status and speech recognition scores over time. Scores for the BKB sentence test (in quiet) are shown for the cochlear implant alone (blackline) and the hybrid (greyline) device (combined acoustic and electrical stimulation).
b. Impedance telemetry in common ground mode from activation until shortly before re-implantation showing increases in impedance measured over time.

An x-ray obtained 1-day post-implantation confirms full insertion of the electrode array into the cochlea as shown in Figure 4.10a. Electrode contacts were superimposed onto the x-ray to illustrate the positioning (Figure 4.10b). A cochlear template defining the position of every quarter turn of the organ of Corti was used to predict the positioning of the electrode contacts after migration (Figure 4.10c). The black dots represent the fully inserted electrode contacts at implantation and the white dots represents the migrated array, showing four extra-cochlear electrodes.

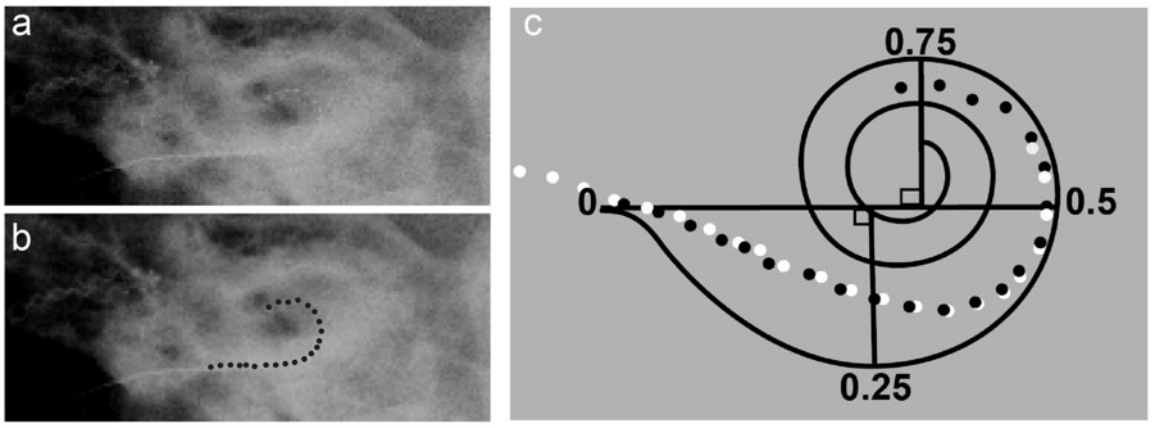


Figure 4.10 **Physical migration of the electrode array out of the cochlea, with four extra-cochlear electrodes at explantation**

- **a.** X-ray micrograph obtained 1-day post-implantation showing the intra-cochlear position of the electrode array.
- **b.** Electrode contact positions superimposed (black).
- **c.** Cochlear template adapted from (Kawano, Seldon and Clark, 1996) defining the position of every quarter turn of the organ of Corti, showing the points from the basal turn. The position of the electrode array post-implantation superimposed (black) based on the postoperative x-ray and the expected position of the migrated array before explantation superimposed (white), showing four extra-cochlear electrodes.

4.4.2 Detail of the tissue and cellular composition across regions of the explant tissue

The main aim of this work is to investigate the tissue response at the tissue-electrode interface in a living person with a CI, undergoing explantation and re-implantation due to a soft failure. The objectives are to characterise the cellular composition and organisation of the tissue surrounding the explanted array and to determine what this tells us about the inflammatory status of the tissue. Histological stains including H&E and trichrome were used to investigate the composition and cellularity of the tissue that formed around the electrode array over 10 months. The tissue was divided into four regions, with R1 furthest from the round window niche and R4 closest to the round window niche. Figure 4.11 shows representative images of H&E stained sections from each region. The density of cells (purple staining) increases from R1-R4. The volume of loose connective tissue (light pink) decreases from R1-R4.

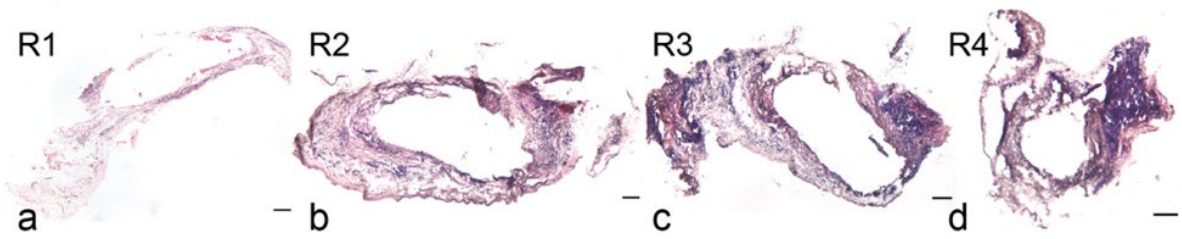


Figure 4.11 **Differences in tissue composition and cellularity between regions one to four**

Low magnification images of sections representative of R1-4 respectively. R1 was furthest away and R4 was closest to the round window niche at the time of explantation. The volume of connective tissue decreases from R1 – R4, whereas the cellularity increases from R1 – R4. Histochemical stain = Haematoxylin and Eosin. Scale bar = 200 μm.

Higher magnification imaging reveals further variability in the tissue composition and cellularity between R1-4 and also within each region, in L1-3 (Figure 4.12). In R1, the cells appear in a stratified organisation with a high density of cells in all layers. R2 and R3 have a similar tissue composition and cellular distribution through all layers, whereby L1 and L2 have a higher cellularity compared to L3 which has a lower cellularity and higher volume of connective tissue. R4, L1 and L2 have a high volume of connective tissue and low cellularity. R4, L3 has a population of cells with a disordered distribution. Differences in cellularity and connective tissue deposition may be giving an indication of the maturity of the tissue.

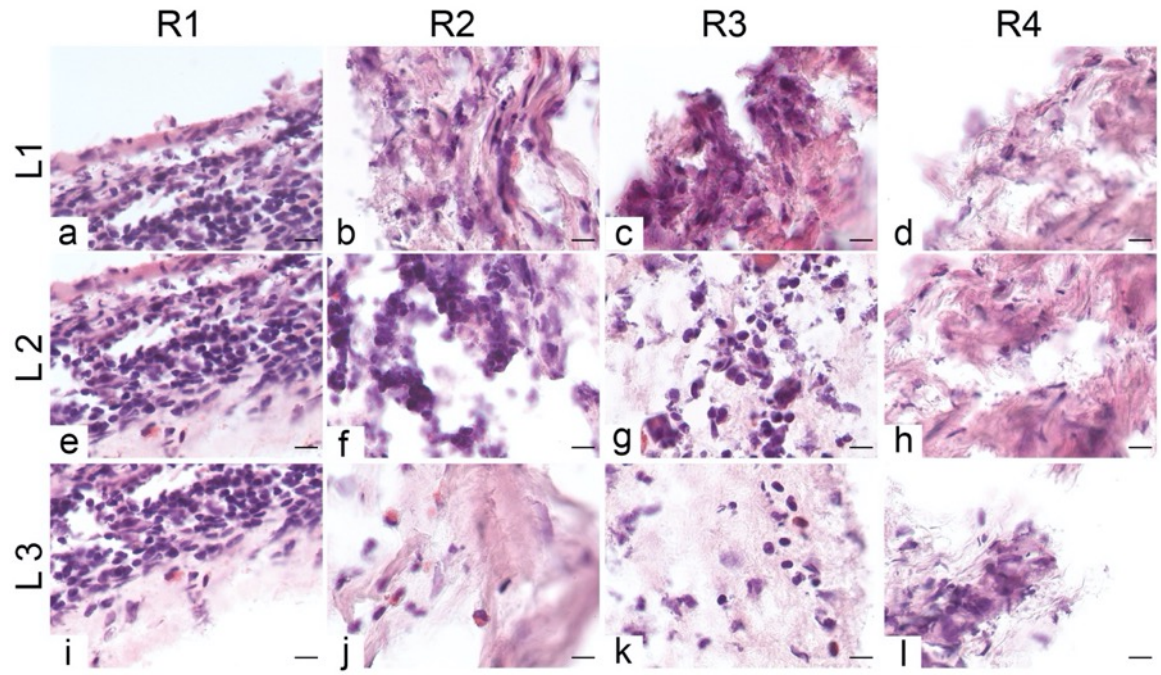


Figure 4.12 **Variable tissue composition and cellularity between regions one to four (R1-4) and within layers one, two and three (L1-3)**

High magnification images of L1-3 across the four regions of explant tissue. L1-3 are the distribution of layers found closest to the electrode array, whereby L1 is adjacent to where the array would have been. The cell density and organisation vary between all layers in all regions. R1 has high cell density in a stratified organisation (**a, e, i**). R4 has low cell density and high volume of loose connective tissue. Histochemical stain = Haematoxylin and Eosin. Scale bar = 10 μm.

Histological staining with MSB trichrome provided insight into the collagen deposition and organisation across the fibrotic tissue. In a trichome stain, cell nuclei are stained blue/black, collagen is blue, muscle is dark red and fibrin is red. Collagen of varying properties were observed across different regions of the tissue and between the layers, with some areas of dense connective tissue and layered collagen fibres, and other areas of loose connective tissue with a lower cell density. Densely layered collagen fibres are found proximal to the electrode array (L1) in R1-2 (Figure 4.13a, b) and loose collagen fibres are found in regions most distal from the electrode array (L3) (Figure 4.13i – l). The collagen organisation is loose and less stratified in R3 and R4, with fewer cells distributed throughout the connective tissue (Figure 4.13g, h, k, l).

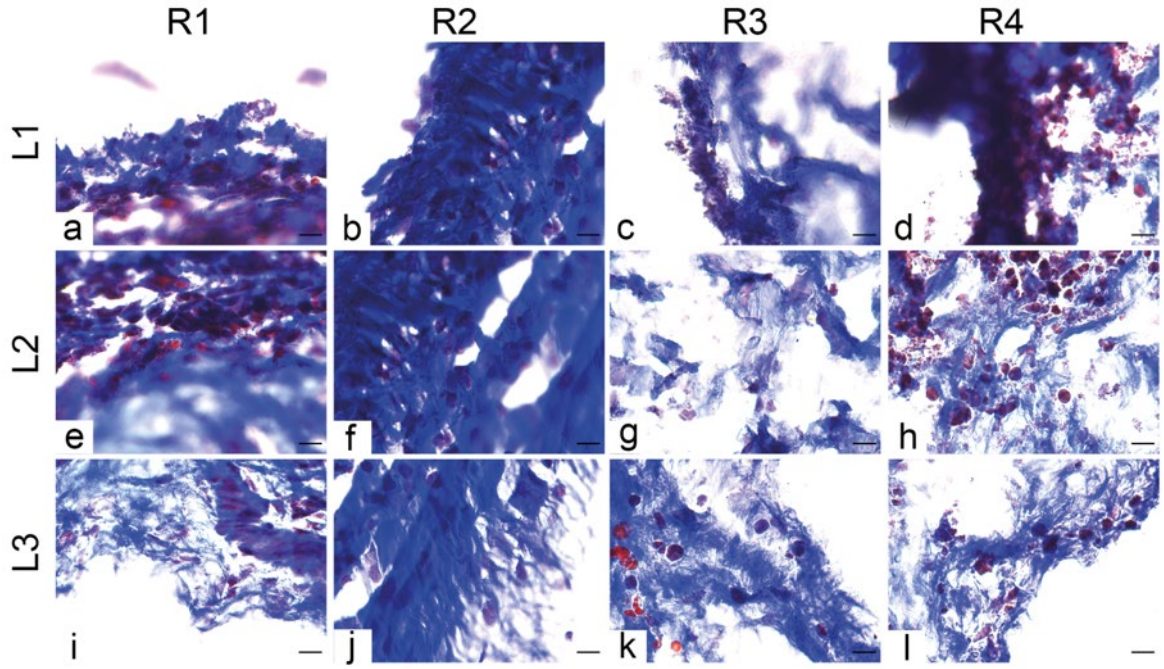


Figure 4.13 **Variable tissue composition and collagen organisation between regions one to four (R1-4) and between layers one to three (L1-3)**

The collagen deposition differs across R1-4 and between L1-3 in the area of the tissue adjacent to where the electrode array would have been. Densely layered collagen fibres are found proximal to the electrode array (L1) and loose collagen fibres are found in regions most distal from the electrode array (L3). Histochemical stain = MSB Trichrome. Scale bar = 10 μm.

4.4.3 Cellular indicators of unresolved, active inflammation

Detailed examination of the cellular composition of the explant tissue stained with H&E, revealed evidence of active inflammation and inflammatory cells. Eosinophils (Figure 4.14a) and neutrophils (Figure 4.14c), granule-containing leukocytes involved in the early stages of an inflammatory response, are found across the tissue. Foreign body giant cells (FBGCs) are also distributed across the tissue as shown in Figure 4.14b. These are a collection of macrophages that are formed as part of a foreign body reaction to implanted devices. Fibrin, a fibrous protein, is found in areas of high cell density (Figure 4.14d).

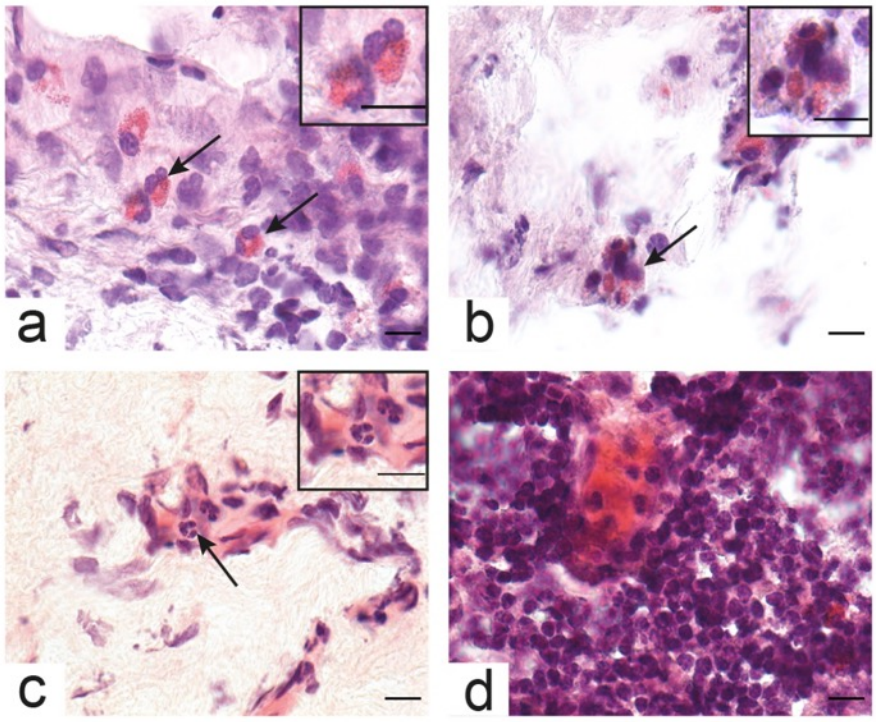


Figure 4.14 **Histological evidence of active inflammation and inflammatory cells**

Inflammatory cells including eosinophils (**a**), foreign body giant cells (**b**) and neutrophils (**c**) are distributed across the tissue. Fibrin, a major component of blood clots (**d**), appears as areas of deep red amongst regions of high cell density. Histochemical stain = Haematoxylin and Eosin. Scale bar = 10 μm.

In order to characterise the cellular composition and organisation, and determine what this tells us about the inflammatory status of the tissue, immunohistochemical analysis of the tissue was carried out using inflammatory cell and signalling markers. Two macrophage markers, CD68 (Abcam, ab783, 1:100) and CD163 (Abcam, ab182422, 1:250) were used to characterise the macrophage populations in the tissue and investigate their function. CD68 identifies phagocytic macrophages and CD163 identifies anti-inflammatory macrophages. CD68-positive macrophages are distributed at the edges of the tissue, as shown in Figure 4.15a and b, closest to where the electrode array had been. CD163-positive macrophages are found at the perimeter of the tissue and in lower numbers compared to CD68-positive macrophages (Figure 4.15d-f). The morphology of both populations appear amoeboid, with a large cell body.

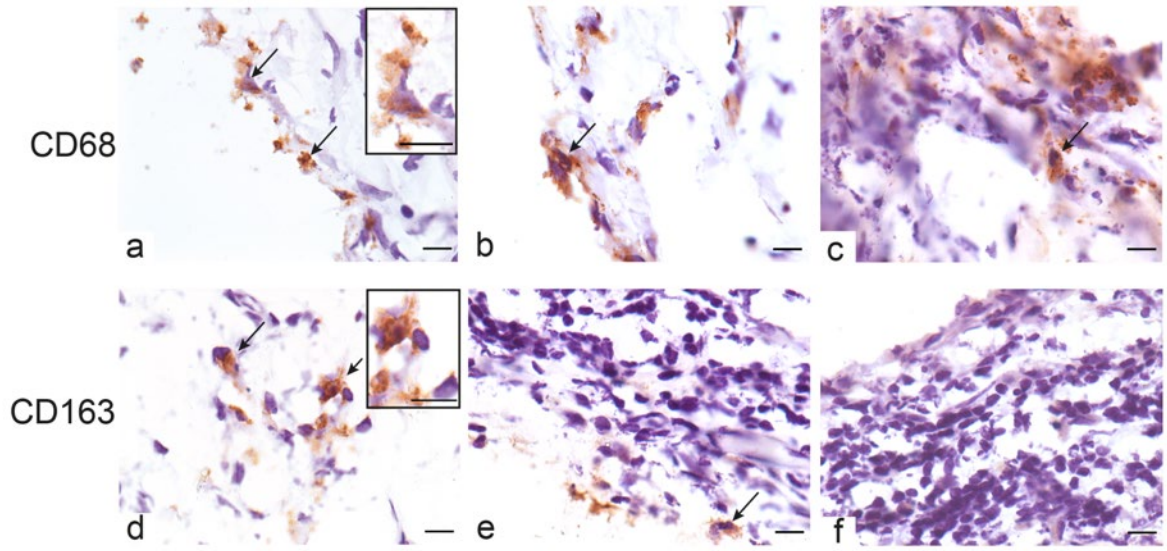


Figure 4.15 **Macrophage populations expressed across different regions of the explant tissue**

Two macrophage markers, CD68 and CD163, have been used to characterise the population of macrophages in the explant tissue. CD68 identified macrophages at the edges of the tissue in close proximity to where the array was in all regions (**a, b**) and in more cellular regions of the tissue (**c**). CD163 identified macrophages that were mostly around the perimeter of the tissue (**d, e**). CD163-positive macrophages were observed in lower numbers compared with CD68, resulting in regions of tissue with no CD163-positive macrophages.

Expression of the pro-inflammatory cytokine, Il-1β (Peprotech, 500-P51, 1:50), was investigated revealing areas of active inflammation with positive Il-1β staining. Positive Il-1β expression is found around the cell bodies of macrophages as indicated by arrows in Figure 4.16. A T-lymphocyte marker, CD3 (Dako, GA503, 1:200), identified clusters of T cells in R4 in areas of the tissue furthest from where the array had been (Figure 4.17).

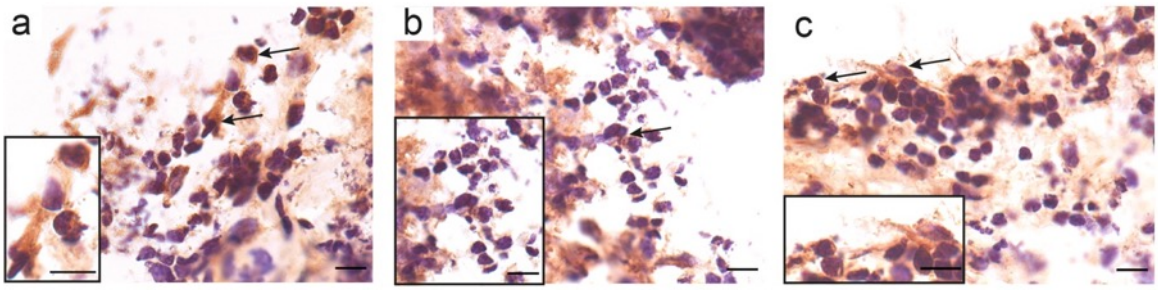


Figure 4.16 **Pro-inflammatory cytokine Il-1β expression in the explant tissue**

Areas of the explant tissue were pro-inflammatory with Il-1β-positive cells, indicated by an arrow. CD68-positive macrophages are likely to be producing and expressing Il-1β in areas of active inflammation.

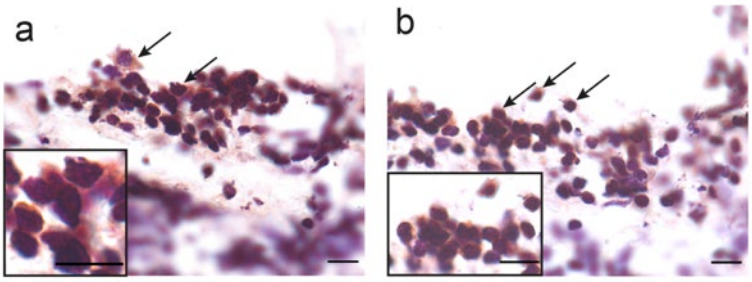


Figure 4.17 **Clusters of T-lymphocytes, labelled with anti-CD3, observed in the explant tissue**

Clusters of CD3-positive cells were observed in R4 in tissue furthest away from the array. T-cells are a type of white blood cell involved in adaptive immunity.

4.4.4 Cellular indicators of ongoing proliferation and angiogenesis

As described in 4.1.4 , the typical wound healing response following cochlear implantation involves inflammation, proliferation then remodelling. Histological and immunohistochemical analysis has shown that areas of the tissue are in the inflammatory phase. Further markers, including Ki-67 (Abcam, ab15580, 1:600), VEGFR2 (Cell Sig. Tech., 55B11, 1:600) and alpha-smooth muscle actin (α-SMA) (Sigma, A2547, 1:50,000) were used to investigate evidence for ongoing tissue and cellular proliferation, associated with the proliferation phase. Ki-67 identifies cells undergoing proliferation as Ki-67 is present during all active stages of the cell cycle but absent in resting cells (Scholzen and Gerdes, 2000). Ki-67-positive cells, at varying stages of the cell cycle, were observed across R2 (Figure 4.18a-c), R3 (Figure 4.18d-e) and R4 (Figure 4.18f). Ki-67 expression is mainly in areas of loose connective tissue with lower cell density, however sparse Ki-67 expression is observed in areas of high cell density close to the round window niche (Figure 4.18f).

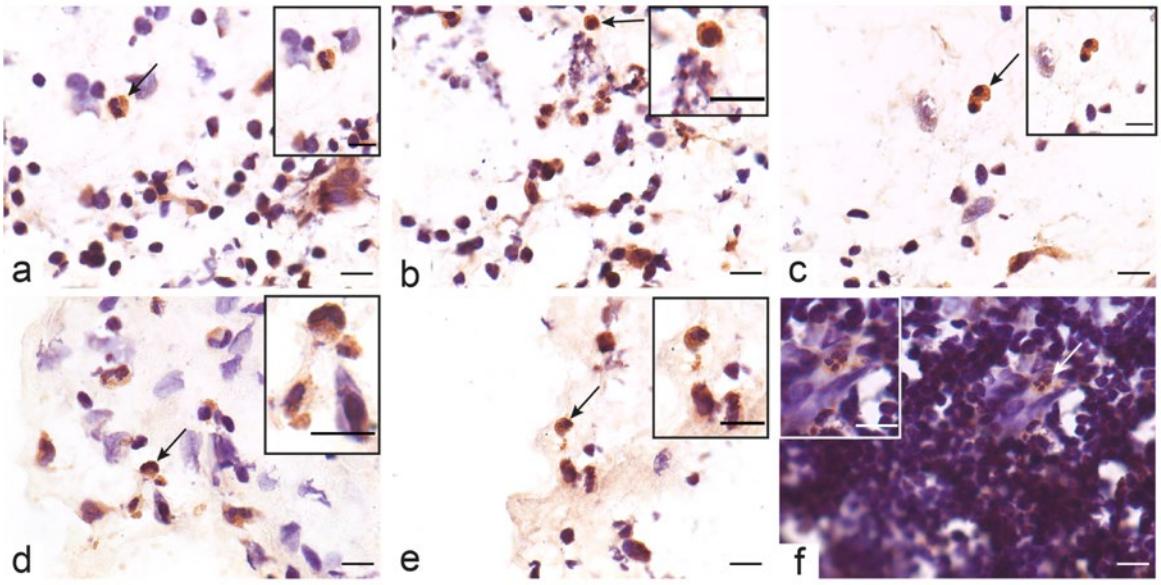


Figure 4.18 **Evidence of ongoing cellular proliferation in the explant tissue**

Proliferating cells, identified with the marker Ki-67, are shown across regions 2 (**a -c**), 3 (**d-e**) and 4 (**f**). Proliferating cells are mostly observed in regions of lower cell density; however, sparse Ki-67-positive expression was observed in the cellular dense region closest to the round window niche in R4.

The formation of new blood vessels, angiogenesis, is part of the proliferation phase. It is required for the delivery of nutrients and oxygen to the active wound site. Vascular endothelial growth factor receptor-2 (VEGFR2) is a major receptor for VEGF-induced signalling in endothelial cells, which line blood vessels. VEGFR2-positive expression is observed around proliferating blood vessels in areas of the tissue most distal to the electrode array path (Figure 4.19). Alpha-smooth muscle actin (α-SMA) is an actin protein found in smooth muscle cells that line blood vessels. Further evidence of blood vessels is shown in areas most distal to where the array had been and in areas of low cell density (Figure 4.20a and b), using the marker for α-SMA.

During the proliferation phase fibroblasts, key cells involved in extra-cellular matrix production and remodelling, differentiate into myofibroblasts. Alpha-smooth muscle actin (α-SMA) identified myofibroblasts in R1, close to the electrode path in areas of higher cell density (Figure 4.20c), and distributed in a linear strand alignment in a region of connective tissue in L3 (Figure 4.20d). Myofibroblasts produce collagen which forms the fibrotic sheath around the electrode array.

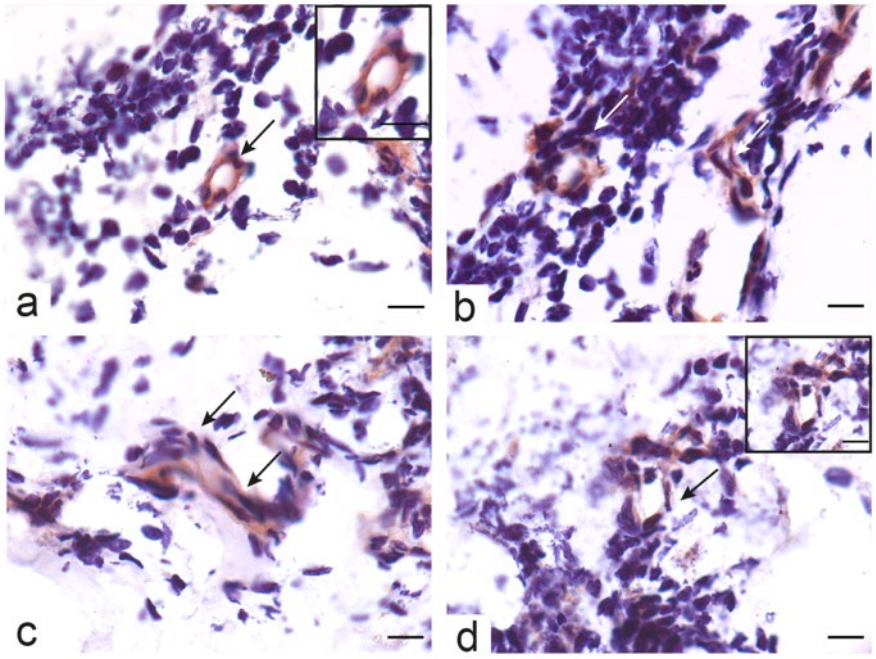


Figure 4.19 **Evidence of active angiogenesis and proliferating blood vessels, using the marker for VEGFR2 expression**

VEGFR2 expression is observed in areas of the tissue most distal to where the array had been and indicates the presence of proliferating blood vessels (highlighted by arrows).

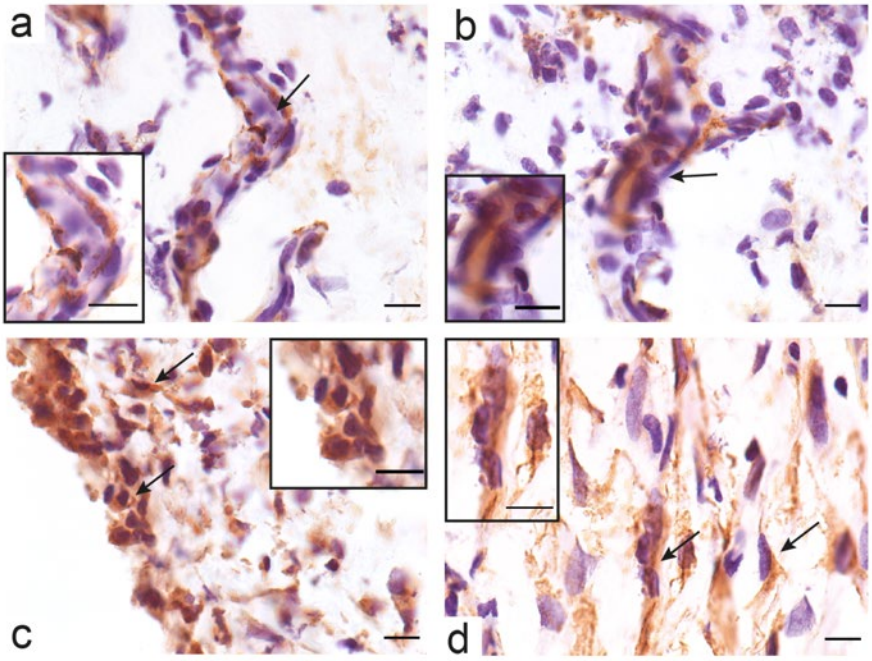


Figure 4.20 **Evidence of ongoing cellular proliferation and myofibroblast expression using the marker alpha-smooth muscle actin (α-SMA)**

Positive staining for α-SMA, an actin protein found in smooth muscle cells that line blood vessels (**a, b**) and myofibroblasts (**c, d**). Blood vessels are observed in areas with lower cellularity and further from where the array had been.

4.4.5 Established a protocol for further explant analysis

This work was the first investigation of the tissue response at the tissue-electrode interface in a living person with a CI and demonstrates that this type of analysis is valuable and feasible. A protocol for further explant analysis has been established (see appendix A.3) to enable a pipeline for routine human explant analysis to provide essential insight into the tissue response to cochlear implantation.

4.5 Discussion

4.5.1 Novel work compared to human temporal bone studies

The tissue response to cochlear implantation has been investigated in human temporal bone studies and animal models. Human temporal bone analysis has provided insight into the gross tissue reaction and inflammatory response on the intracochlear fibrotic sheath and surrounding cochlear tissue at the end of life (Nadol and Eddington, 2004; Li *et al.*, 2007; Nadol, Eddington and Burgess, 2008; Fayad, Makarem and Linthicum, 2009; Seyyedi and Nadol, 2014). Similar findings have been described in animal models, surgically implanted with an electrode array (Bas *et al.*, 2015). This study was the first to investigate the tissue response at the tissue-electrode interface in a living person with a CI, which is advantageous for multiple reasons . A main reason is the ability to capture the tissue response at the time of performance decline/failure, increasing our understanding of the tissue response associated with soft failures and underperformance. This approach enables the correlation of the clinical measures taken across the period of implantation with the biological findings. All previous studies involve post-mortem analysis of temporal bones and often the clinical/performance data is not always recorded accurately and completely. In addition, previous studies have examined the intracochlear tissue response associated with cochlear implantation. A unique aspect of this study is the investigation of the extracochlear fibrotic sheath which formed around an extruding electrode array, providing insight into the extracochlear inflammatory response and may be giving an indication of the type of inflammatory response inside the cochlea. A further advantage of this study was that immunohistochemical investigation of the tissue response, using multiple markers, allowed for detailed cellular and immunological analysis that many of the temporal bone studies lack.

4.5.2 Vast tissue and cellular heterogeneity across the tissue

Histological staining, H&E and trichrome, was used to characterise the composition and organisation of the tissue surrounding the explanted array. The tissue was composed of a heterogeneous mix of cells and a varying composition of connective tissue. The cellularity increased from R1 to R4, which is the area of tissue closest to the round window niche. The tissue composition and cellularity varied within the layers in the mid-region of the tissue, proximal to where the array had been.

The trichrome stain indicated that the tissue was fibrotic with evidence of collagen deposition, of variable properties, across the tissue. Some regions had densely, layered collagen fibres whereas other regions had loose collagen deposition. This may indicate functional differences and varying

stages in development e.g. how mature the collagen is (Coelho and McCulloch, 2016). The properties of dense connective tissue include not very flexible, poorly vascularised, aligned collagen fibres and resistant to stress. Whereas, loose connective tissue is loosely organised, flexible, well vascularised and not resistant to stress. A marker for alpha-smooth muscle actin (α-SMA) identified myofibroblasts organised in a linear strand alignment in areas of loose connective tissue. During the proliferation phase of wound healing, fibroblasts become activated and differentiate into myofibroblasts. These cells play a major role in the synthesis and deposition of the extracellular matrix components including collagen (Micallef *et al.*, 2012). Because of the α-SMA expressed by myofibroblasts, they have contractile properties and play a role in the contraction of tissue required for tissue remodelling (Hinz, 2010). De-regulated and chronic myofibroblast activity can cause overexuberant tissue contraction, inhibiting the resolution of wound healing and formation of scar tissue. In this case of electrode migration, the presence of the myofibroblasts may have contributed to the extrusion of the array out of the cochlea.

4.5.3 Evidence of active, ongoing inflammation and proliferation in the explant tissue

Multiple inflammatory cells were identified in the fibrotic tissue using H&E including eosinophils, neutrophils and foreign body giant cells (FBGCs). Neutrophil recruitment and activation are central to acute inflammation following implantation of a biodevice, in an attempt to destroy/clear the biomaterial using the same mechanisms they would use to clear cellular debris including phagocytosis and degranulation (Nimeri *et al.*, 2002; Christo *et al.*, 2015). Eosinophils release cationic proteins stored in cytoplasmic granules by degranulation, involved in allergic responses (Weller and Spencer, 2017). Eosinophil infiltration was observed in 25% (7/28) of temporal bones (Seyyedi and Nadol, 2014), suggesting an allergic reaction. Presence of neutrophils and eosinophils suggests areas of acute/active inflammation, not what would be expected 10-months after implantation. Persisting presence of neutrophils will prolong inflammation and contribute to the development of a chronic wound (Kruger *et al.*, 2015). FBGCs were observed across the explant tissue. FBGCs are present as part of a foreign body reaction which is an end-stage inflammatory response following implantation. Human temporal bone analysis found this to be common following cochlear implantation and it was suggested that it could be a cause of soft failures (Nadol, Eddington and Burgess, 2008; Seyyedi and Nadol, 2014). The presence of fibrin was further evidence of inflammation as this protein is a component of blood clots which form as a result of inflammatory damage to capillaries.

A panel of immunohistochemical markers were used to investigate the active state of the fibrotic tissue. Inflammatory cell and proliferation markers were used. A population of phagocytic CD68-positive macrophages were identified close to the electrode path and around the edges of the

tissue, similar to previous observations in temporal bones (Nadol *et al.*, 2014; Noonan *et al.*, 2020). Macrophages are recruited to the active wound site in the early inflammatory stages following the release of chemokines due to blood clots. CD68-positive macrophages were observed in areas of fibrosis in the scala tympani and scala vestibuli in cases of cochleostomy and CI-translocation (Noonan *et al.*, 2020). A second macrophage population (CD163-positive) was observed around the perimeter of the tissue and in a lower number (compared to CD68). CD163-positive macrophages have an anti-inflammatory phenotype as CD163 expression is strongly induced by anti-inflammatory mediators including IL-10. CD163-positive macrophages may be trying to downregulate the inflammatory response in some areas of the tissue (Kowal *et al.*, 2011). The macrophage populations in the cochlea change following implantation indicating a role of these cells in the inflammatory response to cochlear implantation (Noonan *et al.*, 2020).

Evidence of inflammatory signalling within the tissue was provided by positive staining of Il-1β in areas populated with cells. Il-1β is produced and released by macrophages during inflammation and is involved in the signalling between cells. T-cells are white blood cells, with roles in adaptive immunity as primary effectors for cell-mediated immunity. Clusters of T-lymphocytes (CD3-positive) were observed in regions distal to the electrode path in the explant tissue. Similar observations were made in human temporal bone analysis with expression identified in the fibrous sheath around the electrode track (Nadol *et al.*, 2014). Presence of T-cells indicate a role for adaptive and innate immunity in the response to cochlear implantation.

In the wound healing response, following inflammation there is proliferation and tissue repair. Several markers including Ki-67, VEGFR2 and α-sma, confirmed the presence of ongoing proliferation across areas of the tissue. Evidence of proliferating cells (Ki-67-positive) were observed across R2 - 4. Ki-67 positive cells were identified in dense cellular areas of R4, the portion of tissue that would have extruded the cochlea last before explantation. This expression is suggestive of continuous growth of the fibrotic sheath during implantation (and migration). Several temporal bone studies have described that the formation of a fibrotic sheath around an electrode array is common (Nadol and Eddington, 2004; Li *et al.*, 2007; Somdas *et al.*, 2007; Seyyedi and Nadol, 2014; Ishai *et al.*, 2017). The severity of fibrotic sheath adjacent to the intracochlear part of the electrode has been shown to be higher in the basal region, close to cochleostomy, compared to the middle or tip of the electrode in multiple studies (Li *et al.*, 2007; Fayad, Makarem and Linthicum, 2009; Seyyedi and Nadol, 2014); likely due to insertion trauma and subsequent inflammation.

The formation of blood vessels was observed in the fibrotic sheath using the markers VEGFR2 and α-sma. VEGFR2 is a major receptor for angiogenic VEGF-induced signalling in endothelial cells.

VEGFR2-positive staining was observed in areas most distal from the electrode path, indicating proliferation of endothelial cells. α-sma expression in smooth muscle cells provided further evidence of blood vessels indicating the presence of a vasculature to the active wound site. Delivery of nutrients, oxygen and immune cells may be facilitating the active inflammatory state of the tissue and lack of tissue maturation.

4.5.4 Correlate biological findings of the tissue response with clinical measures

Following in-depth tissue analysis using a range of inflammation and proliferation markers, we aimed to correlate the biological findings of this migration-related soft failure with the documented clinical indicators to shed light on the potential biological mechanisms behind the soft failure. The clinical measures indicated a continuous migration of the electrode array out of the cochlea during the 10 months of implantation. During this time, a collagenous fibrotic sheath formed around the electrodes. Upon explantation, tissue covering the four most basal electrodes, which were extracochlear at explantation, was attached to the array after removal. We predict that the intracochlear fibrotic sheath remained in the cochlea at explantation. We have shown that tissue growth at the electrode-cochlear interface is associated with rapid changes in impedance, sequential open circuits starting at the basal end and a decrease in speech perception scores. Formation of the fibrotic sheath is part of the typical wound healing response following implantation. The final stage of the response is tissue remodelling where a mature scar tissue forms. However, in this case, the wound healing response is aberrant with ongoing inflammation and proliferation across the fibrotic sheath without the tissue remodelling, despite the length of time post-implantation. The expression of macrophages, neutrophils and the pro-inflammatory cytokine Il-1β, is indicative of a chronic inflammatory response. The expression of proliferating cells, angiogenesis, myofibroblasts and the dense collagen deposition is indicative of chronic proliferation.

In terms of the mechanism which caused the migration of the electrode, there are two potential possibilities. One possibility is that the aberrant wound healing response is contributory to the migration of the array, whereby continuous inflammation and proliferation results in further fibrosis formation forcing the electrode array out of the cochlea. Another possibility is that the gradual movement of the array causes repeated tissue repair, resulting in the lack of tissue maturation and resolution of the response. It is not possible to determine which mechanism is responsible for the migration of the array in this case. Further analysis of the tissue response in soft failure migration cases may help elucidate the mechanism.

4.5.5 Factors contributing to CI performance

Several factors influence CI performance such as patient and intervention factors and the tissue response (Figure 4.21). Patient factors include medical history, cause of deafness, age of deafness, age at implantation and lifestyle. The tissue response includes the type, distribution and function of the cells in the vicinity of the electrode array. Intervention factors include surgical methods such as soft/atraumatic surgery, round window insertion vs cochleostomy and properties of the electrode array. What is not known is whether these factors interact, such as whether an individual's medical history influences the cellular composition resulting in a specific overall tissue response. Is the tissue response associated with identifiable patient factors?

Less is known about how the tissue response affects the function of the CI and whether tissue growth contributes to poor performance over time. An overexuberant inflammatory response can lead to increased tissue growth/fibrosis and in some cases new bone formation. Increased fibrosis around the electrode contacts lead to increases in impedance. Increased electrical impedance can result in inner ear pathology (Choi *et al.*, 2017) and a decrease in functional outcomes (Busby, Plant and Whitford, 2013). In general lower electrical impedance results in better implant performance (Sanderson *et al.*, 2019).

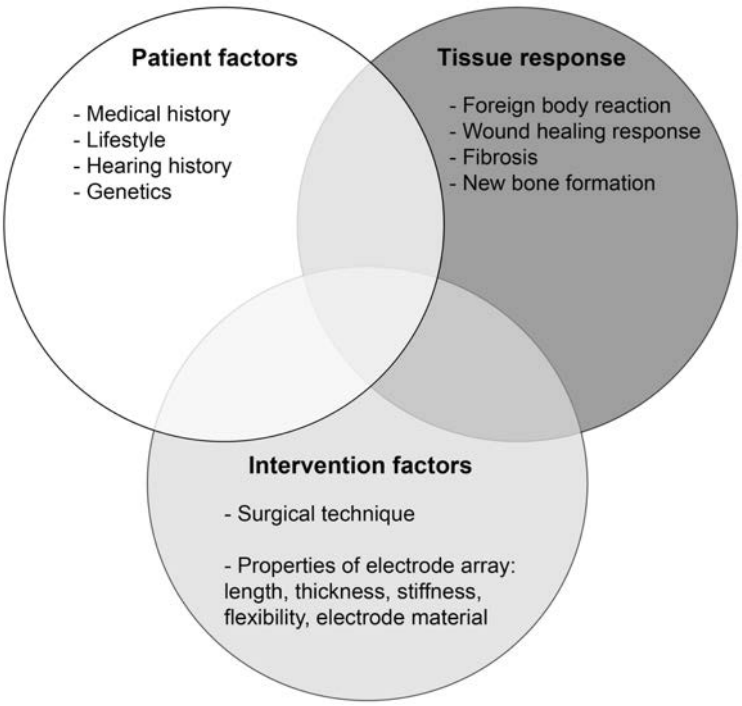


Figure 4.21 **Factors that influence CI performance**

Patient factors, intervention factors and the tissue response to cochlear implantation all contribute CI performance.

4.5.6 Further explant analysis will progress understanding in the field

Correlating the tissue response with the routinely collected clinical measures will shed light on the biological mechanisms behind soft failures and help identify how clinical measures can give an indication of the type of tissue response occurring in people with a CI. It will identify the hallmarks of performance decline and enable earlier intervention to prevent subsequent soft failures and explantation. In addition, understanding how the tissue response correlates with performance may enable the development of therapies or use of existing anti-inflammatory drugs to stabilise/down-regulate an active response to improve performance outcomes.

As evidence suggests that a typical tissue response occurs in most cases after cochlear implantation and is often exacerbated in soft failures; this novel, detailed analysis provides key insight into the cellular profile and mechanism of the fibrotic response (irrespective of failure). This study demonstrates that investigation of fibrosis on explanted devices from living people with CIs is feasible and valuable in building our understanding of the biological response at the tissue-electrode interface, at the time of underperformance.

4.6 Future work

4.6.1 Larger scale systematic explant tissue analysis

In order to gain a deeper understanding of the tissue response at early stages post-implantation, and at the time of underperformance, we have designed a study for larger scale systematic analysis of the tissue response in multiple explants from living people with CIs. Characterising the tissue response to cochlear implantation at the time of underperformance will enable us to investigate the variability in the tissue response between individuals and to ask what factors are contributing to the tissue response. This study aims to investigate the cellular response of the tissue associated with the electrode array at the time of explantation, in several cases where the device is being explanted and a new device is being reimplanted. Individuals experiencing performance decline, who are likely to require explantation and reimplantation, will be identified from the people with CIs at University of Southampton Auditory Implant Service (USAIS). We are aware of a faulty device resulting in several individuals requiring revision surgery. The experimental workflow of the study is illustrated in Figure 4.22. To our knowledge, this is the first study to investigate the cellular response at the tissue-electrode interface in multiple living individuals undergoing explantation. The initial objective is to characterise the tissue structure and cellular composition of any fibrotic tissue that has formed on the electrode array and compare with other explant tissue. Establishing an overview of the tissue response to implantation over time in multiple cases, at the time of underperformance or failure, may enable the identification of immune targets that could enhance performance and increase the lifetime of implants in existing and future people with CIs.

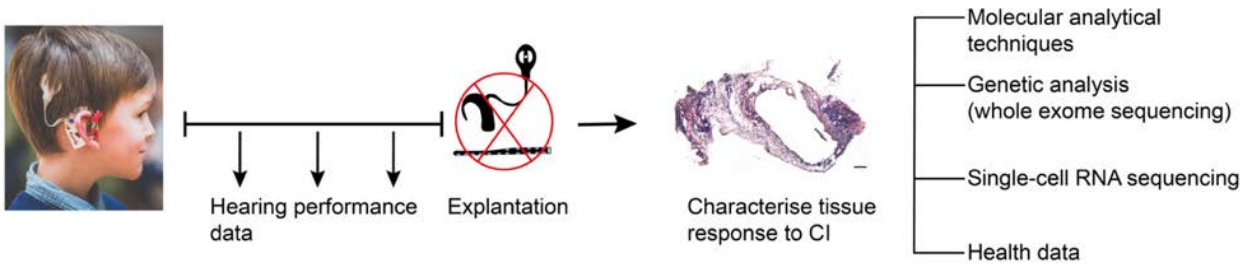


Figure 4.22 **Experimental workflow for a larger scale CI explant tissue analysis study**

The experimental workflow. Routine clinical measures will be collected. Indicators of poor performance or failure may result in explantation of the device. The explanted array and tissue attached will be collected for analysis using methods including

molecular analytical techniques, genetic analysis, single-cell RNA sequencing and health data analysis.

A second objective is to determine if the tissue response is associated with identifiable patient factors. We will determine the variability in the tissue response and cellular composition between the different the individuals undergoing explantation. In addition, clinical (impedance, speech perception, x-ray) and health (medical records, genomics) data will be analysed to unpick the potential mechanisms causing the response identified. We hypothesise that a rampant inflammatory environment, in a large fibrotic sheath, may be associated with an individual who has increased levels of inflammatory markers in their blood and an inflammatory co-morbidity. This work will bridge the gap between performance, clinical measures and the fundamental biology at the level of the tissue-electrode interface as well an overview of the immune status of the individual through genomic analysis.

A further objective is to ascertain whether tissue growth on the electrode array is associated with hearing performance with a CI. Presence of a fibrotic sheath is associated with increased impedance levels (Wilk *et al.*, 2016). Changes in electrode impedance levels are associated with inner ear pathology (Choi *et al.*, 2017) and reduced hearing performance (Busby, Plant and Whitford, 2013). Electrode impedance will be used as a measure of hearing performance. The amount of tissue growth and the characterised cellular environment, will be compared with electrode impedance to determine if there is a correlation between tissue growth/fibrosis and hearing performance in these explant cases.

See appendix A.3 for detailed workflow for each stage of the experimental design. Firstly, we will establish if a fibrotic sheath has formed around the array (and whether any tissue is still attached following explantation). The amount and properties of the tissue will be macroscopically determined before processing and staining the tissue to characterise the biological response at the tissue-electrode interface. Markers specific for cells involved in the different stages of the wound healing response including inflammation, proliferation and maturation will be used (as described in this chapter). We predict that there will be heterogeneity in the tissue response between the people with CIs, due to the multiple factors likely to affect the tissue response. Through the abundance of data that will be collected for each individual, this work may provide insight into how different factors may affect the tissue response to cochlear implantation. Single-cell RNA sequencing and genetic analysis (whole exome sequencing) will be carried out on part of the fibrotic tissue to gain deeper understanding in the immunological profile of the individual people with a CI. Cochlear fluid and blood samples will be obtained during explantation to carry out further genomic analysis to investigate individual patient factors. This in-depth tissue analysis,

alongside the wealth of clinical data will provide essential insight into the biological contribution to CI performance.

There are several benefits of investigating the tissue response on explants from living individuals. This combined analytical approach allows us to consider the cellular environment in the context of hearing performance, and clinical and medical history around the time of explantation, which may inform what factors influence tissue response and CI performance and suggest potential ways to modulate this. The tissue is going to be from relatively early stages post-implantation (in comparison to the human temporal bone studies), particularly in the device faulty group. This will enable us to characterise the early stages of the tissue/fibrotic response to cochlear implantation. In the cases of device failure, we will analyse the tissue response on multiple explants of the same device, enabling us to investigate the variability in tissue response between individuals. And finally, in the people being explanted that are being reimplanted, it will be possible to investigate and follow functional outcomes following reimplantation which may inform future reimplantation protocol.

4.6.2 Conclusion

Analysis of the fibrotic sheath on an explanted electrode array is a novel technique. The published findings described in this chapter (Hough, Sanderson, *et al.*, 2021) demonstrate a new and informative method for investigating the tissue response in living people with a CI. Combining additional information about the person with a CI including clinical, aetiological and medical data is beneficial when trying to establish the reason for the performance decline and potential failure and factors contributing to CI performance. Further explant analysis of the tissue response in multiple people with a CI will build the field's understanding of the tissue response to cochlear implantation and how it may influence performance.

Chapter 5 Establishing a mouse model for cochlear implantation

5.1 Introduction

5.1.1 Back-translation from human work to mouse model

Human temporal bones studies have provided a wealth of information about the tissue response to cochlear implantation that is species specific. Histopathological evidence from post-mortem studies have provided information about the acute and chronic inflammatory response to cochlear implantation (Li *et al.*, 2007; Somdas *et al.*, 2007). These studies have highlighted the presence of immune cells in the fibrotic sheath at the tissue-electrode interface such as B-lymphocytes, T-cells and macrophages containing particulates (Seyyedi and Nadol, 2014). In Chapter 4, we described the novel analysis of tissue from an explanted array from a living person with a CI which added valuable insight into the tissue response associated with poor performance in the early stages following cochlear implantation. Investigation of the fibrosis on the explanted array, associated with a soft failure, indicated an aberrant wound healing response. We aim to back-translate this work by establishing a mouse model for cochlear implantation, allowing for similar investigation of the tissue response at the tissue-electrode interface. Animal models for cochlear implantation allow for in-depth investigation of the cellular and molecular mechanisms involved in the inflammatory response to cochlear implantation. An advantage of using animal models compared to human tissue analysis, is that it is possible to manipulate the system to enable the investigation of various aspects associated with CI performance. These include the tissue response, preservation of residual hearing, electrical stimulation, electrode design and materials. This chapter describes the protocol established for the development of a mouse model that enables the investigation of the tissue response to cochlear implantation and how the response changes following alterations in electrode design.

5.1.2 The need for animal models

Animal models, including the use of cats (Kretzmer *et al.*, 2004), guinea pigs (O'Leary *et al.*, 2013; Wilk *et al.*, 2016; Shepherd *et al.*, 2019), rats (Eshraghi *et al.*, 2005) and mice (Irving *et al.*, 2013; Bas *et al.*, 2015; Claussen *et al.*, 2019) have been developed for the study of biological, electrical and mechanical aspects of cochlear implantation. The majority of studies have involved the use of guinea pigs (Wilk *et al.*, 2016; Yamahara *et al.*, 2018; Shepherd *et al.*, 2019) and cats (Kretzmer *et*

al., 2004) due to their overall larger size and the larger size of their tympanic bulla resulting in easier access to the cochlea. Animal models have been used to investigate the effects of array insertion (Mistry *et al.*, 2014), the tissue response associated (Bas *et al.*, 2015) and the effectiveness of delivering anti-inflammatory drugs (Farhadi *et al.*, 2013; Astolfi *et al.*, 2016; Wilk *et al.*, 2016).

The use of mouse models of implantation has increased due to the many advantages associated with using mice. Mouse models are beneficial due to the wide availability of naturally occurring and genetically-modified mouse strains with well characterised auditory health, the ability to easily measure hearing function through auditory brainstem response (ABR) tests and the wide availability of reagents specific to mice. Due to the small size of mice and anatomical differences compared to other animals, further optimisations are required for implantation to be feasible. Mice have a small cochlea size and a smaller scala tympani length and volume (compared to guinea pigs and cats) (Thorne *et al.*, 1999), resulting in the need for an electrode array with a small diameter and length. Mice have a stapedial artery (SA), running inside the middle ear cavity close the round window niche, making surgical access to the cochlea difficult and with greater risk.

A range of different types of mouse models for cochlear implantation have been established over recent years. These include implantation of a passive electrode array, implantation of an array with stimulation into an undamaged auditory system and finally, implantation of an array with stimulation in a damaged auditory system. Each type of model has contributed useful insight to the field. Implantation with stimulation in a damaged auditory system most closely resembles the human CI situation and therefore has the most translational benefit.

Passive cochlear implantation without electrical stimulation involves the insertion of either a ‘dummy’ electrode made of silicone or nylon (Mistry *et al.*, 2014; Bas *et al.*, 2015; Kopelovich, Robinson, *et al.*, 2015) or an electrode analogue containing metal (platinum/iridium) but is not stimulated (Soken *et al.*, 2013). These models have been pivotal in developing reproducible and viable surgical techniques for implantation in a mouse, in addition to establishing effective methods of measuring hearing function and the tissue response to cochlear implantation. Mistry et al describe the successful insertion of both a dummy electrode (inert fluorocarbon thread) and a specialised array with parylene coated platinum/iridium wire with four electrodes (Mistry *et al.*, 2014) and the initial analysis of the tissue response. Cone beam computed tomography (CBCT) confirmed the position of the array inside the cochlea and histological analysis (toluidine blue) revealed fibrotic-like, collagenous tissue within the scala tympani encapsulating both type of arrays. No cell specific analysis was carried out. ABR was measured before and after implantation

to determine any loss of hearing function due to insertion. Greater mean threshold shifts were measured in the implanted ears post-operatively, however there were no cases of profound hearing loss following insertion (Mistry *et al.*, 2014).

To more closely mirror human cochlear implantation, there is a growing need for animal models for cochlear implantation with electrical stimulation. Electrical stimulation can modify the intracochlear response to cochlear implantation and is therefore important when investigating the tissue response (Kopelovich, Reiss, *et al.*, 2015; Ishai *et al.*, 2017; Needham *et al.*, 2019). Electrical stimulation affects the electrochemistry of the cochlear environment due to the movement of ions in the perilymph. When there is a low charge density, ions move along the electrochemical gradient back and forth in response to a biphasic stimulus. In cases of high charge density, an unbalanced biphasic pulse may result in the production of free oxygen radicals and hydrogen. This is toxic to tissue and can lead to local pH changes (Brummer and Turner, 1977; Huang, Carter and Shepherd, 2001) and platinum dissolution (Shepherd *et al.*, 2019), evidenced in tissue from long-term CI users (Clark *et al.*, 2014; Nadol *et al.*, 2014). Electrode impedance is influenced by electrical stimulation (Newbold *et al.*, 2011, 2014). Due to the complex interaction between the electrical, chemical and biological components involved in cochlear implantation, there is a need for sophisticated mouse models with allow the investigation into these components.

A small number of studies have shown the feasibility of electrical stimulation in mouse models (Irving *et al.*, 2013; Claussen *et al.*, 2019; Navntoft, Marozeau and Barkat, 2019, 2020). A model for acute CI stimulation in deafened C57BL/6 mice has been established (Navntoft, Marozeau and Barkat, 2019), whereby pre- and post-implantation hearing thresholds were measured using acoustic ABR (aABR). 0.05% neomycin was applied in the oval and round window to deafen the animal. Neomycin application significantly increased click-evoked hearing thresholds by 46 dB (Navntoft, Marozeau and Barkat, 2019). An electrode array, containing four platinum/iridium parylene insulated wires in a silicone tube, was inserted into the round window. An animal stimulator platform (ASP) was used to electrically stimulate the array and electric ABR (eABR) was measured following implantation, showing that electrical stimulation could evoke eABR activity.

A model for chronic CI stimulation has been described in adult CBA/J mice (Claussen *et al.*, 2019) involving a multi-method approach to analyse implant functionality (serial impedance and neural response telemetry (NRT), implant positioning and integrity (3D x-ray microscopy) and the tissue response (histology and 3D x-ray microscopy) post-implantation. Two groups of mice (stimulated and non-stimulated) were implanted with an electrode array that closely mimic the materials used in a human array, with a silicone carrier and platinum electrode contacts. Implants

maintained at least one functioning electrode for 35 days in the non-stimulated group and 19.8 days in the stimulated group. 3D x-ray microscopy, used to determine implant positioning within the cochlea and analyse the integrity of the array, demonstrated fractures in lead wires which likely caused the abrupt changes in impedance. Pairing 3D x-ray microscopy and histology provided insight into the tissue response involving inflammation and neo-ossification and enabled quantification the fibrotic response in the scala tympani. This study demonstrated the feasibility of a model of chronic stimulation and highlighted benefits of using a multi-method approach for analysis.

The development and demonstration of the feasibility of these different models of cochlear implantation are essential in driving forward this area of auditory research. The use of the mouse compared to other animals, is preferable due to the ability to easily manipulate genetics and environmental factors in a controlled manner. Utilising mouse models to characterise the tissue response to cochlear implantation will allow for corroboration between human and animal findings to help elucidate the tissue response. Further to this, investigation into the how the tissue response may be influenced by various factors including electrode design, variation in electrical stimulation, surgical method and exposure to previous insults will be beneficial.

5.1.3 Evidence of the tissue response to cochlear implantation from mouse models

The tissue response to cochlear implantation has been investigated in mouse models (Irving *et al.*, 2013; Mistry *et al.*, 2014; Bas *et al.*, 2015). Using histological analysis, several studies have observed a fibrous tissue response and new bone growth in the scala tympani around the electrode array (Irving *et al.*, 2013; Mistry *et al.*, 2014; Claussen *et al.*, 2019). Detailed investigation of the wound healing response following the insertion of an electrode analogue (Bas *et al.*, 2015), demonstrated similar findings to what has been described in post-mortem analysis of human temporal bones and the findings described in Chapter 4. A 2 mm nylon monofilament was inserted into mice for a maximum period of 30 days of implantation. Upregulation of inflammation and proliferation associated genes including Ccl3 and Ccl12 (recruitment of leukocytes), Vegfa (promotes angiogenesis) and Pdgfb (promotes proliferation, differentiation and migration) were measured in cochlear tissue obtained 7 days after implantation (Bas *et al.*, 2015). Evidence of Il-1β (pro-inflammatory) and Arg1 (anti-inflammatory) expression was measured in the tissue using immunofluorescence, indicating the presence of both pro- and anti-inflammatory macrophages at the area of electrode insertion. Analysis of the fibrous tissue composition revealed positive staining of collagen fibres in the scala tympani, and α-sma and collagen type 1A in the tissue that formed around the array (Bas *et al.*, 2015). These findings are similar to the response measured in the explant tissue, with evidence of collagen and α-sma expression

(Chapter 4). These studies provide insight into the wound healing tissue response following cochlear implantation in the mouse and mirror closely to the observations described in human temporal bone studies (Nadol *et al.*, 2014; Seyyedi and Nadol, 2014) and the human explant tissue analysis (Hough, Sanderson, *et al.*, 2021). Further detailed analysis of the wound healing response, following implantation in mouse models for cochlear implantation, is necessary to build upon this understanding.

