# THE REGISTRY

## CONTENTS

## MISSION STATEMENT

The NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry was established in 1992 by the National Institute on Deafness and Other Communication Disorders (NIDCD) of the National Institutes of Health (NIH) to continue and expand upon the former National Temporal Bone Banks (NTBB) Program. The Registry promotes research on hearing and balance disorders and serves as a resource for the public and scientific communities about research on the pathology of the human auditory and vestibular systems.

# Enhancing the Accessibility of Archived Human Temporal Bone Sections for Immunohistochemical Analysis

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The introduction of immunohistochemistry in human otopathology research nearly half a century ago[1,2] has significantly advanced the knowledge of the normal morphology of the human ear and its pathomorphology in various disease states. However, oto-immunohistochemistry on archival decalcified, formalin-fixed, celloidin-embedded tissue sections of human post mortem temporal bones (CE-TB sections)—the primary tissue sources in otopathology research—bears inherent methodological constraints.[3]

Foremost, the prolonged formalin fixation times (2–3 weeks) of the extracted large temporal bone specimens causes excessive formalin-induced cross-linking of tissue proteins, which "masks" them for antibody-antigen binding. For many other tissues, this problem can be overcome by applying heat-induced antigen retrieval (HIAR) methods prior to immunohistochemical protocols, in order to "un-mask" tissue proteins and enhance antibody-antigen binding. However, the delicate CE-TB sections do not mechanically withstand these harsh HIAR procedures, which expose the tissue sections to temperatures between 80–110°C. Consequently, the accessibility of CE-TB sections to immunohistochemical methods remains rather limited.

To enhance the performance of immunohistochemical protocols on CE-TB sections and to broaden the spectrum of antibodies that can be applied in oto-immunohistochemistry, we developed a simple technique that allows the application of HIAR on CE-TB sections.
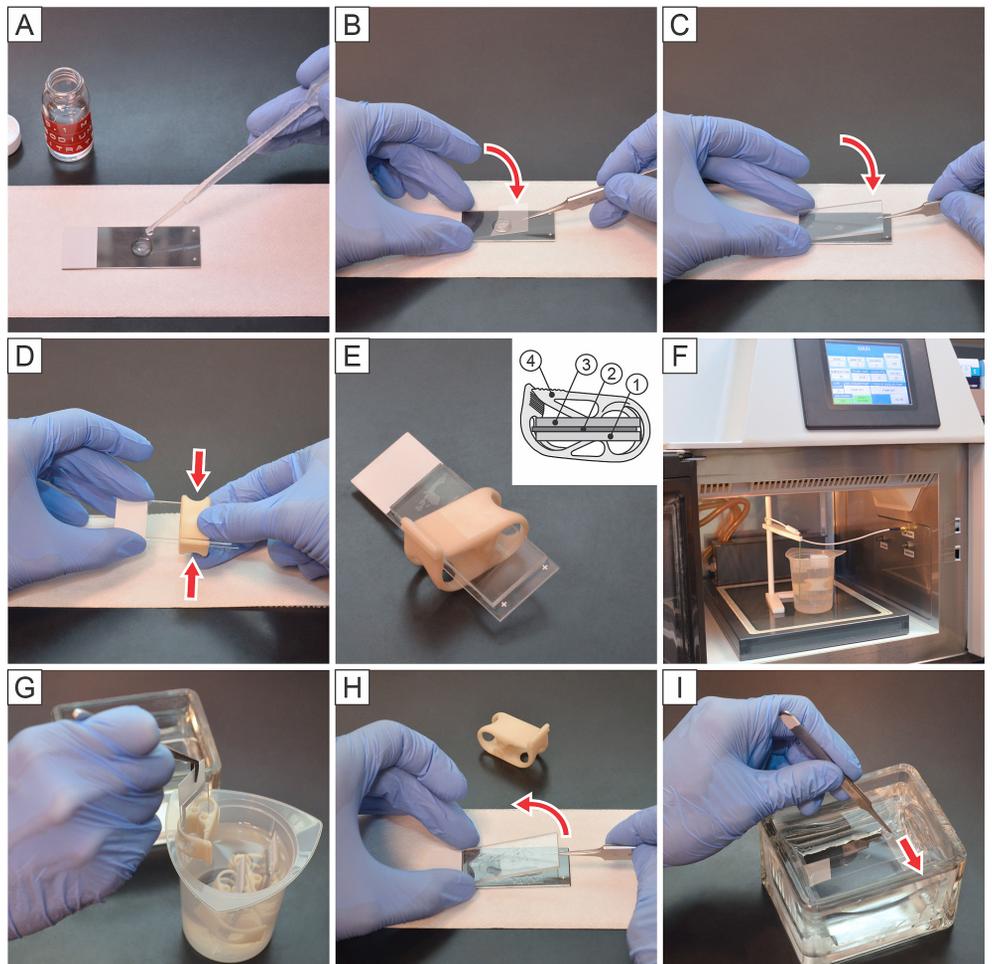
**Figure 1. Step-by-step instructions for pressure coverslipping of mounted tissue sections for HIAR treatment. (A)** Cover the mounted tissue section with antigen retrieval buffer solution. **(B)** Cover the section with a coverslip. **(C)** Place a glass slide on top of the coverslip. **(D)** Press together the two sandwiched glass slides, the tissue section, and the coverslip with a plastic tubing clamp. **(E)** (1) microscope slide with mounted tissue section, (2) coverslip, (3) second microscope slide, (4) plastic tubing clamp. **(F)** Place the pressure-coverslipped slide in a plastic beaker with antigen retrieval buffer solution for heating (e.g., in a microwave oven). **(G)** After heat exposure, transfer the (hot) clamped slide to a glass jar filled with distilled water. **(H)** After a cool-down period (5 min), remove the clamp and the glass slide that resides on top of the coverslip. **(I)** Place the slide in a glass jar filled with distilled water and wait until the coverslip floats off the slide. Proceed with the immunohistochemical labeling protocol. *Adapted from [4], according to the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by-nc/4.0/), under which the original article is distributed.*