5.1.4 Cochlear implant design characteristics and the biological response associated

Electrode arrays can vary in length, shape, flexibility, number of electrode contacts and electrode material. The material of the electrode array that is in the local tissue environment surrounding the scala tympani will affect both the acute and chronic inflammatory response at the tissue-electrode interface.

Electrode arrays are positioned in the scala tympani therefore the electrical stimulation must travel through fluid, bone and connective tissue before reaching and stimulating cells in the auditory nerve. This increases electrode-neuron impedance, degrades signal fidelity and increases the voltage required to excite the neural structures to threshold and initiate action potentials. Research has been carried out to optimise device design to improve hearing quality and to overcome these obstacles, which are specific to cochlear implants due to the fluid-filled environment that they are placed in.

Electrode contact size and shape of the array can affect the tissue response and performance. Three types of arrays with varying shapes exist including straight lateral wall (LW), pre-curved modiolar hugging (MH) and a mid-scala array (Figure 5.1). Straight LW arrays have good insertion depth and give better hearing preservation outcomes, however they can be more prone to partial extrusion. MH arrays curve the modiolus reducing the distance between the electrodes and SGNs and increases the effectiveness at stimulating SGNs. However, with MH arrays there is a risk of damage to basal turn structures. Mid-scalar arrays are designed to sit centrally in the scala tympani and ideally has no contact to either inner or outer wall structures. The design of the array contributes to the pattern and degree of the fibrous sheath (Ishai *et al.*, 2017). Temporal bones implanted with pre-curved electrode arrays, had a significantly higher medial fibrous capsule compared to those implanted with a straight configuration array (Ishai *et al.*, 2017). Pre-curved arrays have a different mode of stimulation to straight arrays, with 16 half-banded electrodes designed to be in close contact with the medial side of the cochlea. This may lead to thicker medial fibrous sheath formation.

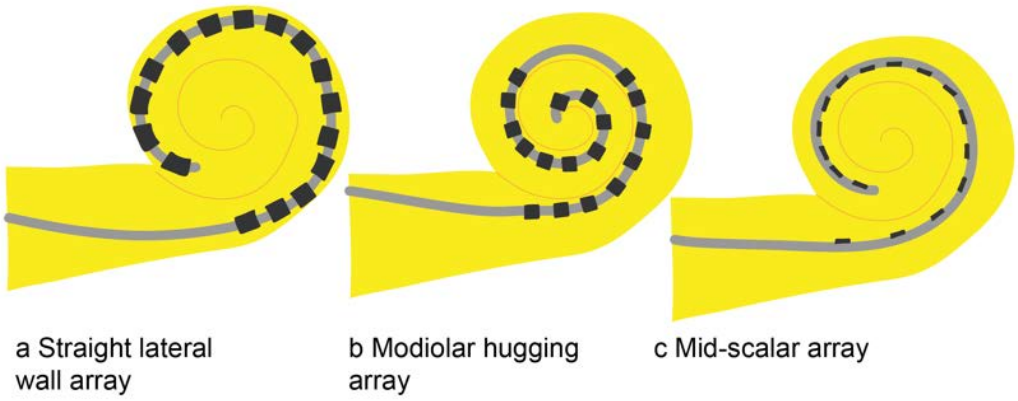


Figure 5.1 **Three types of electrode arrays including straight lateral wall, pre-curved modiolar hugging and mid-scalar array**

Three different types of electrode array design with different properties.

Cochlear implant manufacturers have been exploring the use of additional materials to coat the arrays with the aim to improve CI function and achieve discrete neural stimulation. Poly(3,4-ethylenedioxythiophene) (PEDOT) is a conducting polymer that has been studied in neural probe and prosthetic research. The aim of a conducting polymer coating is to provide a high surface area material, allowing for effective cell and tissue integration and therefore improve the communication between the electrode and tissue (Green *et al.*, 2008). Conductive polymers improve signal-to noise ratio, decrease impedance, reduce scar tissue formation and improve charge transport (Ludwig *et al.*, 2006; Green *et al.*, 2008). PEDOT improves the electrode-fluid charge transfer characteristics (Chikar *et al.*, 2012).

In a guinea pig model for cochlear implantation, the effects of the individual and combined elements of a dual-component CI coating were investigated following CI stimulation (Chikar *et al.*, 2012). The coating contained both PEDOT and an arginine-glycine-aspartic acid (RGD)-functionalised alginate hydrogel, soaked in brain-derived neurotrophic factor (BDNF) solution (Chikar *et al.*, 2012). A reduction in electrode impedance and improved charge delivery was maintained throughout chronic stimulation in the PEDOT/hydrogel implants compared to uncoated Platinum/Iridium implants (Chikar *et al.*, 2012). This was due to the increased surface area of the electrodes with PEDOT coating. BDNF promotes SGN survival following hair cell loss in animal models (Shepherd *et al.*, 2005; Leake *et al.*, 2011). The hydrogel effectively delivered BDNF into the surrounding cochlear fluid, indicating the potential to promote the health of the cochlea and improve overall device performance (Chikar *et al.*, 2012). More extensive research needs to be carried out to investigate the biocompatibility of the materials that may coat the electrode arrays.

The application of corticosteroids is beneficial in reducing inflammation (De Ceulaer *et al.*, 2003; Farhadi *et al.*, 2013; Eshraghi *et al.*, 2019), intracochlear fibrosis and impedance (Wilk *et al.*, 2016). Steroids can be given either pre-, during or post-surgery. Multiple delivery systems are being investigated including local administration at implantation, pre-operative or post-surgery systemic administration or local, gradual release post-administration via an eluting electrode array. Local delivery is expected to provide greater protection to cochlear tissues by penetrating cochlear fluids, in addition to reducing the side effects associated with systemic administration. In animal models of cochlear implantation, topical application of dexamethasone is protective against hearing loss and reduces the inflammatory response following implantation (James *et al.*, 2008; Chang *et al.*, 2009; Eastwood *et al.*, 2010).

The use of dexamethasone (DEX)-eluting electrode arrays is being investigated using animal models, mainly guinea pigs. Evidence suggests they reduce fibrosis surrounding the electrode array and decrease electrical impedance (Farhadi *et al.*, 2013; Y. Liu *et al.*, 2015; Wilk *et al.*, 2016). Several factors associated with electrode insertion trauma were protected against following 3 months implantation with dexamethasone-eluting electrodes including loss of hair cells, damage to neural elements and increases in impedance, fibrosis and hearing thresholds (Bas *et al.*, 2016). 3D immunofluorescence analysis, to investigate the fibrotic response following cochlear implantation, found a reduction in the immune response in the guinea pigs implanted with a dexamethasone-eluting array (Toulemonde *et al.*, 2021). These studies did not involve daily stimulation of the electrodes.

To more closely simulate the human CI, adult guinea pigs were implanted with dexamethasone-eluting arrays which underwent a 4-week period of stimulation (Needham *et al.*, 2019). Four-point impedance is a measure of impedance between two neighbouring intracochlear electrodes when charge is passed between them and gives an accurate indication of the environment at the neural-tissue interface. It was significantly lower in the presence of dexamethasone and this reduction increased as the length of time after implantation increased (Needham *et al.*, 2019). The amount of fibrosis in the cochlea did not differ between the eluting and non-eluting arrays however, the relationship between four-point impedance and fibrosis differed between the DEX-eluting and non-eluting arrays. Four-point impedance showed a strong correlation with fibrotic tissue growth in the non-dexamethasone eluting arrays, meaning that dexamethasone reduced four-point impedance. As there was no difference in the amount of fibrosis between the two groups, the authors suggest that dexamethasone may influence the nature of the fibrosis (rather than amount) and therefore influence the tissue response the tissue-electrode interface (Needham *et al.*, 2019).

The first human study was carried out to compare impedance (monopolar (MP1+2) and 4-point) changes across 24 months post-activation between 10 adults with dexamethasone-eluting arrays and 14 adults with control arrays (Briggs *et al.*, 2020). Both impedance measures were significantly lower over the 2 year period in the dexamethasone group compared to control. This study confirmed the safety and feasibility of dexamethasone-eluting cochlear implants in humans. These findings contribute to the evidence from animal studies suggesting that elution of dexamethasone is the most effective method for reducing fibrosis, impedance and therefore performance. Cochlear, one of the CI manufacturers, are now leading an international clinical study of a CI that releases glucocorticoid drug therapy directly into the cochlea. This is a multi-centre, randomised, blinded study led by Professor Stephen O'Leary, to evaluate the effect of a glucocorticoid releasing CI in up to 120 adults with moderate to profound SNHL (Cochlear, 2021). Monopolar (MP1+2) impedance and speech perception performance will be measured at clinical visits and compared between the two groups. In addition, the rate and type of device related adverse events will be recorded. This larger scale human study will provide essential data into the efficacy and safety of dexamethasone-eluting arrays in humans.

The duration of corticosteroid elution that is required to confer benefit in cochlear implants is not well understood. In a guinea pig model, elevations in ABR and CAP thresholds were measured immediately after implantation however in animals implanted with 1% and 10% dexamethasone base (DXMb) eluting arrays, these threshold shifts decreased progressively until 90 days post-implantation (Bas *et al.*, 2016). In guinea pigs with 10% DXMb-eluting arrays, a significant reduction in the initial increases in ABR and CAP thresholds were measured from day 3, suggesting only a short time is necessary to confer benefit. In the human study (Briggs *et al.*, 2020), the group mean impedance averaged across all electrodes and time points was significantly lower in the dexamethasone-eluting array group compared to control. Measurements were taken from 1-week post-surgery therefore these findings suggest a benefit in reducing impedance from as early as 1 week. Further studies are required to better understand the pharmacokinetics of the corticosteroid elution and the effect on the local tissue response.

The success of CIs in improving hearing function and sound quality is dependent on many variables including the degree of damage to the cochlea prior to implantation, the inflammatory/tissue response to the implant, the materials that make up and coat the electrode array and how this reacts with the cellular environment in the cochlea. This results in increased complexity when investigating the tissue-electrode interface and how to improve overall performance outcomes. A mouse model for cochlear implantation will be established to investigate the tissue response at the tissue-electrode interface. This model will have the capacity to measure the tissue response to arrays of different properties, as well as changing the

stimulation paradigm presented to the mice. This approach will improve upon previous models for several reasons. As discussed, the use of mice over other species presents multiple opportunities to easily manipulate the system using genetic and environmental factors; providing the scope to investigate many aspects contributing to the tissue response and subsequent performance outcomes. The two most used mouse strains for auditory research include CBA/CaJ and C57BL/6J mice. Both CBA/CaJ and C57BL/6J mice have been used in the pilot investigations described in this chapter. However, CBA mice will be preferable in future experiments as C57 exhibit a decline in auditory function with age which will affect hearing function. Compared to the published mouse models for cochlear implantation, this will be the first mouse model whereby custom designed arrays closely mimicking human arrays will be inserted into mice and stimulated. The tissue response will be investigated using a multi-method approach, involving in-depth tissue analysis, and advanced imaging modalities. Establishing a mouse model for the investigation of the tissue response at the tissue-electrode interface and to determine whether changing the material that coats the array, including the presence of dexamethasone, will be beneficial in understanding factors that influence the tissue response. This work will therefore contribute to improving outcomes in people with a CI.

5.2 Aims and objectives

Overarching aim:

Establish a mouse model for cochlear implantation to investigate the biological response at tissue-electrode interface

Objectives:

- Optimise implant design
- Optimise surgical technique
- Establish tissue processing methodology
- Optimise protocol for μCT imaging bone and soft tissue
- Investigate the tissue response to the implant and how it is different between the implant types

5.3 Methods

5.3.1 Mice

C57BL/6J and CBA/CaJ mice were housed in the Ear Institute University College London (UCL) in pairs or small groups. The mice were in a noise-controlled environment and had access to food, water and environmental enrichment.

The minimum sample size that was deemed a priori to be acceptable for inferences to be made was n = 6-8 for each group. This was based on the sample size reported in a mouse study where mice were implanted then the tissue/wound healing response was investigated by measuring F4/80, Il-1β and Arg1 expression (Bas *et al.*, 2015). Similarly in a mouse model for cochlear implantation with chronic stimulation, n = 6 per group were used (Claussen *et al.*, 2019). It was difficult to determine the effect size from previous studies of mouse models for cochlear implantation as the primary outcome measure (number of positively labelled macrophages per unit area) was different. Due to COVID-19 constraints the planned minimum sample size was not obtained.

5.3.2 Tissue processing

Following surgical implantation, the mice were culled by overdose with pentobarbital. The mice were perfused with PBS followed by 4% paraformaldehyde (PFA). Decapitated heads were placed in 10% neutral buffered formalin solution before transferring to 70% ethanol for storage at 4°C.

5.3.2.1 Mouse cochlea dissection

Dissection of the mouse skull for isolation of the cochleae was carried out in a petri dish under a dissecting microscope. A rostro-caudal midline incision was made along the length of the skull using a scalpel to reveal the skull. Bone pliers were used to open the skull at the level of the nasal turbinates before working caudally to remove the skull sections to reveal the brain. A spatula wetted in PBS was used to carefully scoop underneath the olfactory bulb to release the brain from the olfactory nerves. The brain was lifted from the skull-base moving caudally before being released by severing the spinal cord or medulla and was placed in a bijou containing 70% ethanol. The skull base immersed in PBS was transferred to the microscope. The styliform process was located to identify the position of the otic capsule. Bone pliers were used to break squamosal bone anterior to the otic capsule. Soft tissue was cleared from the styliform/otic capsule. Forceps were used to hold one of the auditory bullae, while a second set of forceps were used to apply lateral force (away from the midline) to the top of the styliform process to fracture the otic

capsule from the bulla to reveal the cochlea underneath. Excess skull bone and soft tissue from otic capsule was removed from the cochlea before placing in a labelled bijou with 70% ethanol. This process was repeated for the contralateral cochlea. Dissected cochleae in 70% ethanol were stored at 4°C prior to decalcification.

5.3.2.2 Decalcification, embedding and sectioning

Decalcification was carried out by immersing the cochleae in 0.125 M ethylenediaminetetraacetic acid (EDTA) on a rocker plate set to 40 rev/min for 48 – 55 hours.

To the embed in paraffin, the decalcified cochleae were put into histology cassettes and placed in the tissue processor (Tissue-Tek VIP 5 Jnr, Sakura) and taken to wax. The cochleae were oriented in heated metal moulds using Dumont forceps and a hand-held magnifier, before embedding in a wax block.

Prior to commencing sectioning, the wax blocks were chilled on ice for 15 minutes. Sequential set thickness (either 5, 7 or 10 μm) sections were cut and collected onto either APES and PLL coated glass slides. Sections were dried in a drying oven at 37°C overnight.

Histological staining was carried on tissue sections using haematoxylin. The protocol used was the same protocol used by (Moreno et al., 2011).

5.3.3 Computed tomography (CT)

For the cochlea scans, the cochleae (n=2) were fixed in 10% neutral buffered formalin solution then decalcified and embedded (as above). The wax block was cut down to a small cylinder shape with the cochlea inside. Two wax cylinders were attached to a metal pole, as shown in Figure 5.2, ready to be placed inside the scanner. The scan was performed using Zeiss 160 kVp Versa 510. Scan time was 26 hours for two cochleae (13 hours per cochlea). The resolution was 4.16 μm.

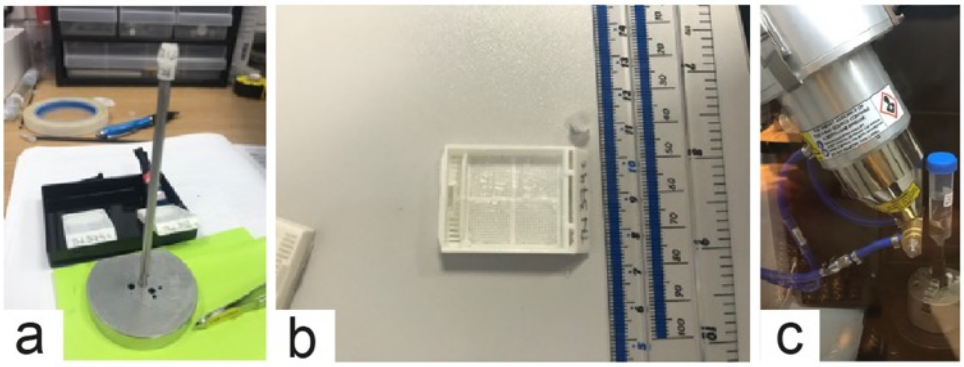


Figure 5.2 **Sample preparation for μCT scanning**

- **a.** Two wax cylinders containing cochleae attached to a metal pole ready to be placed inside the Zeiss 160 kVp Versa 510 scanner.
- **b.** The paraffin wax cylinder containing a cochlea shown to scale against a ruler.
- **c.** Whole calcified, mouse skull placed inside a tube filled with 70% ethanol and placed in the Nikon Med-X scanner.

For the whole head scans with electrodes implanted, the calcified mouse skull was placed into a tube filled with 70% ethanol and positioned in the Nikon Med-X scanner (Figure 5.2c). Three mice (n=3) were implanted and analysed by μCT analysis.

5.3.3.1 Imaging processing and visualisation

FIJI, Avizo and VG Studio Max were used for processing the image data from the μCT scans. FIJI was used as part of the segmentation workflow to crop the image stacks. Avizo was used to carry out a process called segmentation, to highlight the key regions of interest including the cochlea and the array (see A.2 for protocol). Both FIJI and Avizo were used to take measurements of the cochlea, implant and positioning of the implant. VG Studio Max was used to segment the cochlea bone and implant for visualisation.

5.4 Results

5.4.1 Establishing methodology

Due to the wide range of expertise required to carry out this work, a collaborative approach has been taken to establish the methodology for the development of a mouse model for cochlear implantation with the ability to investigate the cellular and molecular components of the tissue response.

5.4.1.1 Optimise implant design

We have been working with Oticon Medical, one of the four leading CI manufacturers, who part fund my PhD project. Oticon designed functional electrode arrays, small enough to be implanted into mice and stimulated. Optimisation of the electrode arrays was carried out, with the aim to satisfy each of the objectives below.

- The easiest surgical implantation, with minimal physical damage to the cochlea and surrounding tissue
- Sufficient insertion of the array into the cochlea
- Secure placing of the electrode array which will remain in place in a living animal
- Stimulation of the electrode in a living mouse

Dummy electrodes, made from silicone, were initially provided. The aim was to surgically implant the dummy electrodes to determine the appropriate dimensions of the array. Constant communication and feedback took place, after the initial dummy electrodes were implanted into mice, to determine the most appropriate dimensions for future models. Through optimisation, there were advances in the design and complexity of these electrode arrays. The implants (shown in Figure 5.3) include both a stimulator electrode and reference electrode, made of silicone and platinum, a connector with the stimulator to be connected and a piece of mesh anchored to the connector. After trying to surgically insert the implant shown in Figure 5.3, further optimisations were discussed with Oticon which included to add a plug or cap to the connector to the keep the pins clear. We suggested adding a sheath/covering to the array to keep the array clean and protect from damage during surgery. During surgery, when trying to insert the electrode towards the round window, there was a tight turn. Therefore, having the reference and active electrodes further apart would be preferable. The angle of array and reference electrode was changed to 45 degrees. In addition, there was difficulty in inserting the electrode into the cochlea due to the tight angle of insertion and difficultly in maintaining visibility of the round window when

implanting. To improve this, the distance from the tip of the array to the shoulder was increased from 1.5 mm to 3-4 mm. The overall length of the array was increased from 12 mm to 14 mm.

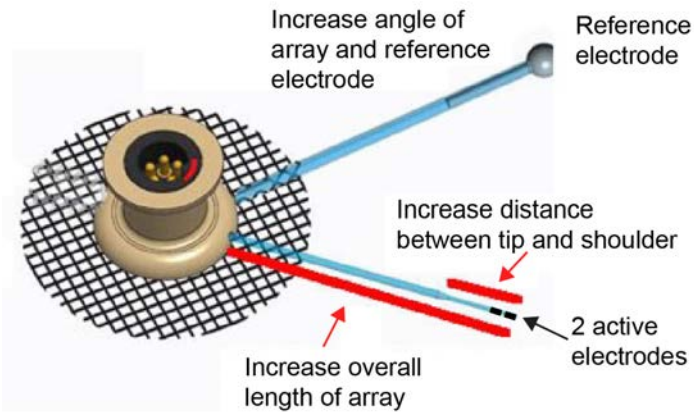


Figure 5.3 **Diagram showing the implant designed by Oticon Medical in the early stages of development**

The components of the implant that will be surgically placed into a mouse including an array with two active electrodes, a reference electrode, connector and mesh.

Further optimisations of the implant design were carried out. Figure 5.4 shows the current design with the dimensions. Figure 5.4c is a photograph of the implant. The implants are fully functional meaning the electrodes can be stimulated using an animal stimulator platform (ASP). The ASP can be used to measure impedance in vivo, following insertion and electrical stimulation. Through working with Oticon Medical the design and production of implantable electrode arrays, which mimic the materials used in human arrays and can be stimulated, has been possible.

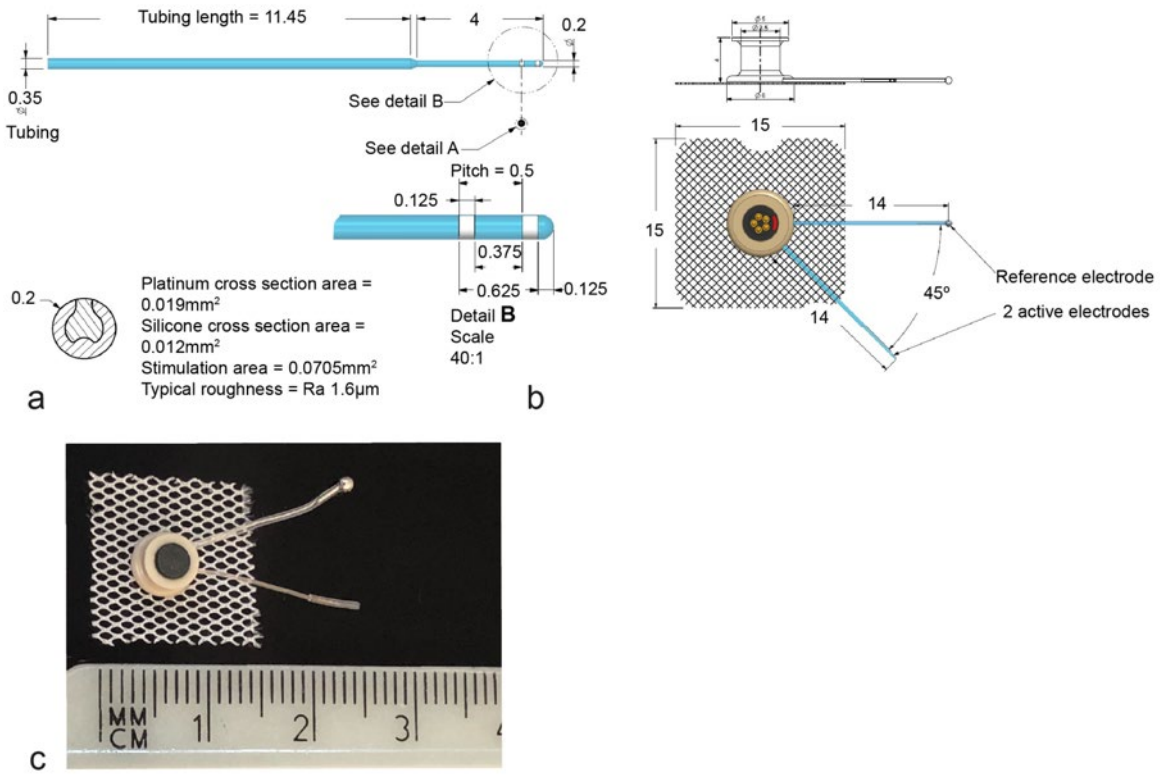


Figure 5.4 **Optimised implant design and dimensions**

- **a.** Schematic of the electrode array design with dimensions.
- **b.** Schematic of the whole implant including the connector, reference electrode and two active electrodes. A square mesh was secured around the connector which will be sutured to the skin to hold it in place.
- **c.** Photograph of array.

A novel aspect of this CI mouse model is the investigation of whether changing the material that coats the electrode array will have an effect on impedance and the tissue response. Further implant optimisation includes the production of electrode arrays comprising of the following materials:

1. Pure silicone + platinum
2. Pure silicone + platinum with PEDOT
3. Pure silicone + platinum with PEDOT and 10% dexamethasone
4. Black platinum
5. PEDOT from LAAS Toulouse

Silicone is the material that usually coats electrode arrays and the electrode array contacts are platinum. In the PEDOT coated implants the silicone will be coated with PEDOT, a conducting polymer. Oticon measured the electrochemical impedance of the electrodes in vitro in saline solution and found that in the electrodes with PEDOT coating, impedance was reduced by 10V

compared to platinum. In the PEDOT coated implant with dexamethasone, dexamethasone will be within the PEDOT coating the silicone and will leak out and into the surrounding environment. Electrode array 4, the black platinum electrode, has increased roughness that significantly reduced impedance when tested in vitro. Electrode array 5 was produced by a lab in Toulouse and the impedance was very stable.

5.4.1.2 Optimise surgical technique

Due to the access of the equipment to carry out ABR and surgical implantation, all mouse surgeries were carried out at UCL. Our collaborator, Dr Lucy Anderson, at UCL had previous experience with implanting electrode arrays into gerbils however the size and anatomy of mice cochleae is significantly different. Considerable time was invested to become competent in surgically implanting an electrode array (or dummy electrode) into the cochleae of mice. The decision was made that Lucy would carry out the surgeries with the primary aim to obtain adequate tissue for μCT and tissue analysis, to be carried out in Southampton. Due to COVID-19, there were issues with availability of mice and access to laboratories which prevented further in vivo experiments being carried out.

Surgical technique was optimised with the aim to comply with the following aims.

- The easiest surgical implantation, with minimal physical damage to the cochlea and surrounding tissue
- Successful recovery of the mouse with the implant in
- Precision and repeatability in additional mice

Many difficulties arose in terms of the best insertion route (round window or cochleostomy), how to determine whether the electrode array was inside the cochlea and the length of insertion, how best to secure the array in place and how best to repair the hole that was made for insertion. Round window insertion was decided to minimise damage to intracochlear structures from drilling. It was decided early on that the left ear will be implanted. To secure the array in place, tissue glue was used. However, in some cases, the glue ran down the array and into the round window. Mesh was used to secure the connector in place. Tissue glue was used to secure the wound. As optimisations occurred and new implants were produced, further difficulties arose due to changes in size, design and material resulting in further adaptions to surgical procedure.

For insertion of the array, a hole is made in the bulla bone (bullostomy), at the top left region, making the round window visible. The array is then inserted through the round window membrane (as shown in Figure 5.5 and Figure 5.6a). Figure 5.6 shows images of the optimal surgical technique. The ground electrode is tucked into neck muscles to secure in place (Figure

5.6b). The implant connector plug is inserted through the skin at the back of neck and placed into the scruff of the animal between the two ears, with the wire coming down the back of the ear (Figure 5.6c). This will allow movement of the mouse in the cage when the stimulator wire is attached to the connector. The initial aim was to have an implantation time of 2 -3 weeks, therefore the mice need to be able to move freely and comfortably with the implant in.

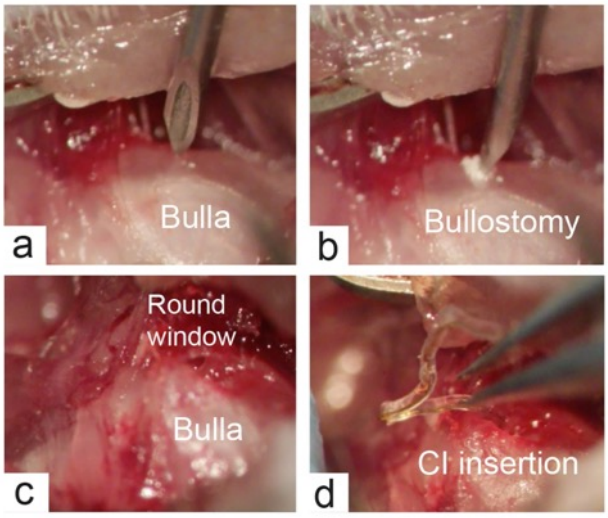


Figure 5.5 **Images of mouse bullostomy and CI insertion**

Images taken from a video of mouse bullostomy and round window insertion of a dummy cochlear implant. Surgery was carried out by Dr Lucy Anderson.

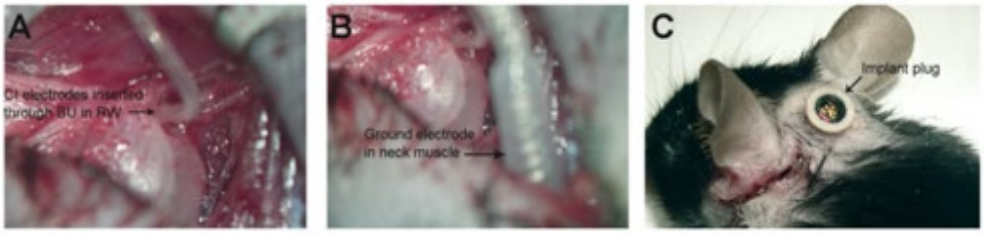


Figure 5.6 **Collection of images showing the optimised surgical technique for the implantation of an electrode array into a mouse for stimulation**

These images were taken during surgical implantation of a custom-made CI into a mouse.

a. The electrode array is inserted through bullostomy into the round window.

b. The ground electrode is tucked into neck muscles.

c. The implant connector plug is inserted through the skin at the back of the neck.

5.4.1.3 Establish tissue processing methodology

Following successful implantation and recovery, the mice will remain housed for 2-3 weeks to allow for the acute stages of an inflammatory response to occur. The mice will then be culled to

carry out analysis of the tissue response associated with implantation. The objective was to establish an effective tissue processing methodology which made the following aims feasible.

- Analysis of the cellular composition and tissue environment in close proximity to the implant in the cochlea
- Analysis of the cellular composition and tissue environment in key regions of the cochlea (organ of Corti, stria vascularis, spiral ganglion neurons) and auditory pathway
- Biomedical imaging analysis of the implant in situ

Careful consideration was carried out for how best to process the implanted mouse heads, as the normal protocol was not applicable for several reasons. The electrode array wire was made of platinum, therefore sectioning paraffin wax embedded tissue on the microtome (as done previously) was not feasible as the blade would not cut through the metal wire. Additionally, if the tissue containing the implant was placed into the tissue processor (Tissue-Tek VIP 5 jnr, Sakura), the silicone casing around the electrode arrays would melt and dissolve. Other sectioning techniques that would cut through the platinum wire were considered but this would require changing the staining technique and add further complications. Additionally, as the aim was to investigate the cellular composition and tissue environment at the tissue-electrode interface, removal of the electrode array before embedding and sectioning was not a suitable method as this could result in damage and change to the tissue morphology in contact with the electrode array.

A method for processing, sectioning and analysing tissue from implanted mice was established. The cochleae were not dissected out as this could risk movement and dislodging of the arrays. The mouse skulls were processed intact (fixed and decalcified) and a sagittal incision was made to produce two halves. The tissue was dehydrated in an ascending concentration of sucrose before embedding in OCT (optical cutting temperature) compound, a matrix that allows tissue to be frozen and sectioned. After embedding in OCT, the tissue was immediately placed in liquid nitrogen to freeze. Following this, the electrode array was gently removed to ensure minimal tissue disruption and damage. Removing the array once the OCT has been frozen creates an electrode track which is visible in the tissue sections and useful to determine where the array had been. Figure 5.7 summaries the steps involved in implanting, processing and analysing the tissue response to cochlear implantation in a mouse model. By carrying out the explant tissue analysis (Chapter 4), a pipeline for tissue staining and analysis has been established which can be utilised in this model.

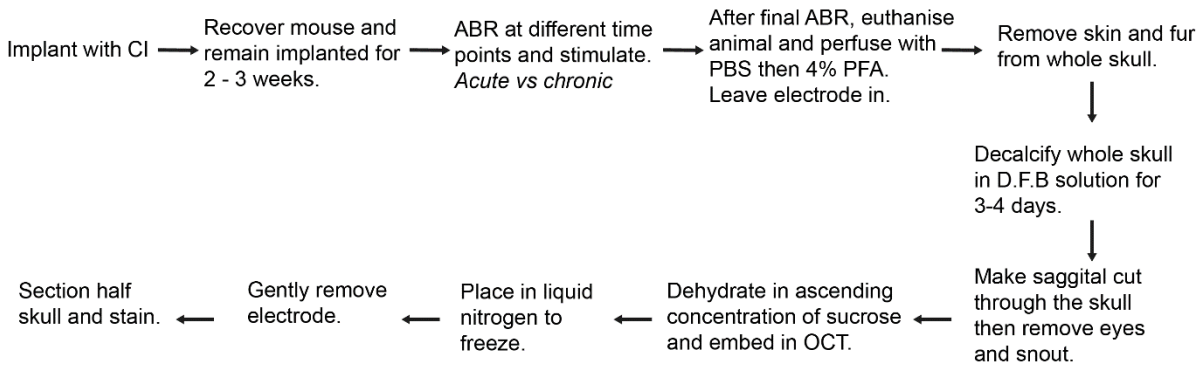


Figure 5.7 **Schematic describing the step-by-step procedure for tissue processing and analysis**

The experimental steps that will be taken from implanting the mice through to collecting, processing and analysing the tissue.

5.4.2 Establishing methodology for micro-computerised tomography (μCT)

5.4.2.1 Pilot investigation of the mouse cochleae using micro-computerised tomography (μCT)

μCT is an x-ray imaging technique, where a single scan will image the samples complete internal 3D structure at high resolution. μCT involves placing a sample in an x-ray beam path and following rotation of the sample during image acquisition, projected x-ray absorption patterns (radiographs) are captured (Katsamenis *et al.*, 2019). This technique will be used to provide 3D structural information about the cochlea pre-implantation and to validate surgical technique post-implantation, as it will visualise the position of the electrode array in the cochlea.

A pilot investigation of a mouse cochlea was carried out to determine the capability of the μCT equipment in Southampton and determine the resolution of a decalcified wax embedded label-free cochlea. Two formalin fixed paraffin embedded cochleae (n=2) (without CI) were imaged in the mu-vis centre on the Zeiss 160 kVp Versa 510 scanner. The cochleae were embedded in a small, plastic tube and imaged on a metal pole as shown in Figure 5.2. The resolution obtained was 4.16 μm. The quality and resolution of the images from the scan were informative and showed detailed morphology of the cochleae. 3D images of the cochleae were obtained (Figure 5.8a and b), highlighting the spiral structure and the cochlea turns. Digital sections of the cochleae are shown in Figure 5.9 indicating the different orientations, where physical sections could be sectioned. The three scalae, the organ of Corti, the tectorial membrane and Reissner's membrane can all be visualised (Figure 5.9c). The capability of this technique in terms of visualising morphology of the mouse cochlea at high resolution was confirmed.

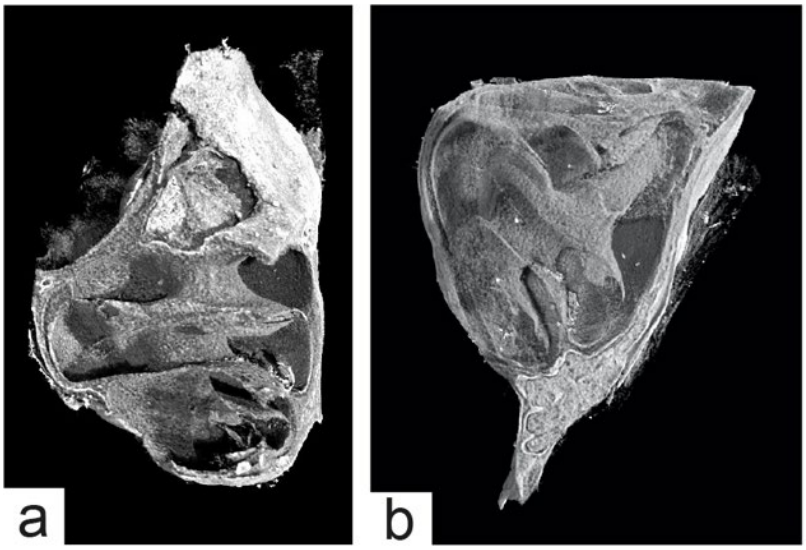


Figure 5.8 **Images from a μCT scan of a young C57 mouse cochlea**

3D images of the cochlea at high resolution. Image processing was carried out using VG Studio Max software.

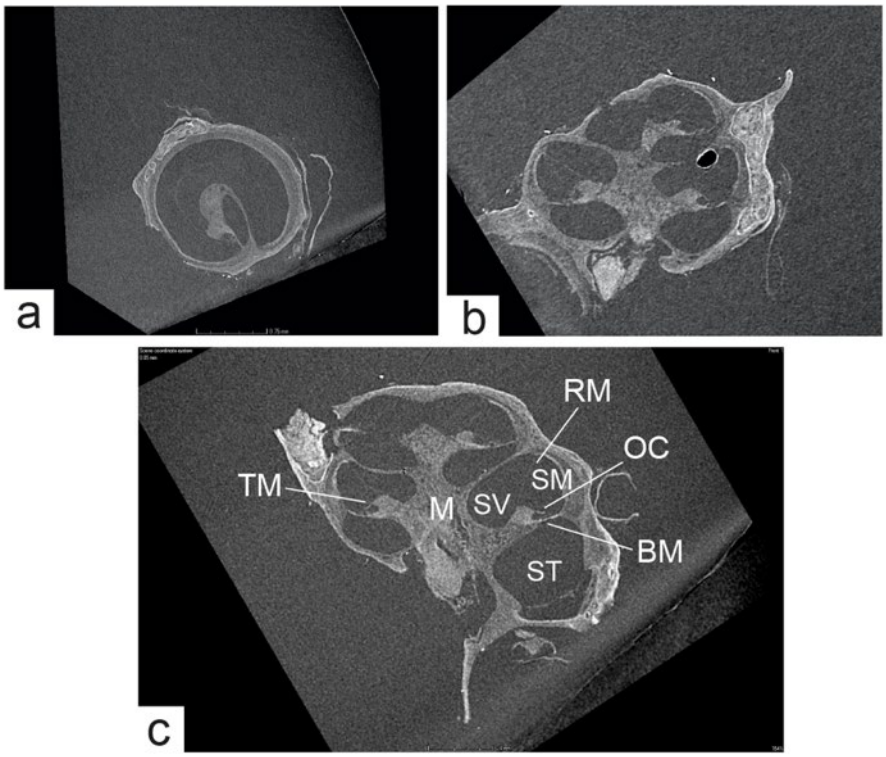


Figure 5.9 **Digital sections of the mouse cochlea at various orientations and planes**

a. Apical view of the cochlea.

b. Mid-modiolar view of the cochlea.

c. Mid-modiolar view with the apex at the top and key regions of the cochlea identified. M = modiolus, TM = tympanic membrane, BM = basilar membrane, OC

= organ of Corti, RM = Reissner's membrane, ST= scala tympani, SM = scala media, SV = scala vestibuli.

We corroborated the 3D (μCT) spatial information with histological (light microscopy) findings, a method known as 3D X-ray histology (Katsamenis *et al.*, 2019), to determine whether this methodology was feasible and could be used in the implanted mice. Figure 5.10 shows a mid-modiolar cochlea section imaged with light microscopy and μCT, whereby key regions of the cochlea can be visualised. This confirms that it is feasible to corroborate the findings from both techniques to gain insight into the tissue and cellular composition of the cochlea.

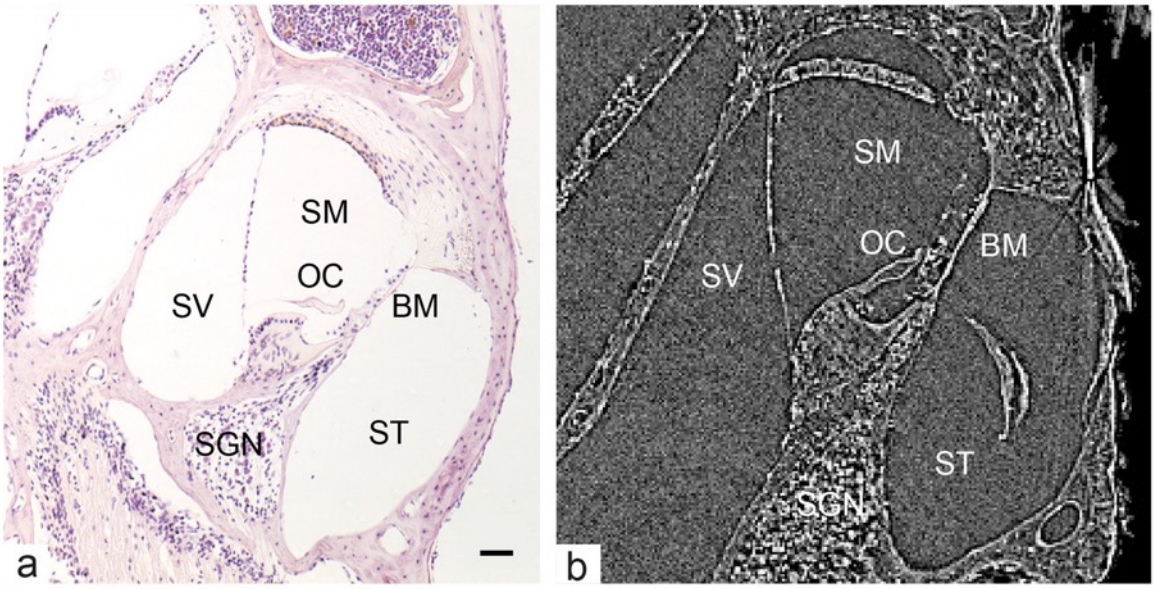


Figure 5.10 **Comparison between a mid-modiolar cochlea section imaged by light microscopy with conventional histology (a) and μCT (b)**

Key regions of the cochlear anatomy, visible in a cochlea section imaged with light microscopy and μCT, are highlighted. Scale bar = 5 μm

5.4.2.2 Optimise protocol for μCT imaging bone and soft tissue

Four scans were carried out (on n=3 samples) on the Nikon Med-X scanner at Southampton General Hospital, with the aim to visualise the array and the cochlea and to identify the positioning of the array. For the scan, the whole mouse head samples were formalin fixed, calcified and bathed in 70% ethanol. Figure 5.2c illustrates how the sample was positioned in the scanner. Table 5.1 details the samples scanned, the resolution and duration of the scan.

Table 5.1 **Detail of the scans of implanted mouse skulls (n=3) carried out on the Nikon Med-X Scanner**

Sample ID	Region scanned	Resolution	Scan time
TN516	Region of interest	6.6 μm	9h
TN516	Whole skull	12 μm	1h30
TN519	Whole skull	12 μm	1h30
TN520	Whole skull	12 μm	1h30

The objectives for carrying out these scans are listed below.

Objectives:

- Determine 3D structural detail of the mouse cochleae to inform device design
- Visualise and understand the position of the array
- Validate surgical technique post-implantation by examining positioning of the array and device integrity

Post-processing of the data was carried out whereby the raw data was processed to enhance and sharpen edges, which resulted in masking the soft tissue. A script was applied to enhance the image by applying Gaussian blur, a mathematical function that softens uneven pixel values in an image by removing the extreme outliers. Due to the amount of bone in the skull and cochlea, there was beam hardening creating lines coming off the specimen.

Figure 5.11 shows a whole mouse skull (12 μm resolution) with the cochlea and array highlighted using segmentation. Segmentation is the process of delineating the areas of interest in imaging, in terms of pixels or voxels, and is a necessary step when creating 3D images. It can be used to distinguish between the different materials which comprise the sample following μCT imaging. Two different orientations of the same sample are shown in Figure 5.12. The cochlea and inserted array have been segmented and visualised using VG Studio Max software, showing the cochlear spiral and the array inserted in the basal scala tympani region (Figure 5.13).

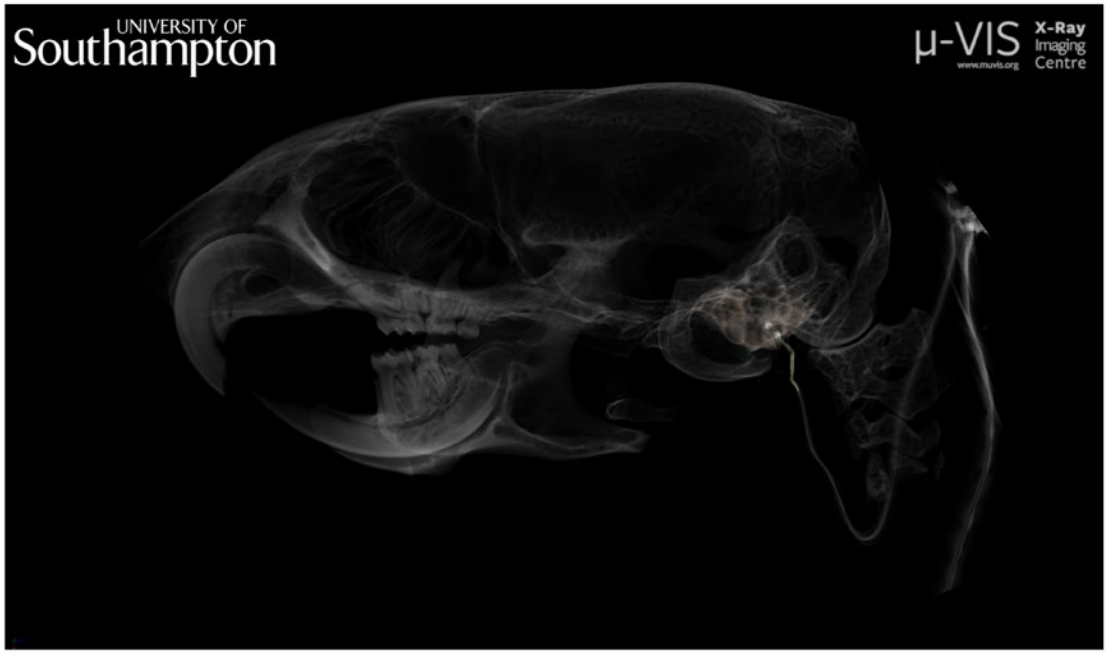


Figure 5.11 **Whole mouse skull with electrode array implanted into the cochlea from a 12 µm resolution scan of TN516 visualised using VG Studio Max software**

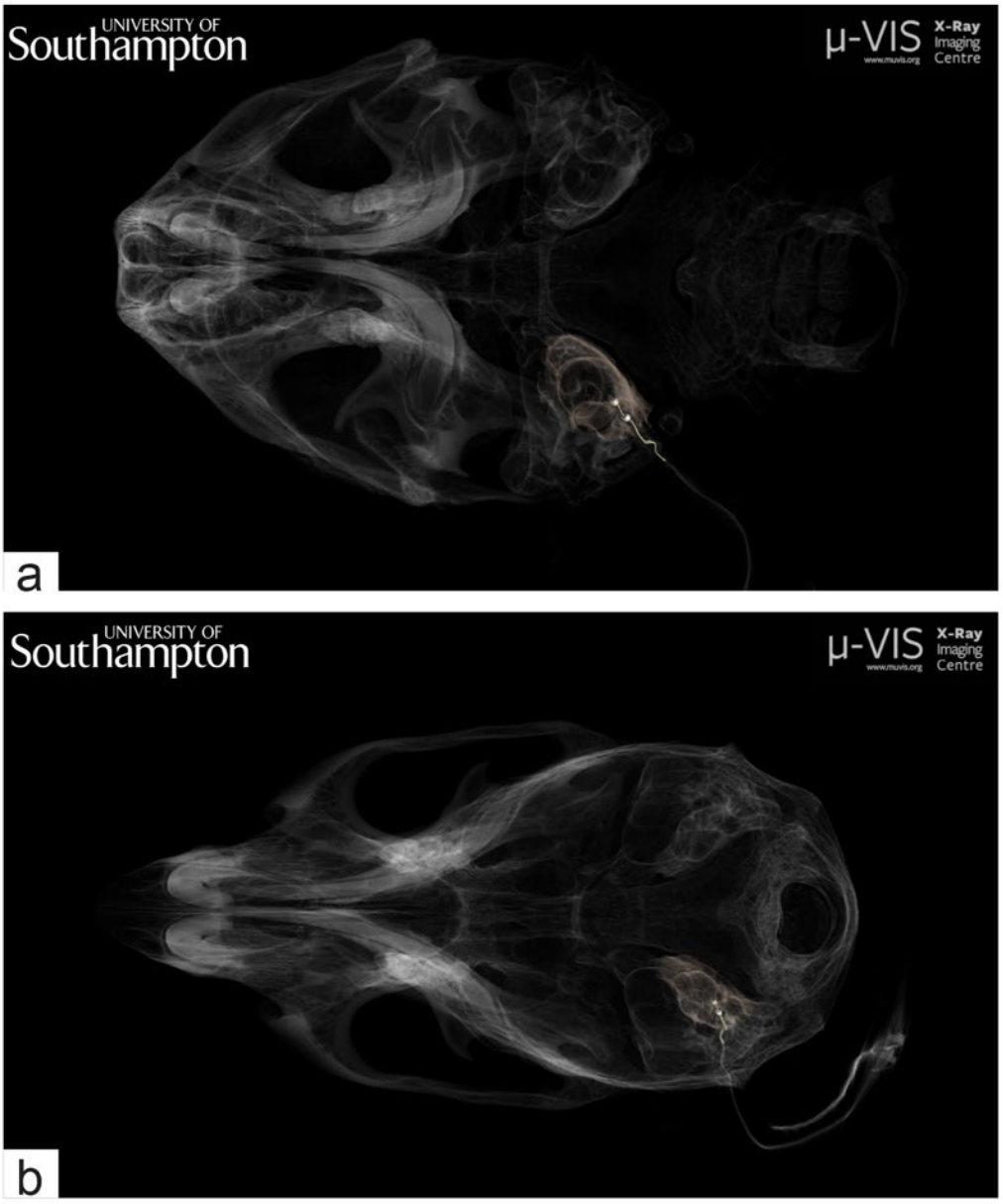


Figure 5.12 **Whole mouse skull with electrode array implanted into the cochlea from a 12 µm resolution scan of TN516 visualised using VG Studio Max software**

a. Below skull orientation

b. Above skull orientation

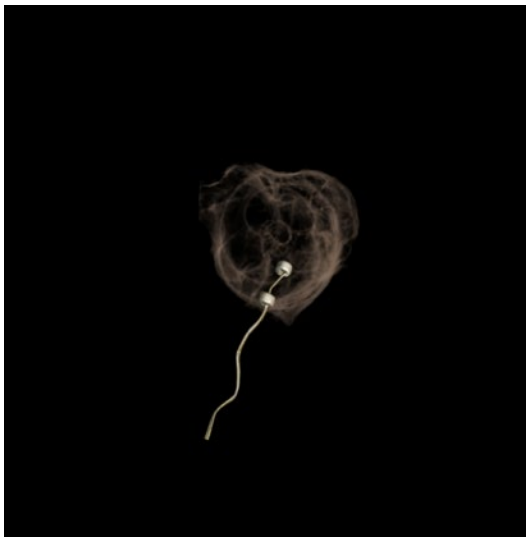


Figure 5.13 **Mouse cochlea with implanted electrode array obtained from a 12 μm resolution scan, visualised using VG Studio Max software**

5.4.2.3 Using Avizo software for effective image processing and visualisation

A protocol was optimised for post-scan processing and segmentation of the scans of the whole mouse skull with an electrode array inserted. The raw data files were imported into FIJI and cropped to reduce the size of the dataset before opening in Avizo. An interactive thresholding tool was used to carry out semi-automatic segmentation of the implant, before carrying out manual segmentation of the bone and then the implant. Figure 5.14 summarises the workflow for the segmentation of bone and the implant using Avizo.

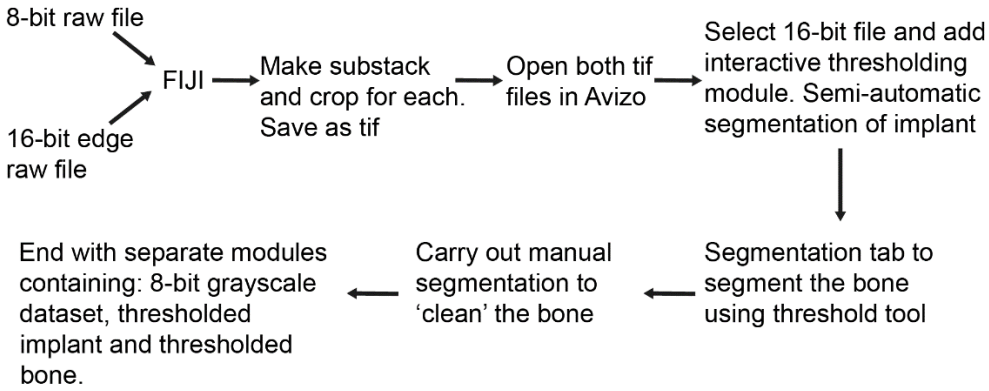


Figure 5.14 **Workflow for the segmentation of bone and the implant using FIJI and Avizo**

Using Avizo, it is possible to view the sample in multiple different formats and clipping planes, enabling 2D and 3D visualisation of the cochlea and implant. A higher resolution scan (6 μm) of the region of interest was carried out on sample TN516. Figure 5.15a shows an ortho-slice of the implanted cochlea, with the array with two electrodes positioned in the scala tympani. Figure 5.15b is a 3D volume render showing a section through the cochlea at the same position as in

Figure 5.15a. Figure 5.15c is a 3D view of the cochlea from a different orientation towards the tip of the cochlea with the array inserted into the base.

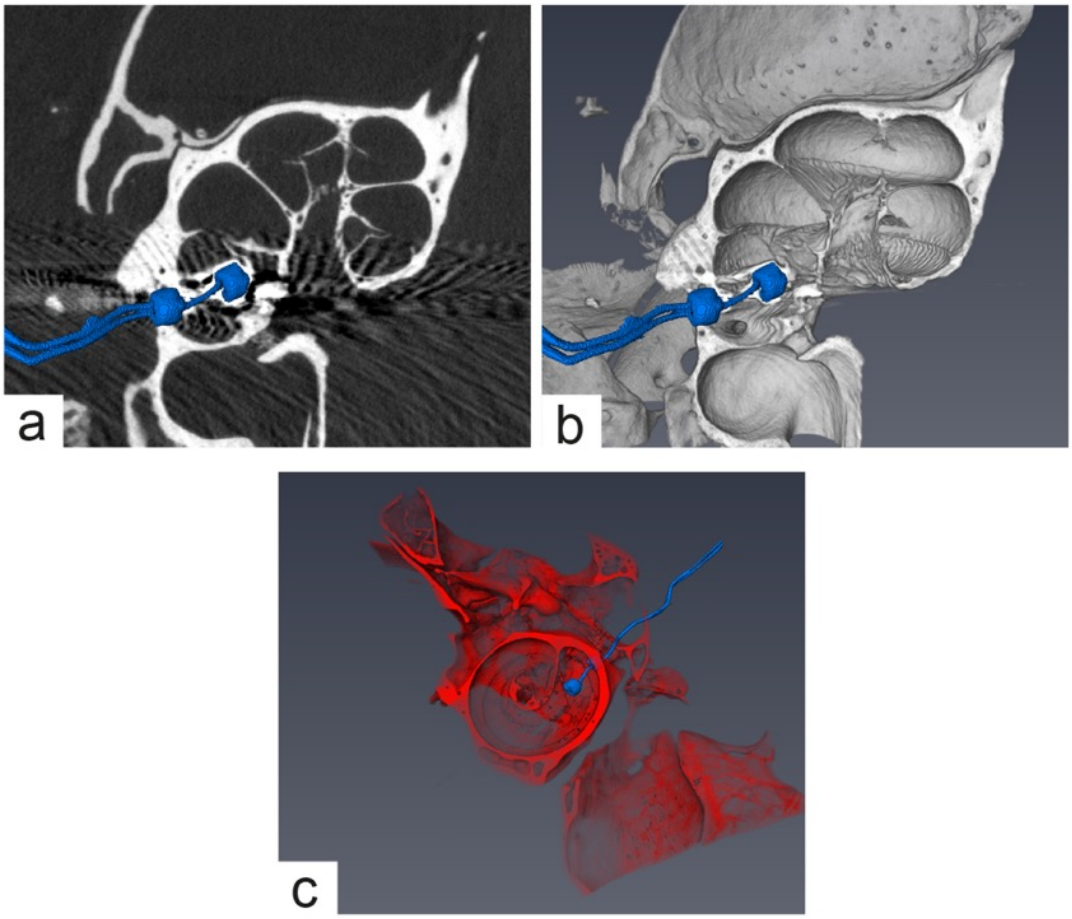


Figure 5.15 **Multiple views of an implanted mouse cochlea from a 6 μm resolution (TN516) scan**

- **a.** An 8-bit ortho slice showing a mid-modiolar view of the cochlea with the array positioned in the basal scala tympani.
- **b.** An 8-bit volume render showing a mid-modiolar view of the cochlea with the array sitting in the basal scala tympani.
- **c.** A 3D view of the segmented mouse cochlea with the electrode array in the basal scala tympani.

The same sample (TN516) was scanned at 12 μm resolution. Figure 5.16 is a panel of images of an 8-bit volume render moving through the YZ plane, indicating the position of the array at different points through the cochlea. A volume render of the segmented cochlea and implant in the XY clipping plane is shown in Figure 5.17.

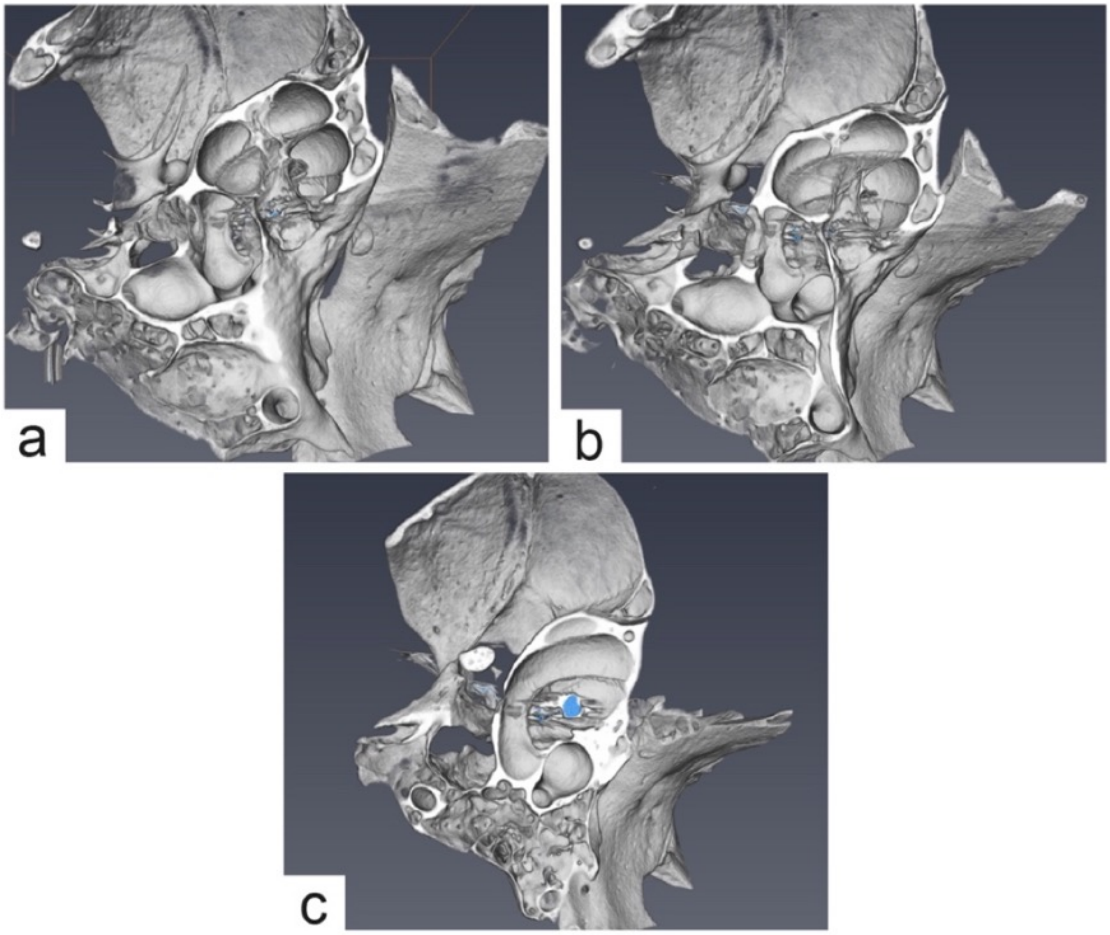


Figure 5.16 **Multiple views of a volume render of an implanted mouse cochlea sitting in the skull from a 12 μm resolution (TN516) scan**

An 8-bit volume render showing the segmented cochlea and implant in a YZ plane, slice 50 (**a**), 51 (**b**), 57 (**c**).

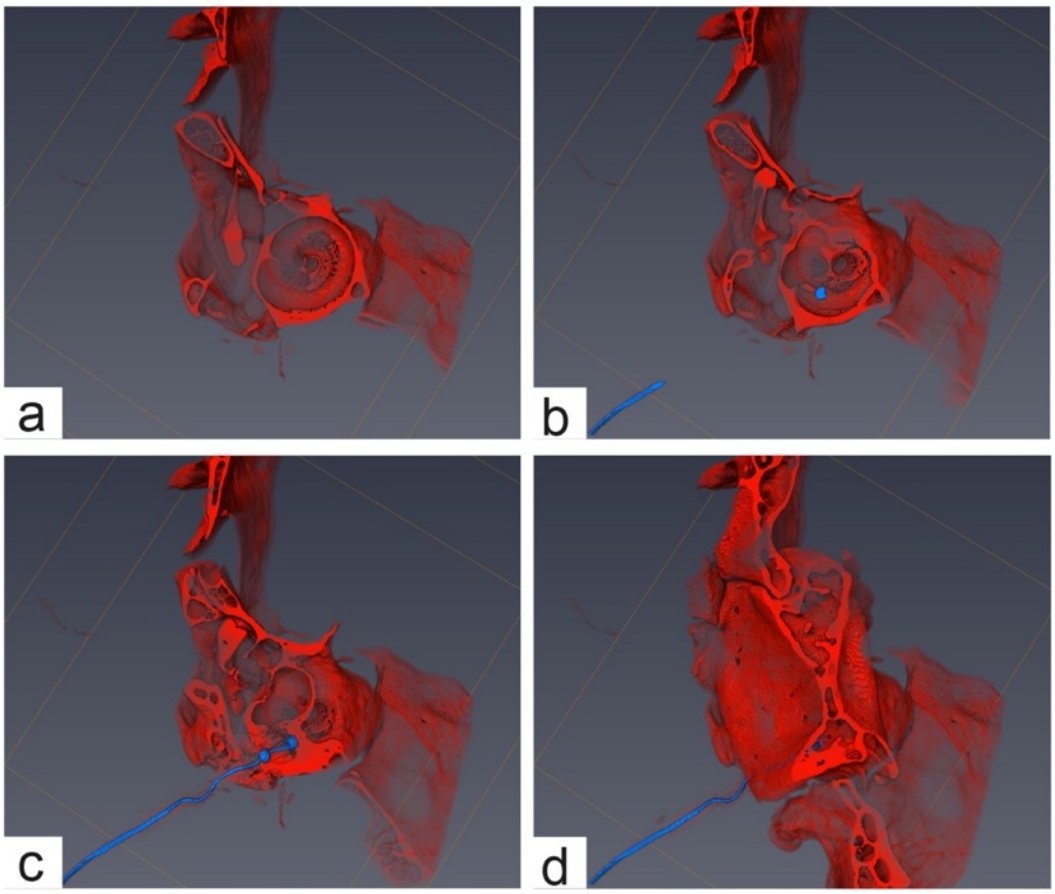


Figure 5.17 **Multiple views of the segmented cochlea and implant in the xy clipping plane, from a 12 μm resolution (TN516) scan**

A volume render of the segmented cochlea, slice 1 (**a**) showing the cochlear spiral. A volume render of the segmented cochlea and implant, slice 51 (**b**), 57 (**c**) and 93 (**d**).

The 12 μm resolution μCT scan of sample TN519 revealed the electrode array was no longer in the cochlea (Figure 5.18). The surgical notes indicate that the round window was located and the bulla was opened up to the left of the tympanic ring, destroying the middle ear. The CI was inserted through the round window but came out of the round window when the connector plug was inserted. Once the plug was in place, the CI was inserted back into the round window. The array must have dislodged following securing the wound.

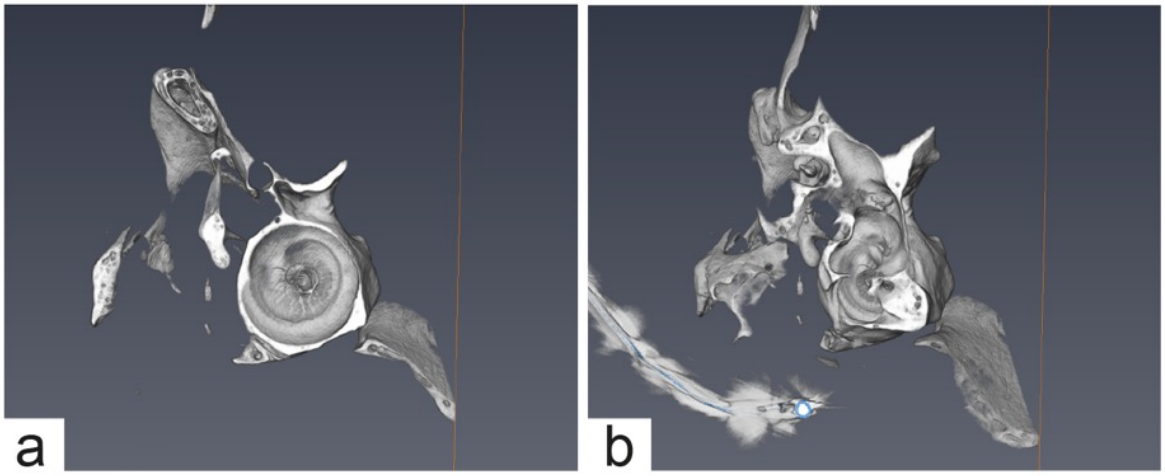


Figure 5.18 **8-bit volume render of the mouse cochlea with the electrode array positioned outside the cochlea, from a 12 μm resolution (TN519) scan**

a. An 8-bit volume render showing the segmented cochlear bone, slice 40.

b. An 8-bit volume render of the mouse cochlea showing the segmented cochlear bone and the electrode array positioned outside of the cochlea, slice 72.

The 12 μm resolution μCT scan of sample TN520 indicated that the electrode array was damaged. It was recorded that the array was newly removed from an autoclaved packet but were missing covers for the electrodes and that the electrodes appeared faint and in poor condition. Figure 5.19 displays the segmented electrode array from sample TN516, with the two electrodes clearly visible (Figure 5.19a) and from sample TN520, showing the damaged array (Figure 5.19b). The electrodes in Figure 5.19b are not clear.

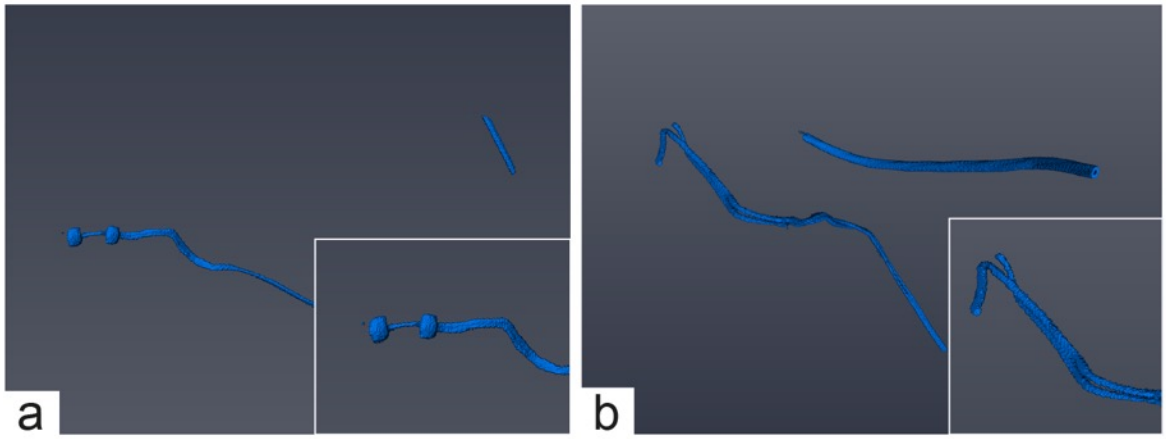


Figure 5.19 **Segmented electrode array that was inserted into the mouse cochlea of sample TN516 (a) and TN520 (b) from a 12 μm resolution scan**

The array in (**a**) shows two electrode contacts, whereas the array in (**b**) appears damaged and the electrodes cannot be visualised.

The positioning of the array, in sample TN520, can be visualised in the 16-bit volume render of the segmented cochlear bone and array. Figure 5.20a and b show the cochlea in the xy clipping plane

and Figure 5.20c and d show the cochlea in the yz clipping plane. The electrode array is positioned in the basal turn of the cochlea.

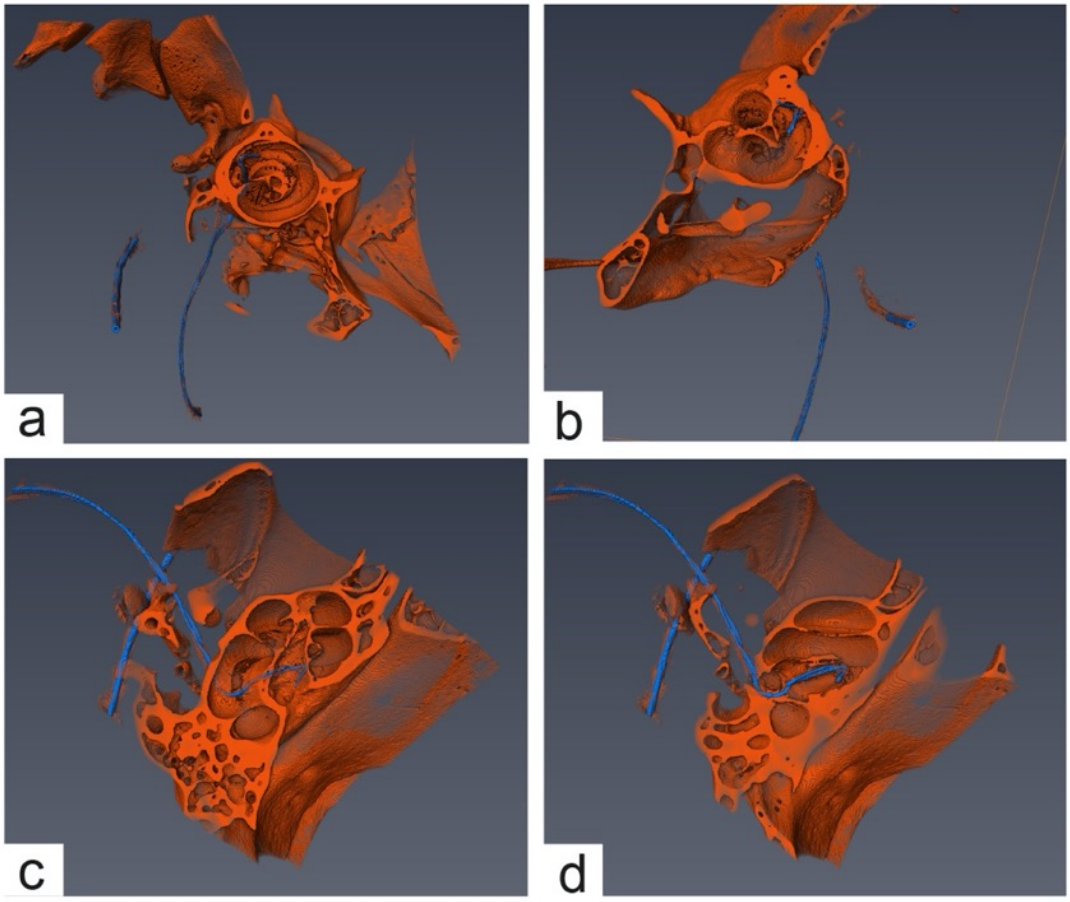


Figure 5.20 **A 16-bit volume render of a substack of the segmented cochlear bone and implant at two different clipping planes, from a 12 μm resolution (TN520) scan**

A 16-bit volume render showing the segmented cochlea and implant at the xy clipping plane, slice 33 (**a**) and 48 (**b**). A 16-bit volume render showing the segmented cochlea and implant at the yz clipping plane, slice 43 (**c**) and 48 (**d**).

Avizo was used to obtain measurements of the segmented array from the 6 μm resolution scan of sample TN516. Figure 5.21a shows the dimensions of the electrode array and Figure 5.21b shows the segmented array at 6 μm resolution and the measurements taken using Avizo of the various array dimensions. The measurements of the segmented electrode array made on Avizo were similar to the dimensions of the array, as shown in Table 5.2, indicating an effective 3D volume segmentation method was used.

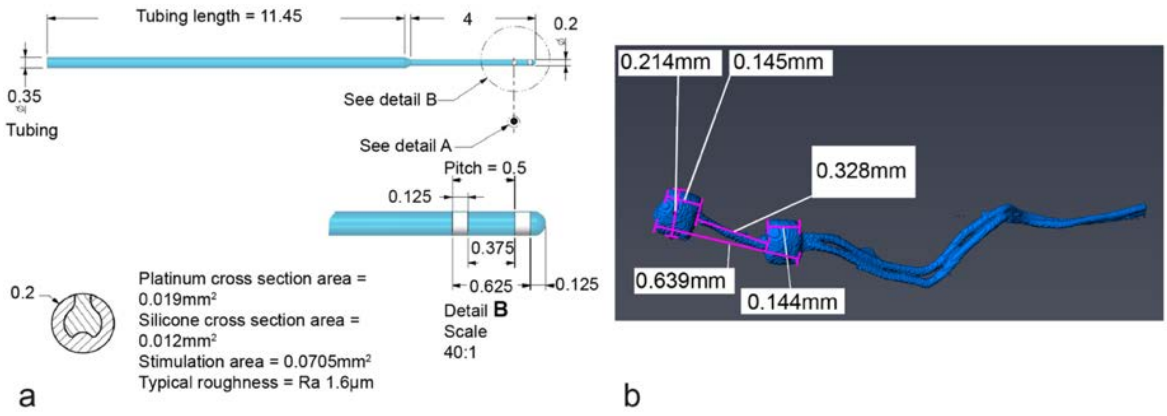


Figure 5.21 **Optimised dimensions of the electrode array alongside measurements obtained of an implanted electrode array using Avizo**

a. Dimensions of the electrode array showing the total tubing length, the distance between and thickness of each active electrode.

b. Measurements of an implanted electrode using segmentation in Avizo, followed by taking measurements.

Table 5.2 **Comparing array dimension with segmented array measurements made using Avizo**

Array dimensions (mm)	**Segmented array measurements (mm)**
0.125	0.145, 144
0.200	0.214
0.625	0.639
0.375	0.328

5.4.3 Investigating the tissue response to cochlear implantation

Further work to investigate the biological response at tissue-electrode interface and how this varies between different implant types was not possible due to limitations imposed by the COVID-19 pandemic. However, the techniques and model described above advances our ability to do this by allowing a combination of histology and immunohistochemistry using a panel of stains and markers associated with the wound healing response (Table 5.3), to carry out detailed analysis of the tissue response. Alongside this, we have evidenced the feasibility of corroborating information from both histology and micro-computed tomography (µCT) in the approach known as 3D X-ray histology to provide greater insight into the cellular and tissue response to cochlear implantation.

All animal experiments including breeding and housing of animals was stopped for a lengthy period of time, as well as lab access. The complexity of this project, whereby aspects of the work was being carried out in Nice (Oticon Medical), UCL and Southampton, made it not possible for the final part of this project to be carried out. This included the implantation of arrays of varied materials, recovery of the mice and then obtaining tissue for analysis of the tissue response. However, the following steps to establish this mouse model were successfully achieved including optimising implant design of a sophisticated functional array, surgical technique and tissue processing methods. μCT image acquisition and processing methodology were established for a mouse cochlea with and without an implanted electrode array. The ability to corroborate 3D μCT imaging with histological findings in a mouse cochlea was evidenced.

Table 5.3 **Histological stains and immunohistochemical markers to investigate the wound healing response**

Inflammation	**Proliferation**	**Maturation**
H&E (Eosinophils, neutrophils, foreign body giant cells (inflammatory cells))	Trichrome (Collagen deposition)	Trichrome (Collagen deposition)
CD68 (Phagocytic macrophage)	Ki-67 (Proliferating cells)	Apoptosis marker
CD163 (Anti-inflammatory macrophage)	VEGFR2 (Angiogenesis)	
IL-1β (Pro-inflammatory cytokine)	Alpha-smooth muscle actin (Myofibroblasts and blood vessels)	
CD3 (T-cell)		

5.5 Discussion

5.5.1 Developments in establishing a mouse model for cochlear implantation

The aim of this work was to establish an effective mouse model for cochlear implantation with stimulation, whereby the response at the tissue-electrode interface to different electrode arrays could be determined. Multiple aspects of the model were optimised including the implant design, surgical technique and tissue processing methodology. Functional electrode arrays which mimic the material and properties of human CIs, were designed and successfully implanted into mice. μCT imaging of the implanted mouse skulls confirmed the insertion and positioning of the array in the cochlea. Avizo was used for image processing, segmentation and visualisation. Measurements taken of the segmented array, using Avizo, confirmed the effectiveness of the imaging and post-processing to visualise the array at an appropriate resolution. The protocol for tissue processing and analysis was established and demonstrated to be informative in Chapter 4. Corroboration of μCT imaging and histological analysis was evidenced. Developments have been made in producing the materials and establishing the surgical, tissue processing and imaging techniques which are an advancement of existing techniques described in literature.

5.5.2 What is now enabled in terms of investigating the tissue response to cochlear implantation in mice?

As described in Chapter 4, as a field we are still limited in our understanding of the cellular and molecular mechanism behind the tissue response to cochlear implantation and the specific factors influencing this response. Several mouse models for cochlear implantation have been established using arrays which are either passive, non-stimulated or stimulated, to investigate multiple aspects of CI design and function. Following implantation and electrical stimulation, detailed analysis of the cellular composition and signalling molecules expressed in the fibrotic tissue at tissue-electrode interface can be carried out using the techniques established here. Further to this, investigation of how changing specific experimental conditions affects the tissue response will be informative and may provide insight into the variability in CI performance measured between individuals. For example, the mice undergoing cochlear implantation could be exposed to additional inflammatory insults to investigate the cumulative effect of cochlear insults on the size and type of tissue response using immunohistochemistry. Measuring hearing function (ABR) alongside impedance, following electrical stimulation in these mice, will provide insight into factors that affect both hearing performance and the tissue response.

5.5.3 Challenges and limitations of mouse models

Several technical challenges exist regarding implant design and surgical technique in the mouse compared to other animals due to smaller size and anatomical differences. In humans, the stapedial artery (SA) does not persist after the third month of prenatal life however in mice it persists and makes cochlear implantation difficult. The SA runs inside the middle ear cavity close the round window niche and obstructs exposure of the round window. Several surgical techniques have been described which allow insertion of the array into the scala tympani without causing damage to the SA, such as a modified dorsal approach (Soken *et al.*, 2013) and post-auricular approach (Mistry *et al.*, 2014). Other methods involve cauterization of the SA to provide access to the round window (Lu, Xu and Shepherd, 2005). This had no significant effect on hearing thresholds or SGN number (Lu, Xu and Shepherd, 2005). Despite this, several groups deem this unnecessary as it could lead to damage of residual hearing or further complications (Soken *et al.*, 2013; Mistry *et al.*, 2014; Navntoft, Marozeau and Barkat, 2019).

5.5.3.1 Limitations

Limitations of the model were evident following the analysis of the 3D volume renders of the μCT scans. One of the inserted arrays appeared damaged which could have been a result of poor production, storage or damage caused at insertion. Additionally surgical technique requires consistent and further practise and optimisation to ensure full insertion, as one of the arrays was no longer positioned in the cochlea at the imaging stage. Surgical implantation into a mouse cochlea requires a high level of skill and precision. For this, practise is important. To ensure this is possible for the future work with this model, large periods of time will be carved specifically for a focused and intense period of animal work. This will increase practise and surgical skill. This was not possible due to COVID-19 and the halt of a sustained period of animal work.

5.5.4 Utilising μCT for high-resolution imaging of cochlear bone and soft tissue

μCT imaging has multiple benefits as a method to be used alongside histological and immunohistochemical techniques. It is a non-destructive imaging technique providing 3D structural and anatomical detail of the sample. This is particularly useful when investigating the tissue response to cochlear implantation, as understanding the overall 3D structure of the cochlea and determining the position of the array within the cochlea is essential.

Our pilot results indicate the feasibility of corroborating information from both histology and μCT imaging, to provide greater insight of the cellular and tissue response to cochlear implantation in the context of the overall 3D cochlea. A similar approach was taken in a mouse model for chronic

implantation (Claussen *et al.*, 2019) whereby 3D x-ray microscopy was used to provide anatomical cochlear detail and intracochlear position of the array and volumetric analysis of the scala tympani and implant tract. Histological investigation provided detail of the cellular response. In this study, an initial image was obtained with the array in situ before removing the array and processing the cochlea by osmication to improve soft tissue contrast. Osmium tetroxide (OsO_4) increases the contrast of unsaturated fatty acids in cell membranes and lipid rich structures (Metscher, 2009). Further x-ray microscopy was carried out to obtain an image series for the volumetric analysis. An advantage of removing the implant then processing the cochlea was increased soft tissue contrast and therefore detailed analysis of the inflammatory response in the scala tympani. Between 27.6 – 38.9% of the mean fractional volume of the scala tympani was occupied by soft tissue and 4.11- 7.37% was occupied by neo-ossification (Claussen *et al.*, 2019). No significant difference was measured between the non-stimulated and stimulated groups, which may suggest the tissue response is due to the implantation of the electrode rather than the stimulation. A limitation of the study is the lack of specific cellular analysis using immunohistochemistry. The novel analysis of the explant tissue described in Chapter 4, demonstrates the capability of investigating specific cellular expression using immunohistochemistry. Combining detailed cellular analysis with the 3D structural information of the mouse cochlea will be informative regarding the tissue response to cochlear implantation.

5.5.4.1 Future work for μCT imaging for the investigation of soft tissue information

We have utilised μCT imaging to visualise the cochlear bone and determine the positioning of the array inside the cochlea. Further development of this methodology would include to troubleshoot the sample preparation and imaging protocol to optimise soft tissue contrast. Alterative post-processing methods will be trialled, to enable investigation of the soft tissue information and therefore analysis of the tissue response following cochlear implantation. Avizo could be used to calculate volumetric measurements such as mean scala volume area, as a reduction in the volume area would be an indication of fibrosis in the scala tympani. In addition, it would be useful to identify the location of gross tissue damage caused by the array as this would inform how effective the surgical technique is and indicate what cochlear structures may be damaged. A challenge of imaging soft tissues is the low x-ray absorption contrast of these specimens. Methods to enhance soft tissue contrast such as placing in osmium tetroxide stain could be used to mitigate this challenge.

μCT imaging could be carried out in vivo at a specific time point following implantation. Comparative μCT imaging in vivo and post-implantation may give an indication of changes in array positioning or the fibrotic tissue response over time.

5.5.4.2 μCT studies in human cochleae

Micro-computerised tomography (μCT) is used to investigate the anatomical structure of the human cochlea (Mei *et al.*, 2018) and to visualise the cochlear bone (Bellos *et al.*, 2014) with a CI inserted. Further method development of μCT imaging in this mouse model, to enable the investigation of the soft tissue response in vivo, may have translational benefits relevant for human cochlear implantation. Will there be aspects of CT imaging that will be compatible enough to resolve the tissue response in the human cochlea? A rate limiting step in humans is the exposure damage associated with x-rays. However, CT/MRI imaging is becoming more feasible in people with a CI as newer implant models are safe to undergo diagnostic x-ray. In people with a CI experiencing a decline in hearing function, combining these imaging modalities with tissue analysis of explanted arrays (as discussed in Chapter 4) will provide insight into the tissue response in vivo and following explantation. It will help elucidate the cause for the decline in performance. These techniques are not routinely carried out at the time of writing, however the larger scale human explant tissue analysis study described in Chapter 4 will begin this work.

Challenges with μCT imaging include artefacts, low contrast and noise. Other 3D imaging modalities such as synchrotron phase-contrast imaging (SR-PCI) can overcome these challenges. SR-PCI combines phase contrast imaging (PCI) with synchrotron radiation (SR) and CT reconstruction. Synchrotron radiation x-ray imaging uses high energy x-ray beams, which pass through dense structures more easily for high resolution visualisation of cochlear bone and the PCI highlights the soft tissue. SR-PCI is advantageous for analysing the cochlea, as it can visualise both bone and soft tissue simultaneously. Recent studies have used SR-PCI to investigate and visualise the cochlear vascular supply (Mei *et al.*, 2020), the spiral ganglion (Li *et al.*, 2020), and sensory cells and nerve fibres (Iyer *et al.*, 2018) and the internal microstructures (Elfarnawany *et al.*, 2017). Utilising high resolution, 3D imaging techniques alongside histological and immunohistochemical techniques will be informative for driving forward the understanding of the tissue response to cochlear implantation in both animal and human studies.

5.6 Future work utilising this mouse model for cochlear implantation

This chapter has described a mouse model for cochlear implantation including a range of techniques which allow detailed analysis of the tissue response to cochlear implantation and how this varies between arrays of different materials. The effect of other variables on the tissue response can be measured such as length of stimulation (acute vs chronic) and genetic or environmental factors such as the exposure to additional inflammatory insults.

Electrical stimulation of the implanted arrays is feasible in this mouse model. The protocols and techniques described in this chapter can be used to continue this work to apply both acute and chronic stimulation and to measure the tissue response associated. Following the implantation of multiple types of arrays and acute and chronic stimulation, the tissue response can be measured using the histological and immunohistochemical techniques (described in 5.4.3). In the published human temporal bone studies, the majority of the studies have used histological techniques to analyse the tissue response (Nadol, Eddington and Burgess, 2008; Seyyedi and Nadol, 2014; Ishai *et al.*, 2017). Some studies include greater specificity by using immunohistochemistry (Noonan *et al.*, 2020; Okayasu *et al.*, 2020). Few studies include 3D imaging techniques (Neilan *et al.*, 2012; Clark *et al.*, 2014). In mouse models for cochlear implantation, mainly histological analysis has been carried out (Irving *et al.*, 2013; Mistry *et al.*, 2014). More detailed characterisation of the wound healing response was described (Bas *et al.*, 2015), however a dummy array (nylon monofilament) was inserted which does not mimic the materials used in human arrays. Recent mouse models for cochlear implantation with chronic stimulation described the use of histology and 3D x-ray microscopy (Claussen *et al.*, 2019). However, immunohistochemistry was not used and there was not the capacity to investigate the tissue response to arrays of varying materials. The benefits of this model to investigate the tissue response to cochlear implantation over other published mouse models and human tissue analysis, are the range of tissue analysis techniques combined with the advanced 2D and 3D imaging techniques leading to the increased data richness which can be obtained from the analysis.

Throughout this research, there has been a focus on the role of macrophages in the auditory system and how they respond to acute and chronic inflammatory insults. The role of macrophages in initiating tissue damage following cochlear insults has been described in Chapter 2. Experimental evidence of macrophage activation across the middle ear and cochlea following chronic inflammation and NTHi infection in the Junbo mouse model was described in Chapter 3. We have proposed the hypothesis of macrophage priming in the auditory system and suggested the potential role for this in tissue damage and loss of function, contributing to progressive hearing loss. Macrophage priming may therefore be a mechanism causing the variability in hearing function between individuals and the variability in performance for people with a CI. It is necessary to investigate how different cochlear insults in the auditory system interact and the effect of cumulative insults on the function of the auditory system, as this may contribute to variable and sub-optimal CI performance. Using the mouse model, cochlear insults can be overlaid such as an immune challenge, a noise insult or aging, which will be beneficial as it will more closely mirror the human condition. This will provide further insight into factors that affect the tissue response, CI performance and the variability in performance seen between individuals. This

will also allow for analysis of the macrophage biology in the auditory system to look for evidence of macrophage priming following cochlear implantation. Detailed investigation of the tissue response to cochlear implantation and a greater understanding of the factors that affect the tissue response and overall performance may help identify therapeutics or lifestyle changes which can be implemented to improve CI performance outcomes.

Chapter 6 Overall discussion

6.1 Rationale for this research

The primary aim of this research was to evaluate the role played by the innate immune system and the local tissue response at the tissue-electrode interface in driving hearing outcomes in cochlear implantation. In order to address this overarching aim, the role of macrophages and the change in their distribution, morphology and abundance following both acute and chronic insults was first investigated to determine how the immune system is or is not activated by cochlear insults. Key overarching research questions include: does innate immune function affect cochlear health i.e., do macrophages influence the trajectory of hearing loss and do macrophages influence CI performance outcomes? In addition, what is the role of macrophages in predisposing tissue in the auditory system to further damage when additional insults are overlaid?

Based on the available evidence in the literature from other organ systems and the current evidence for the role of macrophages in the cochlea, we are proposing that macrophages in the auditory system have the ability to become primed following a cochlear insult. Priming results in a change in phenotype so when the macrophages are exposed to an additional inflammatory challenge, they display an exaggerated inflammatory response. An exaggerated and persistent inflammatory response may result in tissue damage contributing to decreased function of the auditory system and progressive hearing loss. Progressive tissue damage and loss of function, as a result of repeated macrophage activation following cochlear insults such as noise exposure and cochlear implantation over time, could be a mechanism responsible for the variability in both the trajectory of hearing loss between individuals through life and the hearing outcomes experienced by people with a CI.

6.2 Key findings

This research has used a multi-method approach incorporating the following elements e.g., a systematic literature search, explant tissue analysis and animal model development and validation. A combination of both human and mouse tissue was used to investigate the role of macrophages and tissue response to cochlear implantation, particularly in the context of the innate immune system and whether macrophages contribute to the function of the auditory system following cochlear insults. An extensive review of the literature was carried out which involved extracting and synthesising the available literature regarding the role of macrophages following cochlear insults. This search revealed that cochlear macrophages contribute to injury

following both acute and chronic cochlear insults through macrophage activation and changes in their distribution, morphology and abundance across key regions of the cochlea.

To investigate the role of cochlea and middle ear macrophages following a chronic cochlear insult experimentally, the Junbo mouse model for otitis media was used. The macrophage populations, distribution and function in the cochlea and middle ear following chronic middle ear inflammation was characterised for the first time in a model for otitis media. Increased macrophage activation and Il-1β expression was observed in Junbo mice, with inflamed middle ear cavities, suggesting that middle ear inflammation causes macrophage activation across regions of the cochlea which could contribute to tissue damage and progressive loss of function following chronic inflammation. Expression of these markers have been previously described in the literature following acute (noise exposure, cochlear implantation) (Hirose *et al.*, 2005; Bas *et al.*, 2015; Frye, Zhang and Hu, 2018) and chronic (aging) insults (Frye *et al.*, 2017; Noble *et al.*, 2019).

The first published analysis of the fibrotic sheath around an electrode array from a living person with a CI, who was undergoing explantation and reimplantation, was carried out. Underperformance, impedance fluctuations and a migration of the array out of the cochlea was recorded in this soft failure case. Detailed investigation of the tissue and cellular composition revealed an aberrant wound healing response with evidence of ongoing inflammation and proliferation at the same time across the tissue, 10 months after implantation. This was the first published study to characterise the tissue response to cochlear implantation in a living person with a CI undergoing explantation and reimplantation. The tissue structure and cellular composition of the fibrotic sheath on the explanted device was similar to what has been described previously in mouse (Bas *et al.*, 2015) and human CI studies (Nadol, Eddington and Burgess, 2008; Seyyedi and Nadol, 2014). Analysing the tissue encapsulating an explanted array has many benefits over the published human temporal bone studies. Our combined analytical approach correlates the investigation of the cellular environment of the fibrotic sheath with clinical and CI performance data, and medical history for the individual resulting in a rich dataset. This level of analysis allows the exploration of the reason for underperformance, the factors which influence the tissue response and will help understand ways to modulate the tissue response in future to improve performance.

Questions raised from the human explant study regarding the tissue response to cochlear implantation can be answered via an appropriate animal model. We established a mouse model for cochlear implantation, with the capability of combining in-depth tissue analysis and μCT imaging following implantation with custom-made electrode arrays. The use of mouse models for cochlear implantation, alongside human tissue analysis, is beneficial as it is possible to investigate

multiple aspects contributing to the tissue response by easily manipulating the system. Factors including array design, the material that coats the array and the type of stimulation can be altered whilst keeping all other variables constant, to investigate how these factors influence the tissue response. Future work will involve investigating the effect of previous immune challenges on the tissue response and CI performance following implantation.

Utilising this mouse model offers the opportunity to establish and improve the methodology for the investigation of the tissue response at the tissue-electrode interface. The use of histology and immunohistochemistry, alongside μCT imaging, is an effective method to corroborate 3D structural analysis with specific cellular analysis. Further optimisation of this methodology may have translational benefit to enable the investigation of the soft tissue response to cochlear implantation in humans.

6.3 Questions related to these findings

A role of cochlear macrophages following acute and chronic insults has been highlighted and evidenced by changes in the phenotype and function of these cells across key regions of the cochlea following insults. What is less well known is the effect of multiple/cumulative insults on the macrophage populations in the auditory system and whether cochlear macrophages can become primed by inflammatory insults. Do macrophages in the auditory system retain a memory to an inflammatory insult, meaning they display an exaggerated inflammatory response when exposed to an additional insult. Initial evidence suggests there is an interaction between multiple cochlear insults (Kujawa and Liberman, 2006; Zhang *et al.*, 2020), however further investigation is needed. As discussed in Chapter 3, the Junbo mouse model is a suitable model for investigating macrophage priming in the auditory system. We can investigate the effect of chronic inflammation on the macrophage populations and then how chronic inflammation effects how the macrophages respond to further insults such as NTHi infection. This is an effective model to investigate macrophage priming in the auditory system.

As described in Chapter 4, most of what is known about the tissue response to cochlear implantation comes from post-mortem human temporal bone studies whereby histological, and in some studies, immunohistochemical analysis has been carried out on tissue obtained when an individual has died. Therefore, it can be difficult to interpret when the tissue response and damage has occurred and how long it lasted for. Explant analysis from a living person means the tissue response can be investigated at the time of underperformance or failure.

There are considerable gaps in the literature regarding the reasons for the performance variability and underperformance experienced by people with a CI. Soft failures are those not caused by

hardware or surgical factors. Therefore, it is likely that the tissue response to cochlear implantation is playing a role in the decline in performance and failure. The novel methodology for the explant tissue analysis described in Chapter 4 can be used to investigate the innate immune mechanisms involved in soft failures and underperformance.

6.4 Future work

To further investigate the biological mechanisms playing a role in the underperformance or performance variability experienced by people with a CI, there are two main aspects of work to continue with.

6.4.1 Using animal models to investigate the role of macrophages following acute and chronic cochlear insults

Further investigation of fundamental macrophage biology in the auditory system is required, in order to understand the mechanisms influencing the function of the auditory system through life. The Junbo mouse model for NTHi infection will be used to continue investigating the macrophage response in this model for chronic inflammation and then with additional insults overlaid such as NTHi infection. Further experimental groups will be investigated including older Junbo mice which have had the chronic inflammation for longer and Junbo mice with NTHi infection for longer.

The mouse model for cochlear implantation with additional insults overlaid such as noise exposure, otitis media or aging could be used to investigate the cumulative effects of acute and chronic insults to macrophages in the auditory system, particularly in the context of cochlear implantation. Figure 6.1 demonstrates a timeline for the experimental design whereby Junbo mice will be inoculated with NTHi to cause infection. The infection will then be treated before inserting a cochlear implant. This experimental design mirrors the situation in humans whereby an individual may be exposed to multiple insults throughout their lifetime, such as middle ear inflammation and infection, which could cause damage to the auditory system and influence CI performance.

Questions to address:

- What is the cumulative effect of cochlear insults on macrophages in the auditory system?
- Do macrophages display an exaggerated Il-1β response following multiple cochlear insults?
- Do cumulative cochlear insults cause increased inflammation and tissue damage in the cochlea?

- What is the tissue response to cochlear implantation in mice with pre-existing chronic middle ear inflammation?

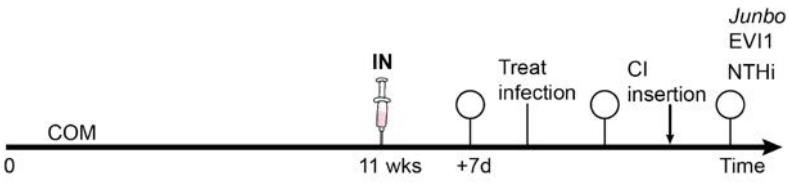


Figure 6.1 **A timeline for the experimental design of a future study whereby Junbo mice are inoculated with NTHi, the infection is treated and resolved, then the mice are inserted with an electrode array into the cochlea**

This timeline mimics a human situation whereby an individual may experience middle ear inflammation and infection in childhood, which resolves/is treated then later in life may receive a CI as a result of severe/profound hearing loss.

6.4.2 Larger scale human explant tissue analysis

Following the study described in Chapter 4, a larger scale human explant tissue analysis study has been designed. The tissue response at the tissue-electrode interface will be analysed in multiple living people with a CI undergoing explantation and reimplantation, as shown in Figure 6.2. Primarily, the tissue structure and cellular composition of the fibrotic sheath on the explanted device will be analysed and compared with findings from other explants from individuals experiencing poor performance. In addition to this a multi-method approach will be taken whereby a collection of clinical, medical and aetiological measures will be recorded from the individual undergoing explantation with the aim to better understand the factors contributing to the tissue response and the poor performance.

Human recruitment numbers for this study will be achieved by collecting explants over a year in an initial single centre study. To increase recruitment numbers and carry out a larger study, a multi-centre study would be desirable and is a goal for this work going forward. Interest from other centres within the UK to contribute to this study and donate explanted arrays, as they are removed, has been expressed. The initial work described in this chapter will involve the investigation of tissue from a few to several explanted cochlear implants with the aim and goal to gain ethical approval for a multi-centre study. This initial single-centre study is important for scaling up the techniques involved and increasing the momentum around this novel technique before expanding to a multi-centre study.

Questions to address:

- What is the tissue response to cochlear implantation in multiple people with a CI, undergoing explantation and reimplantation?
- What factors influence the tissue response?

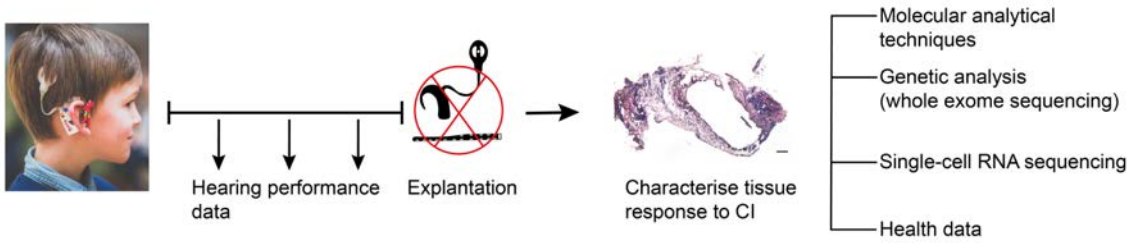


Figure 6.2 **Experimental workflow for a larger scale explant tissue analysis study**

The experimental workflow. Routine clinical measures will be collected from an individual who has undergone implantation. Indicators of poor performance or failure may result in explantation of the device. The explanted array and tissue attached will be collected for analysis using a range of methods including molecular analytical techniques, genetic analysis, single-cell RNA sequencing and health data analysis.

The schematic in Figure 6.3 highlights how the concepts within this research tie together. Investigating the macrophage response to chronic inflammation and cumulative insults is essential in understanding the factors influencing cochlear health through life and therefore how well an individual may do with a CI. Additionally, investigating the tissue response to cochlear implantation in multiple people with a CI experiencing underperformance will provide insight to the factors which have resulted in the tissue response measured and contributed to the performance decline. Mouse models enable accurate manipulation of the experimental design, providing a wealth of information. Human tissue analysis is relevant, species specific and as discussed, analysis of tissue from living people with a CI has multiple advantages compared to human temporal bone studies.

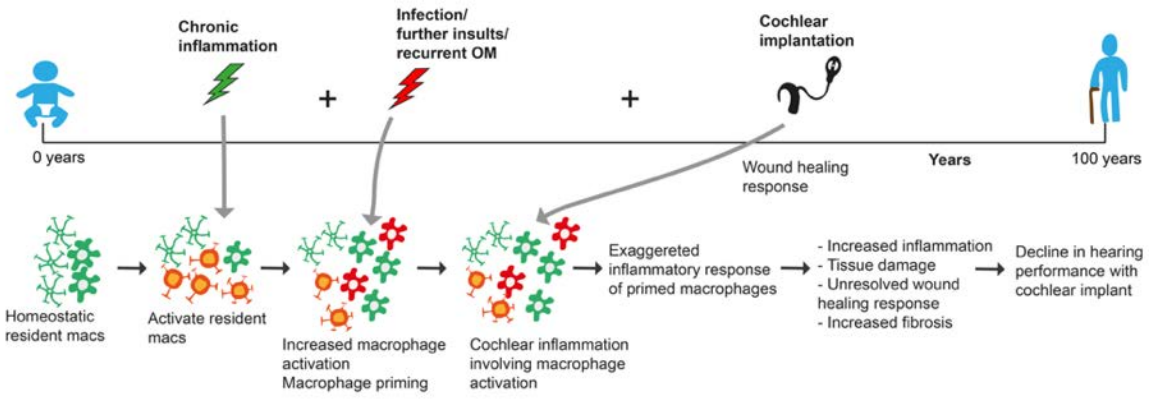


Figure 6.3 **Investigating the role of macrophages in the auditory system following cochlear insults and the potential impact on the tissue response to cochlear implantation and CI performance outcomes**

Schematic demonstrating the ongoing narrative of this research, including the role of macrophages following acute and chronic cochlear insults and how changes to the macrophage populations throughout a lifetime. Changes to macrophage populations over time may influence the function of the auditory system and therefore affect CI performance outcomes.

Continuing research into the function of macrophages in the auditory system using mouse models, alongside the novel human explant tissue analysis in multiple living people with a CI, will bring the field closer to understanding the role of innate immune function on cochlear health and how to modulate this to improve hearing outcomes through life.

Appendix A Experimental protocol

A.1 Table of primary and secondary antibodies and key experimental detail for immunohistochemistry

Target antigen and catalogue information	Primary Antibody	Type	Optimised working dilution	Host	Antigen retrieval	Blocking agent	Secondary antibody	DAB time
Macrophages/ microglia Wako (Alpha labs) 019-19741	Iba1	Polyclonal	1:1000	Rabbit	Microwave citrate buffer 2 mins, open then close, further 1 min, hot citrate buffer 5 mins, cool 5 mins	Normal goat serum 1.5% in PBS	Goat anti-rabbit 1:200. Vector Laboratories. BA-1000	2 min 5s
NTHi MRC Harwell	NTHi-162		1:5000	Rabbit	Two intervals of heating in a microwave for 7 min in distilled water	Normal goat serum (3ml =45μl + 2955 μl)	Goat anti-rabbit 1:200 Vector Laboratories. BA-1000	2 min 50s
Phagocytic macrophages Biorad (FA-11)	CD68	Monoclonal	1:250	Rat	Water bath 20 mins, cool	Normal rabbit serum 5%	Rabbit anti-rat 1:200 Vector Laboratories. BA-4000	2 min 30s

Target antigen and catalogue information	Primary Antibody	Type	Optimised working dilution	Host	Antigen retrieval	Blocking agent	Secondary antibody	DAB time
Microglial cells Abcam (ab209064)	TMEM119	Monoclonal	1:1500 in 3% TBS	Rabbit	Water bath 20 mins 90°C Tris/EDTA pH 9	Normal goat serum 3% in TBS	Goat anti-rabbit 1:200. Vector Laboratories. BA-1000	1 min 45s
Pro-inflammatory cytokine Il-1β Peprotech (500-P51)	Il-1β	Polyclonal	1:50	Rabbit	Hot citrate buffer 3 mins, cool 5 mins, heat 3 min, cool 5 min	Normal goat serum 20%	Goat anti-rabbit 1:200 Vector Laboratories. BA-1000	1 min 5s
Macrophages Abcam (ab783)	CD68	Monoclonal	1:100	Mouse	Hot citrate buffer 5 mins, cool 5 mins	Normal goat serum 1.5% in PBS	Goat anti-mouse 1:1000 Vector Laboratories. BA-9200	2 - 4 min
Macrophages Abcam (ab182422)	CD163	Monoclonal	1:250	Rabbit	Hot citrate buffer 5 mins, cool 5 mins	Normal goat serum 1.5% in PBS	Goat anti-mouse 1:1000 Vector Laboratories. BA-9200	2 – 4 min
Proliferating cells Abcam (ab15580)	Ki-67	Polyclonal	1:600	Rabbit	Hot citrate buffer 5 mins, cool 5 mins	Normal goat serum in 0.01% TX100 0.25% BSA	Goat anti- Rabbit 1:200 (in 0.01% TX100 0.25% BSA) Vector Laboratories. BA-1000	3 min

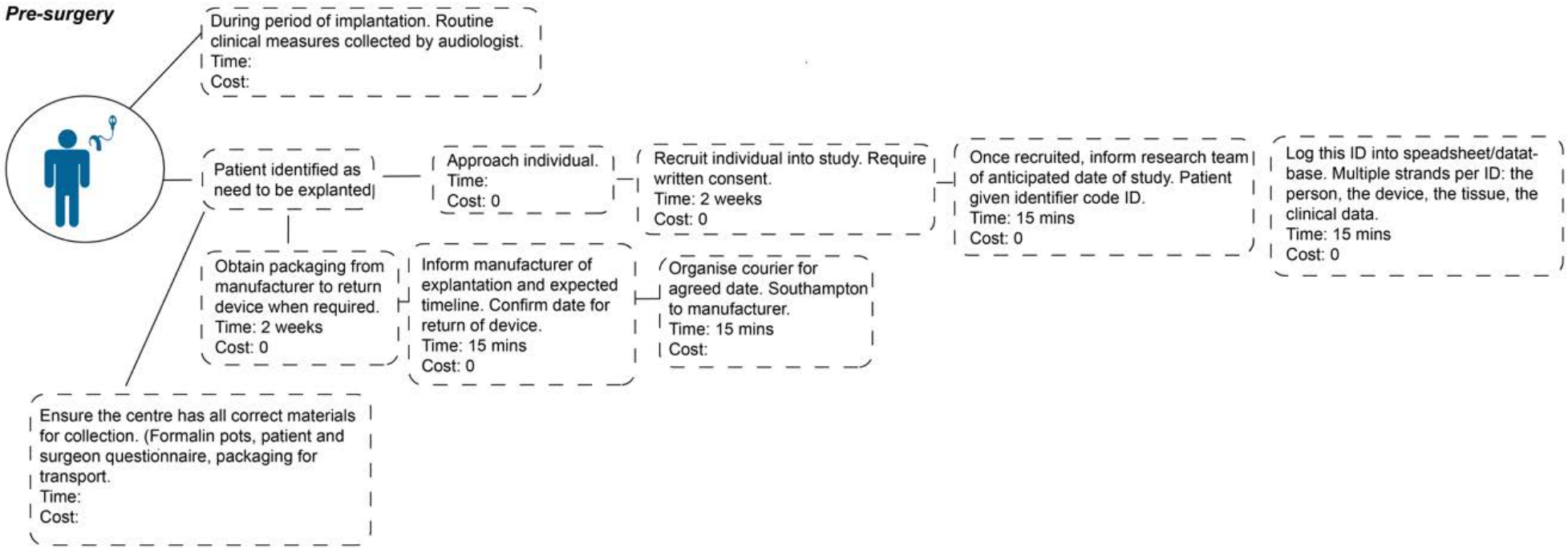
Target antigen and catalogue information	Primary Antibody	Type	Optimised working dilution	Host	Antigen retrieval	Blocking agent	Secondary antibody	DAB time
Myofibroblasts, blood vessels Sigma (A2547)	Alpha-smooth muscle actin clone 1A4	Monoclonal	1:50,000	Mouse	Hot citrate buffer 5 mins, cool 5 mins	Normal goat serum 1.5%	Goat anti- mouse 1:200 (in 0.01% TX100 0.25% BSA) Vector Laboratories. BA-9200	1min 40s
Proliferating blood vessels Cell signalling Technology (55B11)	VEGFR2	Monoclonal	1:600	Rabbit	EDTA with tween microwave until boiling then place slides in and leave 15 min	Normal goat serum 5%	Goat anti-rabbit 1:200 (in TBS Tween) Vector Laboratories. BA-1000	6 min 30s
T-Lymphocytes Dako (GA503)	CD3	Polyclonal	1:200	Rabbit	Hot citrate buffer 3 mins, cool 5 mins, heat 3 min, cool 5 min	Normal goat serum 1.5%	Goat anti-rabbit 1:200 Vector Laboratories. BA-1000	2 min

A.2 Segmentation workflow for Avizo Software

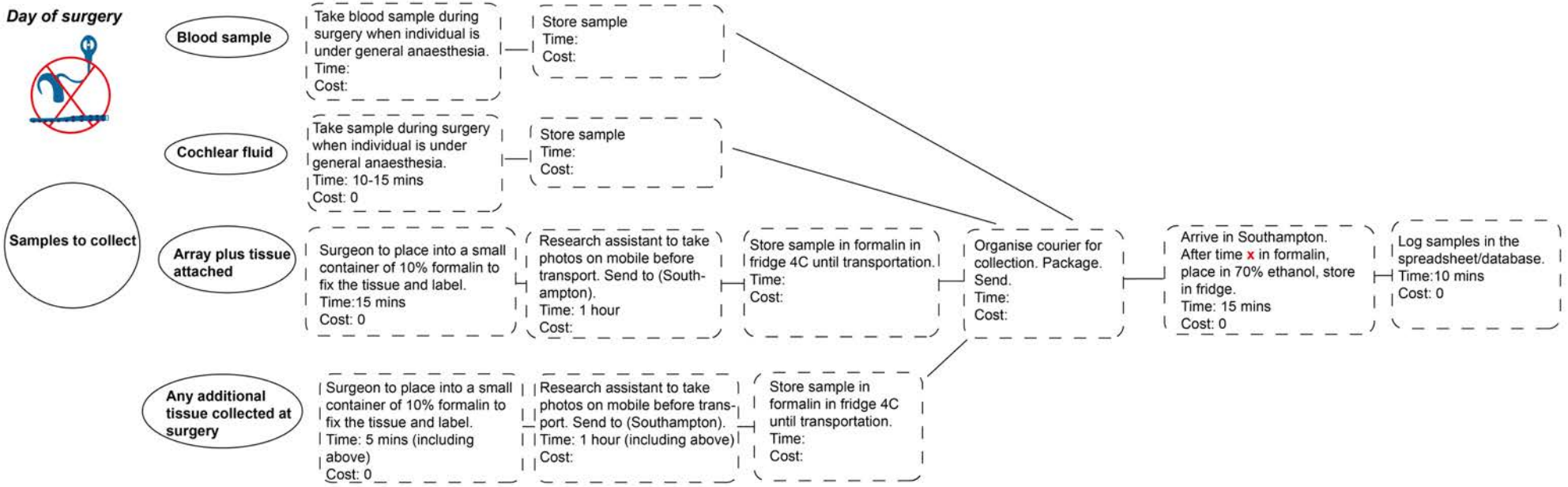
1. Import 16-bit edge and 8-bit raw files into FIJI
2. Select which slices to make substack with (same for each dataset). Image > Stacks > tools > Make substack . Type in which slices
3. Draw a box around just the region of interest then click on other dataset and click edit> selection > Restore selection (to make box on both).
4. Click crop on both then save 8-bit substack and 16-bit substack as tif files
5. Avizo – open both cropped files
6. Select 16-bit and click interactive thresholding module. Use 'preview slice' to move through the dataset. Adjust 'Intensity Range' to select the implant as accurately as possible. (background-white, implant - blue with black around outside. Click Apply to create a thresholded module. Implant = segmented
7. Inactivate interactive thresholding module by clicking yellow square.
8. Duplicate thresholded module and rename 'thresholded implant'
9. Click segmentation tab. In image – select 8-bit
10. Rename 'material' with implant and click lock to protect the implant material. Click 3d to show in 3D
11. Click add to add new material and rename bone.
12. Select the threshold tool in selection. Using the masking slider, select as much bone as possible (roughly 152 – 255)
13. Select masked voxels to see bone in 3D. Click + to add. Bone = segmented
14. Click back to project tab. Duplicate this module containing implant and bone and rename – thresholded implant and bone cleaned
15. Select this module then click segmentation.
16. Remove the bone material around the implant by drawing circled with pen and scrolling through stack and drawing a second circle then click ctrl I to interpolate. Check this is correct then click – to remove this.
17. Continue to remove all bone segmentation that looks wrong.
18. Click back to project view. View vol render.
19. Duplicate thresholded implant and bone cleaned then rename – thresholded implant and bone clean_implant removed
20. Select segmentation window then delete implant material.
21. In project window – now you have the modules containing: 8-bit grayscale dataset, thresholded implant, thresholded bone.
22. Now you can visualise them all separately or together. Change colours etc

A.3 Experimental design for larger scale human explant tissue analysis

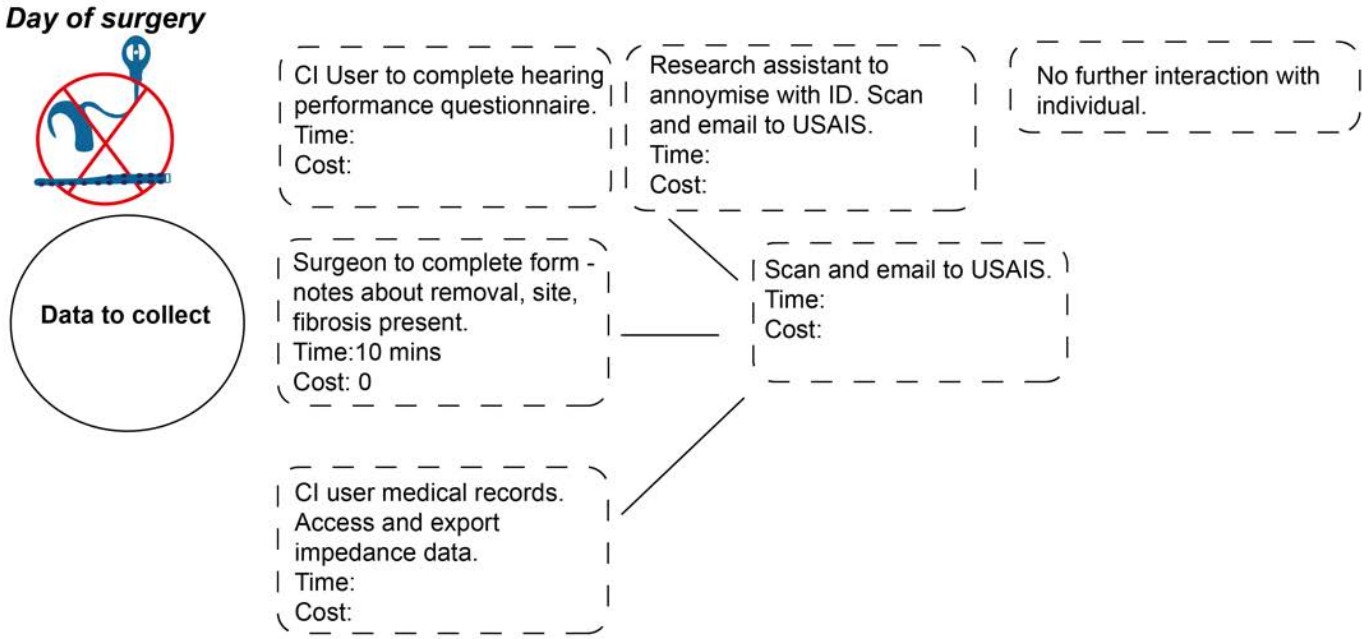
A.3.1 Protocol for the pre-surgery steps in the explant tissue analysis study



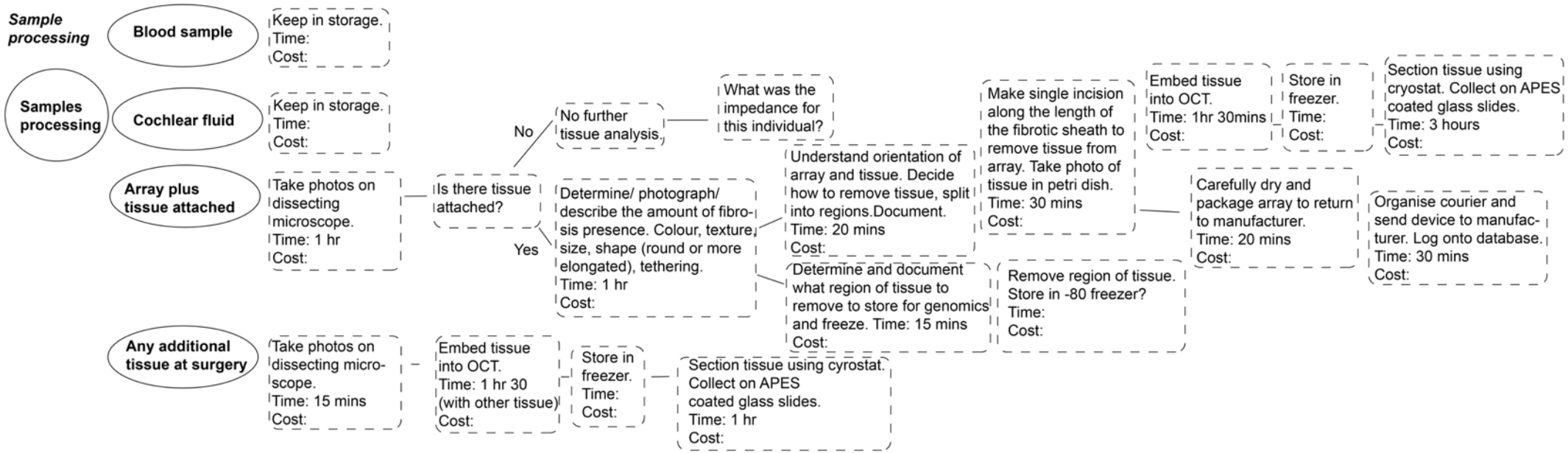
A.3.2 Protocol for the steps to be taken on the day of surgery in the explant tissue analysis study



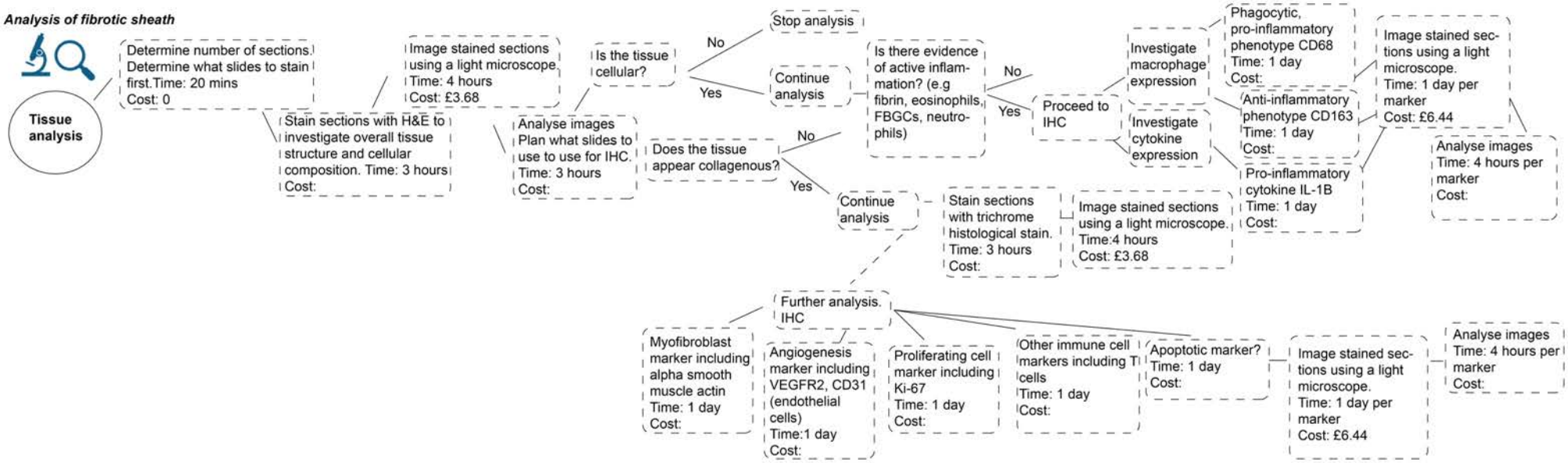
A.3.3 Protocol for the steps to be taken on the day of surgery in terms of data collection in the explant tissue analysis study



A.3.4 Protocol for the sample processing steps in the explant tissue analysis study



A.3.5 Protocol for the analysis of the tissue in the explant tissue analysis study



Appendix B Tables of secondary data extracted from the literature search

B.1 Expression signature markers used to identify macrophages of the auditory system and citations associated with each marker

Type of macrophage	Marker	Citation
Microglia		
	CX3CR1	1: (Li and Barres, 2018)
	CD11b	1: (Li and Barres, 2018)
	$CD45^{low}$	1: (Li and Barres, 2018)
	MHC Class II^{low}	1: (Li and Barres, 2018)
	P2RY12	1: (Li and Barres, 2018)
	SALL1	1: (Li and Barres, 2018)
	TMEM119	1: (Li and Barres, 2018)
Perivascular macrophages (PVMs)		(Li and Barres, 2018)
	CX3CR1	1: (Li and Barres, 2018)
	CD11b	1: (Li and Barres, 2018)
	$CD45^{hi}$	1: (Li and Barres, 2018)
	MERTK	1: (Li and Barres, 2018)
	CD206	1: (Li and Barres, 2018)
	CD163	1: (Li and Barres, 2018)

Type of macrophage	Marker	Citation
	MHC Class II^{hi}	1: (Li and Barres, 2018)
Infiltrating monocytes		
	CD45+Iba1+CX3CR1+	1: (Hirose *et al.*, 2005)
	Ly6C	3: (Frye, Zhang and Hu, 2018), 4: (Dong *et al.*, 2018) 34: (Zhang *et al.*, 2020)
	CCR2	5: (Hirose and Li, 2019), 34: (Zhang *et al.*, 2020)
Cochlear macrophages		
	Iba1	9: (Okano *et al.*, 2008), 10: (Wakabayashi *et al.*, 2010), 11: (Shi, 2010), 12: (Sun *et al.*, 2014), 13: (O'Malley, Nadol and Mckenna, 2016), 8: (Brown *et al.*, 2017), 3: (Frye, Zhang and Hu, 2018), 4: (Dong *et al.*, 2018), 14: (Fuentes-Santamaria *et al.*, 2017), 15: (Chen *et al.*, 2018) 16: (Liu *et al.*, 2018), 17: (Liu *et al.*, 2019), 18: (Noble *et al.*, 2019), 19: (Kishimoto *et al.*, 2019), 34: (Zhang *et al.*, 2020)
	CD45	2: (Hirose *et al.*, 2005), 6: (Tornabene *et al.*, 2006), 20: (Tan, Lee and Ruan, 2008), 11: (Shi, 2010), 10: (Wakabayashi *et al.*, 2010), 26: (Yang *et al.*, 2015), 27: (Vethanayagam *et al.*, 2016), 7: (Frye *et al.*, 2017), 28: (Zhang *et al.*, 2017), 3: (Frye, Zhang and Hu, 2018), 34: (Zhang *et al.*, 2020), 35: (Rai *et al.*, 2020)
	CX3CR1	22: (Sato *et al.*, 2008), 23: (Kaur, Zamani, *et al.*, 2015), 24: (Kaur, Ohlemiller and Warchol, 2018), 25: (Kaur *et al.*, 2019), 5: (Hirose and Li, 2019), 34: (Zhang *et al.*, 2020), 35: (Rai *et al.*, 2020)
	CD68	20: (Tan, Lee and Ruan, 2008), 21: (Nadol *et al.*, 2014), 13: (O'Malley, Nadol and Mckenna, 2016), 8: (Brown *et al.*, 2017), 17: (Liu *et al.*, 2019), 34: (Zhang *et al.*, 2020), 4: (Dong *et al.*, 2018)

Type of macrophage	Marker	Citation
	MHC Class II	26: (Yang *et al.*, 2015), 27: (Vethanayagam *et al.*, 2016)
	F4/80	4: (Dong *et al.*, 2018), 6: (Tornabene *et al.*, 2006), 7: (Frye *et al.*, 2017), 8: (Brown *et al.*, 2017), 34: (Zhang *et al.*, 2020)
	CD163	13: (O'Malley, Nadol and Mckenna, 2016)
	CD11b	8: (Brown *et al.*, 2017), 17: (Liu *et al.*, 2019), 19: (Kishimoto *et al.*, 2019), 35: (Rai *et al.*, 2020)
Perivascular macrophage-like melanocytes (PVM/Ms)		
	F4/80	29: (Zhang *et al.*, 2012), 30: (Zhang *et al.*, 2013), 31: (Neng *et al.*, 2015), 32: (Mizushima *et al.*, 2017), 33: (Jiang *et al.*, 2019)
	CD68	22: (Sato *et al.*, 2008)
	CD11b	22: (Sato *et al.*, 2008)
	CD163	22: (Sato *et al.*, 2008)

B.2 The different animal models of otitis media including the method to induce otitis media and details of the type of otitis media induced

A table detailing the different animal models of otitis media (OM) with the references for the studies included. Last literature search 09-07-21.