The basic principle of this technique is to mechanically press the mounted CE-TB section on the surface of the glass slide during the HIAR procedure, to prevent detachment and tissue damage when the applied heat weakens the adhesive chemical bonds and provokes air bubble formation.

This is achieved by temporally clamping a Teflon sheet and a glass slide on top of the mounted tissue section. An illustrated step-by-step manual of this technique is provided above in Figure 1. The effectiveness of the HIAR procedure in unmasking tissue antigens, improving immunohistochemical labeling results, and preserving the delicate tissue morphology in CE-TB sections is illustrated in Figure 2.

In summary, this "pressurized coverslipping" technique enhances the quality and sensitivity of immunohistochemical protocols on CE-TB sections. It also greatly increases the spectrum of antibodies for application in oto-immunohistochemistry. ●

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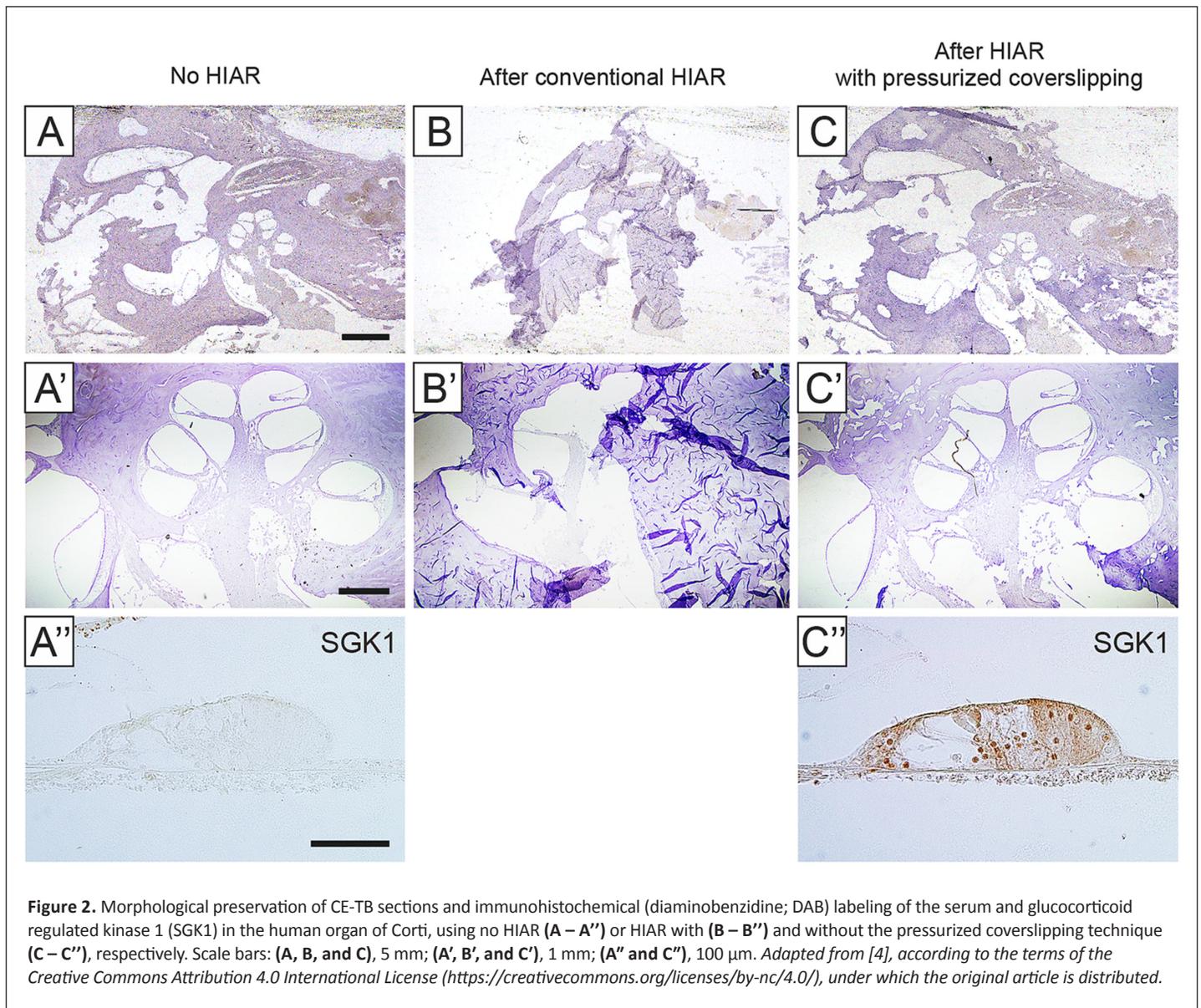
**Figure 2.** Morphological preservation of CE-TB sections and immunohistochemical (diaminobenzidine; DAB) labeling of the serum and glucocorticoid regulated kinase 1 (SGK1) in the human organ of Corti, using no HIAR **(A – A'')** or HIAR with **(B – B'')** and without the pressurized coverslipping technique **(C – C'')**, respectively. Scale bars: **(A, B, and C)**, 5 mm; **(A', B', and C')**, 1 mm; **(A" and C")**, 100 µm. *Adapted from [4], according to the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by-nc/4.0/), under which the original article is distributed.*