Method	Type	Type of OM	Duration of inflammation and or infection	Hearing loss	Example and gene locus	Background	Discovery mechanism	Additional information	Techniques	Reference
Inoculation with antigen										
Inoculation into right middle ear (ME) cavity by retroauricular approach	Treatment	Acute	7 days	SNHL	NA	Guinea pig		100 μL KLH inoculated into right middle ear cavity after opening bulla.	Histology, ABR, ME and perilymph sampling	(Gloddek, Lamm and Haslov, 1992)
Inoculation with bacteria										
Transtympanic injection of heat killed *Streptococcus pneumoniae* type 3	Treatment	Acute	Peak 3 days. Mostly resolved by 7 days.	Not measured	NA	Sprague-Dawley rats	NA	Dose: Unilateral injection approximately 0.05 ml.	IHC (IL-6), mRNA in situ hybridisation	(Forséni *et al.*, 2001)
Injection directly into the middle ear (transbullar) with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*	Treatment	Acute	Mostly resolved by 8 days Dependent on strain, bacteria and dose	Not measured	NA	BALB/C, C57BL/6, Swiss Webster mice	NA	The right bulla was exposed after blunt dissection. The middle ear was inoculated through the bony wall with approximately 0.02 mL of a bacterial suspension with a thin needle.	Clinical observations, otomicroscopy, bacterial samples from blood and middle ears	(Melhus and Ryan, 2003)

Method	Type	Type of OM	Duration of inflammation and or infection	Hearing loss	Example and gene locus	Background	Discovery mechanism	Additional information	Techniques	Reference
Transtympanic injection of heat killed *Streptococcus pneumoniae*	Treatment	Acute	Most elevated between day 3-5.	ABR thresholds elevated from baseline at days 3-5 after both bacterial and PBs inoculation.	NA	BALB/C	NA	Varying concentrations of S. pneumo were used (10^9,10^6,or10^4 bacteria per ml). Injection 5 μL.	Histology, ABR	(MacArthur, Hefeneider, Kempton, Parrish, *et al.*, 2006)
Transtympanic injection of heat killed *Haemophilus influenza* into right middle ear	Treatment	Acute	Maximal inflammation occurs at 3 days and largely recovered by day 7.	Not measured	NA	Balb/c mice 2-5 months of age	NA	Dose: Approximately 5 uL at concentration of 1.5 x 10^9 bacteria/mL (in sterile phosphate-buffered saline).	Cytokine gene expression using prefabricated cDNA arrays Quantitative RT-PCR and ELISA multiplex arrays	(Ghaheri *et al.*, 2007a; Trune *et al.*, 2015)
Injection directly into the middle ear (bulla) of NTHi strain-3655 (biotype II)	Treatment	Acute			NA	48 MyD88-/- mice and 48 WT control (C57BL/6)	NA	ME bulla was fenestrated using a 25-gauge needle. Dose: Concentration of x 10^5–1x 10^6 bacteria/mL. This strain was originally isolated in 1979 from the ME of a child with OM in St. Louis.	Histology, IHC, in vitro macrophage/NTHi interaction	(Hernandez *et al.*, 2008)
Intranasal inoculation and pressure cabin to translocate the inoculum to the middle ear cavity	Treatment	Acute		Not measured	NA	BALB/c mice 7-week old female	NA	Pneumococcal strains and mutants Non-invasive. Infection in both ears.	IL-1β and TNF-αELISA and histology	(Stol *et al.*, 2009)

Appendix B

Method	Type	Type of OM	Duration of inflammation and or infection	Hearing loss	Example and gene locus	Background	Discovery mechanism	Additional information	Techniques	Reference
Transtympanic injection of heat killed *Streptococcus pneumoniae*	Treatment	Acute	Onset of inflammation (24h)	Not measured	NA	Balb/c (10-12 weeks)	NA	Dose: Unilateral injection 5 μL of 10^9.	qRT-PCR, IHC qRT-PCR	(MacArthur *et al.*, 2011; Sautter *et al.*, 2011)
Injection directly into the middle ear (bulla) of NTHi strain-3655 (biotype II)	Treatment	Acute	Bacterial replication and persistence occurred.	Not measured	Na	The Asc-/- knockout mice on a C57BL/6 background	NA	This strain was originally isolated in 1979 from the ME of a child with OM in St. Louis. Mouse bilaterally exposed via a ventral approach and injected with ~5 ul saline containing ~10^4 CFUs of H. influenzae strain 3655 (non-typeable NTHi/biotype II), a clinical isolate	Histology, bacterial clearance, DNA microarrays, immunoblotting, macrophage phagocytosis and NTHI killing assay	(Kurabi *et al.*, 2015)
Injection directly into the middle ear (bulla) of NTHi strain-3655 (biotype II)	Treatment	Acute	Max mucosal thickness between day 2 -3	Not measured	NA	C57B1/6/WB F1 60-90d age were used. Sprague-Dawley rats used for tissue culture	NA	This strain was originally isolated in 1979 from the ME of a child with OM in St. Louis. NTHi injected through the tympanic bulla after a ventral surgical approach.	Histology, OM transcriptome, ME mucosa tissue culture,	(Cho *et al.*, 2016)
Intranasal inoculation of NTHi	Treatment and transgenic	Acute and chronic	Conductive hearing loss		Junbo *Jbo/+* (het) Evi1 (mecom)	CH3/HeN	ENU mutagenesis	Dose: Intranasal inoculation of 5ul per nares of NTHi 162 sr cell suspension concentration 10^8CFU/ml in PBS 1% gelatin.	ME infection rates and titres, histology, IHC, in situ hybridisation, terminal sampling of blood, bulla fluids and nasopharynx, bioluminescent imaging, RT-qPCR of bulla fluids Flow cytometry of bulla fluid, histology and IHC,	(Hood *et al.*, 2016; Vikhe *et al.*, 2018, 2019)

Method	Type	Type of OM	Duration of inflammation and or infection	Hearing loss	Example and gene locus	Background	Discovery mechanism	Additional information	Techniques	Reference
									cytokine and chemokine responses Flow cytometry of bulla fluid, cytokine and chemokine responses	
Injection directly into the middle ear (bulla) of NTHi strain-3655 (biotype II)	Treatment	Acute	Max mucosal thickness between day 2 -3	Not measured	NA	60-90 d old NOD1-/-, NOD2-/- mice on C57BL/6 background and CD57BL/6 WT	NA	A hole was carefully drilled with a 25-gauge needle and 5 ml injected into the ME cavity. This strain was originally isolated in 1979 from the ME of a child with OM in St. Louis.	Histology, bacterial clearance, macrophage phagocytosis and NTHi killing assay, DNA microarrays	(Lee *et al.*, 2019)
Intranasal inoculation of mice with *Bordetella pseudohinzii* or natural transmission from cage mates	Treatment and natural transmission	Acute then chronic		Progressive		C57BL/6J 4-6 wks female	Noticed variation in ABR thresholds in untreated mice. Found middle ear colonisation of natural mouse pathogen B pseudohinzii.	B. pseudohinzii naturally transmits among mice and persists in the middle ear chronically.	Histology, bacterial loads in ME bullae and respiratory tracts ABR, DPOAE	(Dewan *et al.*, 2019)
Injection directly into the middle ear (bulla) of NTHi strain-3655 (biotype II)	Treatment	Acute	Max between day 2-3 and resolved by day 7 in no treatment group		NA	CCR2−/− mice (B6.129S4-Ccr2tm1Ifc/J), age-matched WT mice (C57BL/6J), and C57/WB-F1 hybrid mice	NA	This strain was originally isolated in 1979 from the ME of a child with OM in St. Louis. Approximately 5 µL of an inoculum containing 500–5000 colony-forming units (CFUs) of NTHi were	Gene arrays on mucosal tissue and effusions, histology, IHC ME bacterial culture	(Hur *et al.*, 2021)

Method	Type	Type of OM	Duration of inflammation and or infection	Hearing loss	Example and gene locus	Background	Discovery mechanism	Additional information	Techniques	Reference
								bilaterally injected into the MEs through a puncture in bulla.		
Surgical manipulation										
Severing the soft palate in a rat	Surgical	Acute	Day 5 – many immunocompetent cells in mucosa	Not measured	NA	Female Lewis rats 5 weeks	NA		IHC Phenotype characterisation (APAAP), detection of the incorporated BrdU	(Jecker, Pabst and Westermann, 1996, 2001)
Myringotomy: surgically induced tympanic membrane perforation in chronic OM mouse model - the Junbo mouse	Surgical	Chronic	5 days after myringotomy - investigated	Not measured	NA	WT +/+ and Jbo/+ on a congenic C3H/HeH 6-9 weeks	NA	Is it a good model because in humans, the Eustachian tube is usually normal or only partially obstructed?	PIMO labelling (tissue hypoxia), histology, IHC	(Bhutta, Cheeseman and Brown, 2014)
Eustachian tube ligation: surgical ligation of Eustachian tube	Surgical	Chronic	After 1 month, thickened tympanic membrane and ME effusions.	Not measured	NA	CBA/CaJ 2 months old	NA	ET obstruction was surgically created on left side by placing a small piece of dental material in the tympanic orifice of the ET via a transbullar approach.	Otomicroscopy, histology	(Varsak and Santa Maria, 2016)
Eustachian tube occlusion (transtympanic or transcervical) and inoculation of P. aeruginosa (human pathogen)	Surgical plus treatment	Chronic	Chronic suppurative otitis media	Not measured	NA	CBA/CaJ C57Bl/6J 6-8 weeks	NA	10μl of P. aeruginosa were inoculated into ME bulla through open tympanic membrane wounds. Ofloxacin was unsuccessful in	Real-time infection tracking, fluoroquinolone treatment, in vivo grading of infection, bacterial burden assessment, microscopy, SEM	(Khomtchouk *et al.*, 2020)

Method	Type	Type of OM	Duration of inflammation and or infection	Hearing loss	Example and gene locus	Background	Discovery mechanism	Additional information	Techniques	Reference
								eradicating pathogen from the mice.		
Genetic mutation										
Chemical mutagenesis: typically with injection of the chemical mutagen N-ethyl-N-nitrosourea (ENU)	Transgenic	Chronic	Chronic proliferative OM with effusion.	Conductive hearing loss	Jeff (het) *Jf/+* Fbxo11	C3H/HeN	ENU mutagenesis	The mutation is fully penetrant. Conductive deafness and is associated with raised threshold for a cochlear nerve response.	Hematology and bulla fluid analysis, PIMO labelling, RT-qPCR, Vegfa, Il-1B, Tnf-α protein assays, ABR, histology Western blotting, histology, IHC Histology, 3D reconstruction, physiology and ultrastructure, IHC	(Hardisty *et al.*, 2003; Hardisty-Hughes *et al.*, 2006; Cheeseman *et al.*, 2011)
Chemical mutagenesis: typically with injection of the chemical mutagen N-ethyl-N-nitrosourea (ENU)	Transgenic	Chronic	Spontaneously develop, chronic OM with otorrhea.	Conductive hearing loss	Junbo *Jbo/+* (het) Evi1 (mecom)	CH3/HeN	ENU mutagenesis	(Acute OM following birth that develops into suppurative form of OM)	(Parkinson et al., 2006) - Histology, x-ray analysis, IHC, skeletal preparations, immunology and FACS analysis	(Parkinson *et al.*, 2006; Cheeseman *et al.*, 2011; Hood *et al.*, 2016)
Genetic mutation	Transgenic	Chronic	Spontaneous COM	SNHL and conductive	TLR4	C3H/HeJ 8 – 20 weeks of age		Single amino acid substitution in its toll-like receptor 4 (TLR4) making it insensitive to endotoxin. The disease is not fully penetrant (40 – 50%)	Cytokine expression in gene arrays, IHC ABR, histology (MacArthur et al, 2008) qRT-PCR, IHC (MacArthur et al, 2011) gene chip, whole genome study, ion homeostasis gene evaluation, qRT-PCR (MacArthur et al, 2013)	(MacArthur, Hefeneider, Kempton and Trune, 2006; Ghaheri *et al.*, 2007b, 2007a; MacArthur *et al.*, 2008, 2011; MacArthur, Hausman, Kempton, Sautter, *et al.*, 2013)

Appendix B

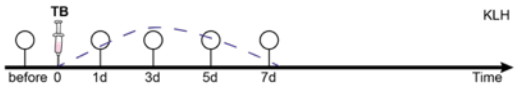
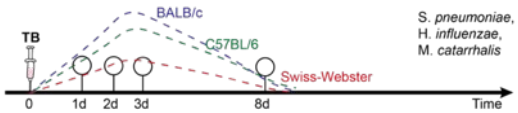
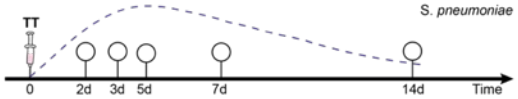
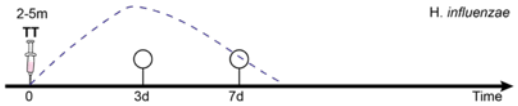
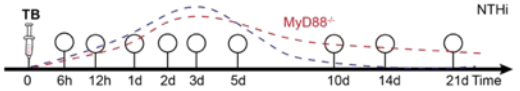
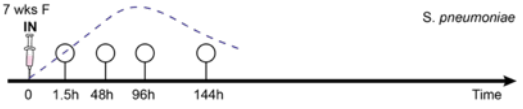
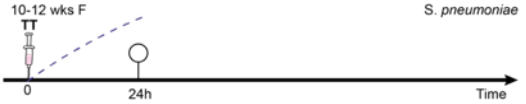
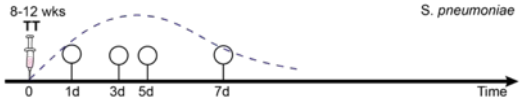
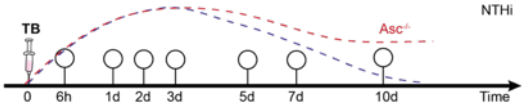
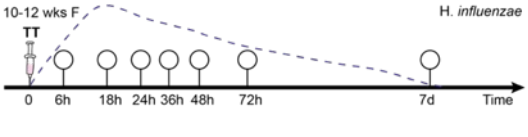
Method	Type	Type of OM	Duration of inflammation and or infection	Hearing loss	Example and gene locus	Background	Discovery mechanism	Additional information	Techniques	Reference
Targeted genetic mutation	Transgenic	Chronic	Chronic effusion starting at around 3 weeks causing conductive hearing loss.	Conductive hearing loss	Tgif1 -/- knockout Tgif1 homozygous	C57BL/6J	Knockout	Fluid in ME cavity and thickened epithelial lining. No cochlea damage. Craniofacial defect and reduced hearing by weaning age. Tgif is a semi-dominant mutation of OM. OM progression from 21 days – 1m, 2m, 4m.	Histology, clickbox, ABR, X-ray analysis, SEM, western blot, zymography, blood and ear effusion collection for Vegf, TNF-α, IL-1β protein assays, ear effusion collection for FACS analysis, IHC	(Tateossian *et al.*, 2013)
Chemical mutagenesis: typically, with injection of the chemical mutagen N-ethyl-N-nitrosourea (ENU)	Transgenic	Chronic	Spontaneously develops conductive hearing loss due to chronic OM.	Conductive hearing loss	Edison (hom) Nisch	C3H/HeH	ENU mutagenesis	Develop a serous or granulocytic effusion, macrophage and neutrophil rich, thickened and inflamed mucoperiosteum. 56% mice display bilateral OM by 20 weeks.	Skull morphology and radiography, ABR, histology, SEM, blood and bulla fluid collection, RT-qPCR, IHC, western blot	(Crompton *et al.*, 2017)

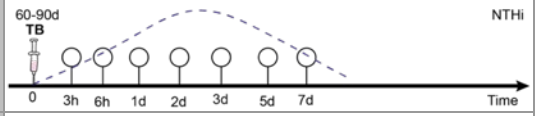
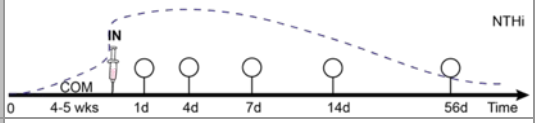
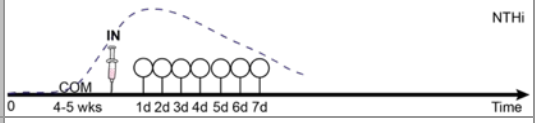
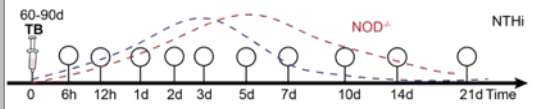
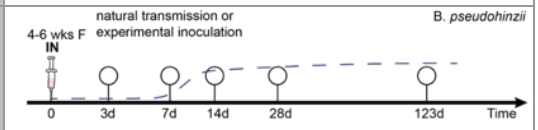
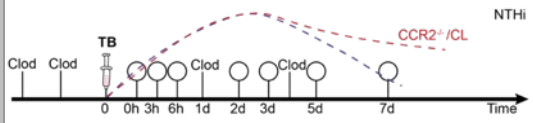
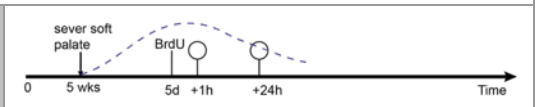
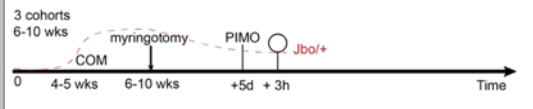
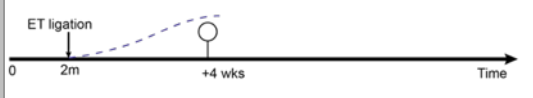
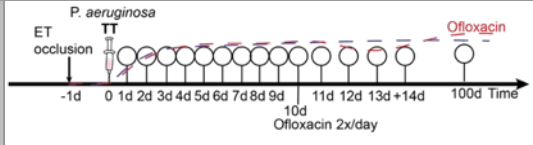
B.3 The experimental design of studies using various mouse models of otitis media

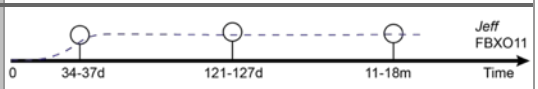
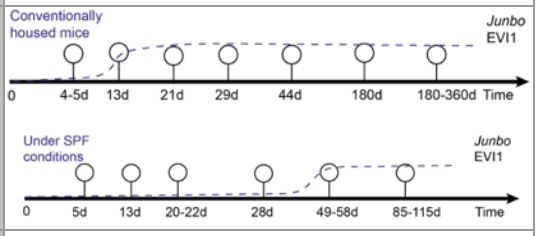
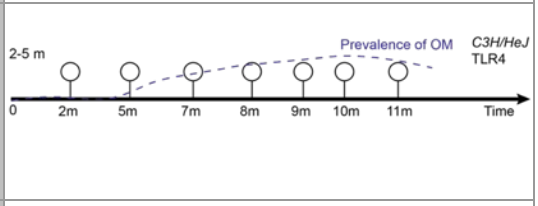
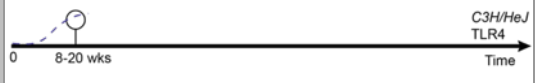
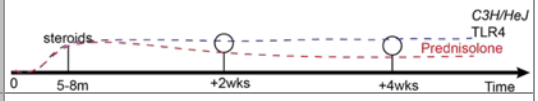
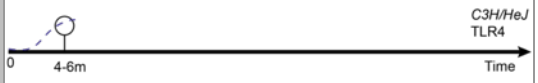
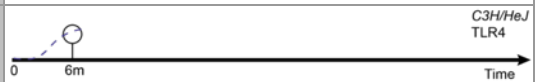
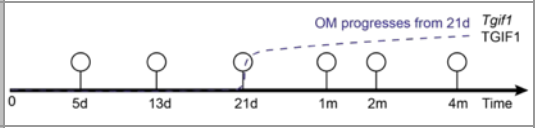
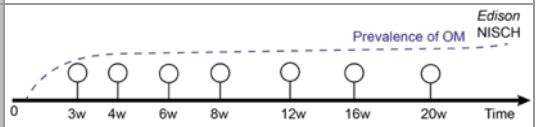
A table describing different models of otitis media (OM) with the experimental design included. The studies have been separated into three groups depending on the way in which the OM was induced; either by treatment, surgical manipulation or genetic manipulation.

Details for the experimental design: **Treatment** – timeline of experimental design showing the age/time at which the animals were exposed to the treatment and the number of measurements taken across the timeline of the experiment. The dashed line (blue) indicates the time course of the middle ear inflammation in the acute model for OM. A red dashed line indicates the change in the time course of the middle ear inflammation in that model of AOM in a mouse with a specific gene knocked-out. **Surgical manipulation** - timeline of experimental design showing the age/time at which the animals were exposed to the surgical manipulation and the number of measurements taken across the timeline of the experiment. The dashed line (blue) indicates the time course of the middle ear inflammation in the model for OM involving surgical manipulation. A red dashed line indicates the change in the time course of the middle ear inflammation in that model of OM in a mouse with a specific gene mutated (Jbo/+). **Genetic mutation** - timeline of experimental design showing the number of measurements taken across the timeline of the experiment in models of chronic otitis media. The dashed line (blue) indicates the time course of the middle ear inflammation in the model for chronic OM induced buy a genetic mutation. Where indicated, the blue dashed line indicates the change in prevalence of the middle ear inflammation across time.

Key: Transtympanic = TT, Transbullar = TB, Intranasal = IN. Top right = name of bacteria, name of mouse mutant, mutated gene . Last literature search 09-07-21.

Number	Citation	Animal and strain	Type of OM	Type	Experimental design
Treatment					
1	(Glodde k, Lamm and Haslov, 1992)	Guinea pig	Acute	Keyhole limpet hemocyanin (KLH) inoculated into middle ear after opening bulla	
2	(Forséni *et al.*, 2001)	Rat Sprague-Dawley	Acute	Transtympanic injection of heat killed *Streptococcus pneumoniae* type 3	
3	(Melhu s and Ryan, 2003)	Mouse BALB/C, C57BL/6, Swiss Webster	Acute	Transbullar injection of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*	
4	(MacAr thur, Hefenei der, Kempto n, Parrish, *et al.*, 2006)	Mouse BALB/C	Acute	Transtympanic injection of heat killed *Streptococcus pneumoniae*	
5	(Ghaher i *et al.*, 2007a)	Mouse BALB/C	Acute	Transtympanic injection of heat killed *Haemophilus influenzae*	
6	(Hernan dez *et al.*, 2008)	Mouse MyD88-/- mice and WT control (C57BL/6)	Acute	Injection of NTHi strain-3655 (biotype II) into middle ear	
7	(Stol *et al.*, 2009)	Mouse BALB/c	Acute	Intranasal inoculation of Pneumococcal strains and mutants and pressure cabin	
8	(MacAr thur *et al.*, 2011)	Mouse BALB/C	Acute	Transtympanic injection of heat killed *Streptococcus pneumoniae*	
9	(Sautter *et al.*, 2011)	Mouse BALB/C	Acute	Transtympanic injection of heat killed *Streptococcus pneumoniae*	
10	(Kurabi *et al.*, 2015)	Mouse Asc-/- knockout mice on a C57BL/6 backgrou nd	Acute	Injection of NTHi strain-3655 (biotype II) into middle ear	
11	(Trune *et al.*, 2015)	Mouse BALB/C	Acute	Transtympanic injection of heat killed *Haemophilus influenzae*	

Number	Citation	Animal and strain	Type of OM	Type	Experimental design
12	(Cho *et al.*, 2016)	Mouse C57B1/6/ WB F1	Acute	Injection of NTHi strain-3655 (biotype II) into middle ear	
13	(Hood *et al.*, 2016)	Mouse Junbo *Jbo/+* (het) CH3/HeN	Acute and chronic	Evi1 mutation and intranasal inoculation of NTHi	
14	(Vikhe *et al.*, 2018)	Mouse Junbo *Jbo/+* (het) CH3/HeN	Acute and chronic	Evi1 mutation and intranasal inoculation of NTHi	
15	(Lee *et al.*, 2019)	Mouse NOD1-/-, NOD2-/- mice on C57BL/6 background and CD57BL/6 WT	Acute	Injection of NTHi strain-3655 (biotype II) into middle ear	
16	(Dewan *et al.*, 2019)	Mouse C57BL/6J	Acute and then chronic	Intranasal inoculation of mice with B. pseudohinzii or natural transmission from cage mates	
17	(Hur *et al.*, 2021)	Mouse CCR2−/− mice (B6.129S4-Ccr2tm1Ifc/J), age-matched WT mice (C57BL/6J), and C57/WB-F1 hybrid mice	Acute	Injection of NTHi strain-3655 (biotype II) into middle ear	
Surgical manipulation					
18	(Jecker, Pabst and Westermann, 2001)	Rat Lewis	Acute	Sever soft palate	
19	(Bhutta, Cheeseman and Brown, 2014)	Mouse WT +/+ and Jbo/+ on a congenic C3H/HeH	Acute	Evi1 mutation and myringotomy	
20	(Varsak and Santa Maria, 2016)	Mouse CBA/CaJ	Acute	Eustachian tube ligation	
21	(Khomtchouk *et al.*, 2020)	Mouse CBA/CaJ and C57Bl/6J	Chronic	Eustachian tube occlusion plus inoculation of P. aeruginosa	
Genetic mutation					

Number	Citation	Animal and strain	Type of OM	Type	Experimental design
22	(Hardisty *et al.*, 2003)	Mouse Jeff (het) *Jf/+* on a congenic C3H/HeH	Chronic	Mutation in Fbxo11	
23	(Parkinson *et al.*, 2006)	Mouse Junbo *Jbo/+* (het) CH3/HeN	Chronic	Mutation in Evi1	
24	(MacArthur, Hefeneider, Kempton and Trune, 2006)	Mouse C3H/HeJ	Chronic	Mutation in TLR4	
25	(Ghaheri *et al.*, 2007b)	Mouse C3H/HeJ	Chronic	Mutation in TLR4	
26	(MacArthur *et al.*, 2008)	Mouse C3H/HeJ	Chronic	Mutation in TLR4	
27	(MacArthur *et al.*, 2011)	Mouse C3H/HeJ	Chronic	Mutation in TLR4	
28	(MacArthur, Hausman, Kempton, Sautter, *et al.*, 2013)	Mouse C3H/HeJ	Chronic	Mutation in TLR4	
29	(Tateosian *et al.*, 2013)	Mouse Tgif1-/- on a congenic C57BL/6J	Chronic	Mutation in Tgif1 gene	
30	(Crompton *et al.*, 2017)	Mouse Edison (hom) on a congenic C3H/HeH	Chronic	Mutation in Nischarin gene	

B.4 Data richness for each human study investigating the tissue response to cochlear implantation

The level of data richness for each of the studies was calculated, based on the different techniques used and the type of data collected in each study. The method used to assign a data richness score is as follows - each method was scored e.g., histology and IHC were given a score of 3, clinical, CT imaging and 3D reconstruction were given a score of 2 etc. and for each study, the data richness score was calculated. The data richness score was used to produce Figure 4.5. Last literature search 30-04-21.

Letter	Reference	Number of TB Years of implantation (average)	Actual years of implantation	Measures collected	Histology 3	IHC 3	IF 3	Clinical 2	CT imaging 2	3D Construction 2	Bacterial assay 1	SEM 1	Fluor microscopy 1	Data richness score	Symbol on figure
a	(Clark *et al.*, 1988)	N = 1 2y	2y	H, C, CT	•			•	•					7	
b	(Nadol, Ketten and Burgess, 1994)	N = 1 10wks	10wks (0.18y)	H	•									3	
c	(Kawano *et al.*, 1998)	N = 5 3.8 y	3 y 2y 8 y 1 y 5 y	H, CT, C, 3D	•			•	•					9	
d	(Nadol *et al.*, 2001)	N = 8 3.5 y	2, 2, 6, 1, 7, 3, 2, 5	H, C	•			•						5	
e	(Nadol and Eddington, 2004)	N = 21 6.3y	2, 2, 1, 5, 3, 2, 7, 5, 9, 3, 6, 12, 12, 12, 12, 4, 4, 8, 7, 7, 10	H	•									3	
f	(Gassner, Shallop and	N = 1 7y	7y	C, H	•			•						5	

Appendix B

Letter	Reference	Number of TB Years of implantation (average)	Actual years of implantation	Measures collected	Histology 3	IHC 3	IF 3	Clinical 2	CT imaging 2	3D Construction 2	Bacterial assay 1	SEM 1	Fluor microscopy 1	Data richness score	Symbol on figure
	Driscoll, 2005)														
g	(Fayad and Linthicum, 2006)	N = 14 12y	0.9, 0.7, 2.7, 3.8, 0.6, 3.3, 12.9, -, 3.3, 9.5, 6.4, 4.4, 9.9, 2.5	C, H	•			•						5	
h	(Li *et al.*, 2007)	N = 12 9y	1, 2.9, 2.6, 5.8, 9.4, 10.4, 17, 6.75, 11.5, 12.2, 11, 15.2	C, H, 3D	•			•		•				7	
i	(Somdas *et al.*, 2007)	N = 7 7.3y	1, 3, 3, 6, 10, 11, 17	H, 3D	•					•				5	
j	(Nadol, Eddington and Burgess, 2008) Soft failure case	N = 9 10.5y (1 failure 13y, 8 control 8y)	13, 8, 7, 3, 12, 12, 10, 2, 2	H, B, C, CT	•			•	•		•			8	
k	(Fayad, Makarem and Linthicum, 2009)	N = 10 6y (range 0.9 - 12.9)	0.9, 2.7,12.9, -, 3.3, 9.5, -, 6.4, 9.9, 2.4	H, 3D	•					•				5	
l	(Lee, Eddington and Nadol, 2011) Revision surgery	N = 4 7.2	5.8, 12.8, 2.6, 9.8	H, C, B	•			•			•			6	

Letter	Reference	Number of TB Years of implantation (average)	Actual years of implantation	Measures collected	Histology 3	IHC 3	IF 3	Clinical 2	CT imaging 2	3D Construction 2	Bacterial assay 1	SEM 1	Fluor microscopy 1	Data richness score	Symbol on figure
m	(Richard *et al.*, 2012)	N = 12 9y (1-25)	0.9, 5.9, 4.5, 7.9, 4.2, 3.4, 1.4, 19.5, 9.7, 6.5, 17.4, 24.5	H, 3D	•					•				5	
n	(Neilan *et al.*, 2012) Soft failure	N =1 3y	3y	H, CT	•				•		•		•	7	
o	(Clark *et al.*, 2014)	N =1 30y	30y	H, CT, 3D, S	•				•	•		•		8	
p	(Nadol *et al.*, 2014)	N = 7 12.4 y (4 – 21)	13, 11, 11, 17, 4, 21, 10	H, **IHC,** C, S	•	•		•				•		9	
q	(Seyyedi and Nadol, 2014)	N = 28 11y (1-23)	11, 12, 1, 12, 12, 9, 6, 6, 17, 7, 7, 12, 12, 11, 12, 8, 23, 8, 18, 8, 8, 7, 13, 12, 20, 3, 13, 11	H	•									3	
r	(Kamakura and Nadol, 2016)	N = 17 11y	11.9, 8.1, 8.75, 5.1, 18.6, 6.7, 12.6, 23, 10.6, 8.5, 29.5, 5.7, 11.6, 12.3, 6.2, 7.9, 2.8	C, H, 3D	•			•		•				7	
s	(Spiers *et al.*, 2016)	N=1 30y	30	H, F	•								•	4	
t	(Ishai *et al.*, 2017)	N = 12 10.2y (2 -24)	8, 8, 9, 12, 3, 2, 2, 24, 13, 12, 18, 11	H, C	•			•						5	

Appendix B

Letter	Reference	Number of TB Years of implantation (average)	Actual years of implantation	Measures collected	Histology 3	IHC 3	IF 3	Clinical 2	CT imaging 2	3D Construction 2	Bacterial assay 1	SEM 1	Fluor microscopy 1	Data richness score	Symbol on figure
u	(Ishiyama *et al.*, 2019)	N = 13 Average 5.5y (grp1 4.4, grp2 6.67)	G1 – N.A, 1, 3, 10, 6, 2 G2 – 8, 8, 1, N/A, 4, 3, 16	H, C, 3D	•			•		•				7	
v	(Noonan *et al.*, 2020)	N = 19 Average 10y 9TB CI – average 10y	16, 18, N/A, 1, NA, 19, NA, 2, 11, NA, 10, NA, 3, NA, 10, NA, NA, NA, NA	**IHC,** IF		•		•						6	
w	(Okayasu *et al.*, 2020)	N =20 From 10 patients unilateral CI 8.4y	12.6, 17.5, 2.8, 6.9, 10.6, 5.1, 9.2, 1, 6.3, 11.9	H, **IHC**	•	•								6	
x	(Hough, Sanderson, *et al.*, 2021) (Explant)	N = 1 10 months		H, **IHC,** C, CT	•	•		•	•					10	

Key for data richness score:

Appendix C Published work

Research paper

Published in Otology and Neurotology

'**Inflammation at the Tissue-Electrode Interface in a Case of Rapid Deterioration in Hearing Performance Leading to Explant After Cochlear Implantation.**'

Hough, K., Sanderson, A., Grasmeder, M., Mitchell, T., Verschuur, C. A., & Newman, T. A. (2021). Inflammation at the Tissue-Electrode Interface in a Case of Rapid Deterioration in Hearing Performance Leading to Explant After Cochlear Implantation. *Otology & Neurotology*, *42*(4), e445–e450. https://doi.org/10.1097/MAO.0000000000003014

Inflammation at the Tissue-Electrode Interface in a Case of Rapid Deterioration in Hearing Performance Leading to Explant After Cochlear Implantation

*Kate Hough, *Alan Sanderson, †Mary Grasmeder, †Tim Mitchell, †Carl A. Verschuur, and ‡Tracey A. Newman

**Faculty of Engineering and Physical Sciences; †Faculty of Engineering and Physical Sciences, Auditory Implant Centre; and ‡Clinical and Experimental Sciences, Faculty of Medicine, Institute for Life Sciences, University of Southampton, Southampton, UK*

Objective: The reasons for soft failure after cochlear implantation require investigation. This study proposes a method to study and characterize the tissue response to the array in a case of soft failure in a person undergoing reimplantation.

Case: The woman in her 50s, with an underlying autoimmune condition, received a cochlear implant using hearing preservation technique after developing profound hearing loss more than 2 kHz with a moderate loss of less than 500 Hz over a 10-year period. The case was identified as a soft failure due to deteriorating performance, discomfort, and migration over the 10 months after implantation. Impedance telemetry, speech perception measures, and audiometric thresholds are described. At explantation there was evidence of fibrosis.

Intervention(s): To use histology and immunohistochemistry to determine the cellular response of the tissue associated with the electrode array at time of explantation.

Main Outcome Measure(s): Identification of the cell types, regional variations, and inflammatory marker expression in the fibrotic tissue associated with the array.

Results: Neutrophils and eosinophils were identified, along with a variable pattern of collagen deposition. CD68 and CD163-positive macrophages and T cells were variably distributed through the tissue and interleukin-1 beta and vascular endothelial growth factor receptor-2 expression was identified.

Conclusions: The expression profile is evidence of active inflammation in the tissue despite the time since implantation. This study is the first to characterize the tissue response to the array in a person undergoing reimplantation, and who can be followed to determine the individual response to arrays. It establishes that the investigation of explanted devices after soft-failure is feasible. **Key Words:** Cochlear implants—Electrode—Explant—Fibrosis—Hearing outcomes—Inflammation—Migration.

Otol Neurotol **42:**e445–e450, 2021.

A small but persistent percentage of cases fail to achieve or maintain the desired hearing outcomes after cochlear implantation (1,2). Soft failure is indicated by a broad assessment battery that allows hardware and medical failures to be ruled out (3). Some soft failures are due to tissue responses to the CI that develop over time (4–6). Surgical trauma, electrode style (7), electrode materials, and anti-inflammatory steroid treatment (8,9) all influence the tissue response. However, the mechanisms of this immune-mediated homeostatic response to implantation are inherently difficult to study in people. This case report describes the performance measures across the period of implantation, and histology of the tissue response at the electrode array, in a case where rapid electrode migration necessitated explantation and reimplantation. This work gives unique insight into the developmental time course of tissue associated with the array during performance changes. Findings add to the existing knowledge gained through postmortem analyses (4,5,10,11) and provide the basis for a feasible protocol for a larger, systematic analysis of soft failures with the aim of reducing the need for explantation.

The individual who has a history of autoimmune disease developed a progressive sensorineural hearing loss over a decade. She was implanted in her early 50s by which time her hearing loss was profound for frequencies of 2 kHz and above and moderate for low frequencies (250 and 500 Hz). Audiometry and speech perception scores met the guidelines for funding for cochlear

Address correspondence and reprint requests to Tracey A. Newman, Ph.D., B85, Life Sciences, Faculty of Medicine, University Road, Southampton, Hants SO17 1BJ, UK; E-mail: t.a.newman@soton.ac.uk

K.H. and A.S. authors have contributed equally to this work.

Sources of funding: EPSRC for PhD studentship funding to K.H. and A.S.

Authorship: All authors listed meet the criteria for having contributed to the production of this manuscript.

The authors disclose no conflicts of interest.

Supplemental digital content is available in the text.

DOI: 10.1097/MAO.0000000000003014

implantation in the UK and a Nucleus CI522 device (Cochlear Europe Ltd, Surrey, UK) was fully inserted through the round window, confirmed by postoperative x-ray, using a hearing preservation technique, including application of steroids. Vicryl ties were used to anchor the electrode array and receiver stimulator, muscle was used to seal the array in the round window. The device was activated 6 weeks later using the ACE processing strategy with a stimulation rate of 900 Hz, pulse width of 37 μs, 10 maxima, and stimulation mode MP1+2. At activation there were only limited changes in audiometric thresholds compared with preoperative levels: the audiometric thresholds at 250 and 500 Hz were 65 and 80 dBHL, compared with 60 dBHL for these frequencies preoperatively. However, the two most basal electrodes (E1 and E2) gave open circuits on impedance telemetry

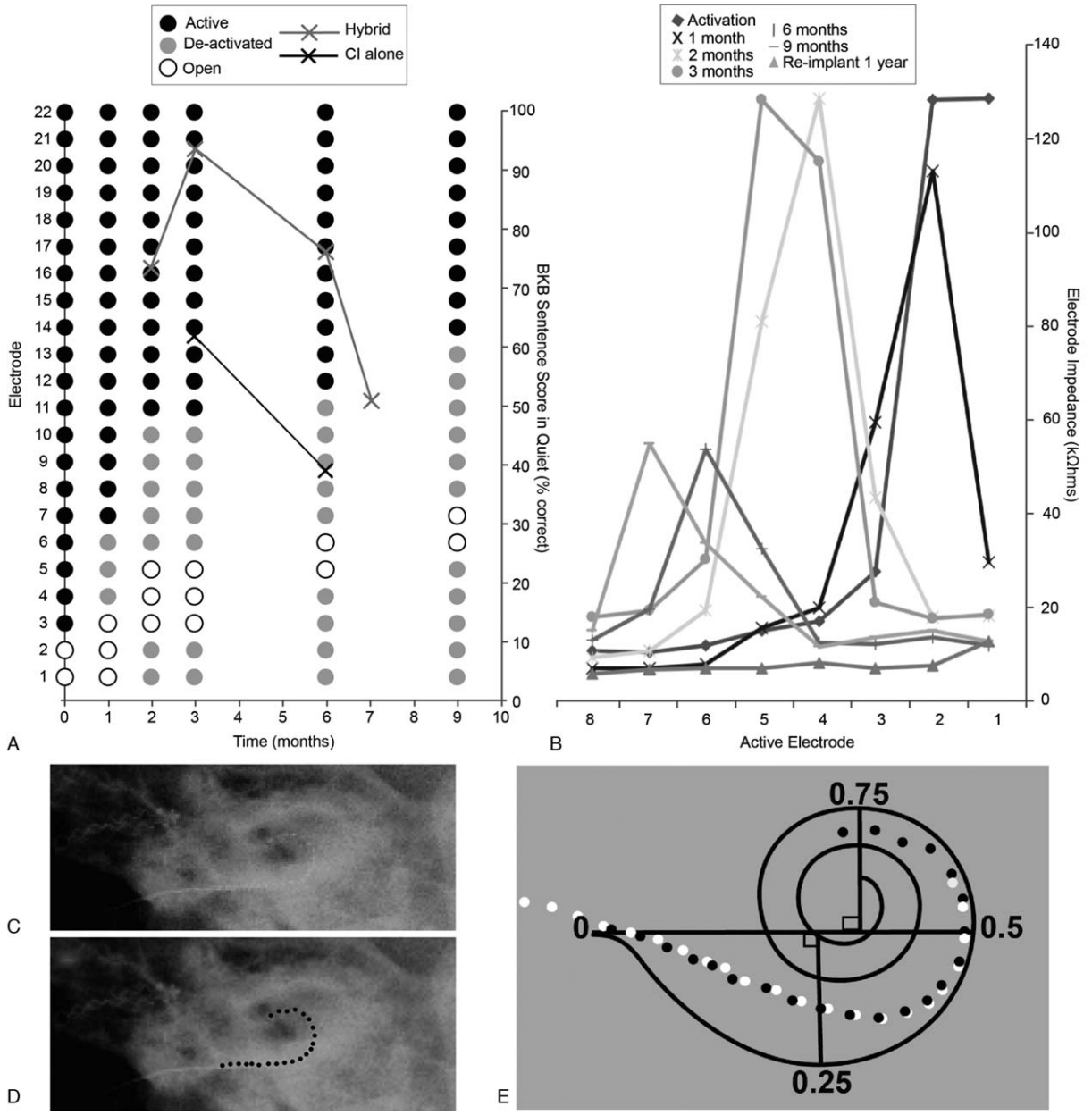


FIG. 1. Clinical indicators of electrode migration concomitant with a deterioration in hearing. *A*, Electrode status and speech recognition scores over time. Scores for the BKB sentence test (in quiet) are shown for the cochlear implant alone (*black line*) and the hybrid (*grey line*) device (combined acoustic and electrical stimulation) post-implant. *B*, Impedance telemetry in common ground mode from activation until shortly before re-implantation. *C*, X-ray micrograph obtained 1-day post-implantation showing the intra-cochlear position of the electrode array. *D*, Electrode contact positions superimposed (black). *E*, Cochlear template adapted from Kawano et al. (1996), defining the position of every quarter turn of the Organ of Corti, showing the points from the basal turn (28). The position of the electrode array post-implantation superimposed (black) based on the postoperative x-ray and the expected position of the migrated array before explantation superimposed (white)—showing four extra-cochlear electrodes.

and so were deactivated. The remaining electrodes had impedances within the normal range. Stimulation threshold measurements were higher for electrodes 3 to 5 (>120 Nucleus current units) than for more apical electrodes (70–80 Nucleus current units). Over 10 months, successive basal electrodes within the cochlea were deactivated (Fig. 1A) due to measures consistent with open circuits, reports of non-auditory sensations, poor loudness growth, and drop in speech perception scores for BKB sentences in quiet (Fig. 1A). Rapid and dramatic (Fig. 1B) increases in impedance were measured over time. The clinical profile suggested electrode migration, which was confirmed by a CT scan 3-months post activation (Fig. 1C–E). Consequently, 10 months after implantation the decision was made to explant and replace with a new device. At explantation the surgeon reported significant fibrosis and mechanical resistance to array removal and observed five extra-cochlear electrodes. A fibrotic sheath was seen (Fig. 2A) on the array. Routine hardware testing confirmed that the device was functional. Accordingly, the case was diagnosed as a soft failure. The patient consented to an investigation of the tissue associated with the device and to the use of their fully anonymised data in this case report.

METHOD

To determine the cellular response to the electrode array in the tissue seen at time of explantation. On removal the device was prepared for histological analysis by tissue fixation in 10% neutral buffered formalin, before immersion in 70% ethanol (v/v). A midline incision was made along the length of the tissue cuff (Fig. 2A), the tissue removed and immersed in 30% sucrose in 0.1% phosphate buffer for cryoprotection before embedding in OCT (Finntek) (12). The device was returned to the manufacturer for integrity testing. Tissue sections (10 μm) were cut using a cryostat (Leica) and collected onto APES coated (13)

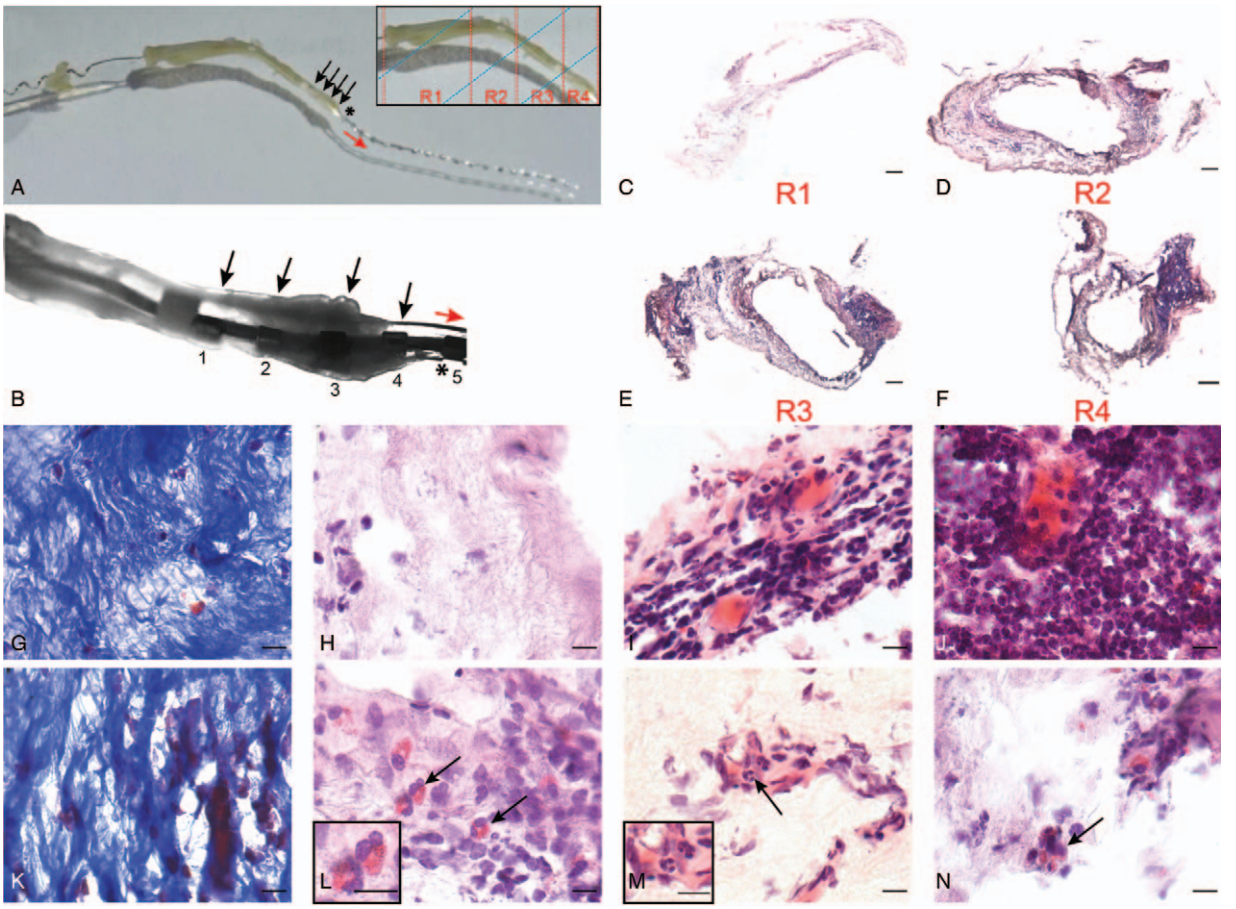


FIG. 2. Explanted electrode with associated fibrotic tissue before tissue removal and after sectioning and histochemical staining to illustrate the variable cellular response and local organization within the fibrotic tissue. Photographs of the full length of electrode array (*A*) showing fibrotic tissue enveloping the basal portion of the array. The insert in (*A*) identifies the four regions of tissue referred to in subsequent images and is shown at higher magnification in *B*, where the black arrows indicate tissue-covered electrode contacts and the numbers refer to the regions in *C–F*. *C–F*, Low magnification (×4) images of sections representative of regions 1 to 4 respectively to illustrate the gross organization of the tissue along its length. *G–N*, higher magnification (×40) images showing the variability in density of cells and volume of connective tissue across and within the regions. *G* and *H*, Illustrate areas of loose connective tissue with sparsely distributed cells. *I*, Areas of dense, organized cells close to position of the electrode array. *J*, Areas packed with cell nuclei and and dense connective tissue in *K* with aligned collagen fibres. *L–N*, Inflammatory cells (*arrows*) including eosinophils (*L*), neutrophils (*M*), and foreign body giant cells (*N*). Histochemical stains; hematoxylin and eosin, except *G*, *K* which are stained using MSB trichrome. Scale bar: *C–F* = 200 μm, *G–N* = 10 μm.

glass slides. To reveal the gross morphology and cellular organization tissue sections collected at four equidistant points (Fig. 2, A and B) along the cuff were stained using hematoxylin and eosin (Harris) and MSB trichrome. Immunohistochemistry (see supplementary Table 1 for detail, http://links.lww.com/MAO/B162) was used to identify macrophages, T cells, interleukin-1β, proliferating cells, and blood vessels (6,14). Endogenous peroxidase was quenched using 1% hydrogen peroxide. Biotinylated secondary antibodies were labeled using streptavidin conjugated horse-radish peroxidase (HRP) detected using diaminobenzidine. Sections were counterstained using hematoxylin, dehydrated through serial alcohols, cover-slipped using DPX mounting media, and air-dried overnight. Images were captured using light microscopy (Q-imaging 2000R digital camera connected to a Nikon eclipse E4000 microscope and Nikon HB-101004F light source).

RESULTS

The four basal electrodes were encapsulated in fibrotic tissue. Beyond this the cuff of fibrotic tissue appeared to have sheared (Fig. 2A, asterisk) at the round window niche on explantation, which is consistent with the resistance to removal noted in the surgical report. The tissue was composed of a heterogeneous mix of cells and extracellular matrix protein across and within the four regions examined (Fig. 2C–F) with an increase in cellularity closest to the round window. Stratification and organization of the cells within the tissue cuff is clear as seen in Figure 2E–K. Eosinophils and neutrophils, evidence of active inflammation (15), can be seen in Figure 2L and M. Collagen deposition differs across the tissue with the spatial organization of the collagen across and within regions suggesting different stages of tissue maturity and potential functional differences (16). Immunohistochemical analysis of the tissue identified CD68-positive cells (Fig. 3A) at the edge of the tissue in close proximity to the position of the electrode array. Interleukin-1β expression, evidence of inflammatory signaling between cells (17), is seen (Fig. 3B). A second macrophage (CD163+ve) population (18) was observed in low numbers (relative to the CD68+ve population) largely around the perimeter of the tissue sections (Fig. 3C). Clusters of T cells (Fig. 3D) were observed

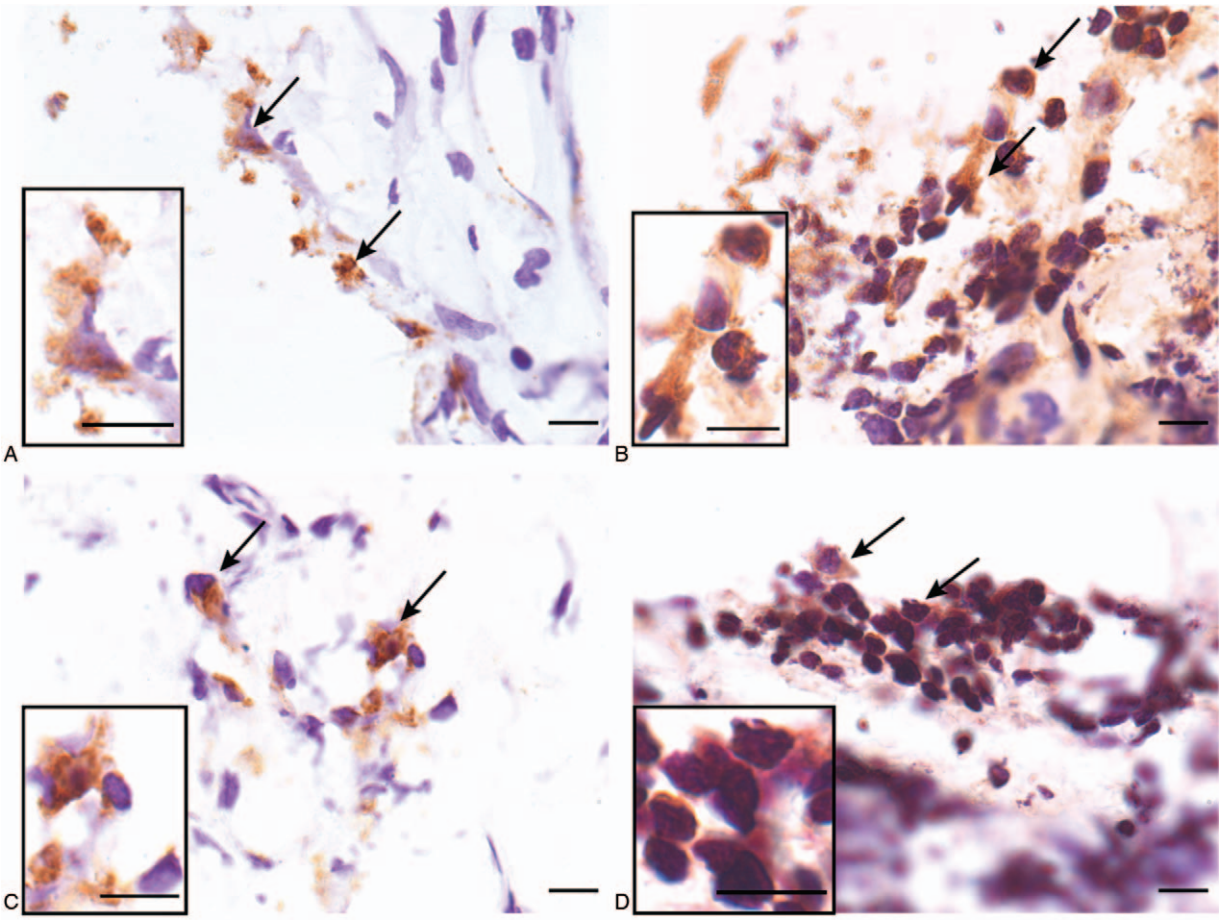


FIG. 3. Variable expression of cellular markers of macrophages and T cells within the explant tissue. Light microscopy images of tissue sections immunolabelled using *A*. anti-CD68, a marker of phagocytic macrophages. CD68-positive cells were observed at the edges of the tissue in close proximity to where the array was in all four regions. *B*, Anti-interleukin 1β, a pro-inflammatory cytokine. *C*, Anti-CD163 a marker of anti-inflammatory macrophages. CD163-positive macrophages were observed in lower number (compared with CD68) and were mostly observed around the perimeter of the tissue. *D*, T-lymphocytes labeled with anti-CD3. Clusters of T cells were observed in region 4 in tissue furthest away from the array. Arrows highlight examples of positively labeled cells. Scale bar: 10 μm.

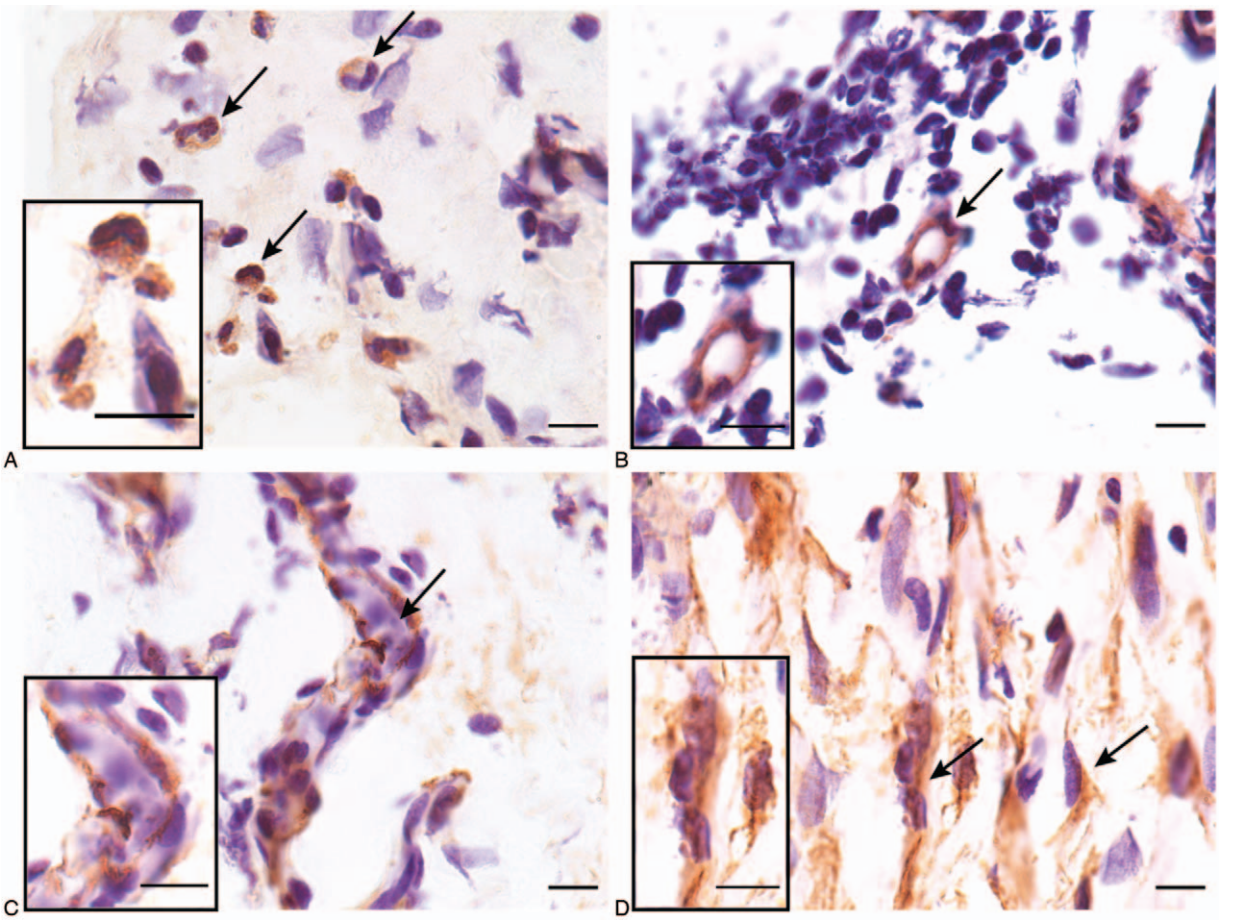


FIG. 4. Evidence of active tissue proliferation and angiogenesis. Light microscopy images of tissue sections immunolabeled using *A.* Anti-Ki-67 a marker of dividing, i.e., proliferating cells. The majority of the Ki-67 positive cells were observed in regions of low cell density and high extracellular matrix. *B*, Vascular endothelial growth factor receptor-2 (VEGFR2) a marker of developing blood vessels, i.e., angiogenesis. VEGFR2 expression was primarily observed in regions distal to the electrode array. *C*, Expression of alpha-smooth muscle actin (α-sma) clearly associated with blood vessel walls and in *D* α-sma positive cells indicating myofibroblasts that do not appear to be associated with vessels. Examples of positively labeled cells are indicated by arrows. Scale bar: 10 μm.

in region 4, most distant from the electrode surface. Evidence of active cell (Fig. 4A, Ki67 expression) and vascular proliferation identified by expression of vascular endothelial growth factor receptor 2 (Fig. 4B) was seen in regions of the tissue. Alpha-smooth muscle actin was clearly associated with vessels (Fig. 4C) and in cells within the regions of connective tissue (Fig. 4D).

DISCUSSION

Tissue growth (fibrosis) at the electrode-cochlear interface in this case is associated with an ascending pattern of sequential open circuits starting at the basal end, altered impedance measures, and an associated drop in BKB sentence scores in quiet. Sequential deactivation of electrodes began at switch-on and continued across the subsequent clinic visits. Macrophages, neutrophils, and interleukin-1β expression identified in the fibrotic tissue on the array are consistent with a chronic inflammatory (19) response, i.e., one that has not resolved despite the length of time (10 mo) since implantation. Whether this aberrant response is contributory to the migration of the array, or a consequence of repeated tissue remodeling due to movement of the array, cannot be determined. Typically, cell-signaling during the time after implantation, the acute phase, would mediate, wound repair, and progress to a collagen-rich dominated response with sparse macrophages at the site expressing anti-inflammatory mediators consistent with a restoration of homeostasis (12,20). The expression of vascular growth factor receptors (VEGFR2) (21) and upregulation of proliferation marker (Ki67) (22) support the active state of the tissue. As the use of implants becomes more common across different organs and tissues, e.g., cochlear implants, vascular stents, and joint prostheses, there is a growing recognition that the immune response to the implant may contribute to complications, or impaired function of the implant (23,24). Whether this is due to the immune response to a particular material is unclear. It is also unclear whether the response to a material varies between individuals, or even within an individual, according to other coexistent conditions (25). This

may be relevant in this case in which the individual was implanted with a Nucleus CI512 device immediately after the explantation of the migrated device. Explantation was made difficult by the presence of fibrotic tissue, but a full insertion was achieved on reimplantation. Significantly, tuning of the new device was straightforward and only the most basal electrode was deactivated. After 1 year of use of the new device, there are no indications of electrode migration and all impedances are in the normal range. Auditory performance has exceeded levels obtained early on with the first implant with 99% correct on the BKB sentence test in quiet and 2.0 dB in adaptive noise.

CONCLUSIONS

This is the first report to combine 1) post-cochlear implant cochlear tissue analysis from a person who has subsequently been reimplanted, 2) the tissue is from a relatively early stage post-implantation (particularly when compared with the majority of cadaveric cases), and 3) the histology is considered in the context of the functional outcomes and clinical history around time of explantation. This determines that investigation of fibrosis on explanted devices from living cases is feasible. The tissue response to implanted arrays is known to be associated with cases of poor performance (26) but little is known about whether clear biomarkers of a detrimental fibrotic response can be identified. There is a need to reduce soft failures, due to the surgical and psychological burden on the person with the implant and the associated healthcare costs (27). By investigating cases that undergo subsequent re-implantation it may be possible to determine the likelihood that a detrimental response to implantation is due to the individual or to some aspect of the implantation process. The establishment of a profile of the immune responses to the implant over time after implantation (such as could be obtained from a population of explanted devices) may identify targets for immune-modulation that could increase the useful lifetime of individual implants.

Acknowledgments: None.

REFERENCES

1. Venail F, Sicard M, Piron JP, et al. Reliability and complications of 500 consecutive cochlear implantations. *Arch Otolaryngol Head Neck Surg* 2008;134:1276–81.
2. Causon A, Verschuur C, Newman TA. Trends in cochlear implant complications: implications for improving long-term outcomes. *Otol Neurotol* 2013;34:259–65.
3. Bourdoncle M, Fargeot C, Poncet C, et al. Analysis and management of cochlear implant explantation in adults. *Eur Ann Otorhinolaryngol Head Neck Dis* 2020. In press.
4. Nadol JB, Eddington DK, Burgess BJ. Foreign body or hypersensitivity granuloma of the inner ear after cochlear implantation: one possible cause of a soft failure? *Otol Neurotol* 2008;29:1076–84.
5. Seyyedi M, Nadol JB. Intracochlear inflammatory response to cochlear implant electrodes in humans. *Otol Neurotol* 2014;35:1545–51.
6. Nadol JB, O'Malley JT, Burgess BJ, et al. Cellular immunologic responses to cochlear implantation in the human. *Hear Res* 2014;318:11–7.
7. Ishai R, Herrmann BS, Nadol JB, et al. The pattern and degree of capsular fibrous sheaths surrounding cochlear electrode arrays. *Hear Res* 2017;348:44–53.
8. Bas E, Bohorquez J, Goncalves S, et al. Electrode array-eluted dexamethasone protects against electrode insertion trauma induced hearing and hair cell losses, damage to neural elements, increases in impedance and fibrosis: a dose response study. *Hear Res* 2016;337:12–24.
9. Needham K, Stathopoulos D, Newbold C, et al. Electrode impedance changes after implantation of a dexamethasone-eluting intracochlear array. *Cochlear Implants Int* 2020;21:98–109.
10. Kamakura T, O'Malley JT, Nadol JB. Preservation of cells of the organ of corti and innervating dendritic processes following cochlear implantation in the human: an immunohistochemical study. *Otol Neurotol* 2018;39:284–93.
11. Okayasu T, Quesnel AM, O'Malley JT, et al. The distribution and prevalence of macrophages in the cochlea following cochlear implantation in the human: an immunohistochemical study using Anti-Iba1 antibody. *Otol Neurotol* 2020;41:e304–16.
12. Bas E, Goncalves S, Adams M, et al. Spiral ganglion cells and macrophages initiate neuro-inflammation and scarring following cochlear implantation. *Front Cell Neurosci* 2015;9:303.
13. Maddox PH, Jenkins D. 3-Aminopropyltriethoxysilane (APES): a new advance in section adhesion. *J Clin Pathol* 1987;40:1256–60.
14. Moreno B, Jukes JP, Vergara-Irigaray N, et al. Systemic inflammation induces axon injury during brain inflammation. *Ann Neurol* 2011;70:932–42.
15. Christo SN, Diener KR, Bachhuka A, et al. Innate immunity and biomaterials at the Nexus: friends or foes. *BioMed Res Int* 2015;2015:23.
16. Coelho NM, McCulloch CA. Contribution of collagen adhesion receptors to tissue fibrosis. *Cell Tissue Res* 2016;365:521–38.
17. Landgraeber S, Jäger M, Jacobs JJ, et al. The pathology of orthopedic implant failure is mediated by innate immune system cytokines. *Mediators Inflamm* 2014;2014:9.
18. Galea I, Felton LM, Waters S, et al. Immune-to-brain signalling: the role of cerebral CD163-positive macrophages. *Neurosci Lett* 2008;448:41–6.
19. Kzhyshkowska J, Gudima A, Riabov V, et al. Macrophage responses to implants: prospects for personalized medicine. *J Leukocyte Biol* 2015;98:953–62.
20. Gurtner GC, Werner S, Barrandon Y, et al. Wound repair and regeneration. *Nature* 2008;453:314–21.
21. Kendall RT, Feghali-Bostwick CA. Fibroblasts in fibrosis: novel roles and mediators. *Front Pharmacol* 2014;5:123.
22. Sun X, Kaufman PD. Ki-67: more than a proliferation marker. *Chromosoma* 2018;127:175–86.
23. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. *Semin Immunol* 2008;20:86–100.
24. Mariani E, Lisignoli G, Borzì RM, et al. Biomaterials: foreign bodies or tuners for the immune response? *Int J Mol Sci* 2019;20:636.
25. Chung D, Kim AH, Parisier S, et al. Revision cochlear implant surgery in patients with suspected soft failures. *Otol Neurotol* 2010;31:1194–8.
26. Foggia MJ, Quevedo RV, Hansen MR. Intracochlear fibrosis and the foreign body response to cochlear implant biomaterials. *Laryngoscope Investig Otolaryngol* 2019;4:678–83.
27. Crowson MG, Semenov YR, Tucci DL, et al. Quality of life and cost-effectiveness of cochlear implants: a narrative review. *Audiol Neurootol* 2017;22:236–58.
28. Kawano A, Seldon LH, Clark GM. Computer-aided three-dimensional reconstruction in human cochlear maps: measurement of the lengths of organ of Corti, outer wall, inner wall, and Rosenthal's canal. *Ann Otol Rhinol Laryngol* 1996;105:701–9.

Appendix C

Review article

Published in Glia

‘Macrophages in the cochlea; an immunological link between risk factors and progressive hearing loss.’

Hough, K., Verschuur, C. A., Cunningham, C., & Newman, T. A. (2021). Macrophages in the cochlea; an immunological link between risk factors and progressive hearing loss. Glia,1–20. **https://doi.org/10.1002/glia.24095**

Received: 27 May 2021 | Revised: 8 September 2021 | Accepted: 9 September 2021

DOI: 10.1002/glia.24095

GLIA

WILEY

REVIEW ARTICLE

Macrophages in the cochlea; an immunological link between risk factors and progressive hearing loss

Kate Hough[1] | Carl A. Verschuur[2] | Colm Cunningham[3] | Tracey A. Newman[4]

[1]Faculty of Engineering and Physical Sciences, University of Southampton, Southampton, UK

[2]Faculty of Engineering and Physical Sciences, Auditory Implant Centre, University of Southampton, Southampton, UK

[3]School of Biochemistry & Immunology, Trinity Biomedical Sciences Institute & Trinity College Institute of Neuroscience (TCIN), Dublin, Ireland

[4]Clinical and Experimental Sciences, Faculty of Medicine, IfLS, University of Southampton, Southampton, UK

Correspondence
Tracey A Newman, B85, Life Sciences, Faculty of Medicine, University Road, Southampton, Hants, SO17 1BJ, UK.
Email: t.a.newman@soton.ac.uk

Abstract

Macrophages are abundant in the cochlea; however, their role in hearing loss is not well understood. Insults to the cochlea, such as noise or insertion of a cochlear implant, cause an inflammatory response, which includes activation of tissue-resident macrophages. Activation is characterized by changes in macrophage morphology, mediator expression, and distribution. Evidence from other organs shows activated macrophages can become primed, whereby subsequent insults cause an elevated inflammatory response. Primed macrophages in brain pathologies respond to circulating inflammatory mediators by disproportionate synthesis of inflammatory mediators. This signaling occurs behind an intact blood–brain barrier, similar to the blood-labyrinth barrier in the cochlea. Local tissue damage can occur as the result of mediator release by activated macrophages. Damage is typically localized; however, if it is to structures with limited ability to repair, such as neurons or hair cells within the cochlea, it is feasible that this contributes to the progressive loss of function seen in hearing loss. We propose that macrophages in the cochlea link risk factors and hearing loss. Injury to the cochlea causes local macrophage activation that typically resolves. However, in susceptible individuals, some macrophages enter a primed state. Once primed, these macrophages can be further activated, as a consequence of circulating inflammatory molecules associated with common co-morbidities. Hypothetically, this would lead to further cochlear damage and loss of hearing. We review the evidence for the role of tissue-resident macrophages in the cochlea and propose that cochlear macrophages contribute to the trajectory of hearing loss and warrant further study.

KEYWORDS

cochlea, hearing loss, immune memory, immune response, inflammation, macrophages, priming

1 | MACROPHAGES IN THE AUDITORY SYSTEM

The identification of the blood-labyrinth barriers (BLB) (Juhn & Rybak, 1981) resulted in the cochlea being regarded as immune-privileged. However, the cochlea has an immune capacity. Immune cells and inflammation have roles in the physiology and pathophysiology of hearing and hearing loss (Fujioka et al., 2006; Keithley et al., 2008; Ma et al., 2000; Wang et al., 2003). Cochlear macrophages and perivascular macrophage-like melanocytes (PVM/Ms) are resident in the cochlea of adult humans (Liu et al., 2018; O'Malley et al., 2016) and mice (Okano et al., 2008). Evidence from studies in

rodents has identified recruitment and infiltration of monocytes from the circulation into the cochlea after acoustic trauma (Hirose et al., 2005; Tan et al., 2008; Tornabene et al., 2006), hair cell ablation (Kaur, Zamani, et al., 2015b), ototoxicity (Sato et al., 2010) and in response to cochlear implantation (Bas et al., 2015; Farhadi et al., 2013). The roles of these populations of cochlear macrophages are still to be fully determined.

Macrophages are pleiotropic immune cells with roles in homeostasis and inflammation (Davies & Taylor, 2015; Ginhoux & Guilliams, 2016; Hoeffel & Ginhoux, 2018). Macrophages detect, phagocytose, and clear cellular debris and pathogens from tissues. They initiate inflammation and the subsequent anti-inflammatory reparative response (or tissue repair) by producing pro- and anti-inflammatory cytokines and chemokines, which signal to surrounding cells. Macrophages can be divided into tissue-resident and infiltrated macrophages. Tissue-resident macrophages reside in tissue or organ systems (Ginhoux & Guilliams, 2016; Krenkel & Tacke, 2017; Mu et al., 2021; Wang et al., 2019b) and primarily carry out homeostatic roles under steady state (healthy) conditions. Monocytes are recruited from the bone-marrow (hematopoietic system) into tissues following an insult or pathology and differentiate into macrophages, so called infiltrated macrophages. The distinction between long-lived resident and tissue-infiltrated macrophages in the cochlea and the value in being able to identify these populations and their origins will be addressed in this review.

1.1 | Hearing and auditory system

The auditory system comprises a number of anatomical structures from the pinna (external ear) to the auditory centres of the central nervous system (CNS). Once sound waves are converted to mechanical energy by the tympanic membrane and middle ear, pressure changes are then converted to neural signals through the action of specialized sensory hair cells within the cochlea. Neural signals are transmitted along the auditory nerve to the brain. The organization of the auditory system and cellular basis of hearing has been reviewed extensively (Cunningham & Tucci, 2017; Raphael & Altschuler, 2003). An overview of the anatomical regions referred to in this review is illustrated in Figure 1. Hearing loss is a widespread problem with multiple causes, with almost 500 million people worldwide thought to have disabling hearing impairment (World Health Organisation, 2020). Understanding the role played by macrophages in driving the pathophysiology of progressive hearing loss is crucial in the development of future therapies. This is particularly timely given the absence of effective pharmacological treatments for the prevention or reversal of hearing loss.

Each stage in the auditory system can be damaged, with resulting impairment of hearing function. The earliest identified sites of injury following an insult are to the synapses between inner hair cells and spiral ganglion neurons (SGNs) (Fernandez et al., 2015; Kujawa & Liberman, 2009; Sergeyenko et al., 2013). Hair cells can become damaged and die following both acute and chronic insults (Bohne & Harding, 2000). In humans, hair cells are terminally differentiated and therefore cannot self-renew following significant damage (Mittal et al., 2017; Shu et al., 2019). Spiral ganglion neurons, which make synaptic connections between the hair cells and the neurons of the cochlear nucleus (Glueckert et al., 2005) (indicated in Figure 1c), can become damaged and degenerate. Damage or atrophy of the stria vascularis disrupts cochlear function (Neng et al., 2015; Zhang et al., 2013). Repeated exposure of the auditory system to insults will cause further damage to these structures, resulting in a gradual loss of function leading to progressive hearing loss. This review will discuss tissue-resident macrophages of the central (microglia) and peripheral (cochlear macrophages) auditory system and propose that tissue-resident cochlear macrophages contribute to the progression of hearing loss.

Cochlear macrophages have functional similarities with microglia in the central nervous system. Both cell types adopt characteristic morphologies and phenotypes, which are plastic and responsive to changes in the local tissue micro-environment. Macrophage morphology is an indicator of function and can identify a change in the state of immune activity (Li & Barres, 2018). The distribution, abundance, and morphology of cochlear macrophages across different regions of the adult cochlea under homeostatic conditions, and after different cochlear insults, have been investigated in both human and animals using mainly histological techniques. A summary of the distribution and morphological characteristics of macrophages in the structures of the auditory system is presented in Figure 1. Figure 2 details the cellular expression profiles of the macrophage populations. Compared to other tissue-resident populations of macrophages, the cellular expression profiles of cochlear macrophages and the markers which have and can be used to identify these cells is less well documented; this needs to be considered when drawing conclusions in the field. This review draws together the data and principal findings that describe the cochlear macrophage populations in steady state and after three common causes of damage or change to the cochlea, namely exposure to noise, age-associated changes, and cochlear implantation (which can be considered an unwanted side-effect of the intervention).

1.2 | Tissue-resident macrophage populations

Tissue-resident macrophage populations with distinct features have been identified and characterized in different tissues, including $CCR2^{-}$ cells in the heart and microglia in the eye and brain (Li & Barres, 2018; Wang et al., 2019b). These resident cells have roles in development, homeostasis, and pathologies including those associated with aging (Li & Barres, 2018; Wang et al., 2019b). Tissue-resident macrophages are distributed across the central (microglia and perivascular macrophages) and peripheral (cochlear macrophages and perivascular macrophage-like melanocyte (PVM/Ms)) auditory system. The central auditory system (part of the CNS) begins at the cochlear nucleus in the brainstem from where pathways project through to the auditory cortex (Figure 1d). Microglia and macrophages reside within the CNS. The methods, current limitations and value of being able to delineate between these cells is discussed below. The peripheral auditory system begins at the proximal auditory nerve leading into the cochlea

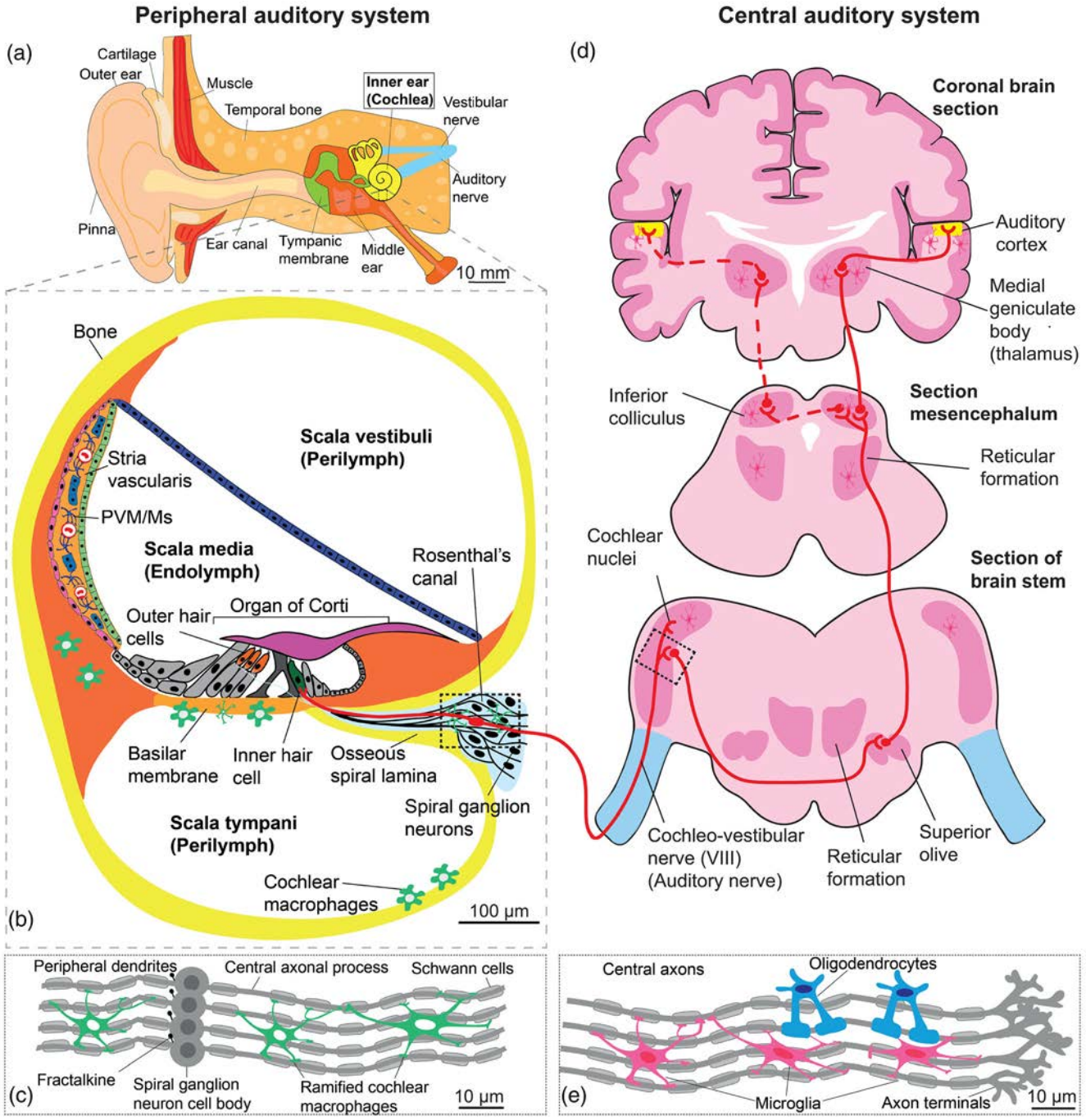
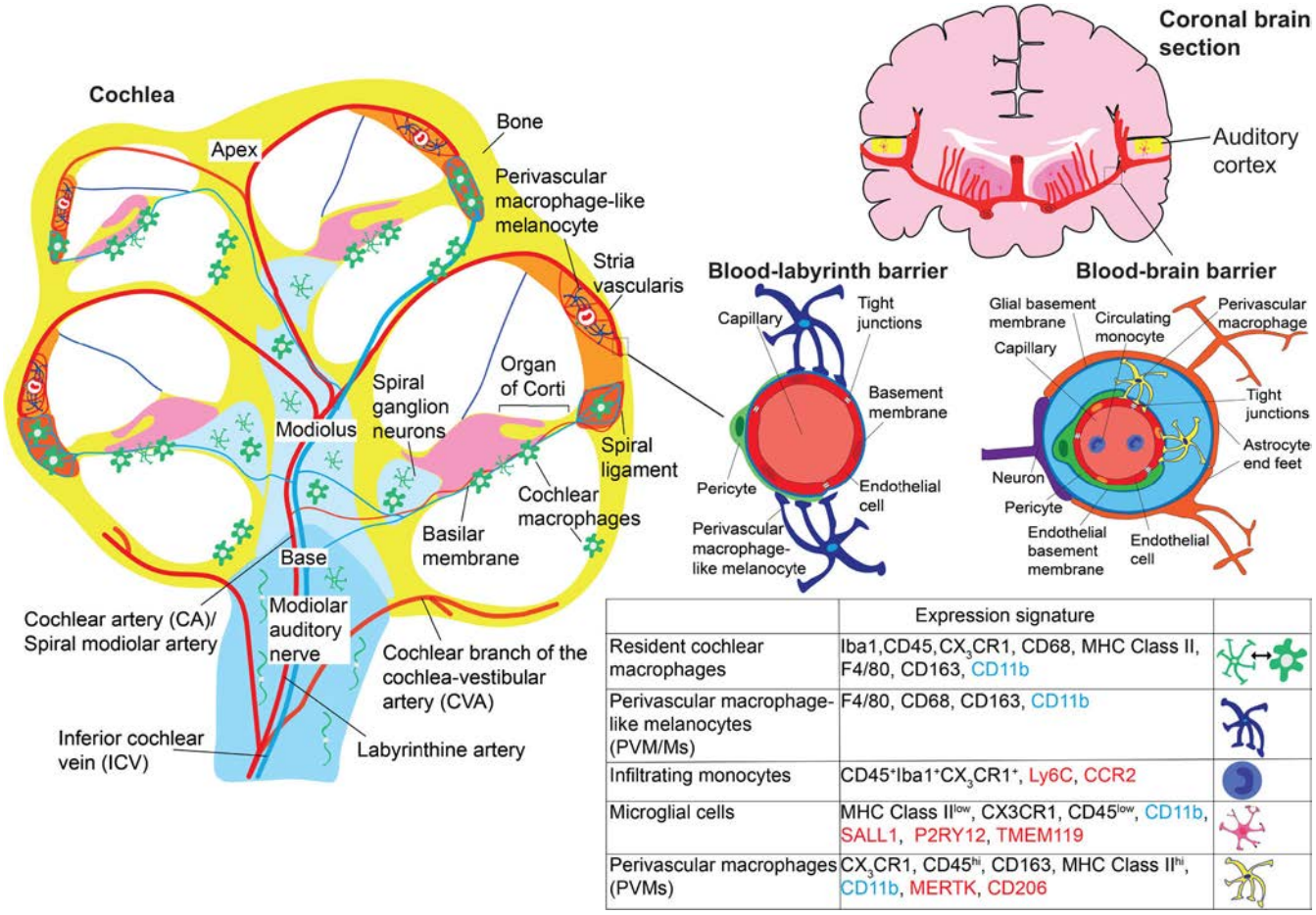


FIGURE 1 Overview of the organization and anatomy of the auditory system, highlighting the distribution of macrophages. The auditory system comprises the peripheral and central regions (left and right columns). The peripheral auditory system (a) consists of the outer, middle, and inner part of the ear or cochlea. Hearing function relies on specialized soft-tissues, cartilage, and bone and associated vascular beds. A high magnification (b) cross-section of a cochlear turn to identify the organ of corti, stria vascularis and Rosenthal's canal. The organ of corti contains the inner and outer hair cells, that detect and amplify sounds in a frequency-dependent manner. The stria vascularis regulates the ionic composition of the cochlear fluids and separates the internal cochlear environment from the systemic circulation. The modiolus, or bony central axis of the cochlea, contains Rosenthal's canal which houses the cell bodies of the spiral ganglion neurons (SGNs) which relay auditory information between the cochlea and central nervous system. An illustration (c) of the cellular organization of a region of the cochlear auditory nerve showing SGNs, which have peripheral and central axonal processes. Peripheral dendrites synapse with the hair cells. Cochlear macrophages have ramified processes that contact adjacent neurons. Centrally (d) the pathways project from the cochlear nucleus in the brainstem to the auditory cortex. The ascending primary auditory pathway relays signals from the cochlea along axons of spiral ganglion neurons to the cochlear nucleus in the brain stem. The auditory fibers cross the midline and relay to the superior olivary complex and inferior colliculus. The final relay is the medial geniculate body in the thalamus before the signal is relayed to the auditory cortex. An illustration (e) of the cellular organization of a region of the distal part of the auditory nerve, with centrally projecting axons of SGNs with axon terminals that synapse in the cochlear nuclei. Function within the auditory pathway relies on resident glial cells including non-myelinating and myelinating Schwann cells, oligodendrocytes and specialized sub-populations of macrophages



	Expression signature	
Resident cochlear macrophages	Iba1,CD45,CX_3CR1, CD68, MHC Class II, F4/80, CD163, CD11b	
Perivascular macrophage-like melanocytes (PVM/Ms)	F4/80, CD68, CD163, CD11b	
Infiltrating monocytes	$CD45^+Iba1^+CX_3CR1^+$, Ly6C, CCR2	
Microglial cells	MHC Class II^{low}, CX3CR1, $CD45^{low}$, CD11b, SALL1, P2RY12, TMEM119	
Perivascular macrophages (PVMs)	CX_3CR1, $CD45^{hi}$, CD163, MHC Class II^{hi}, CD11b, MERTK, CD206	

FIGURE 2 The distribution and expression signature of macrophage sub-populations in the human and murine auditory systems. Cochlear macrophages and microglia are located in different sub-structures and microenvironments across the auditory system (left panel). Perivascular macrophages are integral to the regulation of the permeability of the blood–brain barrier (BBB) and cochlear blood-labyrinth barrier (BLB) (right panel). The origins of the macrophages within sub-structures are reflected in the array of cellular markers which can be used experimentally to identify these cells. Several markers that identify different CNS glial populations have been identified; however, this selectivity has not been defined for macrophages in the peripheral auditory system. Greater delineation of the populations is needed to improve our understanding of the role of these cells in auditory homeostasis and hearing loss. Cell markers used to identify the populations vary in specificity from CD45, a pan leukocyte marker, to the commonly used microglia and macrophage marker Iba1. CD11b (highlighted in blue text) is expressed by all the resident macrophage populations described to date. Markers unique to sub-populations (red text) that could be used to differentiate between macrophage populations. Despite the progress, a definitive list of specific, reliable and experimentally tractable markers for cochlear macrophages is still needed and would be highly beneficial for advancing the field. See Table S1 for the citations associated with each cell marker

(Figure 1b). Macrophages reside within the peripheral auditory system.

Microglial precursors (Figure 2) infiltrate the brain early in the developing embryo. They arise from embryonic yolk sac precursors (Ginhoux et al., 2010) and maintain their population by self-renewal (Ajami et al., 2007; Lawson et al., 1992). The ability to identify the origin and kinetics of these cells in homeostatic or pathological tissue has been important in understanding their responses to other signaling cascades. Perivascular macrophages (PVMs), a second resident population in the brain, are located between the systemic circulation and CNS parenchyma at the blood–brain barrier (Galea et al., 2008; Galea & Perry, 2018; Lapenna et al., 2018; Varatharaj & Galea, 2017). PVMs are yolk sac-derived and are seeded in the brain early in development (Faraco et al., 2017; Goldmann et al., 2016; Lapenna et al., 2018). PVMs and microglia have roles in homeostasis and pathology. Importantly both brain resident populations, microglia and perivascular macrophages, are long-lived and sensitive to epigenetic modification (Haley et al., 2018; Keren-Shaul et al., 2017). This gives them the ability to develop a memory of previous insults to the tissue they reside in. It is this characteristic that may give tissue-resident macrophages a pivotal role in some pathologies.

1.3 | Tissue-resident cochlear macrophages

Cochlear macrophages become activated and change their morphology, abundance, and distribution in response to insults (Hirose et al., 2005; Lang et al., 2006; Liu et al., 2018; Noonan et al., 2020;

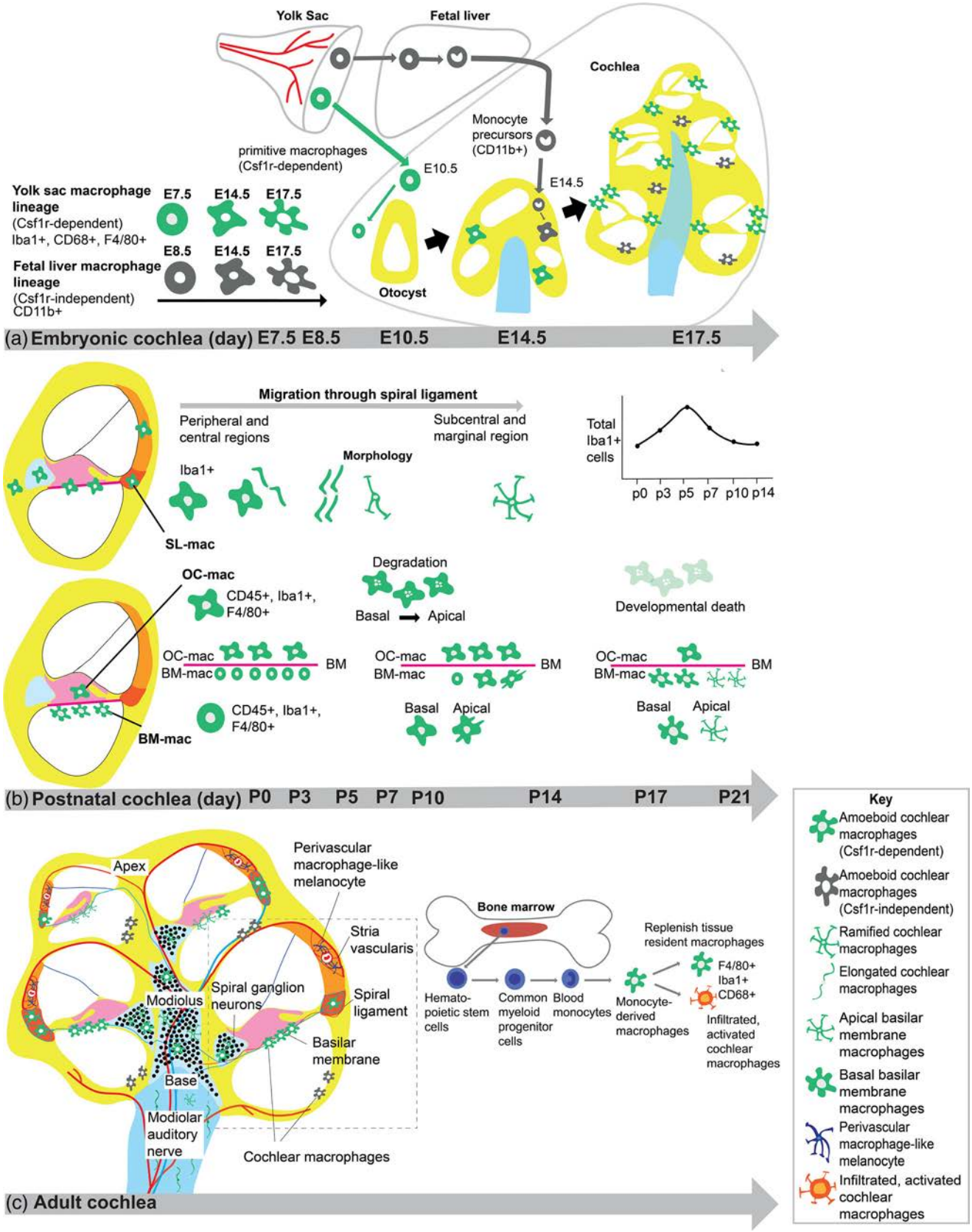


FIGURE 3 Legend on next page.

O'Malley et al., 2016; Okano et al., 2008; Okayasu et al., 2020). It is unclear whether and when these changes are protective or detrimental to cochlear function and this raises several questions, including: what are the roles of cochlear macrophages in injury/repair responses? What are the molecular mediators that control macrophage recruitment, signaling, and turnover and do these contribute to hearing loss? Can macrophages in the cochlea enter a state of altered activation referred to as primed, leaving them susceptible to disproportionate responses to secondary inflammatory insults? In order to improve hearing outcomes for people with hearing loss, we need to identify and understand how best to modulate the auditory system at a cellular level. This requires a major advance in our understanding of the biology of macrophages in the auditory system.

1.4 | Origin of macrophages in the cochlea

The dynamics of macrophage populations in the developing cochlea has been characterized most extensively in the mouse and are summarized in Figure 3 (Chen et al., 2018; Dong et al., 2018; Kishimoto et al., 2019; Okano et al., 2008). Colony stimulating factor 1 (Csf1) signaling, which is integral to the development and maintenance of microglia (Ryan et al., 2001), also controls seeding of the larger population of macrophages in the cochlea during development. A second population derived from the fetal liver, which is csf1r-independent (Kishimoto et al., 2019), is found in the modiolus and intraluminal surface of the perilymphatic space in the embryonic cochlea. Cochlear macrophages and perivascular macrophage-like melanocytes (PVM/Ms), (Ginhoux & Guilliams, 2016; Kishimoto et al., 2019; Okano et al., 2008), are resident in the adult cochlea. Cochlear macrophages persist from early post-natal stages (Dong et al., 2018) and they renew or maintain their population through infiltration of circulating monocytes (Hirose et al., 2005; Lang et al., 2006; Okano et al., 2008; Sato et al., 2010; Shi, 2010; Tan et al., 2008). Whether self-renewal also occurs through proliferation of the resident population, as is seen in the brain (Askew et al., 2017), is not clear. Perivascular macrophage-like melanocytes are found adjacent to blood vessels in the cochlea (Shi, 2010; Zhang et al., 2012). They have a turnover time of around 10 months in mice and are maintained through migration of monocytes into the cochlea (Shi, 2010). PVM/Ms contribute to the maintenance of the endocochlear potential (Zhang et al., 2013; Zhang et al., 2012) and the essential role of these cells in the cochlea is seen in a study where depletion of PVM/Ms in mice causes a reduction in endocochlear potential and hearing loss (Zhang et al., 2012).

2 | DISTRIBUTION AND MORPHOLOGY OF COCHLEAR MACROPHAGES UNDER HOMEOSTATIC CONDITIONS

2.1 | Organ of Corti macrophages

There is conflicting evidence regarding the existence of macrophages in the organ of Corti under homeostatic conditions. This uncertainty is likely due to variability in age at sample collection as much as the species studied and the methods applied to identify these cells. The organ of Corti in the mouse has been reported not to contain macrophages (Hirose et al., 2005; Yang et al., 2015). By contrast, analysis of human temporal bones identified Iba1+ macrophages with differing morphologies in the organ of Corti (Liu et al., 2018, 2019; O'Malley et al., 2016). Super-resolution structured illumination microscopy (SR-SIM) and transmission electron microscopy were used in the analysis of human tissue (Liu et al., 2018, 2019), these techniques have not been reported in the mouse. Macrophages in the organ of Corti are positioned along the perilymphatic side of the basilar membrane at the basal region of the tunnel of Corti and could be described as basilar membrane macrophages (Liu et al., 2018, 2019; O'Malley et al., 2016). In human tissue, these macrophages have been shown to have some cellular processes which penetrate through the basilar membrane (Okayasu et al., 2020) and which could engage in surveillance of synapses, a role demonstrated in brain microglia, at the hair cell: spiral ganglion neuron interface, though this has not been described.

2.2 | Basilar membrane macrophages

Basilar membrane macrophages show morphological differences between the apical and basal regions of the cochlea. Macrophages in the apical region of the sensory epithelium have a ramified morphology with long processes and a small cell body, in contrast amoeboid cells are reported in the basal turn (Frye et al., 2017, 2018; Yang et al., 2015). It is unclear whether this reflects a morphological difference associated with cells that are in steady-state and is due to location, or a difference due to a local change in the tissue associated with

FIGURE 3 Origins and maintenance of macrophages in the cochlea, through development. (a) Csf1r-dependent and csf1r-independent resident macrophages are found in the cochleae of the developing embryo. These subtypes vary in their origin, the markers they express, their abundance and distribution. Figure adapted from (Kishimoto et al., 2019). (b) The distribution and morphology of macrophages changes during postnatal development of the cochlea, this is most marked in the spiral ligament (SL) (Chen et al., 2018) organ of Corti (OC) and basilar membrane (BM) macrophages (Dong et al., 2018). (c) The distribution, morphology, and abundance of macrophages vary depending on the specific site within the tissue and these factors determine the function of the cells under homeostatic conditions and following a cochlear insult. The resident macrophage population is replenished by bone-marrow derived cells. See Figure 2 for a description of each cell type and the markers they express. Whether maintenance or replenishment of any of the resident macrophage populations in the adult cochlea occurs through self-renewal is not yet described in the literature

being in the low or high-frequency regions of the cochlea. High frequency regions of the cochlea appear more vulnerable to age-related changes. The hearing status is not reported for all the studies and would enable a closer correlation between intact sensory function, so a homeostatic state, and macrophage characteristics.

2.3 | Perivascular resident macrophage-like melanocytes

Perivascular resident macrophage-like melanocytes (PVM/Ms) are associated with blood vessels in the healthy adult stria (Neng et al., 2015). These cells have a branched morphology and ensheath the vessel walls with endfeet-like processes (Zhang et al., 2013; Zhang et al., 2012). Low numbers of cochlear macrophages have been reported in the spiral ligament, often around blood vessels (Liu et al., 2018) and amongst the fibrocytes (O'Malley et al., 2016).

2.4 | Spiral ganglion neuron macrophages

A morphologically distinct population of cochlear macrophages is distributed amongst the cell bodies of the spiral ganglion neurons. In the cases reported from a rat and human study, the macrophages are characteristic of homeostatic microglia (Fuentes-Santamaria et al., 2017; Liu et al., 2018). The rat and human modiolar auditory nerve is also populated with elongated macrophages (Figure 1c, 3c) with filamentous processes that contact adjacent neurons (Fuentes-Santamaria et al., 2017; Liu et al., 2019).

2.5 | Characterizing the distinct populations of macrophages in the auditory system

Resident and infiltrated macrophages produce pro-inflammatory and anti-inflammatory cytokines and chemokines following an immune event including IL-1β, TNF-α, TGF-β, CX3CR1, and IL-10. Drawing on the functional similarities between CNS microglia and cochlear macrophages is useful, as it highlights their complex and pivotal functions in tissue homeostasis and in diseased or injured states. Despite the advances, the characterization and identification of the distinct macrophage populations in the cochlea is limited by the relatively small number of published studies and methods used to characterize these cells. Compared to other tissue-resident populations of macrophages, the expression signature of cochlear macrophages and the markers, which can be used to identify these cells is less well documented. The detailed expression profiles of microglia and macrophages of the brain have been characterized using comparative transcriptomic studies (Crotti & Ransohoff, 2016; Li & Barres, 2018). Recent single-cell RNA sequencing of cochlear tissue after noise damage (Rai et al., 2020) and transcriptomic analysis of the C57 mouse cochlea has identified genes associated with macrophage activation (Su et al., 2020). Extending this work to identify and establish the set of markers expressed by cochlear macrophages using multiplex and comparative transcriptomic studies will enable more reliable identification of sub-populations and roles and comparison with other organ systems. To better understand the role of these cells, approaches are needed that enable source and region (cochlear sub-structure) specific macrophage information to be captured. This requires the site of isolated cells to be identified, or the signal to be detected in-situ in the tissue. In the brain microglia and infiltrated monocytes have been shown to display functional differences and contribute to disease pathologies differently, such as after ischemic stroke or in multiple sclerosis (Ritzel et al., 2015; Yamasaki et al., 2014). Markers such as TMEM119, which appears to be specific for microglia in both human and mouse, can be used to distinguish resident microglia from blood-derived macrophages (Bennett et al., 2016; Satoh et al., 2016). Being able to distinguish resident and infiltrated macrophages in the cochlea will help elucidate the precise function of each sub-population.

3 | DISTRIBUTION AND MORPHOLOGY OF COCHLEAR MACROPHAGES AFTER INJURY

Experimental models, predominantly in the mouse, have been used to investigate the distribution, morphology, and potential function of macrophages and the role of inflammation after cochlear insults. Our review of the published evidence shows that macrophage populations across the anatomical regions of the cochlea respond, albeit variably, to tissue injury induced by noise but many of the experimental designs are multifactorial. A challenge, in drawing clear conclusions about the role of macrophages, is the heterogeneity in experimental design between studies, even when considering the consequences of a single type of insult or injury (summarized in Table 2).

3.1 | Variation in noise exposure study design

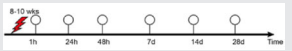
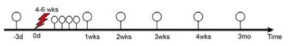
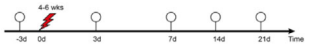
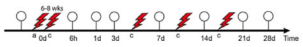
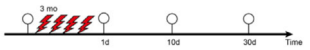
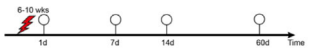
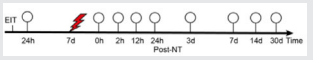
Differences in study design include intensity and duration of noise exposure, age, and strain of mouse, whether the noise exposure caused temporary or reversible threshold shifts and frequency and type of measurements taken. Many of the studies used young mice (4–6 weeks). The immune/macrophage response is likely to be different between older and younger mice since regional, age-dependent differences have been identified in microglial phenotypes between young (4 months) and aged (21 months) C57/BL6 mice (Hart et al., 2012). This may have translational relevance. The level of noise exposure, both intensity and duration, is variable; whereby a significant exposure such as 4 h at 118 dB for four consecutive days, designed to induce long-lasting auditory damage and result in a permanent threshold shift (Fuentes-Santamaria et al., 2017), is likely to elicit a very different inflammatory response compared to lower level noise exposure that only produces temporary threshold shifts (Frye et al., 2018). Although this may be further altered by the age, at which the subject is exposed to the noise or insult (Quraishe et al., 2019). Higher

TABLE 1 The distribution of macrophages across the sub-structures of the cochlea and auditory nerve

Peripheral auditory system								
Anatomical region	**Auditory nerve (AN)**	**Rosenthal's canal (RC)- spiral ganglion neurons (SGNs)**	**Osseous spiral lamina (OSL)**	**Organ of corti (OC)**	**Basilar membrane (BM)**	**Scala tympani (ST)**	**Spiral ligament (SL)**	**Stria vascularis (SV)**
Under homeostatic conditions	Elongated Iba1+ cells with filamentous processes that have terminal podosomes attach to adjacent neurons in the AN[1]***(H)***.	Macrophages with a small cell body with ramified long processes[2]*(R)*. Multiple macrophages amongst the SGNs. Iba1+ cells with pseudopodia and cell projections are observed between the satellite glial cell layer and nerve soma[3]***(H)***. Density of macrophages is positively correlated with the density of spiral ganglion cells[4]***(H)***. Iba1+ cells with a ramified, branching morphology with spider-like extensions[5]*(H)*.	CD163+ and Iba1+ cells are present along OSL[6]***(H)***. Iba1+ and CD68 + present in the OSL[5]***(H)***.	Distinct macrophage populations present during the postnatal developmental period[7]***(M)***. Macrophages are rarely observed under homeostatic conditions[8,9]*(M)*. Human OC contains some active, dendritic, lysosome containing macrophages[1,3,6]***(H)***.	Distinct macrophage populations present during the postnatal developmental period[7]***(M)***. Apical vs basal differences in morphology[10]***(M)***. Located on the perilymphatic side of the BM. Some processes penetrate through BM[4]***(H)***. Located below inner hair cells and at the base of the Hensen cells[6]***(H)***. Few cells present[5]***(H)***.	Macrophages (some PVM/Ms) are observed along the ST wall, usually amoeboid morphology. The cells are CD163+, Iba1+ and CD68 + [6]***(H)***.	Few macrophages observed, often around blood vessels[3]***(H)***. CD163+ and Iba1+ cells are observed amongst various types of fibrocytes[6]***(H)***.	PVM/Ms have branched morphology[11,12]***(M)***. PVM/Ms are tightly associated with endothelial cells[13]***(M)***. PVM/Ms are located near and around blood vessels and intermediate cells[3]***(H)***. Iba1+ and CD68+ cells present in SV[5]***(H)***.

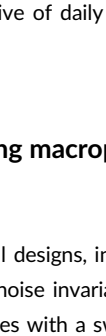
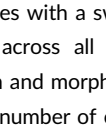
Note: This table summarizes key findings by anatomical location from studies of macrophage distribution and morphology in the cochlea and auditory nerve under homeostatic conditions, that is, in the absence of a deliberate or identified insult. The data are from studies in mouse, rat, and humans. Homeostatic macrophages exhibit a branched, ramified morphology, whereas activated macrophages exhibit an amoeboid morphology, with fewer processes. This morphological characterization of macrophages is indicative, though not definitive, of cellular function. More detailed characterization of the cellular expression patterns and mediator release from the cells is needed to understand macrophage function and heterogeneity. References: 1, (Liu et al., 2019) (H), 2, (Fuentes-Santamaria et al., 2017) (R), 3, (Liu et al., 2018) (H), 4, (Okayasu et al., 2020) (H), 5, (Noonan et al., 2020) (H), 6, (O'Malley et al., 2016) (H), 7, (Dong et al., 2018) (M), 8, (Hirose et al., 2005) (M), 9, (Yang et al., 2015) (M), 10, (Frye et al., 2017) (M), 11, (F. Zhang et al., 2013) (M), 12, (W. Zhang et al., 2012) (M), 13, (Neng et al., 2015) (M). Key: (H) human primary study, (M) mouse primary study, (R) rat primary study.

TABLE 2 Summary of study designs investigating the effect of a single type of insult on macrophages in the auditory system

	Citation	Animal	Strain	Gender	Age	Intensity	Readout/measurement
1	(Hirose et al., 2005)	Mouse	CBA/CaJ	M	8–10 weeks	0, 106, 112, 120 dB	
2	(Sautter et al., 2006)	Mouse	Tg CCL2$^{-/-}$CX3CR1$^{+/GFP}$ & CCR2$^{-/-}$CX3CR1$^{+/GFP}$	M and F	8–10 weeks	0, 106, 112 dB	
3	(Fujioka et al., 2006)	Rat	Sprague Dawley	M	4–6 weeks	124 dB, 4 kHz, 2 h	
4	(B. T. G. Tan et al., 2008)	Mouse	C57BL/6J (M) GFP Tg (F)	M and F	7 months	120 dB, 2 h	
5	(Sato et al., 2008)	Mouse	C57BL/6 & CX3CR1 mutant		10 weeks	112 dB 8–16 kHz, 2 h	
6	(Wakabayashi et al., 2010)	Mouse	C57BL/6J	M	4–6 weeks	124 dB, 4 kHz, 2 h	
7	(F. Zhang et al., 2013)	Mouse	C57BL/6J	M	6–8 weeks	120 dB, 3 h on day 1 and day 2	
8	(Yang et al., 2015)	Mouse	C57BL/6 J, CBA/CaJ & B6.B10ScN-Tlr4lps-del/JthJ	M	4–8 weeks	120 dB, 1–7 kHz, 1 h	
9	(Yang et al., 2015)	Mouse	C57BL/6J	M and F	4–8 weeks	120 dB, 1–7 kHz, 1 h	
10	(Vethanayagam et al., 2016)	Mouse	C57BL/6 J & B6.B10ScN-Tlr4lps-del/JthJ	M and F	4–6 weeks	120 dB, 1–7 kHz, 1 h	
11	(W. J. T. Tan et al., 2016)	Mouse	C57BL/6	M	6–8 weeks	55–65 dB, Acute: 100 dB 24 h Chronic: 90 dB, 2 h per day	
12	(Mizushima et al., 2017)	Mouse	C57BL/6	F	6–10 weeks	121 dB, 4 kHz, 4 h	
13	(Fuentes-Santamaria et al., 2017)	Rat	Wistar	F	3 months	118 dB, 4 h, 4 consecutive days	
14	(Frye et al., 2018)	Mouse	CBA/CaJ	M and F	1–3 months	LLN: 95 dB, 8–16 kHz, 8 h on 16 h off. TN: 120 dB, 1–7 kHz, 1 h	
15	(Kaur et al., 2018)	Mouse	CX3CR1$^{1/1}$ CX3CR1$^{GFP/1}$, CX3CR1$^{GFP/GFP}$	M and F	6–8 weeks	120 dB, 2 h	
16	(Kishimoto et al., 2019)	Mouse	Csf $^{op/op}$ w/ B6C3F1	M and F	4 weeks	120 dB, 8 kHz, 1 h	
17	(Kaur et al., 2019)	Mouse	C57BL/6 (B6) & CX3CR1$^{+/+}$ CX3CR1$^{GFP/+}$ CX3CR1$^{GFP/GFP}$	M and F	6 weeks	90 dB, 8–16 kHz, 2 h	
18	(Mittal et al., 2017)	Guinea pig		M and F		120 dB 6–10 kHz centred at 8 kHz, 2 h	

(Continues)

TABLE 2 (Continued)

	Citation	Animal	Strain	Gender	Age	Intensity	Readout/measurement
19	(Zhang et al., 2020)	Mouse	C57BL/6J & B6.129P2(Cg) Cx3Cr1^{tm1Litt}/J (aka Cx3cr1$^{GFP/GFP}$)	M and F	4–6 weeks	120 dB SPL 1–7 kHz, 1 h (N2- repeated 20 d after first NE)	
20	(Rai et al., 2020)	Mouse	C57BL/6J	M and F	Before 7 weeks	120 dB SPL, 8–16 kHz, 2 h	

Note: The table below summarizes key methodological variables in published studies of noise damage to the auditory system in which the effect on macrophage populations was assessed. Variables are: age, strain, and gender of animal and also the intensity and duration of noise exposure. The timeline indicates the age at which the animals were exposed to noise and the number of measurements taken across the timeline of the experiment.

intensity exposures may provide insight into the damage caused by repeat exposure to a significant event, for example, a blast, whereas the lower level noise is more representative of daily noise exposure associated with certain industries.

3.2 | Pathophysiology involving macrophages following insults to the cochlea

Despite the heterogeneity of experimental designs, insults or trauma to the cochlea such as exposure to loud noise invariably lead to the activation of resident cochlear macrophages with a switch to a more pro-inflammatory, activated, phenotype across all regions of the cochlea. This change in expression pattern and morphology is associated with changes in the distribution and number of cochlear macrophages, as summarized in Table 3. The resulting inflammatory response is associated with damage and degeneration within cochlear structures including sensory cells (Bohne & Harding, 2000), spiral ganglion neurons (Wang et al., 2002) and cochlear atrophy. Perivascular macrophage-like melanocytes (PVM/Ms) in the stria become activated and change their morphology and distribution, resulting in smaller PVM/Ms with shorter processes, which are physically detached from capillary walls (Neng et al., 2015; Noble et al., 2019; Zhang et al., 2013). PVM/Ms are essential for adequate hearing function but persistent activation of PVM/Ms in response to an inflammatory event causes strial dysfunction. Breakdown, or even a partial/transient loss of integrity of the blood-labyrinth barrier, including the intrastrial blood-fluid barrier, enables leakage of molecules and cells from the circulation and contributes to altered or lost hearing.

Exposure to noise results in activation and a greater number of macrophages in the spiral ligament (Fuentes-Santamaria et al., 2017), with significant monocyte infiltration (Hirose et al., 2005; Sautter et al., 2006; Tornabene et al., 2006). The macrophages amongst the spiral ganglion neurons become activated; increasing in number with an amoeboid appearance, indicating a switch to a pro-inflammatory, activated, phenotype (Fuentes-Santamaria et al., 2017; Kaur et al., 2018). Similarly, macrophages in the central portion of the auditory nerve increase in number and appear activated (Fuentes-Santamaria et al., 2017). They also display a shift to more variable morphologies with increased age (Noble et al., 2019). Acute noise exposure results in the infiltration of monocytes to the basilar membrane (Yang et al., 2015) and the activation of macrophage populations in regions adjacent to sensory cells (Frye et al., 2018). Whereas aging results in the activation of tissue-resident macrophages with little monocyte infiltration (Frye et al., 2017).