# Cochlear Histopathology in Human Genetic Hearing Loss: State of the Science and Future Prospects

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Hearing loss is the most common sensory deficit, affecting two to three out of 1,000 newborns and over half of the population above the age of 75.[1] Genetic hearing loss accounts for approximately 50 percent of all congenital cases (Figure 1) and is classified according to its occurrence as part of a recognized syndrome (syndromic hearing loss/SHL) or as an isolated finding (non-syndromic hearing loss/NSHL).[2] NSHL is responsible for 80 percent of genetic hearing loss and can be further subdivided according to pattern of inheritance—dominant, recessive, or X-linked.
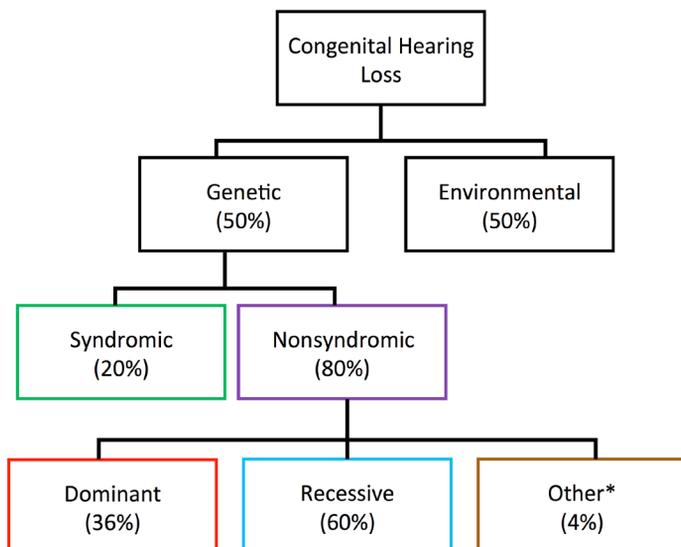


**Figure 1: Causes of congenital hearing loss in developed countries (Van Camp and Smith 2019).** TB histopathology has been reported for three genes implicated in autosomal dominant hearing loss (COCH, GSDME, and MYH9), one gene implicated in recessive hearing loss (GJB2), and eighteen genes implicated in syndromic hearing loss (COL4A5, EYA1, CDH23/PCDH15, PAX3, ERCC6, ERCC8, XPA, XPC, XPD, KCNQ1, TIMM8A, NDS, NF2, GJB2, CHD7, SLC26A4, and PHYH). * = X-linked, mitochondrial, and other causes of hearing loss.

To date, nearly 200 deafness-related loci and causative genes within these loci have been identified; however, the pathophysiology associated with these mutations has rarely been studied at the cellular level in living humans due to the challenges associated with accessing the primary organ of hearing, the cochlea within the inner ear.[3] The cochlea's small size, fragility, and encasement in the densest bone in the body located deep within the skull have made it difficult to resolve human cochlear microanatomy in living people with conventional clinical imaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI). Consequently, two approaches have been used to study the pathology of hereditary hearing loss: 1) post-mortem analysis of human temporal bone (TB) specimens, and 2) the creation of mouse models with the intention of reproducing the human phenotype.

The study of human TBs from autopsies began in the 1920s and today the process remains largely unchanged. Samples are acquired several hours post-mortem, fixed in formalin, decalcified, embedded, and sectioned for preservation and further analysis. Results are presented as case reports that describe TB histopathology for a patient with a known cause of deafness. These studies are difficult to conduct due to the limited availability of TB specimens and the laborious process required to generate hematoxylin and eosin-stained slides, which can take several months to a year. Specimens are infrequently donated to research and few institutions have the infrastructure to process and analyze them. Importantly, these obstacles have limited the study of human TB histopathology to only 22 genes of the nearly 200 genes implicated in deafness (Figure 2).