Damaged spiral ganglion neurons (SGNs) and surrounding resident cells produce pro-inflammatory cytokines that activate and recruit macrophages (Bas et al., 2015; Fuentes-Santamaria et al., 2017). There is evidence that these macrophage responses have a neuroprotective role mediated through fractalkine signaling. Selective hair cell ablation, aminoglycoside ototoxicity, and noise trauma result in fractalkine-dependent recruitment of macrophages to the sensory epithelium and spiral ganglion of the cochlea that is

TABLE 3 Changes in macrophage distribution and morphology in the cochlea and auditory pathway following different cochlear insults including noise exposure, aging, and cochlear implantation

Anatomical region	Auditory nerve (AN)	Rosenthal's canal (RC)- spiral ganglion neurons (SGNs)	Osseous spiral lamina (OSL)	Organ of corti (OC)	Basilar membrane (BM)	Scala tympani (ST)	Spiral ligament (SL)	Stria vascularis (SV)
After noise exposure (NE)	Increase in Iba1 staining. Cells resemble activated microglia[2] ***(R).***	Activated with amoeboid morphology[2] ***(R).*** Increase in the number of macrophages[14] ***(M).***	Increase in the number of macrophages[15] ***(M).***	†	Site of migrating macrophages[8] ***(M).*** Acute NE - infiltration of monocytes[9] ***(M).*** Low level NE- macrophage activation to amoeboid morphology. Increased number in regions adjacent to sensory cells[16] ***(M).*** Increase in the number of macrophages, change from monocyte-like to amoeboid morphology after 20d. Increased number of activated macrophages after second NE[15] ***(M).***	Increase in the number of macrophages on the luminal surface[15] ***(M).***	Increase in the number of macrophages and the number of activated macrophages[2] ***(R).*** Significant monocyte infiltration[8,17,18] ***(M).***	PVM/Ms change morphology. Physical detachment of PVM/Ms from capillary walls[11] ***(M).***
With aging	Number of macrophages increases with age, variable morphologies. Increase in activated macrophages [19] ***(H).***	Worm-like cytoarchitecture located near neuronal soma. Increased interactions between macrophages and glial cell-associated myelinated axonal projections of type I SGN cell bodies[19] ***(H).***	Bipolar architecture and flat encroaching filopodia-like structures suggesting interactions with peripheral neural projections[19] ***(H).***	†	Resident tissue macrophages involved in the pathology rather than the infiltrated macrophages. Number of apical macrophages increases, number of basal macrophages decreases[10,20] ***(M).***	†	Increase in the number of macrophages in the middle and basal turns. Increase in activated macrophages with amoeboid morphology[19] ***(H).***	PVM/Ms are smaller with shorter processes[13] ***(M).*** PVM/Ms are highly ramified in aged human temporal bones[19] ***(H).***
After cochlear implantation	Increase in the infiltration of macrophages following implantation [21] ***(M).***	Monocyte invasion increases rapidly after implantation[21] ***(M).*** Density of macrophages is greater in the basal RC. Transitional, ramified morphology with long processes. Increased number of macrophages 'wrapping' spiral ganglion cells[4] ***(H).***	Higher density of macrophages. More amoeboid morphology[4] ***(H).***	High IL-1β levels on day 14 which then remained stable until day 30 - pro-inflammatory environment[21] ***(M).*** No difference in the distribution of macrophages in the OC after 12 to 210 months after implantation[4] ***(H).***	Few macrophages observed beneath the OC with processes extending into the OC[4,5] ***(H).***	Iba1+ macrophages within areas of fibrosis in the scala tympani[5] ***(H).***	No difference observed in the number of macrophages [4] ***(H).***	PVM/Ms observed near blood vessels. Increased infiltration of monocytes over time[21] ***(M).*** No difference in the number of macrophages [4] ***(H).***

Note: The table summarizes the changes in distribution, morphology and abundance of macrophages following cochlear insults such as noise exposure, aging and cochlear implantation. Inflammation following cochlear insults is often associated with an increase in number and activation status of cochlear macrophages. These changes reflect the role of these cells in the tissue injury and repair response. There is evidence from other pathologies that dysregulated macrophage responses are associated with change or loss of function. Key: † No reporting on this to be found, (H) human primary study, (M) mouse primary study, (R) rat primary study. References: 2, (Fuentes-Santamaria et al., 2017) (R), 14, (Kaur et al., 2018) (M), 15, (C. Zhang et al., 2020) (M), 8, (Hirose et al., 2005) (M), 9, (Yang et al., 2015) (M), 16, (Frye et al., 2018) (M), 17, (Sautter et al., 2006) (M), 18, (Tornabene et al., 2006) (M), 11, (F. Zhang et al., 2013) (M), 19, (Noble et al., 2019) (H), 10, (Frye et al., 2017) (M), 20, (Frye et al., 2018) (M), 13, (Neng et al., 2015) (M), 21, (Bas et al., 2015) (M), 4, (Okayasu et al., 2020) (H), 5, (Noonan et al., 2020) (H).

associated with survival of the spiral ganglion neurons (Kaur, Zamani, et al., 2015b; Kaur et al., 2018, 2019).

An understanding of the precise molecular mediators that influence the macrophage phenotype after a cochlear insult is lacking. Collating information from multiple (animal and human) studies, as displayed in Table 3, to begin to build a consensus understanding of macrophage morphology, distribution, and function across different regions of the cochlea after cochlear insults will enable better understanding of how these cells respond to cochlear insults and potentially contribute to an individual's hearing loss trajectory. Further investigation into other immune insults that could occur throughout a lifespan, such as peripheral and systemic infection and inflammation, and which may influence the function and behavior of cochlear macrophages is necessary.

4 | IMMUNE EVENTS FOLLOWING AN ACUTE INSULT TO THE COCHLEA

Acute insults to the cochlea include noise exposure (Frye et al., 2018; Hirose et al., 2005; Sato et al., 2010), ototoxicity (Kaur et al., 2018; Sato et al., 2010), selective hair cell ablation (Kaur, Zamani, et al., 2015b) and cochlear implant surgery (Bas et al., 2015; Seyyedi & Nadol, 2014). These lead to the rapid onset of sensory cell injury and tissue pathogenesis. These signals to resident cochlear macrophages, switching them to an activated, pro-inflammatory state. Pro-inflammatory macrophages release cytokines such as TNF-α (Keithley et al., 2008) and chemokines including CCL2 (Bas et al., 2015; Sautter et al., 2006; Zhang et al., 2020) which attract and recruit inflammatory, circulating monocytes into the cochlea (Frye et al., 2017; Hirose et al., 2005; Okayasu et al., 2020; Tan et al., 2008; Vethanayagam et al., 2016; Yang et al., 2015). These infiltrated monocytes differentiate into macrophages, in the cochlea, and carry out characteristic immunological functions including phagocytosis (Kaur, Hirose, et al., 2015a), inflammatory mediator production, and antigen presentation (Yang et al., 2015).

4.1 | Time course of macrophage activity

The time course of monocyte infiltration following acute injury is variable, depending on the type, and duration of injury. For example, infiltration beginning between 12 and 24 h, has been described after noise exposure, with a peak at 3–7 days and then a gradual decrease as the inflammation appears to resolve (Hirose et al., 2005; Tornabene et al., 2006; Wakabayashi et al., 2010). By contrast, a continuous presence of monocyte-like macrophages ($CD45^{high}$, $F4/80^{low}$, $Iba1^{low}$) in the cochlea at 20 days after noise exposure has been observed (Zhang et al., 2020). Some of these macrophages have been identified as adopting an anti-inflammatory profile ($Ly6C^{low}$), consistent with a healthy tissue reparative response, where an acute wave of inflammatory activity is closely followed by resolving, anti-inflammatory signaling. Following cochlear implantation, monocyte infiltration to the wound-site increases rapidly at 1 day post-implantation with peak numbers detected at 7 days post-implantation (Bas et al., 2015). The precise role and turnover rate of the newly infiltrated macrophages is not well understood, though there is evidence that some of these cells persist (Okano et al., 2008; Sato et al., 2008). Although cochlear implantation is a single event, the signaling and tissue response may persist to a variable degree. Surgical insertion of an electrode array inside the cochlea causes physical trauma. The inflammatory response to this is likely as has been described above and should resolve as part of a normal wound healing response. However, in some individuals there is a chronic inflammatory response, which promotes the excessive deposition of fibrotic tissue around the array, which develops long after the surgical intervention, leading in some cases to reduced performance with the device (Bas et al., 2015; Hough et al., 2021; Nadol et al., 2008, 2014).

5 | IMMUNE EVENTS ASSOCIATED WITH A CHRONIC INSULT TO THE COCHLEA

Chronic damage/deterioration of the auditory system, such as that due to aging, chronic noise exposure, or after cochlear implantation is thought to trigger a different immune response to an acute event. The effects of cumulative low-grade cochlear insults, such as can occur with aging, have been investigated in a small number of studies (Frye et al., 2017; Noble et al., 2019; Su et al., 2020; Zhang et al., 2017). Cumulative, progressive, sensory cell degeneration and death leads to the activation of resident macrophages, with little infiltration of circulating monocytes (Frye et al., 2017), which parallels the innate immune response seen in chronic compared to acute CNS disease. Activation of resident macrophages is associated with a morphological change to macrophages with a larger cell body and amoeboid shape. The activated, resident macrophages adopt either a pro- or anti-inflammatory profile. Pro-inflammatory macrophages produce and release pro-inflammatory mediators including IL-1β, TNF-α, IL-6, which signal to surrounding cells resulting in further inflammation and cellular damage/apoptosis (Fujioka et al., 2006; Tan et al., 2016; Wang et al., 2019a). Anti-inflammatory macrophages produce anti-inflammatory, tissue reparative, mediators such as IL-10 and arginase (Kalinec et al., 2017).

5.1 | Inflammaging and progressive hearing loss

Chronic inflammation or 'inflammaging' describes low-grade inflammation that can occur in aging tissues and that worsens with age (Franceschi et al., 2006, 2017; Gruver et al., 2007; Watson et al., 2017). It is associated with age-related changes in immune function, characterized by a decreased ability to control the production of pro-inflammatory proteins, which results in an increased inflammatory state in the majority of tissues. Data from cross-sectional aging cohort studies showed that higher levels of circulating markers of inflammation are associated with a more marked age-related hearing loss

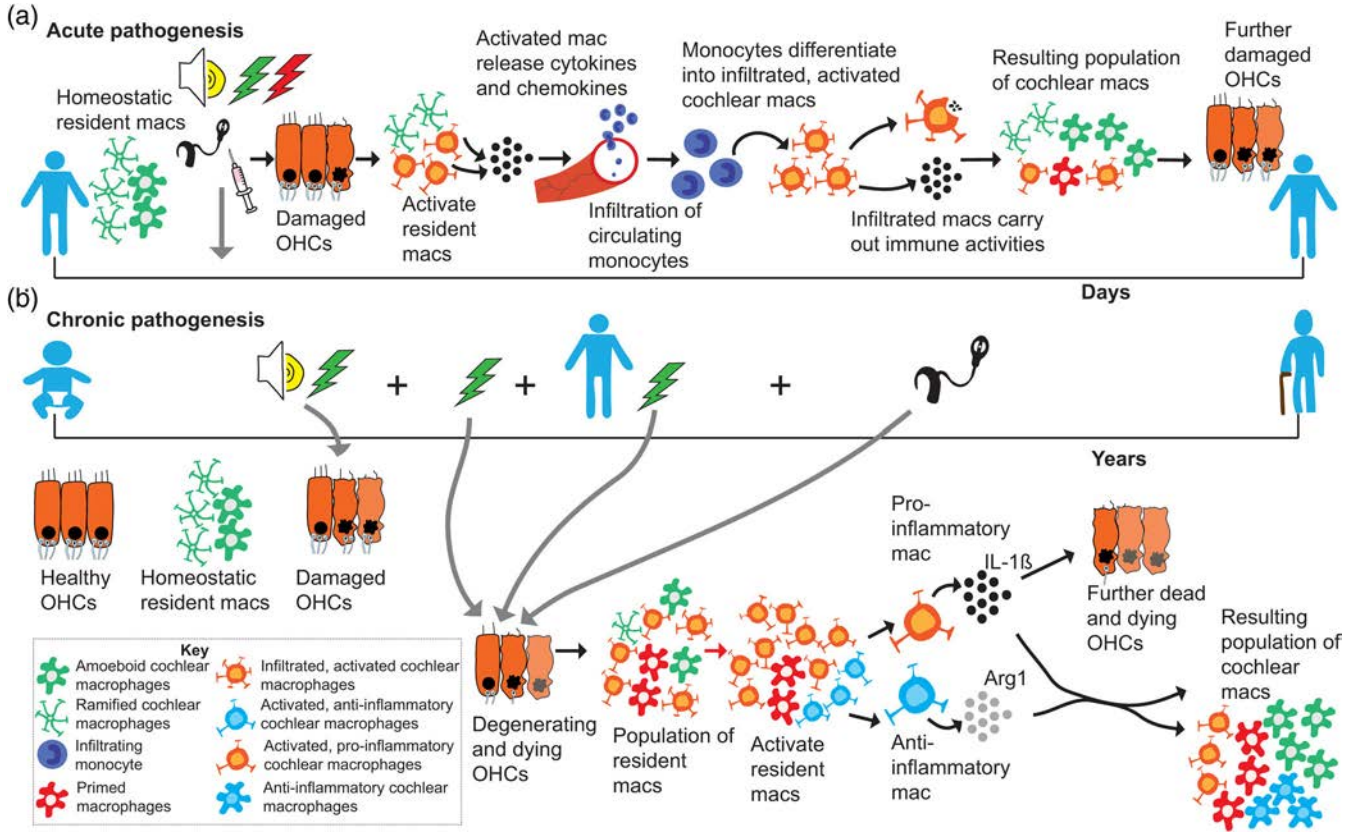


FIGURE 4 A role for macrophage activation in response to acute and chronic insults in the cochlea. A summary of the sequence of immune events that occur in the cochlea following (a) an acute insult and (b) how this may be altered in the context of chronic insults such as repeated noise exposure or age-associated changes, if activated cochlear macrophages become primed. (a) Multiple studies have shown that an acute insult with sensory cell and tissue injury signals to resident cochlear macrophages (and cochlear fibrocytes) resulting in their activation. Activated, pro-inflammatory cochlear macrophages and fibrocytes release cytokines and chemokines which recruit circulating bone-marrow derived monocytes. On entry to the cochlea, the monocytes differentiate into activated macrophages. Further tissue damage may occur due to local release of mediators from these macrophages resulting in cellular dysfunction and hearing loss. Typically, a wave of inflammation is followed by induction of an anti-inflammatory response that results in resolution of the inflammatory response and tissue-remodeling. However, some macrophages may adopt an altered or primed phenotype in response to the initial activating insult. (b) Chronic, cumulative, progressive insults such as repeated noise exposure and/or age cause gradual sensory cell denervation and degeneration. This signals to activate resident cochlear macrophages to express a pro-inflammatory profile and secrete pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. This activation may result in a switch to a primed state, such that the macrophages develop a lower threshold (hyper-responsiveness) to subsequent insults, whereby they mount a more florid inflammatory response, with greater numbers of cells with greater associated tissue damage. Tissue damage in the auditory system is likely to manifest as a progressive loss of hearing function. The precise aspect of the function that is impaired reflects the site within the system that has been injured. References: (a) (Bas et al., 2015; Fuentes-Santamaria et al., 2017; Hirose et al., 2005; Kaur, Zamani, et al., 2015b; Okano et al., 2008; Shi, 2010; Yang et al., 2015) (b) (Frye et al., 2017, 2018; Noble et al., 2019; Zhang et al., 2020)

(ARHL) (Bainbridge et al., 2010; Nash et al., 2014; Simpson et al., 2013; Verschuur et al., 2014; Verschuur et al., 2012). Similarly, data from a longitudinal aging study showed that elevated white blood cell count was positively correlated with degree of hearing loss in older adults (Lassale et al., 2020). A small longitudinal population study from our own group (unpublished 57 adults aged 65–75 at time of recruitment) identified an association between increased activation of macrophages, as detected by the excretion of a metabolite (neopterin) and the likelihood of more marked low frequency hearing loss. The greater the number of times across a 12-month period neopterin was elevated above the basal rate for adults, the greater was the likelihood of more marked low frequency hearing loss 3–4 years later. Much like other organs, the immune system within the auditory system is exposed to multiple insults, such as the effects of co-morbidities across the lifespan. The combination of type, intensity, and temporal sequence of insults, or immunobiography, is unique for each individual (Franceschi et al., 2017; Haley et al., 2018; Netea et al., 2020).

5.2 | Cumulative effect of cochlear insults

The cumulative effect of cochlear insults is not well understood. Investigation of the interactions between noise-induced hearing loss (NIHL) and age-related hearing loss (ARHL) found that early life noise exposure made the cochlea more vulnerable to changes commonly

associated with aging (Kujawa & Liberman, 2006). A widespread loss of spiral ganglion cells (not associated with hair cell death) was seen in older mice that had been noise-exposed that was not evident in unexposed age-matched controls (Kujawa & Liberman, 2006). Evidence from a repeated noise model highlighted the effect of prior macrophage activation on the cochlear response to subsequent acoustic trauma (Zhang et al., 2020). In mice exposed to 120 dB SPL noise for 1 h then re-exposed 20 days later, a greater increase in macrophage populations in the osseous spiral lamina, luminal surface of the scala tympani, and underneath the basilar membrane was measured compared to cell numbers in those regions following only the first noise exposure (Zhang et al., 2020). Additionally, after the second noise exposure (or insult) there was a significant increase in the number of macrophages with an amoeboid morphology; suggestive of a transition to a more pro-inflammatory phenotype following an initial exposure. This is the first published study to closely examine the macrophage distribution, morphology, and phenotype following repeated insults alongside providing evidence for an exaggerated inflammatory response following a prior immune activation in the cochlea (Zhang et al., 2020). The mechanistic links between noise induced hearing loss (NIHL) and age-related hearing loss (ARHL) (Eckert et al., 2021; Mills et al., 1997) are not yet understood but macrophages and their ability to retain immunological memory are a putative link. This is supported by the evidence from the studies that identify the distribution and morphology of macrophages (summarized in Tables 1 and 3) and the immune response to acute and chronic tissue changes seen in NIHL, ARHL, and following cochlear implantation (Figure 4).

5.3 | Priming of tissue-resident macrophages

Microglia, once activated, can persist in a hyper-responsive, pro-inflammatory state making them more susceptible to a secondary inflammatory stimulus, which can trigger a subsequent exaggerated inflammatory response (Perry & Holmes, 2014). The nomenclature of the different activation states of microglia and macrophages is the subject of ongoing discussion in the literature (Haley et al., 2018; Neher & Cunningham, 2019; Perry & Teeling, 2013). The term "primed" is applied to microglia with an immunophenotype associated with memory of previous insults. There is overlap between *priming* and the term *trained*, which is used to describe this state in macrophages in other organ systems. Primed microglia typically show greater expression of inflammatory markers coupled with a lower threshold to switch to a pro-inflammatory state (Lull & Block, 2010). Once triggered, they exhibit an exaggerated inflammatory (with associated local tissue damage) response to immune activation (Lull & Block, 2010; Norden et al., 2015). Primed microglia have been described in models of aging, neurodegenerative disease, and traumatic brain injury (Cunningham et al., 2005; Kokiko-Cochran & Godbout, 2018; Lopez-Rodriguez et al., 2021; Neher & Cunningham, 2019). However, priming of microglia/macrophages in the auditory system has not been described in the literature, despite the similarities in macrophage responses described within the auditory system.

Similarities can be drawn between how macrophages/the immune system respond to insults across different organ systems. Traumatic brain injury (TBI) is defined as the functional disruption of the brain from an impact (blunt) or penetrating injury (Centers for Disease Control and Prevention, 2013). Typical pathological features of TBI include BBB disruption, neuronal and axonal injury, oedema, focal contusion and a widespread inflammatory response including microglial and astrocyte activation, infiltration of peripheral cells and increased production and release of inflammatory molecules (Puntambekar et al., 2018). Many of these pathological features are similar to that following an insult, such as exposure to loud noise, to the cochlea or elsewhere in the auditory pathway.

Figure 4 integrates the findings from key studies and the sequence of immune events that take place in the cochlea during acute and chronic pathogenesis. The presence of primed macrophages has been added based on the hypothesis proposed through this review. The timeline emphasizes the differences in time course between acute (across days) and chronic (across years/lifetime) pathogenesis. This allows for current and future studies to be put into context of where an experimental design sits in relation to the relative contribution of acute and chronic insults, the interaction between both, and the consequences of these insults on immune cell population and tissue damage.

5.4 | Proposing priming of cochlear macrophages

The timeline depicted in Figure 4b demonstrates that a single, acute insult (such as noise exposure) causes damage to OHCs. However, this is happening alongside the accumulation of multiple insults over time, leading to the development of chronic pathology, which involves the slow, progressive degeneration of the sensory cells and the activation of the long-lived resident cochlear macrophages. Based on the understanding of glial biology from other systems, we propose that some of the macrophages involved in this response are likely to have already been exposed to an immunological insult, or been trained, and have therefore adopted a primed, or more responsive state. Upon activation through further cochlear insults or through chronic inflammation associated with aging and age-associated morbidities (which are also risk factors for hearing loss), these macrophages may exhibit an exaggerated inflammatory response and exacerbate cochlear damage and manifest as progressive hearing loss (as depicted through the schematic in Figure 5).

6 | DO MACROPHAGES INFLUENCE THE TRAJECTORY OF HEARING LOSS?

Macrophage phenotype including morphology, expression of markers and the signals they produce and release, the way in which macrophages behave and how they respond to inflammatory insults, play a significant role in both normal and pathological auditory function across a lifetime. Insults, or injury to the cochlea, such as noise

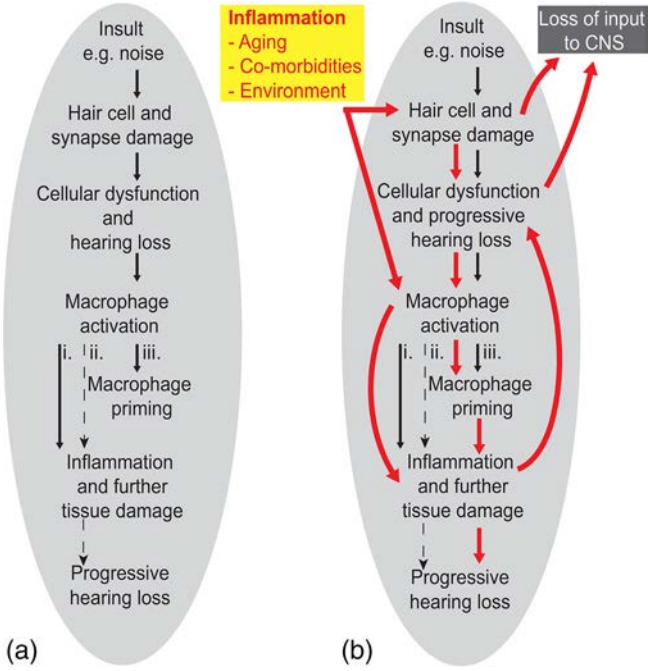


FIGURE 5 Schematic of the hypothesis proposing the role for cochlear macrophages in contributing to the pathology of hearing loss. (a) An insult to the auditory system such as noise overexposure, causes hair cell and synapse damage in the cochlea which results in cellular dysfunction and hearing loss. Damaged sensory cells release mediators which recruit and activate resident cochlear macrophages (i). Activated macrophages will adopt a pro-inflammatory or anti-inflammatory phenotype. Pro-inflammatory macrophages produce and release pro-inflammatory cytokines and chemokines, resulting in inflammation and cellular damage or apoptosis. (ii). Some macrophages may already be in a pro-inflammatory state from a previous insult (primed), therefore upon activation display an exaggerated inflammatory response resulting in further inflammation and damage to tissues in the local area, which will contribute to progressive hearing loss. (iii). Some macrophages may switch to a primed phenotype as a result of this insult. (b) Additional inflammation due to aging, co-morbidities or environmental factors results in further hair cell and synapse damage (and subsequent progressive hearing loss) and further macrophage activation. (iii). Macrophages in an active, pro-inflammatory state as a result of the previous noise exposure may be primed and therefore display an exaggerated inflammatory response to the additional inflammation, causing further tissue damage and progressive hearing loss. Sensory cell and tissue damage and the resulting loss of function of essential cells in the cochlea, causes loss of input to the central auditory pathways. Decreased activation of the ascending and descending central auditory pathway leads to dysfunctional auditory processing

exposure, the chronic deterioration of the auditory system that occurs with aging (Jafari et al., 2019) and conditions causing systemic inflammation are associated with a greater degree of hearing loss and the evidence suggests that the accumulation of insults such as early noise exposure plus overlaid on the effects of aging (Kujawa & Liberman, 2006) or repeated noise exposure (Zhang et al., 2020) may exacerbate this hearing loss. Despite this, the mechanisms responsible for the pathology causing hearing loss are not well understood although inflammation and cochlear macrophages are involved in its pathogenesis.

6.1 | Evidence of priming/innate immune memory in models of disease/aging

Based on the understanding from the fundamental studies discussed in this review and our wider understanding of how macrophages respond to inflammatory insults in chronic conditions such as traumatic brain injury (TBI), Alzheimer's disease (AD), and age-associated changes, we hypothesize that innate immune memory and the priming of cochlear macrophages and microglia in the auditory pathway influences the trajectory of hearing loss.

Priming was first observed in peritoneal macrophages exposed to lipopolysaccharide (LPS). Isolated macrophages produced increased IL-1 and inducible nitric oxide synthase (iNOS) (relative to non-primed macrophages) if the cells were pre-exposed to or 'primed by' interferon gamma (Pace et al., 1983). A study by Cunningham et al demonstrated this priming effect in microglia, where an enhanced immune response in the ME7 prion mouse model of chronic neurodegeneration was measured after administration of LPS. Either direct challenge of the CNS with LPS, or a systemic challenge with LPS, resulted in increased microglial activation and greater inflammatory response in the prion mice compared to wild-type animals (Cunningham et al., 2005). Similar observations have been described in models of aging (Godbout et al., 2005), Wallerian (axonal) degeneration (Palin et al., 2008) and Alzheimer's disease (Holtman et al., 2015; Lopez-Rodriguez et al., 2021).

The proposed interaction between chronic immune state and new acute insults to the auditory system is supported by evidence that the inflammatory response to TBI is influenced by previous and subsequent inflammatory challenges (Kokiko-Cochran & Godbout, 2018), potentially through the mechanism of microglial priming. To date, there are no effective interventions to reliably improve outcome after TBI. A variety of TBI models are being used to investigate the mechanisms and pathology, however, due to the different types and the complexity of TBI, and the array of primary and secondary damage which occurs in human TBI, no single model fully recapitulates all aspects of human TBI. Despite the complexity of understanding these overlapping but distinct mechanisms, all TBI models show the consistent involvement of a microglia/macrophage inflammatory response highlighting these cells as critical mediators in the outcome.

6.2 | Utilizing the auditory system to investigate macrophage biology

Noise injury at a specific volume and frequency can be a traumatic injury to the cochlea and auditory system resulting in a measurable loss of function, which is experimentally reproducible (Fuentes-Santamaria et al., 2017; Kurabi et al., 2017; Zhang et al., 2020). Using a frequency-specific noise to create an injury in the cochlea, it is

possible to reliably map the injury site, due to tonotopy of the cochlea. While with noise injury there is a small frequency shift, this shift is reproducible (Quraishe et al., 2019). The resulting tissue damage and the local cellular response from the insult, in and around the area of damage, can be investigated. Functional readouts following the injury can be obtained to determine the severity of damage to the auditory system and any subsequent additional (or progressive) hearing loss. Significantly, there is also the option to study the effect of a loss and re-establishment of function in the auditory system through the use of hearing aids or cochlear implants. Therefore, utilizing the auditory system to investigate macrophage/microglial priming may be beneficial to inform how these cells behave in other pathologies/diseases.

6.3 | Can macrophages contribute to the individual trajectory of hearing loss through priming?

The evidence is that inflammatory insults induce innate immune memory (trained immunity) and that exposure to insults over time establishes an individual's immunobiography. This influences how they respond to further immune insults across their lifespan. In terms of the auditory system, the integration of immunobiography, trained immunity, and inflammaging may explain why individuals exhibit heterogeneity in their hearing-loss trajectory as they go through life, including both their natural hearing alone or with the addition of a hearing aid or cochlear implant. Macrophage priming/training may occur if the cell has been previously activated and returned to a quiescent state but retained a 'memory' of the event so that it produces exaggerated acute responses to subsequent challenges. Persistent cochlear inflammation may result in a chronic state of activation of macrophages, resulting in a persisting phenotype that is primed. A better understanding of the mechanisms by which trained immunity may contribute to chronic inflammaging with aging, and what factors may exacerbate cochlear inflammation, may enable lifestyle or pharmaceutical interventions to slow down the decline in hearing function with age.

7 | CONCLUSION

This review synthesizes the current knowledge of the distribution, phenotype, and function of macrophages under both homeostatic and pathological conditions in the cochlea. Following an acute insult, which can lead to sensory cell and synaptic damage, cochlear macrophages become activated and initiate a typical immune response involving both pro-inflammatory and anti-inflammatory signaling. The macrophage response and molecular mediators involved in their function following progressive, cumulative insults over time needs further investigation. We propose, based on the evidence from other organs in the body, that macrophages contribute to the progressive loss of hearing experienced by many people worldwide and to the variability in hearing function that exists between individuals. Out of necessity, and reflecting the field, much of the evidence is from studies in mice though we have included human data where available. Greater focus on fundamental macrophage biology in the human auditory system will enable advances in understanding of the mechanisms involved in hearing and therefore how to target these mechanisms to improve hearing outcomes for people with hearing loss.

ACKNOWLEDGMENTS

EPSRC for PhD studentship funding and additional support from Oticon Medical to K Hough.

DATA AVAILABILITY STATEMENT

The majority of the data referred to in the manuscript is already in the public domain, tables that have been assembled during the process of the detailed review of the literature have been included. The data referred to in the unpublished study from our group describing evidence of macrophage activation being associated with hearing loss is being prepared for publication.

ORCID

Kate Hough https://orcid.org/0000-0002-5160-2517

REFERENCES

Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W., & Rossi, F. M. V. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature Neuroscience*, *10*(12), 1538–1543.

Askew, K., Li, K., Olmos-Alonso, A., Garcia-Moreno, F., Liang, Y., Richardson, P., Tipton, T., Chapman, M. A., Riecken, K., Beccari, S., Sierra, A., Molnár, Z., Cragg, M. S., Garaschuk, O., Perry, V. H., & Gomez-Nicola, D. (2017). Coupled proliferation and apoptosis maintain the rapid turnover of microglia in the adult brain. *Cell Reports*, *18*(2), 391–405. https://doi.org/10.1016/j.celrep.2016.12.041

Bainbridge, K. E., Cheng, Y. J., & Cowie, C. C. (2010). Potential mediators of diabetes-related hearing impairment in the U.S. population. *Diabetes Care*, *33*(4), 811–816. https://doi.org/10.2337/dc09-1193

Bas, E., Goncalves, S., Adams, M., Dinh, C. T., Bas, J. M., Van De Water, T. R., & Eshraghi, A. A. (2015). Spiral ganglion cells and macrophages initiate neuro-inflammation and scarring following cochlear implantation. *Frontiers in Cellular Neuroscience*, *9*(303), 1–16. https://doi.org/10.3389/fncel.2015.00303

Bennett, M. L., Bennett, F. C., Liddelow, S. A., Ajami, B., Zamanian, J. L., Fernhoff, N. B., Mulinyawe, S. B., Bohlen, C. J., Adil, A., Tucker, A., Weissman, I. L., Chang, E. F., Li, G., Grant, G. A., Hayden Gephart, M. G., & Barres, B. A. (2016). New tools for studying microglia in the mouse and human CNS. *Proceedings of the National Academy of Sciences*, *113*(12), E1738–E1746. https://doi.org/10.1073/pnas.1525528113

Bohne, B. A., & Harding, G. W. (2000). Degeneration in the cochlea after noise damage: Primary versus secondary events. *The American Journal of Otology*, *21*(4), 505–509.

Centers for Disease Control and Prevention. (2013). CDC grand rounds: Reducing severe traumatic brain injury in. *Morbidity and Mortality Weekly Report*, *62*(27), 549–552.

Chen, P., Chai, Y., Liu, H., Li, G., Wang, L., Yang, T., & Wu, H. (2018). Postnatal development of microglia-like cells in mouse cochlea. *Neural Plasticity*, *2018*, 10–15.

Crotti, A., & Ransohoff, R. M. (2016). Microglial physiology and pathophysiology: Insights from genome-wide transcriptional profiling. *Immunity*, *44*(3), 505–515.

Cunningham, C., Wilcockson, D. C., Campion, S., Lunnon, K., & Perry, V. H. (2005). Central and systemic endotoxin challenges exacerbate the

local inflammatory response and increase neuronal death during chronic neurodegeneration. *Journal of Neuroscience*, *25*(40), 9275–9284.

Cunningham, L., & Tucci, D. (2017). Hearing loss in adults. *New England Journal of Medicine*, *377*(25), 2465–2473.

Davies, L. C., & Taylor, P. R. (2015). Tissue-resident macrophages: Then and now. *Immunology*, *144*(4), 541–548.

Dong, Y., Zhang, C., Frye, M., Yang, W., Ding, D., Sharma, A., Guo, W., & Hu, B. H. (2018). Differential fates of tissue macrophages in the cochlea during postnatal development. *Hearing Research*, *365*, 1–17.

Eckert, M. A., Harris, K. C., Lang, H., Lewis, M. A., Schmiedt, R. A., Schulte, B. A., Steel, K. P., Vaden, K. I., & Dubno, J. R. (2021). Translational and interdisciplinary insights into presbyacusis: A multidimensional disease. *Hearing Research*, *402*, 108109.

Faraco, G., Park, L., Anrather, J., & Iadecola, C. (2017). Brain perivascular macrophages: Characterization and functional roles in health and disease. *Journal of Molecular Medicine*, *95*(11), 1143–1152.

Farhadi, M., Jalessi, M., Salehian, P., Ghavi, F. F., Emamjomeh, H., Mirzadeh, H., Imani, M., & Jolly, C. (2013). Dexamethasone eluting cochlear implant: Histological study in animal model. *Cochlear Implants International*, *14*(1), 45–50.

Fernandez, K. A., Jeffers, P. W. C., Lall, K., Liberman, M. C., & Kujawa, S. G. (2015). Aging after noise exposure: Acceleration of cochlear synaptopathy in "recovered" ears. *Journal of Neuroscience*, *35*(19), 7509–7520.

Franceschi, C., Bonafe, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., & De Benedicts, G. (2006). Inflamm-aging: An evolutionary perspective on Immunosenescence. *Annals of the New York Academy of Sciences*, *908*(1), 244–254.

Franceschi, C., Salvioli, S., Garagnani, P., De Eguileor, M., Monti, D., & Capri, M. (2017). Immunobiography and the heterogeneity of immune responses in the elderly: A focus on inflammaging and trained immunity. *Frontiers in Immunology*, *8*, 1–11.

Frye, M. D., Yang, W., Zhang, C., Xiong, B., & Hu, B. H. (2017). Dynamic activation of basilar membrane macrophages in response to chronic sensory cell degeneration in aging mouse cochleae. *Hearing Research*, *344*, 125–134.

Frye, M. D., Zhang, C., & Hu, B. H. (2018). Lower level noise exposure that produces only TTS modulates the immune homeostasis of cochlear macrophages. *Journal of Neuroimmunology*, *323*, 152–166.

Fuentes-Santamaria, V., Alvarado, J. C., Melgar-Rojas, P., Gabaldón-Ull, M. C., Miller, J. M., & Juiz, J. M. (2017). The role of glia in the peripheral and central auditory system following noise overexposure: Contribution of TNF-α and IL-1β to the pathogenesis of hearing loss. *Frontiers in Neuroanatomy*, *11*, 1–26.

Fujioka, M., Kanzaki, S., Okano, H. J., Masuda, M., Ogawa, K., & Okano, H. (2006). Proinflammatory cytokines expression in noise-induced damaged cochlea. *Journal of Neuroscience Research*, *83*, 575–583.

Galea, I., Felton, L. M., Waters, S., Van Rooijen, N., Perry, V. H., & Newman, T. A. (2008). Immune-to-brain signalling: The role of cerebral CD163-positive macrophages. *Neuroscience Letters*, *448*(1), 41–46.

Galea, I., & Perry, V. H. (2018). The blood-brain interface: A culture change. *Brain Behavior and Immunity*, *68*, 11–16.

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M. F., Conway, S. J., Ng, L. G., Stanley, E. R., Samokhvalov, I. M., & Merad, M. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science*, *330*, 841–845.

Ginhoux, F., & Guilliams, M. (2016). Tissue-resident macrophage ontogeny and homeostasis. *Immunity*, *44*, 439–449.

Glueckert, R., Pfaller, K., Kinnefors, A., Rask-Andersen, H., & Schrott-Fischer, A. (2005). The human spiral ganglion: New insights into ultrastructure, survival rate and implications for cochlear implants. *Audiology and Neurotology*, *10*(5), 258–273.

Godbout, J. P., Chen, J., Abraham, J., Richwine, A. F., Berg, B. M., Kelley, K. W., & Johnson, R. W. (2005). Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system. *FASEB Journal*, *19*(10), 1329–1331.

Goldmann, T., Wieghofer, P., Jordão, M. J. C., Prutek, F., Hagemeyer, N., Frenzel, K., Amann, L., Staszewski, O., Kierdorf, K., Krueger, M., Locatelli, G., Hochgerner, H., Zeiser, R., Epelman, S., Geissmann, F., Priller, J., Rossi, F. M. V., Bechmann, I., Kerschensteiner, M., … Prinz, M. (2016). Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nature Immunology*, *17*(7), 797–805.

Gruver, A., Hudson, L., & Sempowski, G. (2007). Immunosenescence of ageing. *Journal of Pathology*, *211*, 144–156.

Haley, M. J., Brough, D., Quintin, J., & Allan, S. M. (2018). Microglial priming as trained immunity in the brain. *Neuroscience*, *405*, 47–54.

Hart, A. D., Wyttenbach, A., Hugh Perry, V., & Teeling, J. L. (2012). Age related changes in microglial phenotype vary between CNS regions: Grey versus white matter differences. *Brain, Behavior, and Immunity*, *26*(5), 754–765.

Hirose, K., Discolo, C. M., Keasler, J. R., & Ransohoff, R. (2005). Mononuclear phagocytes migrate into the murine cochlea after acoustic trauma. *Journal of Comparative Neurology*, *489*(2), 180–194.

Hoeffel, G., & Ginhoux, F. (2018). Fetal monocytes and the origins of tissue-resident macrophages. *Cellular Immunology*, *330*, 5–15.

Holtman, I. R., Raj, D. D., Miller, J. A., Schaafsma, W., Yin, Z., Brouwer, N., Wes, P. D., Möller, T., Orre, M., Kamphuis, W., Hol, E. M., Boddeke, E. W. G. M., & Eggen, B. J. L. (2015). Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: A co-expression meta-analysis. *Acta Neuropathologica Communications*, *3*, 31.

Hough, K., Sanderson, A., Grasmeder, M., Mitchell, T., Verschuur, C., & Newman, T. (2021). Inflammation at the tissue-electrode interface in a case of rapid deterioration in hearing performance leading to explant after cochlear implantation. *Otology & Neurotology*, *42*, e445–e450.

Jafari, Z., Kolb, B. E., & Mohajerani, M. H. (2019). Age-related hearing loss and tinnitus, dementia risk, and auditory amplification outcomes. *Ageing Research Reviews*, *56*, 100963.

Juhn, S. K., & Rybak, L. P. (1981). Labyrinthine barriers and Cochlear homeostasis. *Acta Oto-Laryngologica*, *91*(1–6), 529–534.

Kalinec, G. M., Lomberk, G., Urrutia, R. A., & Kalinec, F. (2017). Resolution of Cochlear inflammation: Novel target for preventing or ameliorating drug-, noise- and age-related hearing loss. *Frontiers in Cellular Neuroscience*, *11*(192), 1–24.

Kaur, T., Clayman, A. C., Nash, A. J., Schrader, A. D., Warchol, M. E., & Ohlemiller, K. K. (2019). Lack of fractalkine receptor on macrophages impairs spontaneous recovery of ribbon synapses after moderate noise trauma in C57BL/6 mice. *Frontiers in Neuroscience*, *13*, 1–16.

Kaur, T., Hirose, K., Rubel, E. W., & Warchol, M. E. (2015a). Macrophage recruitment and epithelial repair following hair cell injury in the mouse utricle. *Frontiers in Cellular Neuroscience*, *9*, 1–9.

Kaur, T., Ohlemiller, K. K., & Warchol, M. E. (2018). Genetic disruption of fractalkine signaling leads to enhanced loss of cochlear afferents following ototoxic or acoustic injury. *Journal of Comparative Neurology*, *526*(5), 824–835.

Kaur, T., Zamani, D., Tong, L., Rubel, E. W., Ohlemiller, K. K., Hirose, K., & Warchol, M. E. (2015b). Fractalkine signaling regulates macrophage recruitment into the cochlea and promotes the survival of spiral ganglion neurons after selective hair cell lesion. *Journal of Neuroscience*, *35*(45), 15050–15061.

Keithley, E. M., Wang, X., & Barkdull, G. C. (2008). Tumor necrosis factor can induce recruitment of inflammatory cells to the cochlea. *Otology & Neurotology*, *29*(6), 854–859.

Keren-Shaul, H., Spinrad, A., Weiner, A., Colonna, M., Schwartz, M., Amit, I., Keren-shaul, H., Spinrad, A., Weiner, A., Matcovitch-natan, O., & Dvir-szternfeld, R. (2017). A unique microglia type

associated with restricting development of Alzheimer's disease. *Cell*, *169*(7), 1–15.

Kishimoto, I., Okano, T., Nishimura, K., Motohashi, T., & Omori, K. (2019). Early development of resident macrophages in the mouse cochlea depends on yolk sac hematopoiesis. *Frontiers in Neurology*, *10*, 1–13.

Kokiko-Cochran, O. N., & Godbout, J. P. (2018). The inflammatory continuum of traumatic brain injury and Alzheimer's disease. *Frontiers in Immunology*, *9*(672), 1–18. https://doi.org/10.3389/fimmu.2018.00672

Krenkel, O., & Tacke, F. (2017). Liver macrophages in tissue homeostasis and disease. *Nature Reviews Immunology*, *17*(5), 306–321. https://doi.org/10.1038/nri.2017.11

Kujawa, S. G., & Liberman, M. C. (2006). Acceleration of age-related hearing loss by early noise exposure: Evidence of a misspent youth. *Journal of Neuroscience*, *26*(7), 2115–2123. https://doi.org/10.1523/JNEUROSCI.4985-05.2006

Kujawa, S. G., & Liberman, M. C. (2009). Adding insult to injury: Cochlear nerve degeneration after "temporary" noise-induced hearing loss. *Journal of Neuroscience*, *29*(45), 14077–14085. https://doi.org/10.1523/JNEUROSCI.2845-09.2009

Kurabi, A., Keithley, E. M., Housley, G. D., Ryan, A. F., & Wong, A. C. Y. (2017). Cellular mechanisms of noise-induced hearing loss. *Hearing Research*, *349*, 129–137. https://doi.org/10.1016/j.heares.2016.11.013

Lang, H., Ebihara, Y., Schmiedt, R. A., Minamiguchi, H., Zhou, D., Smythe, N., Liu, L., Ogawa, M., Schulte, B. A., & Carolina, S. (2006). Contribution of bone marrow hematopoietic stem cells to adult mouse inner ear: Mesenchymal cells and Fibrocytes. *The Journal of Comparative Neurology*, *496*, 187–201. https://doi.org/10.1002/cne.20929

Lapenna, A., De Palma, M., & Lewis, C. E. (2018). Perivascular macrophages in health and disease. *Nature Reviews Immunology*, *18*(11), 689–702. https://doi.org/10.1038/s41577-018-0056-9

Lassale, C., Vullo, P., Cadar, D., Batty, G. D., Steptoe, A., & Zaninotto, P. (2020). Association of inflammatory markers with hearing impairment: The English longitudinal study of ageing. *Brain, Behavior, and Immunity*, *83*, 112–119. https://doi.org/10.1016/j.bbi.2019.09.020

Lawson, L. J., Perry, V. H., & Gordon, S. (1992). Turnover of resident microglia in the normal adult mouse brain. *Neuroscience*, *48*(2), 405–415.

Li, Q., & Barres, B. A. (2018). Microglia and macrophages in brain homeostasis and disease. *Nature Reviews Immunology*, *18*(4), 225–242. https://doi.org/10.1038/nri.2017.125

Liu, W., Molnar, M., Garnham, C., Benav, H., & Rask-Andersen, H. (2018). Macrophages in the human cochlea: Saviors or predators—A study using super-resolution immunohistochemistry. *Frontiers in Immunology*, *9*, 1–16. https://doi.org/10.3389/fimmu.2018.00223

Liu, W., Nordström, C. K., Danckwardt-Lilliestöm, N., & Rask-Andersen, H. (2019). Human inner ear immune activity: A super-resolution immunohistochemistry study. *Frontiers in Neurology*, *10*, 1–14. https://doi.org/10.3389/fneur.2019.00728

Lopez-Rodriguez, A. B., Hennessy, E., Murray, C. L., Nazmi, A., Delaney, H. J., Healy, D., Fagan, S. G., Rooney, M., Stewart, E., Lewis, A., De Barra, N., Scarry, P., Riggs-miller, L., Boche, D., Cunningham, M. O., & Cunningham, C. (2021). Acute systemic inflammation exacerbates neuroinflammation in Alzheimer's disease: IL-1β drives amplified responses in primed astrocytes and neuronal network dysfunction. *Alzheimer's & Dementia*, 1–21. https://doi.org/10.1002/alz.12341

Lull, M. E., & Block, M. L. (2010). Microglial activation and chronic neurodegeneration. *Neurotherapeutics*, *7*(4), 354–365. https://doi.org/10.1016/j.nurt.2010.05.014

Ma, C. L., Billings, P., Harris, J. P., & Keithley, E. M. (2000). Characterization of an experimentally induced inner ear immune response. *Laryngoscope*, *110*(3 I), 451–456.

Mills, J. H., Boettcher, F. A., & Dubno, J. R. (1997). Interaction of noise-induced permanent threshold shift and age-related threshold shift. *The Journal of the Acoustical Society of America*, *101*(3), 1681–1686.

Mittal, R., Nguyen, D., Patel, A. P., Debs, L. H., Mittal, J., Yan, D., Eshraghi, A. A., Van De Water, T. R., & Liu, X. Z. (2017). Recent advancements in the regeneration of auditory hair cells and hearing restoration. *Frontiers in Molecular Neuroscience*, *10*, 1–16. https://doi.org/10.3389/fnmol.2017.00236

Mu, X., Li, Y., & Fan, G. C. (2021). Tissue-resident macrophages in the Control of infection and resolution of inflammation. *Shock*, *55*(1), 14–23.

Nadol, J. B., Eddington, D. K., & Burgess, B. J. (2008). Foreign body or hypersensitivity granuloma of the inner ear after Cochlear implantation: One possible cause of a soft failure? *Otology and Neurotology*, *29*(8), 1076–1084.

Nadol, J. B., O'Malley, J. T., Burgess, B. J., & Galler, D. (2014). Cellular immunologic responses to cochlear implantation in the human. *Hearing Research*, *318*, 11–17.

Nash, S. D., Cruickshanks, K. J., Zhan, W., Tsai, M. Y., Klein, R., Chappell, R., Javier Nieto, F., Klein, B. E. K., Schubert, C. R., Dalton, D. S., & Tweed, T. S. (2014). Long-term assessment of systemic infammation and the cumulative incidence of age-related hearing impairment in the epidemiology of hearing loss study. *The Journals of Gerontology: Series A Biological Sciences and Medical Sciences*, *69 A*(2), 207–214.

Neher, J. J., & Cunningham, C. (2019). Priming microglia for innate immune memory in the brain. *Trends in Immunology*, *40*(4), 358–374. https://doi.org/10.1016/j.it.2019.02.001

Neng, L., Zhang, J., Yang, J., Zhang, F., Lopez, I. A., Dong, M., & Shi, X. (2015). Structural changes in the strial blood–labyrinth barrier of aged C57BL/6 mice. *Cell and Tissue Research*, *361*(3), 685–696.

Netea, M. G., Domínguez-Andrés, J., Barreiro, L. B., Chavakais, T., Divangahi, M., Fuchs, E., Joosten, L. A. B., Van Der Meer, J. W. M., Mhlanga, M. M., Willem, J. M., Riksen, N. P., Schlitzer, A., Schultze, J. L., Stabell Benn, C., Sun, J. C., Xavier, R. J., & Latz, E. (2020). Defining trained immunity and its role in health and disease. *Nature Reviews Immunology*, *20*, 1–17.

Noble, K. V., Liu, T., Matthews, L. J., Schulte, B. A., & Lang, H. (2019). Age-related changes in immune cells of the human cochlea. *Frontiers in Neurology*, *10*, 1–13.

Noonan, K. Y., Lopez, I. A., Ishiyama, G., & Ishiyama, A. (2020). Immune response of macrophage population to cochlear implantation: Cochlea immune cells. *Otology and Neurotology*, *41*(9), 1288–1295.

Norden, D. M., Muccigrosso, M. M., & Godbout, J. P. (2015). Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease. *Neuropharmacology*, *96*, 29–41.

O'Malley, J. T., Nadol, J. B., & Mckenna, M. J. (2016). Anti CD163+, Iba1+, and CD68+ cells in the adult human inner ear: Normal distribution of an unappreciated class of macrophages/microglia and implications for inflammatory otopathology in humans. *Otology & Neurotology*, *14*, 99–108.

Okano, T., Nakagawa, T., Kita, T., Kada, S., Yoshimoto, M., Nakahata, T., & Ito, J. (2008). Bone marrow-derived cells expressing Iba1 are constitutively present as resident tissue macrophages in the mouse cochlea. *Journal of Neuroscience Research*, *86*(8), 1758–1767.

Okayasu, T., Quesnel, A. M., O'Malley, J. T., Kamakura, T., & Nadol, J. B. (2020). The distribution and prevalence of macrophages in the cochlea following Cochlear implantation in the human: An Immunohistochemical study using anti-Iba1 antibody. *Otology and Neurotology*, *41*(3), e304–e316.

Pace, J. L., Russell, S. W., Torres, B. A., Johnson, H. M., & Gray, P. W. (1983). Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing. *The Journal of Immunology*, *130*(5), 2011–2013.

Palin, K., Cunningham, C., Forse, P., Perry, V. H., & Platt, N. (2008). Systemic inflammation switches the inflammatory cytokine profile in CNS Wallerian degeneration. *Neurobiology of Disease*, *30*(1), 19–29.

Perry, V. H., & Holmes, C. (2014). Microglial priming in neurodegenerative disease. *Nature Reviews Neurology*, *10*(4), 217–224.

Perry, V. H., & Teeling, J. (2013). Microglia and macrophages of the central nervous system: The contribution of microglia priming and systemic inflammation to chronic neurodegeneration. *Seminars in Immunopathology*, *35*, 601–612.

Puntambekar, S. S., Saber, M., Lamb, B. T., & Kokiko-Cochran, O. N. (2018). Cellular players that shape evolving pathology and neurodegeneration following traumatic brain injury. *Brain, Behavior, and Immunity*, *71*, 9–17.

Quraishe, S., Newman, T., & Anderson, L. (2019). Auditory temporal acuity improves with age in the male mouse auditory thalamus: A role for perineuronal nets? *Journal of Neuroscience Research*, *98*(9), 1780–1799.

Rai, V., Wood, M. B., Feng, H., Schabla, N. M., Tu, S., & Zuo, J. (2020). The immune response after noise damage in the cochlea is characterized by a heterogeneous mix of adaptive and innate immune cells. *Scientific Reports*, *10*(1), 1–17.

Raphael, Y., & Altschuler, R. A. (2003). Structure and innervation of the cochlea. *Brain Research Bulletin*, *60*(5–6), 397–422.

Ritzel, R. M., Patel, A. R., Grenier, J. M., Crapser, J., Verma, R., Jellison, E. R., & McCullough, L. D. (2015). Functional differences between microglia and monocytes after ischemic stroke. *Journal of Neuroinflammation*, *12*(106), 1–12.

Ryan, G. R., Dai, X. M., Dominguez, M. G., Tong, W., Chuan, F., Chisholm, O., Russell, R. G., Pollard, J. W., & Richard Stanley, E. (2001). Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1op/Csf1op) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood*, *98*(1), 74–84.

Sato, E., Shick, H. E., Ransohoff, R. M., & Hirose, K. (2008). Repopulation of Cochlear macrophages in murine hematopoietic progenitor cell chimeras: The role of CX3CR1. *The Journal of Comparative Neurology*, *506*, 930–942.

Sato, E., Shick, H. E., Ransohoff, R. M., & Hirose, K. (2010). Expression of fractalkine receptor CX3CR1 on cochlear macrophages influences survival of hair cells following ototoxic injury. *Journal of the Association for Research in Otolaryngology*, *11*(2), 223–234.

Satoh, J., Kino, Y., Asahina, N., Takitani, M., Miyoshi, J., Ishida, T., & Saito, Y. (2016). TMEM119 marks a subset of microglia in the human brain. *Neuropathology*, *36*(1), 39–49. https://doi.org/10.1111/neup.12235

Sautter, N. B., Shick, E. H., Ransohoff, R. M., Charo, I. F., & Hirose, K. (2006). CC chemokine receptor 2 is protective against noise-induced hair cell death: Studies in CX3CR1+/GFP mice. *Journal of the Association for Research in Otolaryngology*, *7*(4), 361–372.

Sergeyenko, Y., Lall, K., Charles Liberman, M., & Kujawa, S. G. (2013). Age-related cochlear synaptopathy: An early-onset contributor to auditory functional decline. *Journal of Neuroscience*, *33*(34), 13686–13694.

Seyyedi, M., & Nadol, J. B. (2014). Intracochlear inflammatory response to cochlear implant electrodes in humans. *Otology and Neurotology*, *35*(9), 1545–1551.

Shi, X. (2010). Resident macrophages in the cochlear blood-labyrinth barrier and their renewal via migration of bone-marrow-derived cells. *Cell and Tissue Research*, *342*(1), 21–30.

Shu, Y., Li, W., Huang, M., Quan, Y. Z., Scheffer, D., Tian, C., Tao, Y., Liu, X., Hochedlinger, K., Indzhykulian, A. A., Wang, Z., Li, H., & Chen, Z. Y. (2019). Renewed proliferation in adult mouse cochlea and regeneration of hair cells. *Nature Communications*, *10*(1), 1–15.

Simpson, A. N., Matthews, L. J., & Dubno, J. R. (2013). Lipid and C-reactive protein levels as risk factors for hearing loss in older adults. *Otolaryngology-Head and Neck Surgery*, *148*(4), 664–670. https://doi.org/10.1177/0194599812473936

Su, Z., Xiong, H., Liu, Y., Pang, J., Lin, H., Zhang, W., & Zheng, Y. (2020). Transcriptomic analysis highlights cochlear inflammation associated with age-related hearing loss in C57BL/6 mice using next generation sequencing. *Peer J*, *8*, 1–18. https://doi.org/10.7717/peerj.9737

Tan, B. T. G., Lee, M. M. G., & Ruan, R. (2008). Bone marrow-derived cells that home to acoustic deafened cochlea preserved their hematopoietic identity. *Journal of Comparative Neurology*, *509*(2), 167–179. https://doi.org/10.1002/cne.21729

Tan, W. J. T., Thorne, P. R., & Vlajkovic, S. M. (2016). Characterisation of cochlear inflammation in mice following acute and chronic noise exposure. *Histochemistry and Cell Biology*, *146*(2), 219–230. https://doi.org/10.1007/s00418-016-1436-5

Tornabene, S. V., Sato, K., Pham, L., Billings, P., & Keithley, E. M. (2006). Immune cell recruitment following acoustic trauma. *Hearing Research*, *222*, 115–124. https://doi.org/10.1016/j.heares.2006.09.004

Varatharaj, A., & Galea, I. (2017). The blood-brain barrier in systemic inflammation. *Brain Behavior and Immunity*, *60*, 1–12. https://doi.org/10.1016/j.bbi.2016.03.010

Verschuur, C. A., Dowell, A., Syddall, H. E., Ntani, G., Simmonds, S. J., Baylis, D., Gale, C. R., Walsh, B., Cooper, C., Lord, J. M., & Aihie Sayer, A. (2012). Markers of inflammatory status are associated with hearing threshold in older people: Findings from the Hertfordshire ageing study. *Age and Ageing*, *41*(1), 92–97. https://doi.org/10.1093/ageing/afr140

Verschuur, C., Agyemang-Prempeh, A., & Newman, T. A. (2014). Inflammation is associated with a worsening of presbycusis: Evidence from the MRC national study of hearing. *International Journal of Audiology*, *53*(7), 469–475. https://doi.org/10.3109/14992027.2014.891057

Vethanayagam, R. R., Yang, W., Dong, Y., & Hu, B. H. (2016). Toll-like receptor 4 modulates the cochlear immune response to acoustic injury. *Cell Death and Disease*, *7*(6), 1–11. https://doi.org/10.1038/cddis.2016.156

Wakabayashi, K., Fujioka, M., Kanzaki, S., Okano, H. J. H., Shibata, S., Yamashita, D., Masuda, M., Mihara, M., Ohsugi, Y., Ogawa, K., & Okano, H. J. H. (2010). Blockade of interleukin-6 signaling suppressed cochlear inflammatory response and improved hearing impairment in noise-damaged mice cochlea. *Neuroscience Research*, *66*(4), 345–352. https://doi.org/10.1016/j.neures.2009.12.008

Wang, W., Zhang, L. S., Zinsmaier, A. K., Patterson, G., Leptich, E. J., Shoemaker, S. L., Yatskievych, T. A., Gibboni, R., Pace, E., Luo, H., Zhang, J., Yang, S., & Bao, S. (2019a). Neuroinflammation mediates noise-induced synaptic imbalance and tinnitus in rodent models. *PLoS Biology*, *17*(6), e3000307.

Wang, X., Truong, T., Billings, P. B., Harris, J. P., & Keithley, E. M. (2003). Blockage of immune-mediated inner ear damage by etanercept. *Otology & Neurotology*, *24*(10), 52–57.

Wang, Y., Hirose, K., & Liberman, M. C. (2002). Dynamics of noise-induced cellular injury and repair in the mouse cochlea. *Journal of the Association for Research in Otolaryngology*, *3*(3), 248–268.

Wang, Z., Koenig, A. L., Lavine, K. J., & Apte, R. S. (2019b). Macrophage plasticity and function in the eye and heart. *Trends in Immunology*, *40*(9), 825–841.

Watson, N., Ding, B., Zhu, X., & Frisina, R. D. (2017). Chronic inflammation—Inflammaging—In the ageing cochlea: A novel target for future presbycusis therapy. *Ageing Research Reviews*, *40*, 142–148.

World Health Organisation, W. (2020). Deafness and hearing loss

Yamasaki, R., Lu, H., Butovsky, O., Ohno, N., Rietsch, A. M., Cialic, R., Wu, P. M., Doykan, C. E., Lin, J., Cotleur, A. C., Kidd, G., Zorlu, M. M., Sun, N., Hu, W., Liu, L. P., Lee, J. C., Taylor, S. E., Uehlein, L., Dixon, D., … Ransohoff, R. M. (2014). Differential roles of microglia and monocytes in the inflamed central nervous system. *Journal of Experimental Medicine*, *211*(8), 1533–1549.

Yang, W., Vethanayagam, R. R., Dong, Y., Cai, Q., & Hu, B. H. (2015). Activation of the antigen presentation function of mononuclear phagocyte populations associated with the basilar membrane of the cochlea after acoustic overstimulation. *Neuroscience*, *303*, 1–15.

Zhang, C., Frye, M. D., Sun, W., Sharma, A., Manohar, S., Salvi, R., & Hu, B. H. (2020). New insights on repeated acoustic injury: Augmentation of cochlear susceptibility and inflammatory reaction resultant of prior acoustic injury. *Hearing Research*, *393*, 107996.

Zhang, C., Sun, W., Li, J., Xiong, B., Frye, M. D., Ding, D., Salvi, R., Kim, M. J., Someya, S., & Hu, B. H. (2017). Loss of sestrin 2 potentiates the early onset of age-related sensory cell degeneration in the cochlea. *Neuroscience*, *361*, 179–191. https://doi.org/10.1016/j.neuroscience.2017.08.015

Zhang, F., Dai, M., Neng, L., Zhang, J. H., Zhi, Z., Fridberger, A., & Shi, X. (2013). Perivascular macrophage-like melanocyte responsiveness to acoustic trauma-a salient feature of strial barrier associated hearing loss. *FASEB Journal*, *27*(9), 3730–3740. https://doi.org/10.1096/fj.13-232892

Zhang, W., Dai, M., Fridberger, A., Hassan, A., Degagne, J., Neng, L., Zhang, F., He, W., Ren, T., Trune, D., Auer, M., & Shi, X. (2012). Perivascular-resident macrophage-like melanocytes in the inner ear are essential for the integrity of the intrastrial fluid-blood barrier. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(26), 10388–10393. https://doi.org/10.1073/pnas.1205210109

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Hough, K., Verschuur, C. A., Cunningham, C., & Newman, T. A. (2021). Macrophages in the cochlea; an immunological link between risk factors and progressive hearing loss. *Glia*, 1–20. https://doi.org/10.1002/glia.24095

List of References

Aarhus, L. *et al.* (2015) 'Childhood otitis media: a cohort study with 30-year follow-up of hearing (the HUNT study)', *Ear and Hearing*, 36(3), pp. 302–308. doi: 10.1097/AUD.0000000000000118.

Aarhus, L., Homøe, P. and Engdahl, B. (2020) 'Otitis media in childhood and disease in adulthood: A 40-year follow-up study', *Ear and Hearing*, 41(1), pp. 67–71. doi: 10.1097/AUD.0000000000000729.

Action on Hearing Loss (2015) 'Hearing matters', *The Hearing Journal*. doi: 10.1097/01.hj.0000422322.88697.f3.

Adunka, O. and Kiefer, J. (2006) 'Impact of electrode insertion depth on intracochlear trauma', *Otolaryngology - Head and Neck Surgery*, 135(3), pp. 374–382. doi: 10.1016/j.otohns.2006.05.002.

Ajami, B. *et al.* (2007) 'Local self-renewal can sustain CNS microglia maintenance and function throughout adult life', *Nature Neuroscience*, 10(12), pp. 1538–1543. doi: 10.1038/nn2014.

Akkerman, A. E. *et al.* (2005) 'Analysis of under- and overprescribing of antibiotics in acute otitis media in general practice', *Journal of Antimicrobial Chemotherapy*, 56(3), pp. 569–574. doi: 10.1093/jac/dki257.

Alenzi, S. *et al.* (2021) 'Systematic review of postcochlear implant electrode migration: what is known?', *Otology & Neurotology*, 42(2), pp. 208–216. doi: 10.1097/MAO.0000000000002921.

Alexander, T. H. *et al.* (2009) 'Safety of high-dose corticosteroids for the treatment of autoimmune inner ear disease', *Otology & Neurotology*, 30(4), pp. 443–448. doi: 10.1097/MAO.0b013e3181a52773.

Allen, E. K. *et al.* (2013) 'A genome-wide association study of chronic otitis media with effusion and recurrent otitis media identifies a novel susceptibility locus on chromosome 2', *JARO - Journal of the Association for Research in Otolaryngology*, 14(6), pp. 791–800. doi: 10.1007/s10162-013-0411-2.

Anderson, J. M., Rodriguez, A. and Chang, D. T. (2008) 'Foreign body reaction to biomaterials', *Seminars in Immunology*, 20, pp. 86–100. doi: 10.1016/j.smim.2007.11.004.

Anthwal, N. and Thompson, H. (2016) 'The development of the mammalian outer and middle ear', *Journal of Anatomy*, 228(2), pp. 217–232. doi: 10.1111/joa.12344.

Arazna, M., Pruchniak, M. P. and Demkow, U. (2013) 'Neutrophil extracellular traps in bacterial infections: Strategies for escaping from killing', *Respiratory Physiology and Neurobiology*. Elsevier B.V., 187(1), pp. 74–77. doi: 10.1016/j.resp.2013.03.002.

Ars, B. *et al.* (2012) 'Insights in the physiology of the human mastoid: message to the surgeon', *Journal of International Advanced Otology*, 8(2), pp. 296–310.

Astolfi, L. *et al.* (2016) 'Cochlear implant and inflammation reaction: Safety study of a new steroid-eluting electrode', *Hearing Research*, 336, pp. 44–52. doi: 10.1016/j.heares.2016.04.005.

Baba, T. and Mukaida, N. (2014) 'Role of macrophage inflammatory protein (MIP)-1α/CCL3 in leukemogenesis', *Molecular and Cellular Oncology*, 1(1), pp. 1–5. doi: 10.4161/mco.29899.

Bailey, M. T. *et al.* (2020) 'Immunization with a biofilm-disrupting nontypeable haemophilus influenzae vaccine antigen did not alter the gut microbiome in chinchillas, unlike pral delivery of a broad-spectrum antibiotic commonly used for otitis media', *American Society for Microbiology*, 5(2), pp. 1–13. doi: 10.1128/msphere.00296-20.

Bainbridge, K. E., Cheng, Y. J. and Cowie, C. C. (2010) 'Potential mediators of diabetes-related hearing impairment in the U.S. population', *Diabetes Care*, 33(4), pp. 811–816. doi: 10.2337/dc09-1193.

Bakaletz, L. O. (2009) 'Chinchilla as a robust, reproducible and polymicrobial model of otitis media and its prevention', *Expert Review of Vaccines*, 8(8), pp. 1063–1082. doi: 10.1586/erv.09.63.

Bakaletz, L. O., DeMaria, T. F. and Lim, D. J. (1987) 'Phagocytosis and killing of bacteria by middle ear macrophages', *Archives of Otolaryngology--Head and Neck Surgery*, 113(2), pp. 138–144. doi: 10.1001/archotol.1987.01860020030007.

Balkany, T. J. *et al.* (2005) 'Cochlear implant soft failures consensus development conference statement', *Otology & Neurotology*, 26, pp. 815–818. doi: 10.1179/cim.2005.6.3.105.

Barron, C. L. *et al.* (2020) 'Identification of essential biofilm proteins in middle ear fluids of otitis media with effusion patients', *Laryngoscope*, 130(3), pp. 806–811. doi: 10.1002/lary.28011.

Bas, E. *et al.* (2015) 'Spiral ganglion cells and macrophages initiate neuro-inflammation and scarring following cochlear implantation', *Frontiers in Cellular Neuroscience*, 9(303), pp. 1–16. doi: 10.3389/fncel.2015.00303.

Bas, E. *et al.* (2016) 'Electrode array-eluted dexamethasone protects against electrode insertion trauma induced hearing and hair cell losses, damage to neural elements, increases in impedance

and fibrosis: A dose response study', *Hearing Research*, 337, pp. 12–24. doi: 10.1016/j.heares.2016.02.003.

Bassiouni, M. *et al.* (2019) 'Expression of the microglial marker TMEM119 in the postnatal and adult cochlea', in *Laryngorhinootologie*. New York. doi: 10.1055/s-0039-1686339.

Batuk, M. O. *et al.* (2019) 'Twenty years of experience in revision cochlear implant surgery: Signs that indicate the need for revision surgery to audiologists', *Journal of Laryngology and Otology*, 133(10), pp. 903–907. doi: 10.1017/S0022215119001919.

Beeson, P. (1946) 'Development of tolerance to typhoid bacterial pyrogen and its abolition by reticulo-endothelial blockade', *Proc Soc Exp Biol Med.*, 61, pp. 248–40.

Bellos, C. *et al.* (2014) 'Reconstruction of cochlea based on micro-CT and histological images of the human inner ear', *BioMed Research International*, 2014. doi: 10.1155/2014/485783.

Bennett, M. L. *et al.* (2016) 'New tools for studying microglia in the mouse and human CNS', *Proceedings of the National Academy of Sciences*, 113(12), pp. E1738–E1746. doi: 10.1073/pnas.1525528113.

Beyea, J. A. *et al.* (2016) 'Cochlear implants in adults: effects of age and duration of deafness on speech recognition', *Otology and Neurotology*, 37(9), pp. 1238–1245. doi: 10.1097/MAO.0000000000001162.

Bhutta, M. F. (2012) 'Mouse models of otitis media: strengths and limitations', *Otolaryngology - Head and Neck Surgery (United States)*, 147(4), pp. 611–614. doi: 10.1177/0194599812449986.

Bhutta, M. F. (2014) 'Epidemiology and pathogenesis of otitis media: construction of a phenotype landscape', *Audiology and Neurotology*, 19(3), pp. 210–223. doi: 10.1159/000358549.

Bhutta, M. F. (2015) 'Evolution and otitis media: a review, and a model to explain high prevalence in indigenous populations', *Human Biology*, 87(2), pp. 92–108. doi: doi: 10.13110/humanbiology.87.2.0092.

Bhutta, M. F., Lambie, J., *et al.* (2017) 'A mouse-to-man candidate gene study identifies association of chronic otitis media with the loci TGIF1 and FBXO11', *Scientific Reports*, 7(1), pp. 1–7. doi: 10.1038/s41598-017-12784-8.

Bhutta, M. F., Thornton, R. B., *et al.* (2017) 'Understanding the aetiology and resolution of chronic otitis media from animal and human studies', *Disease Models & Mechanisms*, 10(11), pp. 1289–1300. doi: 10.1242/dmm.029983.

Bhutta, M. F., Cheeseman, M. T. and Brown, S. D. M. (2014) 'Myringotomy in the Junbo mouse model of chronic otitis media alleviates inflammation and cellular hypoxia', *Laryngoscope*, 124(9). doi: 10.1002/lary.24698.

Biswas, S. K. and Lopez-Collazo, E. (2009) 'Endotoxin tolerance: new mechanisms, molecules and clinical significance', *Trends in Immunology*, 30(10), pp. 475–487. doi: 10.1016/j.it.2009.07.009.

Blamey, P. and Artieres, F. (2013) 'Factors affecting auditory performance of postlinguistically deaf adults using cochlear implants: an update with 2251 patients', *Audiology and Neurotology*, 18, pp. 36–47. doi: 10.1159/000343189.

Bluestone, C. D. and Doyle, W. J. (1988) 'Anatomy and physiology of eustachian tube and middle ear related to otitis media', *Journal of Allergy and Clinical Immunology*, 81(5), pp. 997–1003. doi: https://doi.org/10.1016/0091-6749(88)90168-6.

Bohne, B. A. and Harding, G. W. (2000) 'Degeneration in the cochlea after noise damage: primary versus secondary events.', *The American journal of otology*, 21(4), pp. 505–509.

Bourdoncle, M. *et al.* (2020) 'Analysis and management of cochlear implant explantation in adults', *European Annals of Otorhinolaryngology, Head and Neck Diseases*. Elsevier Masson SAS, 137(6), pp. 459–465. doi: 10.1016/j.anorl.2020.04.001.

Briggs, R. *et al.* (2020) 'Comparison of electrode impedance measures between a dexamethasone-eluting and standard Cochlear™ Contour Advance® electrode in adult cochlear implant recipients', *Hearing Research*. Elsevier B.V, 390, p. 107924. doi: 10.1016/j.heares.2020.107924.

Brinkmann, V. *et al.* (2004) 'Neutrophil extracellular traps kill bacteria', *Science*, 303(5663), pp. 1532–1535. doi: 10.1126/science.1092385.

Brown, K. D. *et al.* (2009) 'Incidence and indications for revision cochlear implant surgery in adults and children', *Laryngoscope*, 119, p. 152. doi: 10.1002/lary.20012.

Brown, L. N. *et al.* (2017) 'Macrophage-mediated glial cell elimination in the postnatal mouse cochlea', *Frontiers in Molecular Neuroscience*, 10(December), pp. 1–20. doi: 10.3389/fnmol.2017.00407.

Brown, S. D. M. and Hardisty, R. E. (2003) 'Mutagenesis strategies for identifying novel loci associated with disease phenotypes', *Seminars in Cell and Developmental Biology*, 14(1), pp. 19–24. doi: 10.1016/S1084-9521(02)00168-4.

Browning, G. *et al.* (2010) 'Grommets (ventilation tubes) for hearing loss associated with otitis

media with effusion in children', *Cochrane Database of Systematic Reviews*, 1(1), pp. 1–46. doi: 10.1002/14651858.CD001801.pub3.

Brummer, S. B. and Turner, M. J. (1977) 'Electrochemical considerations for safe electrical stimulation of the nervous system with platinum electrodes', *IEEE Transactions on Biomedical Engineering*, BME-24(1), pp. 59–63. doi: 10.1109/TBME.1977.326218.

Buchman, C. A., Copeland, B. J., *et al.* (2004) 'Cochlear implantation in children with inner ear malformations', *The Laryngoscope*, 114(3), pp. 205–214. doi: 10.1177/000348940611500309.

Buchman, C. A., Higgins, C. A., *et al.* (2004) 'Revision cochlear implant surgery in adult patients with suspected device malfunction', *Otology and Neurotology*, 25(4), pp. 504–510. doi: 10.1097/00129492-200407000-00018.

Büchner, A. *et al.* (2017) 'Investigation of the effect of cochlear implant electrode length on speech comprehension in quiet and noise compared with the results with users of electro-acoustic-stimulation, a retrospective analysis', *PLoS ONE*, 12(5), pp. 1–16. doi: 10.1371/journal.pone.0174900.

Bullen, A. *et al.* (2019) 'Localized disorganization of the cochlear inner hair cell synaptic region after noise exposure', *The Company of Biologists*. doi: 10.1242/bio.038547.

Busby, P., Plant, K. and Whitford, L. (2013) 'Electrode impedance in adults and children using the Nucleus 24 cochlear implant system', *Cochlear Implants International*, 3(2), pp. 87–103. doi: 10.1179/cim.2002.3.2.87.

Cai, Q. *et al.* (2014) 'Molecular profile of cochlear immunity in the resident cells of the organ of Corti', *Journal of neuroinflammation*, 11, p. 173. doi: 10.1186/s12974-014-0173-8.

Cai, T. and McPherson, B. (2017) 'Hearing loss in children with otitis media with effusion: a systematic review', *International Journal of Audiology*, 56(2), pp. 65–76. doi: 10.1080/14992027.2016.1250960.

Campagnola, P. J. and Dong, C. Y. (2011) 'Second harmonic generation microscopy: Principles and applications to disease diagnosis', *Laser and Photonics Reviews*, 5(1), pp. 13–26. doi: 10.1002/lpor.200910024.

Casselbrant, M. L. *et al.* (2003) 'Evidence-based otitis media', *Epidemiology*, 3, pp. 147–62.

Causon, A. (2015) *Contemporary biological factors in cochlear implantation and hearing preservation*. University of Southampton.

Causon, A., Verschuur, C. and Newman, T. A. (2015) 'A retrospective analysis of the contribution of reported factors in cochlear implantation on hearing preservation outcomes', *Otology & Neurotology*, 36(7), pp. 1137–45. doi: 10.1097/MAO.0000000000000753.

Causon, A., Verschuur, C. and Newman, T. a (2013) 'Trends in cochlear implant complications: implications for improving long-term outcomes.', *Otology & Neurotology*, 34(2), pp. 259–65. doi: 10.1097/MAO.0b013e31827d0943.

Centers for Disease Control and Prevention (CDC) (2013) 'CDC grand rounds: reducing severe traumatic brain Injury in the United States', *MMWR. Morbidity and mortality weekly report*, 62(27), pp. 549–552. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23842446%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4604945.

De Ceulaer, G. *et al.* (2003) 'Long-term evaluation of the effect of intracochlear steroid deposition on electrode impedance in cochlear implant patients', *Otology and Neurotology*, 24(5), pp. 769–774. doi: 10.1097/00129492-200309000-00014.

Chang, A. *et al.* (2009) 'Factors influencing the efficacy of round window dexamethasone protection of residual hearing post-cochlear implant surgery', *Hearing Research*. Elsevier B.V., 255(1–2), pp. 67–72. doi: 10.1016/j.heares.2009.05.010.

Chang, M. Y. *et al.* (2017) 'The effect of systemic steroid on hearing preservation after cochlear implantation via round window approach: a guinea pig model', *Otology and Neurotology*, 38(7), pp. 962–969. doi: 10.1097/MAO.0000000000001453.

Cheeseman, M. T. *et al.* (2011) 'HIF-VEGF pathways are critical for chronic otitis media in Junbo and Jeff mouse mutants', *PLoS Genetics*, 7(10). doi: 10.1371/journal.pgen.1002336.

Chen, C. Y., Halpin, C. and Rauch, S. D. (2003) 'Oral steroid treatment of sudden sensorineural hearing loss: A ten year retrospective analysis', *Otology and Neurotology*, 24(5), pp. 728–733. doi: 10.1097/00129492-200309000-00006.

Chen, P. *et al.* (2018) 'Postnatal development of microglia-like cells in mouse cochlea', *Neural Plasticity*, pp. 10–15. doi: 10.1155/2018/1970150.

Chen, X. *et al.* (2012) 'Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure', *Nature Protocols*. Nature Publishing Group, 7(4), pp. 654–669. doi: 10.1038/nprot.2012.009.

Chikar, J. A. *et al.* (2012) 'The use of a dual PEDOT and RGD-functionalized alginate hydrogel

coating to provide sustained drug delivery and improved cochlear implant function', *Biomaterials*, 33(7), pp. 1982–1990. doi: 10.1016/j.biomaterials.2011.11.052.

Cho, C. G. *et al.* (2016) 'Both canonical and non-canonical NF-κB activation contribute to the proliferative response of the middle ear mucosa during bacterial infection', *Innate Immunity*, 22(8), pp. 626–634. doi: 10.1177/1753425916668581.

Choi, J. *et al.* (2017) 'Electrode impedance fluctuations as a biomarker for inner ear pathology after cochlear implantation', *Otology and Neurotology*, 38(10), pp. 1433–1439. doi: 10.1097/MAO.0000000000001589.

Christo, S. N. *et al.* (2015) 'Innate immunity and biomaterials at the nexus: friends or foes', *BioMed Research International*, 2015. doi: 10.1155/2015/342304.

Chung, D. *et al.* (2010) 'Revision cochlear implant surgery in patients with suspected soft failures', *Otology and Neurotology*, 31(8), pp. 1194–1198. doi: 10.1097/MAO.0b013e3181f0c631.

Clark, G. M. *et al.* (1988) 'The histopathology of the human temporal bone and auditory central nervous system following cochlear implantation in a patient: Correlation with psychophysics and speech perception results', *Acta Oto-Laryngologica*, 105(S448), pp. 1–65. doi: 10.3109/00016488809098972.

Clark, G. M. *et al.* (1995) 'Cochlear implantation: osteoneogenesis, electrode-tissue impedance, and residual hearin', *Ann Otol Rhinol Laryngol Suppl.*, 166, pp. 40–42.

Clark, G. M. *et al.* (2014) 'Biomedical studies on temporal bones of the first multi-channel cochlear implant patient at the University of Melbourne', *Cochlear Implants International*, 15, pp. S1–S15. doi: 10.1179/1754762814Y.0000000087.

Claussen, A. D. *et al.* (2019) 'A mouse model of cochlear implantation with chronic electric stimulation', *PLoS ONE*, 14(4), pp. 1–23. doi: 10.1371/journal.pone.0215407.

Cochlear (2021) *The CI-DEX Study*. Available at: https://clinicaltrials.gov/ct2/show/NCT04750642 (Accessed: 12 August 2021).

Coelho, N. M. and McCulloch, C. A. (2016) 'Contribution of collagen adhesion receptors to tissue fibrosis', *Cell and Tissue Research*. Cell and Tissue Research, 365(3), pp. 521–538. doi: 10.1007/s00441-016-2440-8.

Connell, S. S. *et al.* (2008) 'Electrode migration after cochlear implantation', *Otology and Neurotology*, 29(2), pp. 156–159. doi: 10.1097/mao.0b013e318157f80b.

Consortium, M. G. S. (2002) 'Initial sequencing and comparative analysis of the mouse genome', *Nature*, 420. doi: 10.1038/nature01262.

Côté, M. *et al.* (2007) 'Cochlear reimplantation: Causes of failure, outcomes, and audiologic performance', *Laryngoscope*, 117(7), pp. 1225–1235. doi: 10.1097/MLG.0b013e31805c9a06.

Crompton, M. *et al.* (2017) 'A mutation in Nischarin causes otitis media via LIMK1 and NF-κB pathways', *PLOS Genetics*, 13(8). doi: 10.1371/journal.pgen.1006969.

Crotti, A. and Ransohoff, R. M. (2016) 'Microglial physiology and pathophysiology: insights from genome-wide transcriptional profiling', *Immunity*. Elsevier Inc., 44(3), pp. 505–515. doi: 10.1016/j.immuni.2016.02.013.

Cullington, H. (2021) *BCIG Annual UK Data Collection*. Available at: https://www.bcig.org.uk/wp-content/uploads/2021/08/2-8-21-BCIG-CI-data.pdf (Accessed: 19 July 2021).

Cunningham, C. *et al.* (2005) 'Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration', *Journal of Neuroscience*, 25(40), pp. 9275–9284. doi: 10.1523/JNEUROSCI.2614-05.2005.

Cureoglu, S. *et al.* (2004) 'Cochlear changes in chronic otitis media', *Laryngoscope*, 114(4), pp. 622–626. doi: 10.1097/00005537-200404000-00006.

Dahmani-Causse, M. *et al.* (2011) 'Morphologic examination of the temporal bone by cone beam computed tomography: Comparison with multislice helical computed tomography', *European Annals of Otorhinolaryngology, Head and Neck Diseases*. Elsevier Masson SAS, 128(5), pp. 230–235. doi: 10.1016/j.anorl.2011.02.016.

Dawes, P. *et al.* (2014) 'Cigarette smoking, passive smoking, alcohol consumption, and hearing loss', *JARO - Journal of the Association for Research in Otolaryngology*, 15(4), pp. 663–674. doi: 10.1007/s10162-014-0461-0.

Dawson, M. A. and Kouzarides, T. (2012) 'Cancer epigenetics: From mechanism to therapy', *Cell*. Elsevier Inc., 150(1), pp. 12–27. doi: 10.1016/j.cell.2012.06.013.

Dewan, K. K. *et al.* (2019) 'A model of chronic, transmissible otitis media in mice', *PLoS pathogens*, 15(4), p. e1007696. doi: 10.1371/journal.ppat.1007696.

Dietz, A. *et al.* (2016) 'Electrode migration after cochlear implant surgery: more common than expected?', *European Archives of Oto-Rhino-Laryngology*, 273(6), pp. 1411–1418. doi: 10.1007/s00405-015-3716-4.

Dong, Y. *et al.* (2018) 'Differential fates of tissue macrophages in the cochlea during postnatal development', *Hearing Research*, pp. 1–17. doi: 10.1016/j.heares.2018.05.010.

Donlan, R. M. (2002) 'Biofilms: microbial life on surfaces', *Emerging Infectious Diseases*, 8(9), pp. 881–890. doi: 10.3201/eid0809.020063.

Duan, Y. Y., Clark, G. M. and Cowan, R. S. C. (2004) 'A study of intra-cochlear electrodes and tissue interface by electrochemical impedance methods in vivo', *Biomaterials*, 25(17), pp. 3813–3828. doi: 10.1016/j.biomaterials.2003.09.107.

Dubey, S. P., Larawin, V. and Molumi, C. P. (2010) 'Intracranial spread of chronic middle ear suppuration', *American Journal of Otolaryngology - Head and Neck Medicine and Surgery*. Elsevier Inc., 31(2), pp. 73–77. doi: 10.1016/j.amjoto.2008.10.001.

Eastwood, H. *et al.* (2010) 'Round window delivery of dexamethasone ameliorates local and remote hearing loss produced by cochlear implantation into the second turn of the guinea pig cochlea', *Hearing Research*. Elsevier B.V., 265(1–2), pp. 25–29. doi: 10.1016/j.heares.2010.03.006.

Echteler, S. M. (1992) 'Developmental segregation in the efferent projections to auditory hair cells in the gerbil', *Proceedings of the National Academy of Sciences*, 89(4), pp. 6324–6327. doi: 10.1002/cne.10931.

Echteler, S. M., Magardino, T. and Rontal, M. (2005) 'Spatiotemporal patterns of neuronal programmed cell death during postnatal development of the gerbil cochlea', *Developmental Brain Research*, 157(2), pp. 192–200. doi: 10.1016/j.devbrainres.2005.04.004.

Echteler, S. M. and Nofsinger, Y. C. (2000) 'Development of ganglion cell topography in the postnatal cochlea', *Journal of Comparative Neurology*, 425(3), pp. 436–446. doi: 10.1002/1096-9861(20000925)425:3<436::AID-CNE8>3.0.CO;2-1.

EDA (2021) *Experimental Design Assistant: RRID:SCR_017019*. Available at: https://eda.nc3rs.org.uk.

Elfarnawany, M. *et al.* (2017) 'Micro-CT versus synchrotron radiation phase contrast imaging of human cochlea', *Journal of Microscopy*, 265(3), pp. 349–357. doi: 10.1111/jmi.12507.

Emmett, S. D. and Francis, H. W. (2015) 'The socioeconomic impact of hearing loss in U.S. adults', *Otology and Neurotology*, 36(3), pp. 545–550. doi: 10.1097/MAO.0000000000000562.

Eshraghi, A. A. *et al.* (2005) 'Pattern of hearing loss in a rat model of cochlear implantation trauma', *Otology and Neurotology*, 26(3), pp. 442–447. doi:

10.1097/01.mao.0000169791.53201.e1.

Eshraghi, A. A. *et al.* (2019) 'Otoprotection to implanted cochlea exposed to noise trauma with dexamethasone eluting electrode', *Frontiers in Cellular Neuroscience*, 13(November), pp. 1–11. doi: 10.3389/fncel.2019.00492.

Fang, L. *et al.* (2020) 'Analysis of inflammatory and homeostatic roles of tissue-resident macrophages in the progression of cholesteatoma by RNA-seq', *Immunological Investigations*. Taylor & Francis, 00(00), pp. 1–13. doi: 10.1080/08820139.2020.1781161.

Faraco, G. *et al.* (2017) 'Brain perivascular macrophages: characterization and functional roles in health and disease', *Journal of Molecular Medicine*. Journal of Molecular Medicine, 95(11), pp. 1143–1152. doi: 10.1007/s00109-017-1573-x.

Farhadi, M. *et al.* (2013) 'Dexamethasone eluting cochlear implant: histological study in animal model', *Cochlear Implants International*, 14(1), pp. 45–50. doi: 10.1179/1754762811Y.0000000024.

Fayad, J. N. and Linthicum, F. H. (2006) 'Multichannel cochlear implants: relation of histopathology to performance', *Laryngoscope*, 116(8), pp. 1310–1320. doi: 10.1097/01.mlg.0000227176.09500.28.

Fayad, J. N., Makarem, A. O. and Linthicum, F. H. (2009) 'Histopathologic assessment of fibrosis and new bone formation in implanted human temporal bones using 3D reconstruction', *Otolaryngology - Head and Neck Surgery*, 141(2), pp. 247–252. doi: 10.1016/j.otohns.2009.03.031.

Feng, S. *et al.* (2011) 'Matrix metalloproteinase-2 and -9 secreted by leukemic cells increase the permeability of blood-brain barrier by disrupting tight junction proteins', *PLoS ONE*, 6(8). doi: 10.1371/journal.pone.0020599.

Fernandez, K. A. *et al.* (2015) 'Aging after noise exposure: acceleration of cochlear synaptopathy in "recovered" ears', *Journal of Neuroscience*, 35(19), pp. 7509–7520. doi: 10.1523/JNEUROSCI.5138-14.2015.

Fetoni, A. R. *et al.* (2015) 'Cochlear injury and adaptive plasticity of the auditory cortex', *Frontiers in Aging Neuroscience*, 7(FEB), pp. 1–7. doi: 10.3389/fnagi.2015.00008.

Foggia, M. J., Quevedo, R. V. and Hansen, M. R. (2019) 'Intracochlear fibrosis and the foreign body response to cochlear implant biomaterials', *Laryngoscope Investigative Otolaryngology*, 4(6), pp. 678–683. doi: 10.1002/lio2.329.

Forséni, M. *et al.* (2001) 'Detection and localization of interleukin-6 in the rat middle ear during experimental acute otitis media, using mRNA in situ hybridization and immunohistochemistry', *International Journal of Pediatric Otorhinolaryngology*, 57(2), pp. 115–121. doi: 10.1016/S0165-5876(00)00444-4.

Forséni, M., Bagger-Sjobach, D. and Huhcrantz, M. (2001) 'A study of inflammatory mediators in the human tympanosclerotic middle ear', *Archives of Otolaryngology - Head and Neck Surgery*, 127(5), pp. 559–564. doi: 10.1001/archotol.127.5.559.

Foster, S. L., Hargreaves, D. C. and Medzhitov, R. (2007) 'Gene-specific control of inflammation by TLR-induced chromatin modifications', *Nature*, 447(7147), pp. 972–978. doi: 10.1038/nature05836.

Franceschi, C. *et al.* (2006) 'Inflamm-aging: An evolutionary perspective on immunosenescence', *Annals of the New York Academy of Sciences*, 908(1), pp. 244–254. doi: 10.1111/j.1749-6632.2000.tb06651.x.

Franceschi, C. *et al.* (2017) 'Immunobiography and the heterogeneity of immune responses in the elderly: A focus on inflammaging and trained immunity', *Frontiers in Immunology*, 8(AUG), pp. 1–11. doi: 10.3389/fimmu.2017.00982.

Franceschi, C. and Campisi, J. (2014) 'Chronic inflammation (Inflammaging) and its potential contribution to age-associated diseases', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 69, pp. S4–S9. doi: 10.1093/gerona/glu057.

Fransen, E. *et al.* (2008) 'Occupational noise, smoking, and a high body mass index are risk factors for age-related hearing impairment and moderate alcohol consumption is protective: A European population-based multicenter study', *JARO - Journal of the Association for Research in Otolaryngology*, 9(3), pp. 264–276. doi: 10.1007/s10162-008-0123-1.

Fredelius, L. and Rask-Andersen, H. (1990) 'The role of macrophages in the disposal of degeneration products within the organ of corti after acoustic overstimulation', *Acta Oto-Laryngologica*, 109(1–2), pp. 76–82. doi: 10.3109/00016489009107417.

del Fresno, C. *et al.* (2009) 'Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients', *The Journal of Immunology*, 182(10), pp. 6494–6507. doi: 10.4049/jimmunol.0803350.

Freudenberg, M. A. and Galanos, C. (1988) 'Induction of tolerance to lipopolysaccharide (LPS)-D-

galactosamine lethality by pretreatment with LPS is mediated by macrophages', *Infection and Immunity*, 56(5), pp. 1352–1357. doi: 10.1128/iai.56.5.1352-1357.1988.

Frye, M. D. *et al.* (2017) 'Dynamic activation of basilar membrane macrophages in response to chronic sensory cell degeneration in aging mouse cochleae', *Hearing Research*. Elsevier B.V, 344, pp. 125–134. doi: 10.1016/j.heares.2016.11.003.

Frye, M. D., Zhang, C. and Hu, B. H. (2018) 'Lower level noise exposure that produces only TTS modulates the immune homeostasis of cochlear macrophages', *Journal of Neuroimmunology*, 323(May), pp. 152–166. doi: 10.1016/j.jneuroim.2018.06.019.

Fuentes-Santamaria, V. *et al.* (2017) 'The role of glia in the peripheral and central auditory system following noise overexposure: contribution of TNF-α and IL-1β to the pathogenesis of hearing loss', *Frontiers in Neuroanatomy*, 11(February), pp. 1–26. doi: 10.3389/fnana.2017.00009.

Fujioka, M. *et al.* (2006) 'Proinflammatory cytokines expression in noise-induced damaged cochlea', *Journal of Neuroscience Researchesearch*, 83, pp. 575–583. doi: 10.1002/jnr.

Galea, I. *et al.* (2008) 'Immune-to-brain signalling: The role of cerebral CD163-positive macrophages', *Neuroscience Letters*. Elsevier Ireland Ltd, 448(1), pp. 41–46. doi: 10.1016/j.neulet.2008.09.081.

Galea, I. and Perry, V. H. (2018) 'The blood-brain interface: a culture change', *Brain Behavior and Immunity*, 68, pp. 11–16. doi: 10.1016/j.bbi.2017.10.014.

Gassner, H. G., Shallop, J. O. N. K. and Driscoll, C. L. W. (2005) 'Long-term clinical course and temporal bone histology after cochlear implantation', *Cochlear Implants International*, 6(2), pp. 67–76. doi: 10.1179/cim.2005.6.2.67.

Gates, G. A. *et al.* (1993) 'The relation of hearing in the elderly to the presence of cardiovascular disease and cardiovascular risk factors', *Archives of Otolaryngology--Head and Neck Surgery*, 119(2), pp. 156–161. doi: 10.1001/archotol.1993.01880140038006.

Geyik, M. F. *et al.* (2002) 'Acute bacterial meningitis as a complication of otitis media and related mortality factors', *Yonsei Medical Journal*, pp. 573–578. doi: 10.3349/ymj.2002.43.5.573.

Ghaheri, B. A. *et al.* (2007a) 'Cochlear cytokine gene expression in murine acute otitis media', *Otolaryngology - Head and Neck Surgery*, 137(2), pp. 332–337. doi: 10.1016/j.otohns.2007.03.020.

Ghaheri, B. A. *et al.* (2007b) 'Cochlear cytokine gene expression in murine chronic otitis media',

Otolaryngology - Head and Neck Surgery, 137(2), pp. 332–337. doi: 10.1016/j.otohns.2007.03.020.

Giebink, G. S. *et al.* (1976) 'Experimental otitis media due to streptococcus pneumoniae: Immunopathogenic response in the chinchilla', *Journal of Infectious Diseases*, 134(6), pp. 595–604. doi: 10.1093/infdis/134.6.595.

Giebink, G. S. *et al.* (1980) 'Experimental otitis media after nasal inoculation of Streptococcus pneumoniae and influenza A virus in chinchillas', *Infection and Immunity*, 30(2), pp. 445–450. doi: 10.1128/iai.30.2.445-450.1980.

Giese, A. P. J. *et al.* (2020) 'Genomics of otitis media (OM): molecular genetics approaches to characterize disease pathophysiology', *Frontiers in Genetics*, 11(April). doi: 10.3389/fgene.2020.00313.

Ginhoux, F. *et al.* (2010) 'Fate mapping analysis reveals that adult microglia derive from primitive macrophages', *Science*, 330(November), pp. 841–845. doi: 10.1126/science.1194637.

Ginhoux, F. and Guilliams, M. (2016) 'Tissue-resident macrophage ontogeny and homeostasis', *Immunity*, 44, pp. 439–449. doi: https://doi.org/10.1016/j.immuni.2016.02.024.

Gloddek, B., Lamm, K. and Haslov, K. (1992) 'Influence of middle ear immune response on the immunological state and function of the inner ear', *Laryngoscope*, pp. 177–181. doi: 10.1288/00005537-199202000-00013.

Glueckert, R. *et al.* (2005) 'The human spiral ganglion: new insights into ultrastructure, survival rate and implications for cochlear implants', *Audiology and Neurotology*, 10(5), pp. 258–273. doi: 10.1159/000086000.

Godbout, J. P. *et al.* (2005) 'Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system', *FASEB Journal*, 19(10), pp. 1329–1331. doi: 10.1096/fj.05-3776fje.

Goldmann, T. *et al.* (2016) 'Origin, fate and dynamics of macrophages at central nervous system interfaces', *Nature Immunology*, 17(7), pp. 797–805. doi: 10.1038/ni.3423.

Goycoolea, M. V. *et al.* (1980) 'Permeability of round window membrane in otitis media', *Archives of Otolaryngology--Head and Neck Surgery*, 106(8), pp. 895–897. doi: 10.1001/archotol.1988.01860200079023.

Grasmeder, M. *et al.* (2021) 'Piloting the recording of electrode voltages (REVS) using surface

electrodes as a test to identify cochlear implant electrode migration, extra-cochlear electrodes and basal electrodes causing discomfort', *Cochlear Implants International*. Taylor & Francis. doi: 10.1080/14670100.2020.1863701.

Green, K. M. J. *et al.* (2004) 'Complications following adult cochlear implantation: Experience in Manchester', *Journal of Laryngology and Otology*, 118(6), pp. 417–420. doi: 10.1258/002221504323219518.

Green, R. A. *et al.* (2008) 'Conducting polymers for neural interfaces: Challenges in developing an effective long-term implant', *Biomaterials*, 29(24–25), pp. 3393–3399. doi: 10.1016/j.biomaterials.2008.04.047.

Gruver, A., Hudson, L. and Sempowski, G. (2007) 'Immunosenescence of ageing', *Journal of Pathology*, 211, pp. 144–156. doi: 10.1002/path.2104.

Gu, X. *et al.* (2014) 'Detection of bacterial biofilms in different types of chronic otitis media', *European Archives of Oto-Rhino-Laryngology*, 271(11), pp. 2877–2883. doi: 10.1007/s00405-013-2766-8.

Gumus, B. *et al.* (2020) 'Analysis of cochlear implant revision surgeries', *European Archives of Oto-Rhino-Laryngology*, (0123456789). doi: 10.1007/s00405-020-06121-5.

Gundogan, O. *et al.* (2013) 'Therapeutic efficacy of the combination of intratympanic methylprednisolone and oral steroid for idiopathic sudden deafness', *Otolaryngology - Head and Neck Surgery (United States)*, 149(5), pp. 753–758. doi: 10.1177/0194599813500754.

Gupta, S. *et al.* (2019) 'Type 2 diabetes and the risk of incident hearing loss', *Diabetologia*, 62(2), pp. 281–285. doi: 10.1007/s00125-018-4766-0.

Haake, S. M. *et al.* (2009) 'Dexamethasone protects auditory hair cells against TNFa-initiated apoptosis via activation of PI3K/Akt and NFkB signaling', *Hearing Research*. Elsevier B.V., 255(1–2), pp. 22–32. doi: 10.1016/j.heares.2009.05.003.

Haley, M. J. *et al.* (2018) 'Microglial priming as trained immunity in the brain', *Neuroscience*. IBRO. doi: 10.1016/j.neuroscience.2017.12.039.

Hall-Stoodley, L. *et al.* (2006) 'Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media', *JAMA*, 296(2), pp. 202–211. doi: 10.1001/jama.296.2.202.

Hardisty-Hughes, R. E. *et al.* (2006) 'A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse', *Human Molecular Genetics*, 15(22), pp. 3273–3279. doi: 10.1093/hmg/ddl403.

Hardisty, R. E. *et al.* (2003) 'The deaf mouse mutant Jeff (Jf) is a single gene model of otitis media', *JARO - Journal of the Association for Research in Otolaryngology*, 4(2), pp. 130–138. doi: 10.1007/s10162-002-3015-9.

Harris, J. P. and Ryan, A. F. (1985) 'Effect of a middle ear immune response on inner ear antibody levels', *Annals of Otology, Rhinology and Laryngology*, 94(2 I), pp. 202–206. doi: 10.1177/000348948509400222.

Hart, A. D. *et al.* (2012) 'Age related changes in microglial phenotype vary between CNS regions: grey versus white matter differences', *Brain, Behavior, and Immunity*. Elsevier Inc., 26(5), pp. 754–765. doi: 10.1016/j.bbi.2011.11.006.

He, P. *et al.* (2018) 'Association of socioeconomic status with hearing loss in Chinese working-aged adults: A population-based study', *PLoS ONE*, 13(3), pp. 1–12. doi: 10.1371/journal.pone.0195227.

Henzen, C. *et al.* (2000) 'Suppression and recovery of adrenal response after short-term, high-dose glucocorticoid treatment', *Lancet*, 355(9203), pp. 542–545. doi: 10.1016/S0140-6736(99)06290-X.

Hernandez, M. *et al.* (2008) 'Myeloid differentiation primary response gene 88 is required for the resolution of otitis media', *Journal of Infectious Diseases*, 198(12), pp. 1862–1869. doi: 10.1086/593213.

Hernandez, M. *et al.* (2015) 'The transcriptome of a complete episode of acute otitis media', *BMC Genomics*, 16(1), pp. 1–16. doi: 10.1186/s12864-015-1475-7.

Hinz, B. (2010) 'The myofibroblast: paradigm for a mechanically active cell', *Journal of Biomechanics*. Elsevier, 43(1), pp. 146–155. doi: 10.1016/j.jbiomech.2009.09.020.

Hirose, K. *et al.* (2005) 'Mononuclear phagocytes migrate into the murine cochlea after acoustic trauma', *Journal of Comparative Neurology*, 489(2), pp. 180–194. doi: 10.1002/cne.20619.

Hirose, K. *et al.* (2014) 'Systemic lipopolysaccharide induces cochlear inflammation and exacerbates the synergistic ototoxicity of kanamycin and furosemide', *JARO - Journal of the Association for Research in Otolaryngology*, 15(4), pp. 555–570. doi: 10.1007/s10162-014-0458-8.

Hirose, K. and Li, S. Z. (2019) 'The role of monocytes and macrophages in the dynamic permeability of the blood-perilymph barrier', *Hearing Research*, 374, pp. 49–57. doi: 10.1016/j.heares.2019.01.006.

Hochmair, I. *et al.* (2003) 'Deep electrode insertion in cochlear implants: apical morphology,

electrodes and speech perception results', *Acta Oto-Laryngologica*, 123(5), pp. 612–617. doi: 10.1080/00016480310001844.

Holden, L. K. *et al.* (2013) 'Factors affecting open-set word recognition in adults with cochlear implants', *Ear and Hearing*, 34(3), pp. 342–360. doi: 10.1097/AUD.0b013e3182741aa7.

Holtman, I. R. *et al.* (2015) 'Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: a co-expression meta-analysis', *Acta neuropathologica communications*. ???, 3, p. 31. doi: 10.1186/s40478-015-0203-5.

Honeder, C. *et al.* (2016) 'Effects of sustained release dexamethasone hydrogels in hearing preservation cochlear implantation', *Hearing Research*, 341, pp. 43–49. doi: 10.1016/j.heares.2016.08.001.

Hong, W. *et al.* (2009) 'Survival of bacterial biofilms within neutrophil extracellular traps promotes nontypeable haemophilus influenzae persistence in the chinchilla model for otitis media', *Journal of Innate Immunity*, 1(3), pp. 215–224. doi: 10.1159/000205937.

Hood, D. *et al.* (2016) 'A new model for non-typeable Haemophilus influenzae middle ear infection in the Junbo mutant mouse', *Disease Models & Mechanisms*, 9(1), pp. 69–79. doi: 10.1242/dmm.021659.

Hough, K., Sanderson, A., *et al.* (2021) 'Inflammation at the tissue-electrode interface in a case of rapid deterioration in hearing performance leading to explant after cochlear implantation', *Otology & Neurotology*, 42(4), pp. e445–e450. doi: 10.1097/MAO.0000000000003014.

Hough, K., Verschuur, C. A., *et al.* (2021) 'Macrophages in the cochlea; an immunological link between risk factors and progressive hearing loss', *Glia*, (September), pp. 1–20. doi: 10.1002/glia.24095.

Hu, B. H., Zhang, C. and Frye, M. D. (2018) 'Immune cells and non-immune cells with immune function in mammalian cochleae', *Hearing Research*, 362, pp. 14–24. doi: 10.1016/j.heares.2017.12.009.

Huang, C. *et al.* (2017) 'The ketone body metabolite β-hydroxybutyrate induces an antidepression-associated ramification of microglia via HDACs inhibition-triggered Akt-small RhoGTPase activation', *Glia*, 66(2), pp. 256–278. doi: 10.1002/glia.23241.

Huang, C. Q., Carter, P. M. and Shepherd, R. K. (2001) 'Stimulus induced pH changes in cochlear implants: An in vitro and in vivo study', *Annals of Biomedical Engineering*, 29(9), pp. 791–802. doi: 10.1114/1.1397793.

Huang, Y. *et al.* (2016) 'TLR2 promotes macrophage recruitment and Streptococcus pneumoniae clearance during mouse otitis media', *Pediatric Research*, 80(6), pp. 886–893. doi: 10.1038/pr.2016.154.

Hur, D. G. *et al.* (2021) 'Macrophage depletion in CCR2-/- mice delays bacterial clearance and enhances neutrophil infiltration in an acute otitis media model', *The Journal of infectious diseases*, 223(2), pp. 333–341. doi: 10.1093/infdis/jiaa353.

Hutchinson, K., Alessio, H. M. and Baiduc, R. R. (2010) 'Association between cardiovascular health and hearing function: pure-tone and distortion product otoacoustic emission measures', *American Journal of Audiology*, 19, pp. 26–35. doi: 10.1017/CBO9781107415324.004.

van Ingen, G. *et al.* (2016) 'Genome-wide association study for acute otitis media in children identifies FNDC1 as disease contributing gene', *Nature Communications*, 7(1), pp. 1–7. doi: 10.1038/ncomms12792.

Irving, S. *et al.* (2013) 'Cochlear implantation for chronic electrical stimulation in the mouse', *Hearing Research*, 306, pp. 37–45. doi: 10.1016/j.heares.2013.09.005.

Ishai, R. *et al.* (2017) 'The pattern and degree of capsular fibrous sheaths surrounding cochlear electrode arrays', *Hearing Research*. Elsevier B.V, 348, pp. 44–53. doi: 10.1016/j.heares.2017.02.012.

Ishiyama, A. *et al.* (2019) 'Temporal bone histopathology of first-generation cochlear implant electrode translocation', *Otology and Neurotology*, 40(6), pp. E581–E591. doi: 10.1097/MAO.0000000000002247.

Iyer, J. S. *et al.* (2018) 'Visualizing the 3D cytoarchitecture of the human cochlea in an intact temporal bone using synchrotron radiation phase contrast imaging', *Biomedical Optics Express*, 9(8), p. 3757. doi: 10.1364/boe.9.003757.

James, C. J. *et al.* (2019) 'Early sentence recognition in adult cochlear implant users', *Ear and Hearing*, 40(4), pp. 905–917. doi: 10.1097/AUD.0000000000000670.

James, D. P. *et al.* (2008) 'Effects of round window dexamethasone on residual hearing in a guinea pig model of cochlear implantation', *Audiology and Neurotology*, 13(2), pp. 86–96. doi: 10.1159/000111780.

Jecker, P., Pabst, R. and Westermann, J. (1996) 'The mucosa of the middle ear and eustachian tube in the young rat: number of granulocytes, macrophages, dendritic cells, NK cells and T and B lymphocytes in healthy animals and during otitis media', *Acta Oto-Laryngologica*, 116(3), pp. 443–

450. doi: 10.3109/00016489609137871.

Jecker, P., Pabst, R. and Westermann, J. (2001) 'Proliferating macrophages, dendritic cells, natural killer cells, T and B lymphocytes in the middle ear and Eustachian tube mucosa during experimental acute otitis media in the rat', *Clinical and Experimental Immunology*, 126(3), pp. 421–425. doi: 10.1046/j.1365-2249.2001.01543.x.

Jensen, R. G., Koch, A. and Homøe, P. (2013) 'The risk of hearing loss in a population with a high prevalence of chronic suppurative otitis media', *International Journal of Pediatric Otorhinolaryngology*. Elsevier Ireland Ltd, 77(9), pp. 1530–1535. doi: 10.1016/j.ijporl.2013.06.025.

Jia, H. *et al.* (2018) 'Intraoperative conebeam CT for assessment of intracochlear positioning of electrode arrays in adult recipients of cochlear implants', *American Journal of Neuroradiology*, 39(4), pp. 768–774. doi: 10.3174/ajnr.A5567.

Jiang, Y. *et al.* (2019) 'Lipopolysaccharide disrupts the cochlear blood-labyrinth barrier by activating perivascular resident macrophages and up-regulating MMP-9', *International Journal of Pediatric Otorhinolaryngology*, 127(August). doi: 10.1016/j.ijporl.2019.109656.

Joglekar, S. *et al.* (2010) 'Cochlear pathology in human temporal bones with otitis media', *Acta Oto-Laryngologica*, 130(4), pp. 472–476. doi: 10.3109/00016480903311252.

Johnsson, L.-G. (1974) 'Sequence of degeneration of Corti's organ and its first-order neurons', *Ann Otol*, 83, pp. 294–303.

Juhn, S. K. (1997) 'Effects of inflammatory mediators on middle ear pathology and on inner ear function', *Annals of the New York Academy of Sciences*, 830, pp. 130–142. doi: 10.1111/j.1749-6632.1997.tb51885.x.

Juhn, S. K. and Rybak, L. P. (1981) 'Labyrinthine barriers and cochlear homeostasis', *Acta Oto-Laryngologica*, 91(1–6), pp. 529–534. doi: 10.3109/00016488109138538.

Juneau, R. A. *et al.* (2011) 'Nontypeable haemophilus influenzae initiates formation of neutrophil extracellular traps', *Infection and Immunity*, 79(1), pp. 431–438. doi: 10.1128/IAI.00660-10.

Kalinec, G. M. *et al.* (2017) 'Resolution of cochlear inflammation: novel target for preventing or ameliorating drug- , noise- and age-related hearing loss', *Frontiers in Cellular Neuroscience*, 11(192), pp. 1–24. doi: 10.3389/fncel.2017.00192.

Kamakura, T. and Nadol, J. B. (2016) 'Correlation between word recognition score and intracochlear new bone and fibrous tissue after cochlear implantation in the human', *Hearing*

Research. Elsevier B.V, 339, pp. 132–141. doi: 10.1016/j.heares.2016.06.015.

Kaminska, B., Mota, M. and Pizzi, M. (2016) 'Signal transduction and epigenetic mechanisms in the control of microglia activation during neuroinflammation', *Biochimica et Biophysica Acta - Molecular Basis of Disease*. Elsevier B.V., 1862(3), pp. 339–351. doi: 10.1016/j.bbadis.2015.10.026.

Kanotra, S. *et al.* (2021) 'Intratympanic dexamethasone in sudden sensorineural hearing loss', *Indian Journal of Otolaryngology and Head and Neck Surgery*. Springer India. doi: 10.1007/s12070-021-02713-7.

Karamert, R. *et al.* (2019) 'Assessment of cochlear implant revision surgeries in a cohort of 802 patients', *Otology and Neurotology*, 40(4), pp. 464–470. doi: 10.1097/MAO.0000000000002152.

Kasemodel, A. L. P. *et al.* (2020) 'Sensorineural hearing loss in the acute phase of a single episode of acute otitis media', *Brazilian Journal of Otorhinolaryngology*. Associação Brasileira de Otorrinolaringologia e Cirurgia Cérvico-Facial, 86(6), pp. 767–773. doi: 10.1016/j.bjorl.2019.06.001.

Katsamenis, O. L. *et al.* (2019) 'X-ray micro-computed tomography for nondestructive three-dimensional (3D) x-ray histology', *The American journal of pathology*, 189(8), pp. 1608–1620. doi: 10.1016/ j.ajpath.2019.05.004.

Kaur, T., Zamani, D., *et al.* (2015) 'Fractalkine signaling regulates macrophage recruitment into the cochlea and promotes the survival of spiral ganglion neurons after selective hair cell lesion', *Journal of Neuroscience*, 35(45), pp. 15050–15061. doi: 10.1523/JNEUROSCI.2325-15.2015.

Kaur, T., Hirose, K., *et al.* (2015) 'Macrophage recruitment and epithelial repair following hair cell injury in the mouse utricle', *Frontiers in Cellular Neuroscience*, 9(April), pp. 1–9. doi: 10.3389/fncel.2015.00150.

Kaur, T. *et al.* (2019) 'Lack of fractalkine receptor on macrophages impairs spontaneous recovery of ribbon synapses after moderate noise trauma in C57BL/6 mice', *Frontiers in Neuroscience*, 13(JUN), pp. 1–16. doi: 10.3389/fnins.2019.00620.

Kaur, T., Ohlemiller, K. K. and Warchol, M. E. (2018) 'Genetic disruption of fractalkine signaling leads to enhanced loss of cochlear afferents following ototoxic or acoustic injury', *Journal of Comparative Neurology*, 526(5), pp. 824–835. doi: 10.1002/cne.24369.

Kawano, A. *et al.* (1998) 'Intracochlear factors contributing to psychophysical percepts following cochlear implantation', *Acta Oto-Laryngologica*, 118(3), pp. 313–326. doi:

10.1080/00016489850183386.

Kawano, A., Seldon, L. H. and Clark, G. M. (1996) 'Computer-aided three-dimensional reconstruction in human cochlear maps: measurement of the lengths of organ of Corti, outer wall, inner wall, and Rosenthal's canal', *Annals of Otology, Rhinology and Laryngology*, 105(9), pp. 701–709. doi: 10.1177/000348949610500906.

Kaya, E. *et al.* (2013) 'Investigation of the presence of biofilms in chronic suppurative otitis media, nonsuppurative otitis media, and chronic otitis media with cholesteatoma by scanning electron microscopy', *The Scientific World Journal*, 2013. doi: 10.1155/2013/638715.

Keithley, E. M. *et al.* (1990) 'Comparison of immune-mediated models of acute and chronic otitis media', *Oto-Rhino-Laryngology*, 247, pp. 247–251. doi: 10.1007/BF00178996.

Keithley, E. M., Wang, X. and Barkdull, G. C. (2008) 'Tumor necrosis factor can induce recruitment of inflammatory cells to the cochlea', *Otology & Neurotology*, 29(6), pp. 854–859. doi: 10.1097/MAO.0b013e31818256a9.

Keren-Shaul, H. *et al.* (2017) 'A unique microglia type associated with restricting development of Alzheimer' s disease', *Cell*, 169(7), pp. 1–15. doi: 10.1016/j.cell.2017.05.018.

Kettenmann, H. *et al.* (2011) 'Physiology of microglia', *Physiological Reviews*, 91(2), pp. 461–553. doi: 10.1152/physrev.00011.2010.

Khomtchouk, K. M. *et al.* (2020) 'A novel mouse model of chronic suppurative otitis media and its use in preclinical antibiotic evaluation', *Science Advances*, 6(33), pp. 1–11. doi: 10.1126/sciadv.abc1828.

Kilkenny, C. *et al.* (2010) 'Improving bioscience research reporting: The arrive guidelines for reporting animal research', *PLoS Biology*, 8(6), pp. 6–10. doi: 10.1371/journal.pbio.1000412.

Kim, S. Y. *et al.* (2020) 'Evaluating reasons for revision surgery and device failure rates in patients who underwent cochlear implantation surgery', *JAMA Otolaryngology - Head and Neck Surgery*, 146(5), pp. 414–420. doi: 10.1001/jamaoto.2020.0030.

Kimura, K. S. *et al.* (2020) 'Outcomes of revision cochlear implantation', *Otology & Neurotology*, 41, pp. e705-711. doi: 10.1097/mao.0000000000002659.

Kishimoto, I. *et al.* (2019) 'Early development of resident macrophages in the mouse cochlea depends on yolk sac hematopoiesis', *Frontiers in Neurology*, 10(October), pp. 1–13. doi: 10.3389/fneur.2019.01115.

Klein, J. O. *et al.* (2017) 'Recent advances in otitis media. Definitions, terminology and classification of otitis media', *Annals of Otology, Rhinology & Laryngology*, 111(3_suppl), pp. 8–18. doi: 10.1177/00034894021110s304.

Kokiko-Cochran, O. N. and Godbout, J. P. (2018) 'The inflammatory continuum of traumatic brain injury and Alzheimer's disease', *Frontiers in Immunology*, 9(672). doi: 10.3389/fimmu.2018.00672.

Kong, K. and Coates, H. L. C. (2009) 'Natural history, definitions, risk factors and burden of otitis media', *MJA*, 191(9), pp. 39–43. doi: 10.5694/j.1326-5377.2009.tb02925.x.

Kopelovich, J. C., Robinson, B. K., *et al.* (2015) 'Acoustic hearing after murine cochlear implantation: effects of trauma and implant type', *Annals of Otology, Rhinology and Laryngology*, 124(12), pp. 931–939. doi: 10.1177/0003489415592162.

Kopelovich, J. C., Reiss, L. A. J., *et al.* (2015) 'Hearing loss after activation of hearing preservation cochlear implants might be related to afferent cochlear innervation injury', *Otology and Neurotology*, 36(6), pp. 1035–1044. doi: 10.1097/MAO.0000000000000754.

Kowal, K. *et al.* (2011) 'CD163 and its role in inflammation', *Folia Histochemica et Cytobiologica*, 49(3), pp. 365–374. doi: 10.5603/FHC.2011.0052.

Kretzmer, E. A. *et al.* (2004) 'An animal model for cochlear implants', *Archives of Otolaryngology - Head and Neck Surgery*, 130(5), pp. 499–508. doi: 10.1001/archotol.130.5.499.

Krieg, N. R. and Hoffman, S. (1986) 'Microaerophil and oxygen toxicty', *Ann. Rev. Microbiol.*, 40, pp. 107–30.

Kruger, P. *et al.* (2015) 'Neutrophils: between host defence, immune modulation, and tissue Injury', *PLoS Pathogens*, 11(3), pp. 1–22. doi: 10.1371/journal.ppat.1004651.

Kujawa, S. G. and Liberman, M. C. (2006) 'Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth', *Journal of Neuroscience*, 26(7), pp. 2115–2123. doi: 10.1523/JNEUROSCI.4985-05.2006.

Kujawa, S. G. and Liberman, M. C. (2009) 'Adding Insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss', *Journal of Neuroscience*, 29(45), pp. 14077–14085. doi: 10.1523/JNEUROSCI.2845-09.2009.

Kujawa, S. G. and Liberman, M. C. (2015) 'Synaptopathy in the noise-exposed and aging cochlea: primary neural degeneration in acquired sensorineural hearing loss', *Hearing Research*. Elsevier B.V., 330, pp. 191–199. doi: 10.1016/j.heares.2015.02.009.

Kunda, L. D. *et al.* (2006) 'Silicone allergy: a new cause for cochlear implant extrusion and its management', *Otology and Neurotology*, 27(8), pp. 1078–1082. doi: 10.1097/01.mao.0000235378.64654.4d.

Kuo, C. L. *et al.* (2015) 'Updates and knowledge gaps in cholesteatoma research', *BioMed Research International*, 2015. doi: 10.1155/2015/854024.

Kurabi, A. *et al.* (2015) 'The inflammasome adaptor ASC contributes to multiple innate immune processes in the resolution of otitis media', *Innate Immunity*, 21(2), pp. 203–214. doi: 10.1177/1753425914526074.

Kurata, N. *et al.* (2016) 'Histopathologic evaluation of vascular findings in the cochlea in patients with presbycusis', *JAMA Otolaryngology - Head and Neck Surgery*, 142(2), pp. 173–178. doi: 10.1001/jamaoto.2015.3163.

Kurokawa, M. *et al.* (1998) 'The oncoprotein Evi-1 represses TGF-B signalling by inhibiting Smad3', *Nature*, 394(6688), pp. 92–96. doi: 10.1038/27945.

Lampikoski, H. *et al.* (2012) 'Mastoid biofilm in chronic otitis media', *Otology and Neurotology*, 33(5), pp. 785–788. doi: 10.1097/MAO.0b013e318259533f.

Lane, C. *et al.* (2019) 'Cochlear implant failures and reimplantation: A 30-year analysis and literature review', *Laryngoscope*. doi: 10.1002/lary.28071.