The other method for studying human hearing loss is through comparative genetics using mouse models. Mouse models for hearing loss were first developed in the 1920s and are useful because of the similarities between mouse and human cochleae.[4]
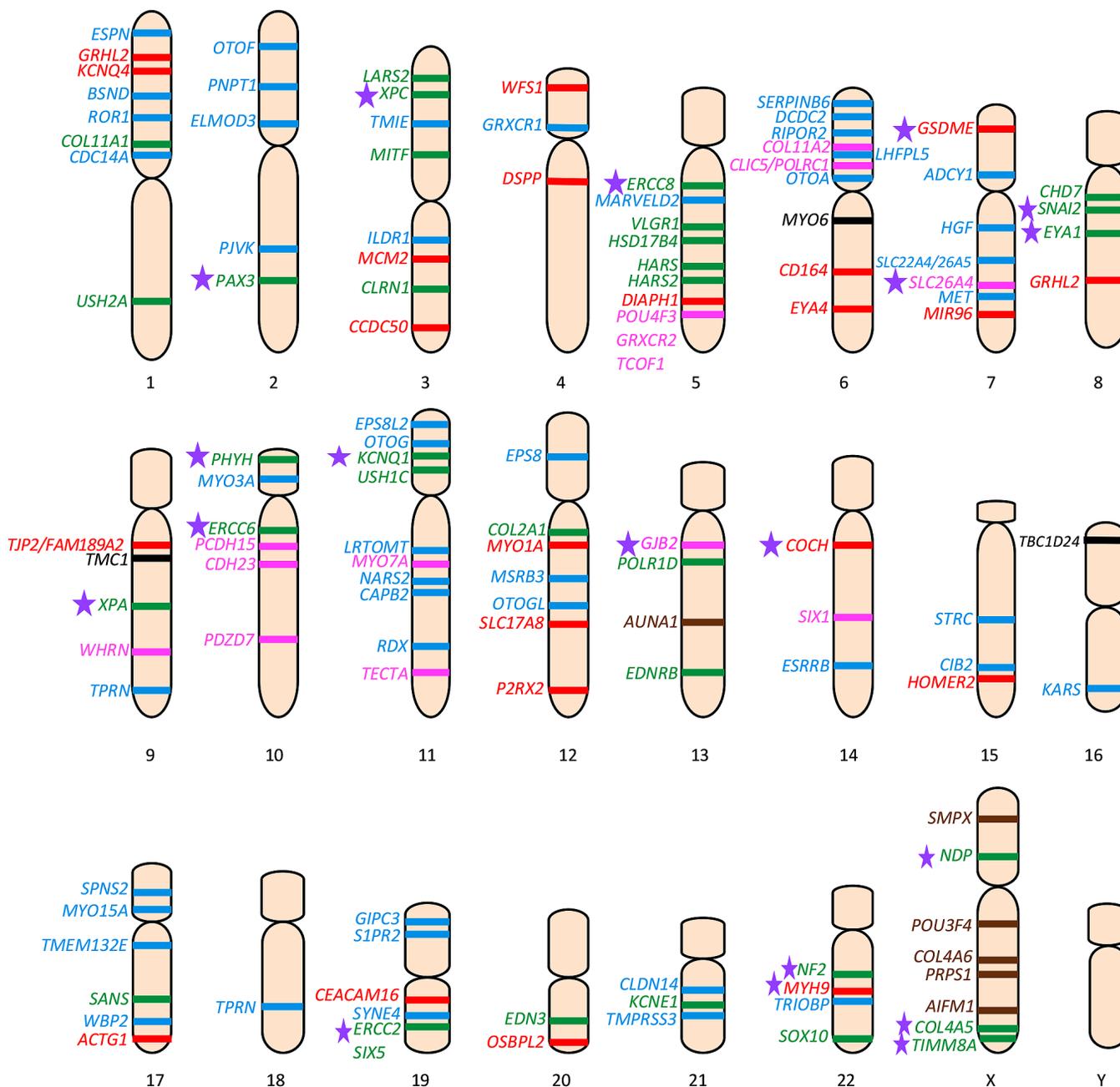
**Figure 2: Chromosomal locations of genes associated with mutation-induced SNHL.** The genes are classified according to the type of genetic hearing loss that they are associated with and the color convention is the same as in Figure 1. Those with corresponding human TB histopathology are identified with a purple star. *Modified from Dror and Avraham 2010 (Dror et al., 2010). Note: Several syndromes were diagnosed according to clinical findings. In these cases, while a particular genetic mutation is suggested as the cause, this has not always been confirmed by molecular testing.*

**Table 1:** Genetic causes of syndromic hearing loss (SHL).

| Syndrome | Human cochlear pathology | Syndrome | Human cochlear pathology |
|---|---|---|---|
| **Usher Syndrome**[12,37-40] | • Patchy atrophy or fibrosis of stria vascularis<br>• Degenerate organ of Corti at basal turn<br>• Reduction in numbers of spiral ganglion cells<br>• Atrophied nerve fibers<br>• Normal stria vascularis | **Neurofibromatosis**[48-52] | • Atrophy of stria vascularis<br>• Loss of hair cells<br>• Loss of cochlear neurons<br>• Cochlear hydrops<br>• No inner ear abnormalities<br>• Neuronal loss<br>• Neo-ossification of basal segment<br>• Separation of cochlear nerve fibers<br>• Invasion of cochlea by tumor<br>• Diminished blood supply to cochlea |
| **Alport Syndrome**[41] | • Mild loss of hair cells at basal turn<br>• Mild, patchy atrophy of stria vascularis<br>• Partial loss of cochlear neurons at basal turn<br>• Apical endolymphatic hydrops<br>• No inner ear abnormalities<br>• Severe loss of hair cells | | |
| **Branchial-oto-renal Syndrome**[4] | • Mild atrophy of stria vascularis<br>• Loss of cochlear neurons and spiral ganglion cells at basal turn<br>• Apical endolymphatic hydrops<br>• Dislocation of spiral ganglion cells to the fundus of the internal auditory canal | **Keratosis-Ichthyosis-Deafness Syndrome**[53-57] | • Cochleosaccular dysplasia<br>• Hypercellularity of the stria vascularis<br>• Partial collapse of Reissner's membrane<br>• Normal stria vascularis<br>• Normal population of spiral ganglion cells<br>• Melanin deposits in the stria vascularis |
| **Jervell and Lange-Nielsen Syndrome**[43] | • Patchy atrophy or fibrosis of stria vascularis<br>• Damaged inner and outer hair cells<br>• Reduction in numbers of ganglion cells<br>• Atrophied nerve fibers | **Xeroderma Pigmentosum**[58-60] | • No inner ear abnormalities<br>• Patchy atrophy of the stria vascularis<br>• Severe atrophy of cochlear neurons |
| **Waardenburg Syndrome**[44] | • Atrophy of stria vascularis<br>• Absent organ of Corti<br>• Reduction in numbers of ganglion cells<br>• Atrophied nerve fibers<br>• Absent ganglion cells | **Cockayne Syndrome**[61-64] | • Patchy loss of inner and outer hair cells<br>• Severe atrophy of the stria vascularis<br>• Moderate to severe reduction of spiral ganglion neurons |
| **Mohr-Tranebjaerg Syndrome**[45,46] | • Near total loss of cochlear neurons<br>• Mild atrophy of stria vascularis<br>• Atrophy of spiral ganglion cells<br>• Apical endolymphatic hydrops | **CHARGE Syndrome**[65,66] | • Cochlear hypoplasia<br>• Absent organ of Corti<br>• Reduction of spiral ganglion cells<br>• No inner ear abnormalities |
| **Norrie Disease**[45,47] | • Severe degeneration of organ of Corti at all turns<br>• Eosinophilic proteinaceous material in perilymphatic spaces<br>• Endolymphatic hydrops<br>• Severe degeneration of tectorial membrane<br>• Cellular loss in the stria vascularis and deposits of lamellar material in the subepithelial space<br>• Loss of spiral ganglion cells<br>• Fibrous tissue deposits between remaining spiral ganglion cells | **Pendred Syndrome**[67,68] | • Hair cell degeneration<br>• Degeneration of stria vascularis<br>• Endolymphatic hydrops<br>• Normal spiral ganglion cell count<br>• Severe atrophy of the stria vascularis |

Mouse mutants are generated through spontaneous mutations, radiation or chemically-induced mutations, or transgenic or knock-out mutations.[4] These models have been critically helpful for understanding the pathophysiology of many forms of hereditary deafness; however, the scarcity of human TB histopathology has made comparative studies between the two species challenging, and importantly, "men are not simply big mice."[5]

We recently conducted a comprehensive review of all published reports on the physiologic and morphologic manifestations of mutation-induced SNHL in humans, the latter ascertained via histopathologic assessment of TBs from patients with known mutation-induced SNHL.[6] In addressing the limitations of both of these methods, the review underscores the need for alternative and improved methods for studying and evaluating human hereditary deafness.