Lang, H. *et al.* (2006) 'Contribution of bone marrow hematopoietic stem cells to adult mouse inner ear: mesenchymal cells and fibrocytes', *The Journal of Comparative Neurology*, 496, pp. 187–201. doi: 10.1002/cne.20929.

Di Laora, A. *et al.* (2019) 'Cochlear implant electrode migration due to cholesterol granuloma: cues from a case', *The Journal of International Advanced Otology*, pp. 2018–2020. doi: 10.5152/iao.2019.6072.

Lapenna, A., De Palma, M. and Lewis, C. E. (2018) 'Perivascular macrophages in health and disease', *Nature Reviews Immunology*, 18(11), pp. 689–702. doi: 10.1038/s41577-018-0056-9.

Lassale, C. *et al.* (2020) 'Association of inflammatory markers with hearing impairment: the english longitudinal study of ageing', *Brain, Behavior, and Immunity*. Elsevier, 83, pp. 112–119. doi: 10.1016/j.bbi.2019.09.020.

Lawson, L. J., Perry, V. H. and Gordon, S. (1992) 'Turnover of resident microglia in the normal adult mouse brain', *Neuroscience*, 48(2), pp. 405–415. doi: 10.1016/0306-4522(92)90500-2.

Layfield, E. *et al.* (2020) 'Failure and revision surgery after cochlear implantation in the adult population: a 10-year single-institution retrospective and systematic review of the literature', *Otology and Neurotology*, 41. doi: 10.1097/MAO.0000000000002940.

Lazard, D. S. *et al.* (2012) 'Pre-, per- and postoperative factors affecting performance of postlinguistically deaf adults using cochlear implants: a new conceptual model over time', *PLoS ONE*, 7(11), pp. 1–11. doi: 10.1371/journal.pone.0048739.

Leake, P. A. *et al.* (2011) 'Brain-derived neurotrophic factor promotes cochlear spiral ganglion cell survival and function in deafened, developing cats', *Journal of Comparative Neurology*, 519(8), pp. 1526–1545. doi: 10.1002/cne.22582.

Lee, J. *et al.* (2019) 'NOD1/NOD2-mediated recognition of non-typeable Haemophilus influenzae activates innate immunity during otitis media', *Innate Immunity*, 25(8), pp. 503–512. doi: 10.1177/1753425919872266.

Lee, J., Eddington, D. K. and Nadol, J. B. (2011) 'The histopathology of revision cochlear implantation', *Audiology and Neurotology*, 16(5), pp. 336–346. doi: 10.1159/000322307.

Lee, M. R. *et al.* (2009) 'Biofilm presence in humans with chronic suppurative otitis media', *Otolaryngology - Head and Neck Surgery*. Elsevier Inc., 141(5), pp. 567–571. doi: 10.1016/j.otohns.2009.08.010.

Lenarz, M. *et al.* (2012) 'Long-term performance of cochlear implants in postlingually deafened adults', *Otology & Neurotology*, 147(1), pp. 112–118. doi: 10.1177/0194599812438041.

Li, H. *et al.* (2020) 'Synchrotron radiation-based reconstruction of the human spiral ganglion: implications for cochlear implantation', *Ear and hearing*, 41(1), pp. 173–181. doi: 10.1097/AUD.0000000000000738.

Li, P. M. M. C. *et al.* (2007) 'Analysis of intracochlear new bone and fibrous tissue formation in human subjects with cochlear implants', *Annals of Otology, Rhinology & Laryngology*, 116(10), pp. 731–738. doi: 10.1177/000348940711601004.

Li, Q. and Barres, B. A. (2018) 'Microglia and macrophages in brain homeostasis and disease', *Nature Reviews Immunology*. Nature Publishing Group, 18(4), pp. 225–242. doi: 10.1038/nri.2017.125.

Li, X. *et al.* (2021) 'Clinical analysis of intratympanic injection of dexamethasone for treating sudden deafness', *International Journal of General Medicine*, 14, pp. 2575–2579. doi: 10.2147/IJGM.S304123.

Liberman, L. D. and Liberman, M. C. (2015) 'Dynamics of cochlear synaptopathy after acoustic overexposure', *JARO - Journal of the Association for Research in Otolaryngology*, 16(2), pp. 205–219. doi: 10.1007/s10162-015-0510-3.

Liberman, M. C. (2017) 'Noise-induced and age-related hearing loss: new perspectives and potential therapies', *F1000Research*, 6(0), p. 927. doi: 10.12688/f1000research.11310.1.

Liberman, M. C. and Kujawa, S. G. (2017) 'Cochlear synaptopathy in acquired sensorineural hearing loss: manifestations and mechanisms', *Hearing Research*, 349, pp. 138–147. doi: 10.1016/j.heares.2017.01.003.

Lieberthal, A. S. *et al.* (2013) 'The diagnosis and management of acute otitis media', *Pediatrics*, 131(3). doi: 10.1542/peds.2012-3488.

Lim, D. J. (1976) 'Functional morphology of the mucosa of the middle ear and Eustachian tube', *Annals of Otology, Rhinology and Laryngology*, 85(Sup.25), pp. 36–43. doi: 10.1177/00034894760850s209.

Lim, D. J. (1979) 'Normal and pathological mucosa of the middle ear and Eustachian tube', *Clinical Otolaryngology & Allied Sciences*, 4(3), pp. 213–232. doi: 10.1111/j.1365-2273.1979.tb01888.x.

Lim, D. J. and Hussl, B. (1969) 'Human middle ear epithelium', *Archives of Otolaryngology - Head and Neck Surgery*, 89(6), pp. 835–849. doi: 10.1001/archotol.1969.00770020837009.

Lim, H. J. *et al.* (2011) 'Foreign body reaction after cochlear implantation', *International Journal of Pediatric Otorhinolaryngology*, 75(11), pp. 1455–1458. doi: 10.1016/j.ijporl.2011.08.004.

Liu, W. *et al.* (2015) 'The pre- and post-somatic segments of the human type I spiral ganglion neurons - structural and functional considerations related to cochlear implantation', *Neuroscience*, 284(December), pp. 470–482. doi: 10.1016/j.neuroscience.2014.09.059.

Liu, W. *et al.* (2018) 'Macrophages in the human cochlea: saviors or predators— a study using super-resolution immunohistochemistry', *Frontiers in Immunology*, 9(February), pp. 1–16. doi: 10.3389/fimmu.2018.00223.

Liu, W. *et al.* (2019) 'Human inner ear immune activity: A super-resolution immunohistochemistry study', *Frontiers in Neurology*, 10(JUL), pp. 1–14. doi: 10.3389/fneur.2019.00728.

Liu, Y.-C. *et al.* (2016) 'Assessment of complications due to intratympanic injections', *World Journal of Otorhinolaryngology-Head and Neck Surgery*. Elsevier Ltd, 2(1), pp. 13–16. doi: 10.1016/j.wjorl.2015.11.001.

Liu, Y. *et al.* (2015) 'Effects of a dexamethasone-releasing implant on cochleae: A functional, morphological and pharmacokinetic study', *Hearing Research*. Elsevier B.V, 327, pp. 89–101. doi: 10.1016/j.heares.2015.04.019.

Livingston, G. *et al.* (2017) 'The lancet commissions dementia prevention, intervention, and care', *The Lancet*, 390(17), pp. 2673–2734. doi: 10.1016/S0140-6736(17)31363-6.

Lopez-Rodriguez, A. B. *et al.* (2021) 'Acute systemic inflammation exacerbates neuroinflammation in Alzheimer's disease: IL-1β drives amplified responses in primed astrocytes and neuronal network dysfunction', *Alzheimer's & Dementia*, (March), pp. 1–21. doi: 10.1002/alz.12341.

Louw, L. (2010) 'Acquired cholesteatoma pathogenesis: Stepwise explanations', *Journal of Laryngology and Otology*, 124(6), pp. 587–593. doi: 10.1017/S0022215109992763.

Lu, W., Xu, J. and Shepherd, R. K. (2005) 'Cochlear implantation in rats: a new surgical approach', *Hearing Research*, 205(1–2), pp. 115–122. doi: 10.1016/j.heares.2005.03.010.

Ludwig, K. A. *et al.* (2006) 'Chronic neural recordings using silicon microelectrode arrays electrochemically deposited with a poly(3,4-ethylenedioxythiophene) (PEDOT) film', *Journal of Neural Engineering*, 3(1), pp. 59–70. doi: 10.1088/1741-2560/3/1/007.

Luers, J. C. and Hüttenbrink, K. B. (2016) 'Surgical anatomy and pathology of the middle ear', *Journal of Anatomy*, 228(2), pp. 338–353. doi: 10.1111/joa.12389.

Lull, M. E. and Block, M. L. (2010) 'Microglial activation and chronic neurodegeneration', *Neurotherapeutics*, 7(4), pp. 354–365. doi: 10.1016/j.nurt.2010.05.014.

Ma, C. L. *et al.* (2000) 'Characterization of an experimentally induced inner ear immune response', *Laryngoscope*, 110(3 I), pp. 451–456. doi: 10.1097/00005537-200003000-00024.

MacArthur, C. J., Hefeneider, S. H., Kempton, J. B. and Trune, D. R. (2006) 'C3H/HeJ mouse model for spontaneous chronic otitis media', *Laryngoscope*, 116(7), pp. 1071–1079. doi: 10.1097/01.mlg.0000224527.41288.c4.

MacArthur, C. J., Hefeneider, S. H., Kempton, J. B., Parrish, S. K., *et al.* (2006) 'Evaluation of the mouse model for acute otitis media', *Hearing Research*, 219(1–2), pp. 12–23. doi: 10.1016/j.heares.2006.05.012.

MacArthur, C. J. *et al.* (2008) 'Control of chronic otitis media and sensorineural hearing loss in C3H/HeJ mice: glucocorticoids vs mineralocorticoids', *Otolaryngology - Head and Neck Surgery*, 139(5), pp. 646–653. doi: 10.1016/j.otohns.2008.07.029.

MacArthur, C. J. *et al.* (2011) 'Altered expression of middle and inner ear cytokines in mouse otitis media', *Laryngoscope*, 121(2), pp. 365–371. doi: 10.1002/lary.21349.

MacArthur, C. J., Hausman, F., Kempton, J. B., Sautter, N., *et al.* (2013) 'Inner ear tissue remodeling and ion homeostasis gene alteration in murine chronic otitis media', *Otology and Neurotology*, 34(2), pp. 338–346. doi: 10.1097/MAO.0b013e31827b4d0a.

MacArthur, C. J., Hausman, F., Kempton, J. B., Choi, D., *et al.* (2013) 'Otitis media impacts hundreds of mouse middle and inner ear genes', *PLoS ONE*, 8(10). doi: 10.1371/journal.pone.0075213.

Maeda, K. *et al.* (2005) 'Dexamethasone inhibits tumor necrosis factor-α-induced cytokine secretion from spiral ligament fibrocytes', *Hearing Research*, 202(1–2), pp. 154–160. doi: 10.1016/j.heares.2004.08.022.

Makary, C. A. *et al.* (2011) 'Age-related primary cochlear neuronal degeneration in human temporal bones', *JARO - Journal of the Association for Research in Otolaryngology*, 12(6), pp. 711–717. doi: 10.1007/s10162-011-0283-2.

Marchant, C. D. *et al.* (1984) 'Course and outcome of otitis media in early infancy: a prospective study', *The Journal of Pediatrics*, 104(6), pp. 826–831. doi: 10.1016/S0022-3476(84)80475-8.

Van Der Marel, K. S. *et al.* (2012) 'Electrode migration in cochlear implant patients: not an exception', *Audiology and Neurotology*, 17(5), pp. 275–281. doi: 10.1159/000338475.

Massa, H. M. *et al.* (2015) 'Middle ear and eustachian tube mucosal immunology', *Mucosal Immunology: Fourth Edition*, 2–2(January), pp. 1923–1942. doi: 10.1016/B978-0-12-415847-4.00101-4.

Masuda, M. *et al.* (2012) 'Correlations of inflammatory biomarkers with the onset and prognosis of idiopathic sudden sensorineural hearing loss', *Otology and Neurotology*, 33, pp. 1142–1150. doi: 10.1097/MAO.0b013e3182755fbb.

Matt, S. M., Lawson, M. A. and Johnson, R. W. (2016) 'Aging and peripheral lipopolysaccharide can modulate epigenetic regulators and decrease IL-1β promoter DNA methylation in microglia', *Neurobiology of Aging*. Elsevier Inc, 47, pp. 1–9. doi: 10.1016/j.neurobiolaging.2016.07.006.

Medvedev, A. E., Kopydlowski, K. M. and Vogel, S. N. (2000) 'Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression', *The Journal of Immunology*, 164(11), pp. 5564–5574. doi: 10.4049/jimmunol.164.11.5564.

Mei, X. *et al.* (2018) 'Human inner ear blood supply revisited: the Uppsala collection of temporal bone—an international resource of education and collaboration', *Upsala Journal of Medical Sciences*. Taylor & Francis, 123(3), pp. 131–142. doi: 10.1080/03009734.2018.1492654.

Mei, X. *et al.* (2020) 'Vascular supply of the human spiral ganglion: novel three-dimensional analysis using synchrotron phase-contrast imaging and histology', *Scientific reports*, 10(1), p. 5877. doi: 10.1038/s41598-020-62653-0.

Melhus, Å. and Ryan, A. F. (2003) 'A mouse model for acute otitis media', *Apmis*, 111(10), pp. 989–994. doi: 10.1034/j.1600-0463.2003.1111012.x.

Menyuk, P. (1986) 'Language development in a social context', *The Journal of Pediatrics*, 109(1), pp. 217–224. doi: 10.1016/S0022-3476(86)80606-0.

Metscher, B. D. (2009) 'Micro CT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues', *BMC Physiology*, 9(1). doi: 10.1186/1472-6793-9-11.

Meyerhoff, W. L., Kim, C. S. and Paparella, M. M. (1978) 'Pathology of chronic otitis media', *Annals of Otology, Rhinology & Laryngology*, 87. doi: https://doi.org/10.1177/000348947808700602.

Micallef, L. *et al.* (2012) 'The myofibroblast, multiple origins for major roles in normal and pathological tissue repair', *Fibrogenesis & Tissue Repair*, 5(Suppl 1:55), pp. 1–5. doi: 10.1186/1755-1536-5-S1-S5.

Mijovic, T., Zeitouni, A. and Colmegna, I. (2013) 'Autoimmune sensorineural hearing loss: the otology-rheumatology interface', *Rheumatology (United Kingdom)*, 52(5), pp. 780–789. doi: 10.1093/rheumatology/ket009.

Mistry, N. *et al.* (2014) 'Cochlear implantation in the mouse via the round window: effects of array insertion', *Hearing Research*. Elsevier B.V., 312, pp. 81–90. doi: 10.1016/j.heares.2014.03.005.

Mitchell, P. *et al.* (2009) 'Relationship of Type 2 diabetes to the prevalence, incidence and progression of age-related hearing loss', *Diabetic Medicine*, 26(5), pp. 483–488. doi: 10.1111/j.1464-5491.2009.02710.x.

Mittal, R. *et al.* (2014) 'Role of innate immunity in the pathogenesis of otitis media', *International Journal of Infectious Diseases*. International Society for Infectious Diseases, 29, pp. e259–e267. doi: 10.1016/j.ijid.2014.10.015.

Mittal, R. *et al.* (2017) 'Recent advancements in the regeneration of auditory hair cells and

hearing restoration', *Frontiers in Molecular Neuroscience*, 10(July). doi: 10.3389/fnmol.2017.00236.

Mittmann, P. *et al.* (2015) 'Electrode migration in patients with perimodiolar cochlear implant electrodes', *Audiology and Neurotology*, 20(6), pp. 349–353. doi: 10.1159/000435873.

von Mitzlaff, C. *et al.* (2020) 'Electrode migration after cochlear implantation', *Cochlear Implants International*. Taylor & Francis. doi: 10.1080/14670100.2020.1833516.

Mizrahi, A. *et al.* (2014) 'Non typable-Haemophilus influenzae biofilm formation and acute otitis media', *BMC Infectious Diseases*, 14(1), pp. 1–10. doi: 10.1186/1471-2334-14-400.

Mizushima, Y. *et al.* (2017) 'Macrophage recruitment, but not interleukin 1 beta activation, enhances noise-induced hearing damage', *Biochemical and Biophysical Research Communications*, 493(2), pp. 894–900. doi: 10.1016/j.bbrc.2017.09.124.

Moberly, A. C. *et al.* (2016) 'The enigma of poor performance by adults with cochlear implants', *Otology and Neurotology*, 37(10), pp. 1522–1528. doi: 10.1097/MAO.0000000000001211.

Monasta, L. *et al.* (2012) 'Burden of disease caused by otitis media: systematic review and global estimates', *PLoS ONE*, 7(4). doi: 10.1371/journal.pone.0036226.

Moriyama, S. *et al.* (2009) 'Formation of biofilm by Haemophilus influenzae isolated from pediatric intractable otitis media', *Auris Nasus Larynx*, 36(5), pp. 525–531. doi: 10.1016/j.anl.2008.10.010.

Murillo-Cuesta, S. *et al.* (2010) 'Melanin precursors prevent premature age-related and noise-induced hearing loss in albino mice', *Pigment Cell and Melanoma Research*, 23(1), pp. 72–83. doi: 10.1111/j.1755-148X.2009.00646.x.

Murphy, T. F. *et al.* (2013) 'Panel 5: microbiology and immunology panel', *Otolaryngology - Head and Neck Surgery (United States)*, 148(4 SUPPL.). doi: 10.1177/0194599812459636.

Murphy, T. F. and Kirkham, C. (2002) 'Biofilm formation by nontypeable Haemophilus influenzae: strain variability, outer membrane antigen expression and role of pili', *BMC Microbiology*, 2, pp. 1–8. doi: 10.1186/1471-2180-2-7.

Musiek, F. E. and Baran, J. A. (2018) *The auditory system: anatomy, physiology, and clinical correlates; second addition*. Plural Publishing Inc.

Myburgh, H. C. *et al.* (2016) 'Otitis media diagnosis for developing countries using tympanic membrane image-analysis', *EBioMedicine*. The Authors, 5, pp. 156–160. doi:

10.1016/j.ebiom.2016.02.017.

Nadol, J. B. *et al.* (2001) 'Histopathology of cochlear implants in humans', *Annals of Otology, Rhinology and Laryngology*, 110(9), pp. 883–891. doi: 10.1177/000348940111000914.

Nadol, J. B. *et al.* (2014) 'Cellular immunologic responses to cochlear implantation in the human', *Hearing Research*, 318, pp. 11–17. doi: 10.1016/j.heares.2014.09.007.

Nadol, J. B. and Eddington, D. K. (2004) 'Histologic evaluation of the tissue seal and biologic response around cochlear implant electrodes in the human', *Otology and Neurotology*, 25(3), pp. 257–262. doi: 10.1097/00129492-200405000-00010.

Nadol, J. B., Eddington, D. K. and Burgess, B. J. (2008) 'Foreign body or hypersensitivity granuloma of the inner ear after cochlear implantation: one possible cause of a soft failure?', *Otology and Neurotology*, 29(8), pp. 1076–1084. doi: 10.1097/MAO.0b013e31818c33cf.

Nadol, J. B., Ketten, D. R. and Burgess, B. J. (1994) 'Otopathology in a case of multichannel cochlear implantation', *Laryngoscope*, pp. 299–303. doi: 10.1288/00005537-199403000-00010.

Nash, S. D. *et al.* (2011) 'The prevalence of hearing impairment and associated risk factors: the beaver dam offspring study', *Archives of Otolaryngology - Head and Neck Surgery*, 137(5), pp. 432–439. doi: 10.1001/archoto.2011.15.

Nash, S. D. *et al.* (2014) 'Long-term assessment of systemic infammation and the cumulative incidence of age-related hearing impairment in the epidemiology of hearing loss study', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 69 A(2), pp. 207–214. doi: 10.1093/gerona/glt075.

Navntoft, C. A., Marozeau, J. and Barkat, T. R. (2019) 'Cochlear implant surgery and electrically-evoked auditory brainstem response recordings in C57BL/6 mice', *Journal of Visualized Experiments*, 2019(143), pp. 1–9. doi: 10.3791/58073.

Navntoft, C. A., Marozeau, J. and Barkat, T. R. (2020) 'Ramped pulse shapes are more efficient for cochlear implant stimulation in an animal model', *Scientific Reports*, 10(1), pp. 1–17. doi: 10.1038/s41598-020-60181-5.

Needham, K. *et al.* (2019) 'Electrode impedance changes after implantation of a dexamethasone-eluting intracochlear array', *Cochlear Implants International*. Taylor & Francis, 0(0), pp. 1–12. doi: 10.1080/14670100.2019.1680167.

Neher, J. J. and Cunningham, C. (2019) 'Priming microglia for innate immune memory in the

brain', *Trends in Immunology*, 40(4), pp. 358–374. doi: 10.1016/j.it.2019.02.001.

Neilan, R. E. *et al.* (2012) 'Cochlear implant device failure secondary to cholesterol granuloma–mediated cochlear erosion', *Otology & Neurotology*, 33(5), pp. 733–735. doi: 10.1097/MAO.0b013e3182544fff.

Neng, L. *et al.* (2013) 'Endothelial cell, pericyte, and perivascular resident macrophage-type melanocyte interactions regulate cochlear intrastrial fluid-blood barrier permeability', *JARO - Journal of the Association for Research in Otolaryngology*, 14(2), pp. 175–185. doi: 10.1007/s10162-012-0365-9.

Neng, L. *et al.* (2015) 'Structural changes in the strial blood–labyrinth barrier of aged C57BL/6 mice', *Cell and Tissue Research*, 361(3), pp. 685–696. doi: 10.1007/s00441-015-2147-2.

Netea, M. G. *et al.* (2020) 'Defining trained immunity and its role in health and disease', *Nature Reviews Immunology*, pp. 1–17. doi: 10.1038/s41577-020-0285-6.

Newbold, C. *et al.* (2011) 'Electrical stimulation causes rapid changes in electrode impedance of cell-covered electrodes', *Journal of Neural Engineering*, 8(3). doi: 10.1088/1741-2560/8/3/036029.

Newbold, C. *et al.* (2014) 'Impedance changes in chronically implanted and stimulated cochlear implant electrodes', *Cochlear Implants International*, 15(4), pp. 191–199. doi: 10.1179/1754762813Y.0000000050.

NICE (2019) 'Cochlear implants for children and adults with severe to profound deafness', *NICE Technol. Apprais. Guid.*, pp. 1–41. Available at: https://www.nice.org.uk/guidance/ta566.

NIH (2021) *NIH, Cochlear Implants*, *NIH Publication No. 00-4798*. Available at: https://www.nidcd.nih.gov/health/cochlear-implants (Accessed: 17 August 2021).

Nimeri, G. *et al.* (2002) 'The influence of plasma proteins and platelets on oxygen radical production and F-actin distribution in neutrophils adhering to polymer surfaces', *Biomaterials*, 23(8), pp. 1785–1795. doi: 10.1016/S0142-9612(01)00305-2.

Noble, K. V. *et al.* (2019) 'Age-related changes in immune cells of the human cochlea', *Frontiers in Neurology*, 10(AUG), pp. 1–13. doi: 10.3389/fneur.2019.00895.

Noonan, K. Y. *et al.* (2020) 'Immune response of macrophage population to cochlear implantation: cochlea immune cells', *Otology and Neurotology*, 41(9), pp. 1288–1295. doi: 10.1097/MAO.0000000000002764.

Norden, D. M., Muccigrosso, M. M. and Godbout, J. P. (2015) 'Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease', *Neuropharmacology*. Elsevier Ltd, 96(PA), pp. 29–41. doi: 10.1016/j.neuropharm.2014.10.028.

Novotny, L. A. *et al.* (2019) 'Biofilm biology and vaccine strategies for otitis media due to nontypeable haemophilus influenzae', *Journal of Pediatric Infectious Diseases*, 14(2), pp. 69–77. doi: 10.1055/s-0038-1660818.

O'Connell, B. P. *et al.* (2016) 'Electrode location and angular insertion depth are predictors of audiologic outcomes in cochlear implantation', *Otology and Neurotology*, 37(8), pp. 1016–1023. doi: 10.1097/MAO.0000000000001125.

O'Leary, S. J. *et al.* (2013) 'Relations between cochlear histopathology and hearing loss in experimental cochlear implantation', *Hearing Research*, 298, pp. 27–35. doi: 10.1016/j.heares.2013.01.012.

O'Malley, J. T., Nadol, J. B. and Mckenna, M. J. (2016) 'Anti CD163+, Iba1+, and CD68+ cells in the adult human inner ear: normal distribution of an unappreciated class of macrophages/microglia and implications for inflammatory otopathology in humans', *Otology & Neurotology*, (14), pp. 99–108. doi: 10.1097/MAO.0000000000000879.

Ohlemiller, K. K. *et al.* (2009) 'Absence of strial melanin coincides with age-associated marginal cell loss and endocochlear potential decline', *Hearing Research*, 249(1–2), pp. 1–14. doi: 10.1016/j.heares.2008.12.005.

Okano, T. *et al.* (2008) 'Bone marrow-derived cells expressing Iba1 are constitutively present as resident tissue macrophages in the mouse cochlea', *Journal of Neuroscience Research*, 86(8), pp. 1758–1767. doi: 10.1002/jnr.21625.

Okano, T. (2014) 'Immune system of the inner ear as a novel therapeutic target for sensorineural hearing loss', *Frontiers in Pharmacology*, 5 AUG(September), pp. 1–8. doi: 10.3389/fphar.2014.00205.

Okano, T. and Kishimoto, I. (2019) 'Csf1 signaling regulates maintenance of resident macrophages and bone formation in the mouse cochlea', *Frontiers in Neurology*, 10(November), pp. 1–8. doi: 10.3389/fneur.2019.01244.

Okayasu, T. *et al.* (2020) 'The distribution and prevalence of macrophages in the cochlea following cochlear implantation in the human: an immunohistochemical study using anti-Iba1 antibody', *Otology and Neurotology*, 41(3), pp. e304–e316. doi: 10.1097/MAO.0000000000002495.

Ostuni, R. *et al.* (2013) 'Latent enhancers activated by stimulation in differentiated cells', *Cell*. Elsevier Inc., 152(1–2), pp. 157–171. doi: 10.1016/j.cell.2012.12.018.

Pace, J. L. *et al.* (1983) 'Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing.', *The Journal of Immunology*, 130(5), pp. 2011–2013.

Palin, K. *et al.* (2008) 'Systemic inflammation switches the inflammatory cytokine profile in CNS Wallerian degeneration', *Neurobiology of Disease*, 30(1), pp. 19–29. doi: 10.1016/j.nbd.2007.11.012.

Papp, Z., Rezes, S. and Jokay, I. (2014) 'Sensorineural hearing loss in chronic otitis media', *Otology & Neurotology*, 24, pp. 141–144. doi: 10.5631/jibirinsuppl1986.1993.supplement62_91.

Paradise, J. L. *et al.* (1997) 'Otitis media in 2253 Pittsburgh-area infants: prevalence and risk factors during the first two years of life', *Pediatrics*, 99(3), pp. 318–333. doi: 10.1542/peds.99.3.318.

Park, K., Ueno, K. and Lim, D. J. (1992) 'Developmental anatomy of the eustachian tube and middle ear in mice', *American Journal of Otolaryngology--Head and Neck Medicine and Surgery*, 13(2), pp. 93–100. doi: 10.1016/0196-0709(92)90005-E.

Parkinson, N. *et al.* (2006) 'Mutation at the Evi1 locus in Junbo mice causes susceptibility to otitis media', *PLoS Genetics*, 2(10), p. e149. doi: 10.1371/journal.pgen.0020149.

Patnaik, U. *et al.* (2016) 'Cochlear re-implantation: Lessons learnt and the way ahead', *Acta Oto-Laryngologica*, 136(6), pp. 564–567. doi: 10.3109/00016489.2015.1136430.

Patuzzi, R. (2011) 'Ion flow in stria vascularis and the production and regulation of cochlear endolymph and the endolymphatic potential', *Hearing Research*. Elsevier B.V., 277(1–2), pp. 4–19. doi: 10.1016/j.heares.2011.01.010.

Penido, N. D. O. *et al.* (2005) 'Intracranial complications of otitis media: 15 years of experience in 33 patients', *Otolaryngology - Head and Neck Surgery*, 132(1), pp. 37–42. doi: 10.1016/j.otohns.2004.08.007.

Perry, V. H. and Holmes, C. (2014) 'Microglial priming in neurodegenerative disease', *Nature Reviews Neurology*, 10(4), pp. 217–224. doi: 10.1038/nrneurol.2014.38.

Perry, V. H. and Teeling, J. (2013) 'Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration', *Semin Immunopathol*, 35, pp. 601–612. doi: 10.1007/s00281-013-0382-8.

Pirvola, U. *et al.* (1997) 'Expression of neurotrophins and TrK receptors in the developing, adult, and regenerating avian cochlea', *Journal of Neurobiology*, 33(7), pp. 1019–1033. doi: 10.1002/(SICI)1097-4695(199712)33:7<1019::AID-NEU11>3.0.CO;2-A.

Post, J. C. *et al.* (1995) 'Molecular analysis of bacterial pathogens in otitis media With effusion', *JAMA: The Journal of the American Medical Association*, 273(20), pp. 1598–1604. doi: 10.1001/jama.1995.03520440052036.

Post, J. C. *et al.* (1996) 'PCR-based detection of bacterial DNA after antimicrobial treatment is indicative of persistent, viable bacteria in the chinchilla model of otitis media', *American Journal of Otolaryngology - Head and Neck Medicine and Surgery*, 17(2), pp. 106–111. doi: 10.1016/S0196-0709(96)90005-8.

Post, J. C. (2001) 'Direct evidence of bacterial biofilms in otitis media', *Laryngoscope*, 111, pp. 2083–2094. doi: 10.1002/lary.25291.

Prasad, S. C. *et al.* (2013) 'Current trends in the management of the complications of chronic otitis media with cholesteatoma', *Current Opinion in Otolaryngology and Head and Neck Surgery*, 21(5), pp. 446–454. doi: 10.1097/MOO.0b013e3283646467.

Puig, C. *et al.* (2014) 'Increased biofilm formation by nontypeable Haemophilus influenzae isolates from patients with invasive disease or otitis media versus strains recovered from cases of respiratory infections', *Applied and Environmental Microbiology*, 80(22), pp. 7088–7095. doi: 10.1128/AEM.02544-14.

Puntambekar, S. S. *et al.* (2018) 'Cellular players that shape evolving pathology and neurodegeneration following traumatic brain injury', *Brain, Behavior, and Immunity*, 71, pp. 9–17. doi: 10.1016/j.bbi.2018.03.033.

Quraishe, S., Newman, T. and Anderson, L. (2019) 'Auditory temporal acuity improves with age in the male mouse auditory thalamus: A role for perineuronal nets?', *Journal of Neuroscience Research*, 98(9), pp. 1780–1799. doi: 10.1002/jnr.24537.

Qureshi, S. T. *et al.* (1999) 'Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4)', *Journal of Experimental Medicine*, 189(4), pp. 615–625. doi: 10.1084/jem.189.4.615.

Rader, T. *et al.* (2016) 'Management of cochlear implant electrode migration', *Otology and Neurotology*, 37(9), pp. e341–e348. doi: 10.1097/MAO.0000000000001065.

Rai, V. *et al.* (2020) 'The immune response after noise damage in the cochlea is characterized by a heterogeneous mix of adaptive and innate immune cells', *Scientific Reports*. Nature Publishing

Group UK, 10(1), pp. 1–17. doi: 10.1038/s41598-020-72181-6.

Raphael, Y. and Altschuler, R. A. (2003) 'Structure and innervation of the cochlea', *Brain Research Bulletin*, 60(5–6), pp. 397–422. doi: 10.1016/S0361-9230(03)00047-9.

Ravikumar, S. B., Vishwakarma, R. and Keshri, A. (2018) 'Cochlear implant device failure in the postoperative period: an institutional analysis', *Asian Journal of Neurosurgery*, 13(2), pp. 1066–1070. doi: 10.4103/ajns.AJNS_93_17.

Rayamajhi, P. *et al.* (2020) 'A clinical profile of revision cochlear implant surgery: MERF experience', *Cochlear Implants International*. Taylor & Francis, (September). doi: 10.1080/14670100.2020.1823128.

Richard, C. *et al.* (2012) 'Round window versus cochleostomy technique in cochlear implantation: Histologic findings', *Otology and Neurotology*, 33(7), pp. 1181–1187. doi: 10.1097/MAO.0b013e318263d56d.

Ritzel, R. M. *et al.* (2015) 'Functional differences between microglia and monocytes after ischemic stroke', *Journal of Neuroinflammation*, 12(106), pp. 1–12. doi: 10.1186/s12974-015-0329-1.

Rivas, A. *et al.* (2008) 'Revision cochlear implantation surgery in adults: Indications and results', *Otology and Neurotology*, 29(5), pp. 639–648. doi: 10.1097/MAO.0b013e31817e5d31.

Rosenfeld, R. M. *et al.* (2016) *Clinical Practice Guideline*, *Otolaryngology - Head and Neck Surgery (United States)*. doi: 10.1177/0194599815623467.

Ryan, A. F. *et al.* (2020) 'Single-cell transcriptomes reveal a complex cellular landscape in the middle ear and differential capacities for acute response to infection', *Frontiers in Genetics*, 11(April), pp. 1–19. doi: 10.3389/fgene.2020.00358.

Ryan, G. R. *et al.* (2001) 'Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1op/Csf1op) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis', *Blood*, 98(1), pp. 74–84. doi: 10.1182/blood.V98.1.74.

Rye, M. S. *et al.* (2012) 'Genome-wide association study to identify the genetic determinants of otitis media susceptibility in childhood', *PLoS ONE*, 7(10). doi: 10.1371/journal.pone.0048215.

Saeed, S. *et al.* (2014) 'Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity', *Science*, 345(6204). doi: 10.1126/science.1251086.

Saeed, S. R. *et al.* (2014) 'The use of cone-beam computed tomography to determine cochlear implant electrode position in human temporal bones', *Otology and Neurotology*, 35(8), pp. 1338–

1344. doi: 10.1097/MAO.0000000000000295.

Salt, A. N. and Hirose, K. (2018) 'Communication pathways to and from the inner ear and their contributions to drug delivery', *Hearing Research*, 362, pp. 25–37. doi: 10.1016/j.heares.2017.12.010.

Sanderson, A. P. *et al.* (2019) 'Exploiting routine clinical measures to inform strategies for better hearing performance in cochlear implant users', *Frontiers in Neuroscience*, 12(January), pp. 1–14. doi: 10.3389/fnins.2018.01048.

Sandhir, R., Onyszchuk, G. and Berman, N. E. J. (2008) 'Exacerbated glial response in the aged mouse hippocampus following controlled cortical impact injury', *Experimental Neurology*, 213(2), pp. 372–380. doi: 10.1016/j.expneurol.2008.06.013.

Santos-Cortez, R. L. P. *et al.* (2018) 'FUT2 variants confer susceptibility to familial otitis media', *American Journal of Human Genetics*, 103(5), pp. 679–690. doi: 10.1016/j.ajhg.2018.09.010.

Sato, E. *et al.* (2008) 'Repopulation of cochlear macrophages in murine hematopoietic progenitor cell chimeras: the role of CX3CR1', *J Comp Neurol*, 506, pp. 930–942. doi: 10.1002/cne.

Sato, E. *et al.* (2010) 'Expression of fractalkine receptor CX3CR1 on cochlear macrophages influences survival of hair cells following ototoxic injury', *JARO - Journal of the Association for Research in Otolaryngology*, 11(2), pp. 223–234. doi: 10.1007/s10162-009-0198-3.

Satoh, J. ichi *et al.* (2016) 'TMEM119 marks a subset of microglia in the human brain', *Neuropathology*, 36(1), pp. 39–49. doi: 10.1111/neup.12235.

Saunders, J., Murray, M. and Alleman, A. (2011) 'Biofilms in chronic suppurative otitis media and cholesteatoma: Scanning electron microscopy findings', *American Journal of Otolaryngology - Head and Neck Medicine and Surgery*. Elsevier Inc., 32(1), pp. 32–37. doi: 10.1016/j.amjoto.2009.09.010.

Sautter, N. B. *et al.* (2006) 'CC chemokine receptor 2 is protective against noise-induced hair cell death: Studies in CX3CR1+/GFP mice', *JARO - Journal of the Association for Research in Otolaryngology*, 7(4), pp. 361–372. doi: 10.1007/s10162-006-0051-x.

Sautter, N. B. *et al.* (2011) 'Tissue remodeling in the acute otitis media mouse model', *International Journal of Pediatric Otorhinolaryngology*, 75(11), pp. 1368–1371. doi: 10.1016/j.ijporl.2011.07.026.

Schachern, P. A. *et al.* (2017) 'Neutrophil extracellular traps and fibrin in otitis media: analysis of

human and chinchilla temporal bones', *JAMA Otolaryngology - Head and Neck Surgery*, 143(10), pp. 990–995. doi: 10.1001/jamaoto.2017.1320.

Schilder, A. G. M. *et al.* (2016) 'Otitis media', *Nature Reviews Disease Primers*. Macmillan Publishers Limited, 2, pp. 1–19. doi: 10.1038/nrdp.2016.63.

Scholes, S. *et al.* (2018) 'Socioeconomic differences in hearing among middle-aged and older adults: cross-sectional analyses using the Health Survey for England', *BMJ Open*, 8(2), pp. 1–10. doi: 10.1136/bmjopen-2017-019615.

Scholzen, T. and Gerdes, J. (2000) 'The Ki-67 protein: from the known and the unknown', *Journal of Cellular Physiology*, 182(3), pp. 311–322. doi: 10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9.

Sergeyenko, Y. *et al.* (2013) 'Age-related cochlear synaptopathy: An early-onset contributor to auditory functional decline', *Journal of Neuroscience*, 33(34), pp. 13686–13694. doi: 10.1523/JNEUROSCI.1783-13.2013.

Seyyedi, M. and Nadol, J. B. (2014) 'Intracochlear inflammatory response to cochlear implant electrodes in humans', *Otology and Neurotology*, 35(9), pp. 1545–1551. doi: 10.1097/MAO.0000000000000540.

Shaul, C. *et al.* (2019) 'Electrical impedance as a biomarker for inner ear pathology following lateral wall and peri-modiolar cochlear implantation', *Otology & Neurotology*, 40(5), pp. e518–e526. doi: 10.1097/MAO.0000000000002227.

Shaw, A. C. *et al.* (2010) 'Aging of the innate immune system', *Current Opinion in Immunology*. Elsevier Ltd, 22(4), pp. 507–513. doi: 10.1016/j.coi.2010.05.003.

Shepherd, R. K. *et al.* (2005) 'Chronic depolarization enhances the trophic effects of brain-derived neurotrophic factor in rescuing auditory neurons following a sensorineural hearing loss', *Journal of Comparative Neurology*, 486(2), pp. 145–158. doi: 10.1002/cne.20564.

Shepherd, R. K. *et al.* (2019) 'Chronic intracochlear electrical stimulation at high charge densities results in platinum dissolution but not neural loss or functional changes in vivo', *Journal of Neural Engineering*. IOP Publishing, 16(2). doi: 10.1088/1741-2552/aaf66b.

Shi, X. (2010) 'Resident macrophages in the cochlear blood-labyrinth barrier and their renewal via migration of bone-marrow-derived cells', *Cell and Tissue Research*, 342(1), pp. 21–30. doi: 10.1007/s00441-010-1040-2.

Shi, X. (2016) 'Pathophysiology of the cochlear intrastrial fluid-blood barrier', *Hearing Research*. Elsevier B.V., 338, pp. 52–63. doi: 10.1016/j.heares.2016.01.010.

Shu, Y. *et al.* (2019) 'Renewed proliferation in adult mouse cochlea and regeneration of hair cells', *Nature Communications*, 10(1), pp. 1–15. doi: 10.1038/s41467-019-13157-7.

Silva, M. D. and Sillankorva, S. (2019) 'Otitis media pathogens– a life entrapped in biofilm communities', *Critical Reviews in Microbiology*. Taylor & Francis, 45(5–6), pp. 595–612. doi: 10.1080/1040841X.2019.1660616.

Simões, E. A. *et al.* (2010) 'Otitis media and its consequences: beyond the earache', *The Lancet Infectious Diseases*, 10(3), pp. 195–203. doi: 10.1016/s1473-3099(10)70012-8.

Simpson, A. N., Matthews, L. J. and Dubno, J. R. (2013) 'Lipid and C-reactive protein levels as risk factors for hearing loss in older adults', *Otolaryngology - Head and Neck Surgery (United States)*, 148(4), pp. 664–670. doi: 10.1177/0194599812473936.

Skelly, D. T. *et al.* (2019) 'Acute transient cognitive dysfunction and acute brain injury induced by systemic inflammation occur by dissociable IL-1-dependent mechanisms', *Molecular Psychiatry*, 24(10), pp. 1533–1548. doi: 10.1038/s41380-018-0075-8.

Slattery, W. H. *et al.* (2005) 'Oral steroid regimens for idiopathic sudden sensorineural hearing loss', *Otolaryngology - Head and Neck Surgery*, 132(1), pp. 5–10. doi: 10.1016/j.otohns.2004.09.072.

Van Sluyters, R. C. and Obernier, J. A. (2003) *Guidelines for the care and use of mammals in neuroscience and behavioral research*, *Contemporary Topics in Laboratory Animal Science*. National Academies Press. doi: 10.17226/10732.

So, H. *et al.* (2007) 'Cisplatin cytotoxicity of auditory cells requires secretions of proinflammatory cytokines via activation of ERK and NF-κB', *JARO - Journal of the Association for Research in Otolaryngology*, 8(3), pp. 338–355. doi: 10.1007/s10162-007-0084-9.

Soken, H. *et al.* (2013) 'Mouse cochleostomy: A minimally invasive dorsal approach for modeling cochlear implantation', *Laryngoscope*, 123(12). doi: 10.1002/lary.24174.

Somdas, M. A. *et al.* (2007) 'Quantitative evaluation of new bone and fibrous tissue in the cochlea following cochlear implantation in the human', *Audiology and Neurotology*, 12(5), pp. 277–284. doi: 10.1159/000103208.

Sorrentino, T. *et al.* (2009) 'Cochlear reimplantations: technical and surgical failures', *Acta Oto-*

Laryngologica, 129(4), pp. 380–384. doi: 10.1080/00016480802552576.

Spiers, K. *et al.* (2016) 'An X-ray fluorescence microscopic analysis of the tissue surrounding the multi-channel cochlear implant electrode array', *Cochlear Implants International*. Taylor & Francis, 17(3), pp. 129–131. doi: 10.1080/14670100.2016.1157943.

Stabenau, K. A. *et al.* (2021) 'RNA sequencing and pathways analyses of middle ear epithelia from patients with otitis media', *Laryngoscope*, pp. 1–8. doi: 10.1002/lary.29551.

Steel, K. P. and Barkway, C. (1989) 'Another role for melanocytes: their importance for normal stria vascularis development in the mammalian inner ear.', *Development (Cambridge, England)*, 107(3), pp. 453–63. doi: 10.1016/0168-9525(90)90041-4.

Stevens, S. M. *et al.* (2019) 'Is hard failure still a common indication for revision surgery in adult cochlear implant recipients?', *Otology and Neurotology*, 40(3), pp. 321–327. doi: 10.1097/MAO.0000000000002118.

Stol, K. *et al.* (2009) 'Development of a non-invasive murine infection model for acute otitis media', *Microbiology*, 155(12), pp. 4135–4144. doi: 10.1099/mic.0.033175-0.

Su, Z. *et al.* (2020) 'Transcriptomic analysis highlights cochlear inflammation associated with age-related hearing loss in C57BL/6 mice using next generation sequencing', *PeerJ*, 8, pp. 1–18. doi: 10.7717/peerj.9737.

Subramaniam, V., Ashkar, A. and Rai, S. (2020) 'Cochlear dysfunction in chronic otitis media and its determinants', *Iranian Journal of Otorhinolaryngology*, 32(2), pp. 79–84. doi: 10.22038/ijorl.2019.35045.2158.

Suhling, M. C. *et al.* (2016) 'The impact of electrode array length on hearing preservation in cochlear implantation', *Otology and Neurotology*, 37(8), pp. 1006–1015. doi: 10.1097/MAO.0000000000001110.

Sun, S. *et al.* (2014) 'Inhibition of the activation and recruitment of microglia-like cells protects against neomycin-induced ototoxicity', *Molecular Neurobiology*, 51(1), pp. 252–267. doi: 10.1007/s12035-014-8712-y.

Suzuki, T. *et al.* (2006) 'Age-dependent degeneration of the stria vascularis in human cochleae', *Laryngoscope*, 116(10), pp. 1846–1850. doi: 10.1097/01.mlg.0000234940.33569.39.

Takahashi, M., Peppard, J. and Harris, J. P. (1989) 'Immunohistochemical study of murine middle ear and eustachian tube', *Acta Oto-Laryngologica*, 107(1–2), pp. 97–103. doi:

10.3109/00016488909127485.

Tan, B. T. G., Lee, M. M. G. and Ruan, R. (2008) 'Bone marrow-derived cells that home to acoustic deafened cochlea preserved their hematopoietic identity', *Journal of Comparative Neurology*, 509(2), pp. 167–179. doi: 10.1002/cne.21729.

Tan, W. J. (2013) 'Noise-induced cochlear inflammation', *World Journal of Otorhinolaryngology*, 3(3), p. 89. doi: 10.5319/wjo.v3.i3.89.

Tan, W. J. T., Thorne, P. R. and Vlajkovic, S. M. (2016) 'Characterisation of cochlear inflammation in mice following acute and chronic noise exposure', *Histochemistry and Cell Biology*. Springer Berlin Heidelberg, 146(2), pp. 219–230. doi: 10.1007/s00418-016-1436-5.

Tateossian, H. *et al.* (2013) 'Otitis media in the Tgif knockout mouse implicates TGFβ signalling in chronic middle ear inflammatory disease', *Human Molecular Genetics*, 22(13), pp. 2553–2565. doi: 10.1093/hmg/ddt103.

Teele, David W, Klein Jerome O, Rosner, B. (1989) 'Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study', *Journal of infectious diseases*, 160(1), pp. 83–94.

Thorne, M. *et al.* (1999) 'Cochlear fluid space dimensions for six species derived from reconstructions of three-dimensional magnetic resonance images', *Laryngoscope*, 109(10), pp. 1661–1668. doi: 10.1097/00005537-199910000-00021.

Tian, C. *et al.* (2017) 'Genome-wide association and HLA region fine-mapping studies identify susceptibility loci for multiple common infections', *Nature Communications*. Springer US, 8(1). doi: 10.1038/s41467-017-00257-5.

Tornabene, S. V *et al.* (2006) 'Immune cell recruitment following acoustic trauma', *Hearing Research*, 222, pp. 115–124. doi: 10.1016/j.heares.2006.09.004.

Tos, M. (1980) 'Middle ear epithelia in chronic secretory otitis media', *Archives of Otolaryngology*, 106(10), pp. 593–597. doi: 10.1001/archotol.1980.00790340001001.

Toulemonde, P. *et al.* (2021) 'Evaluation of the efficacy of dexamethasone-eluting electrode array on the post-implant cochlear fibrotic reaction by three-dimensional immunofluorescence analysis in mongolian gerbil cochlea', *Journal of Clinical Medicine*, 10(15), p. 3315. doi: 10.3390/jcm10153315.

Trune, D. R. *et al.* (2015) 'Correlative mRNA and protein expression of middle and inner ear

inflammatory cytokines during mouse acute otitis media', *Hearing Research*. Elsevier B.V, 326, pp. 49–58. doi: 10.1016/j.heares.2015.04.006.

Trune, D. R. and Zheng, Q. Y. (2009) 'Mouse models for human otitis media', *Brain Research*, 1277, pp. 90–103. doi: 10.1016/j.brainres.2009.02.047.

Tsai, Y. J. *et al.* (2011) 'Intratympanic injection with dexamethasone for sudden sensorineural hearing loss', *Journal of Laryngology and Otology*, 125(2), pp. 133–137. doi: 10.1017/S0022215110002124.

Urík, M., Tedla, M. and Hurník, P. (2021) 'Pathogenesis of retraction pocket of the tympanic membrane-A narrative review', *Medicina (Lithuania)*, 57(5). doi: 10.3390/medicina57050425.

Vandevyver, S. *et al.* (2013) 'New insights into the anti-inflammatory mechanisms of glucocorticoids: an emerging role for glucocorticoid-receptor-mediated transactivation', *Endocrinology*, 154(3), pp. 993–1007. doi: 10.1210/en.2012-2045.

Vanneste, P. and Page, C. (2019) 'Otitis media with effusion in children: pathophysiology, diagnosis, and treatment', *Journal of Otology*, 14(2), pp. 33–39. doi: 10.1016/j.joto.2019.01.005.

Varatharaj, A. and Galea, I. (2017) 'The blood-brain barrier in systemic inflammation', *Brain Behavior and Immunity*, 60, pp. 1–12. doi: 10.1016/j.bbi.2016.03.010.

Varsak, Y. K. and Santa Maria, P. L. (2016) 'Mouse model of experimental Eustachian tube occlusion: a surgical technique', *Acta Oto-Laryngologica*, 136(1), pp. 12–17. doi: 10.3109/00016489.2015.1082191.

Venail, F. *et al.* (2008) 'Reliability and complications of 500 consecutive cochlear implantations', *Archives of Otolaryngology - Head and Neck Surgery*, 134(12), pp. 1276–1281. doi: 10.1001/archoto.2008.504.

Venekamp, R. P., Damoiseaux, R. A. M. J. and Schilder, A. G. M. (2014) 'Acute otitis media in children', *BMJ Clinical Evidence*, 09(301), pp. 1–21. Available at: http://sl4tb4rv5r.search.serialssolutions.com/.

Verschuur, C. A. *et al.* (2012) 'Markers of inflammatory status are associated with hearing threshold in older people: findings from the Hertfordshire ageing study', *Age and Ageing*, 41(1), pp. 92–97. doi: 10.1093/ageing/afr140.

Verschuur, C., Agyemang-Prempeh, A. and Newman, T. A. (2014) 'Inflammation is associated with a worsening of presbycusis: Evidence from the MRC national study of hearing', *International*

Journal of Audiology, 53(7), pp. 469–475. doi: 10.3109/14992027.2014.891057.

Vethanayagam, R. R. *et al.* (2016) 'Toll-like receptor 4 modulates the cochlear immune response to acoustic injury', *Cell Death and Disease*, 7(6). doi: 10.1038/cddis.2016.156.

Viana, L. M. *et al.* (2015) 'Cochlear neuropathy in human presbycusis: Confocal analysis of hidden hearing loss in post-mortem tissue', *Hearing Research*, 327, pp. 78–88. doi: 10.1016/j.heares.2015.04.014.

Vikhe, P. P. *et al.* (2018) 'Cellular content plays a crucial role in NTHi infection of pre-inflamed Junbo mouse middle ear', *Cellular Microbiology*, (July). doi: 10.1111/cmi.12960.

Vikhe, P. P. *et al.* (2019) 'Cellular immune response against nontypeable haemophilus influenzae infecting the preinflamed middle ear of the junbo mouse', *Infection and Immunity*, 87(12), pp. 1–17. doi: 10.1128/IAI.00689-19.

Villanueva, A. R., Hattner, R. S. and Frost, H. M. (1964) 'A tetrachrome stain for fresh, mineralized bone sections, useful in the diagnosis of bone diseases', *Biotechnic and Histochemistry*, 39(2), pp. 87–94. doi: 10.3109/10520296409061213.

Vos, T. *et al.* (2015) 'Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: A systematic analysis for the Global Burden of Disease Study 2013', *The Lancet*, 386(9995), pp. 743–800. doi: 10.1016/S0140-6736(15)60692-4.

Wakabayashi, K. *et al.* (2010) 'Blockade of interleukin-6 signaling suppressed cochlear inflammatory response and improved hearing impairment in noise-damaged mice cochlea', *Neuroscience Research*. Elsevier Ireland Ltd and Japan Neuroscience Society, 66(4), pp. 345–352. doi: 10.1016/j.neures.2009.12.008.

Wang, J. T. *et al.* (2014) 'Rates of revision and device failure in cochlear implant surgery: A 30-year experience', *Laryngoscope*, 124(10), pp. 2393–2399. doi: 10.1002/lary.24649.

Wang, W. *et al.* (2019) 'Neuroinflammation mediates noise-induced synaptic imbalance and tinnitus in rodent models', *PLoS biology*, 17(6), p. e3000307. doi: 10.1371/journal.pbio.3000307.

Wang, X. *et al.* (2003) 'Blockage of immune-mediated inner ear damage by etanercept', *Otology & Neurotology*, 24(10), pp. 52–57. doi: 10.1097/00129492-200301000-00012.

Wang, Y., Hirose, K. and Liberman, M. C. (2002) 'Dynamics of noise-induced cellular injury and repair in the mouse cochlea', *JARO - Journal of the Association for Research in Otolaryngology*,

3(3), pp. 248–268. doi: 10.1007/s101620020028.

Wang, Z. *et al.* (2019) 'Macrophage plasticity and function in the eye and heart', *Trends in Immunology*. Elsevier Ltd, 40(9), pp. 825–841. doi: 10.1016/j.it.2019.07.002.

Watanabe, S. *et al.* (2019) 'The role of macrophages in the resolution of inflammation', *Journal of Clinical Investigation*, 129(7), pp. 2619–2628. doi: 10.1172/JCI124615.

Watson, D. W. and Kim, Y. B. (1963) 'Modification of host responses to bacterial endotoxins', *Journal of Experimental Medicine*, 118(3), pp. 425–446.

Watson, N. *et al.* (2017) 'Chronic inflammation – inflammaging – in the ageing cochlea: A novel target for future presbycusis therapy', *Ageing Research Reviews*, 40(August), pp. 142–148. doi: 10.1016/j.arr.2017.10.002.

Weise, J. B. *et al.* (2005) 'Impact to the head increases cochlear implant reimplantation rate in children', *Auris Nasus Larynx*, 32(4), pp. 339–343. doi: 10.1016/j.anl.2005.03.017.

Weller, P. F. and Spencer, L. A. (2017) 'Functions of tissue-resident eosinophils', *Nature Reviews Immunology*, 17(12), pp. 746–760. doi: 10.1038/nri.2017.95.

Wells, H. R. R., Newman, T. A. and Williams, F. M. K. (2020) 'Genetics of age-related hearing loss', *Journal of Neuroscience Research*. doi: 10.1002/jnr.24549.

Wendeln, A.-C. *et al.* (2018) 'Innate immune memory in the brain shapes neurological disease hallmarks', *Nature*, 556(7701), pp. 332–338. doi: 10.1038/s41586-018-0023-4.

Widmann, G. *et al.* (2020) 'Pre- and post-operative imaging of cochlear implants: a pictorial review', *Insights into Imaging*. Insights into Imaging, 11(1). doi: 10.1186/s13244-020-00902-6.

Wijaya, C. *et al.* (2019) 'Revision cochlear implantation: The Irish experience', *Cochlear Implants International*. Taylor & Francis, 20(6), pp. 281–287. doi: 10.1080/14670100.2019.1647372.

Wilgus, T. A., Roy, S. and McDaniel, J. C. (2013) 'Neutrophils and wound repair: positive actions and negative reactions', *Advances in Wound Care*, 2(7), pp. 379–388. doi: 10.1089/wound.2012.0383.

Wilk, M. *et al.* (2016) 'Impedance changes and Ffbrous tissue growth after cochlear implantation are correlated and can be reduced using a dexamethasone-eluting electrode', *PLoS ONE*, 11(2), pp. 1–19. doi: 10.1371/journal.pone.0147552.

Williamson, I. G. *et al.* (1994) 'The natural history of otitis media with effusion-a three-year study

of the incidence and prevalence of abnormal tympanograms in four south west hampshire infant and first schools', *The Journal of Laryngology & Otology*, 108(11), pp. 930–934. doi: 10.1017/S0022215100128567.

Winskel, H. (2006) 'The effects of an early history of otitis media on children's language and literacy skill development', *British Journal of Educational Psychology*, 76(4), pp. 727–744. doi: 10.1348/000709905X68312.

Woo, J. I. *et al.* (2010) 'Spiral ligament fibrocyte-derived MCP-1/CCL2 contributes to inner ear inflammation secondary to nontypeable H. influenzae-induced otitis media', *BMC Infectious Diseases*, 10, pp. 1–10. doi: 10.1186/1471-2334-10-314.

Xu, H. X., Joglekar, S. S. and Paparella, M. M. (2009) 'Labyrinthitis Ossificans', *Otology & Neurotology*, 30, pp. 579–580. doi: 10.1097/MAO.0b013e31819fe81c.

Xu, X. *et al.* (2012) 'EVI1 acts as an inducible negative-feedback regulator of NFkB by inhibiting p65 acetylation', *The Journal of Immunology*, 188(12), pp. 6371–6380. doi: 10.4049/jimmunol.1103527.

Yamahara, K. *et al.* (2018) 'Hearing preservation at low frequencies by insulin-like growth factor 1 in a guinea pig model of cochlear implantation', *Hearing Research*, 368, pp. 92–108. doi: 10.1016/j.heares.2018.07.004.

Yamasaki, R. *et al.* (2014) 'Differential roles of microglia and monocytes in the inflamed central nervous system', *Journal of Experimental Medicine*, 211(8), pp. 1533–1549. doi: 10.1084/jem.20132477.

Yang, W. *et al.* (2015) 'Activation of the antigen presentation function of mononuclear phagocyte populations associated with the basilar membrane of the cochlea after acoustic overstimulation', *Neuroscience*, 303, pp. 1–15. doi: 10.1016/j.neuroscience.2015.05.081.

Yen, Y. C. *et al.* (2015) 'Higher risk of developing sudden sensorineural hearing loss in patients with chronic otitis media', *JAMA Otolaryngology - Head and Neck Surgery*, 141(5), pp. 429–435. doi: 10.1001/jamaoto.2015.102.

Zeitler, D. M., Budenz, C. L. and Roland, J. T. (2009) 'Revision cochlear implantation', *Current Opinion in Otolaryngology and Head and Neck Surgery*, 17(5), pp. 334–338. doi: 10.1097/MOO.0b013e32832dd6ac.

Zhang, C. *et al.* (2017) 'Loss of sestrin 2 potentiates the early onset of age-related sensory cell degeneration in the cochlea', *Neuroscience*, 361, pp. 179–191. doi:

10.1016/j.neuroscience.2017.08.015.

Zhang, C. *et al.* (2020) 'New insights on repeated acoustic injury: Augmentation of cochlear susceptibility and inflammatory reaction resultant of prior acoustic injury', *Hearing Research*. Elsevier B.V, 393, p. 107996. doi: 10.1016/j.heares.2020.107996.

Zhang, F. *et al.* (2013) 'Perivascular macrophage-like melanocyte responsiveness to acoustic trauma-a salient feature of strial barrier associated hearing loss', *FASEB Journal*, 27(9), pp. 3730–3740. doi: 10.1096/fj.13-232892.

Zhang, J. *et al.* (2015) 'Lipopolysaccharide-induced middle ear inflammation disrupts the cochlear intra-strial fluid-blood barrier through down-regulation of tight junction proteins', *PLoS ONE*, 10(3), pp. 1–20. doi: 10.1371/journal.pone.0122572.

Zhang, W. *et al.* (2012) 'Perivascular-resident macrophage-like melanocytes in the inner ear are essential for the integrity of the intrastrial fluid-blood barrier.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(26), pp. 10388–93. doi: 10.1073/pnas.1205210109.

Zuijlen Van, D. A. *et al.* (2001) 'National differences in incidence of acute mastoiditis: relationship to prescribing patterns of antibiotics for acute otitis media?', *Pediatric Infectious Disease Journal*, 20(2), pp. 140–144. doi: 10.1097/00006454-200102000-00004.