To date, nearly 150 genes have been implicated in nonsyndromic hearing loss.[3] Of these, just over one third display dominant inheritance and the remainder are recessive. Only one gene associated with autosomal recessive NSHL (GJB2) and three genes associated with autosomal dominant NSHL (COCH, GSDME, and MYH9) have corresponding human TB histopathology reports. Findings from these TB specimens included near total agenesis of the organ of Corti, hair cells, stria vascularis (GJB2), cochlear infiltrates (COCH), degeneration of spiral ganglion neurons (GSDME), and absence of the cochlear duct (MYH9).[7-11]

SHL is characterized by its co-occurrence with other clinical signs of disease. Approximately 20 percent of cases of genetic hearing loss are syndromic.[3] Some of the earliest reports of human TB histopathology describe this.[12] Fourteen syndromes with documented histopathology have been reported (Table 1).

Because of the challenges associated with acquiring and processing human TB specimens for study, our understanding of the pathophysiology of hearing loss relies heavily on the study of mouse models. It is important to consider that while mouse models allow for comparative genomics, they are often not used to study the same genetic changes that are seen in humans.

Of the 22 genes and syndromes for which human TB histopathology has been reported, we identified 17 with corresponding mouse models. Similarities and differences between the mouse and human phenotypes should thus be interpreted with caution. This is most evident in the mouse models for COCH, NF2, autosomal dominant GJB2, and MYH9, which did not faithfully mimic human inner ear pathology.[13-25] The COCH knockout model, for example, had normal hearing until 21 months, and subsequent hearing loss at very high frequencies.[16,26] When translating this age to humans, it would be analogous to 71 years, which is significantly older than the typical age of the onset of hearing loss in humans.[8-10,27,28] Additionally, there

were no observable histopathologic changes in the knockout model.[16,26] This suggests that the associated deafness phenotype is not caused by COCH haploinsufficiency, but by a dominant negative or gain-of-function effect in nonsensory regions of the inner ear.[16,26] A similar discrepancy exists between the mouse and human models of NF2.[14]

Human TB histopathology is most notable for inner ear damage, including cochlear degeneration, atrophy of the stria vascularis, loss of hair cells, and loss of spiral ganglion cells.[14] In stark contrast, the most recent NF2 knockout mouse model does not show structural abnormalities in the cochlea.[14] The quantity and nature of the differences between histopathologic findings from mouse models of hearing loss vs. human cases motivate the development of novel, improved methods for assessing the pathophysiology underlying human hereditary hearing loss.

Though there are many challenges associated with studying hearing loss in humans, recent technological developments, particularly in high resolution cochlear imaging and intracochlear fluid sampling, inspire hope that there may soon be significantly improved clinical tools for diagnosing hereditary deafness and understanding its underlying pathophysiology.

Currently, there is no alternative to studying human TBs via histologic preparation because conventional clinical imaging modalities cannot sufficiently resolve cochlear microstructures. To address these concerns, several high-resolution fluorescence-, phase contrast-, coherence-, and X-ray-based imaging techniques have been applied to the cochlea. In 2018, we introduced the concept and demonstrated the feasibility of a virtual human cochlear whole mount with the use of synchrotron radiation phase contrast imaging (SR-PCI) to provide 3D visualization of sensory cells and nerve fibers in the cochlea's sensory epithelium in non-decalcified, unstained, three-dimensionally intact human TBs (Figure 3).[29]

In addition to improvements in techniques for high-resolution imaging of the inner ear, efforts are underway to collect and analyze perilymph from living patients to infer the structural and functional integrity of cells.[30–32] We have recently developed a novel microneedle device for controlled and reliable human perilymph sampling, which brings us one step closer to clinical liquid biopsy of the inner ear.[33] This future diagnostic tool would allow us to infer the molecular mechanisms responsible for different forms of acquired and progressive hearing loss *in vivo*.

Improvements in cellular-level diagnostics of the inner ear have taken place alongside major advances in gene therapy.[34] In 2017, Landegger, et al., demonstrated that a synthetic AAV viral vector, called Anc80, has significantly improved efficiency of transducing inner and outer hair cells compared to other viral
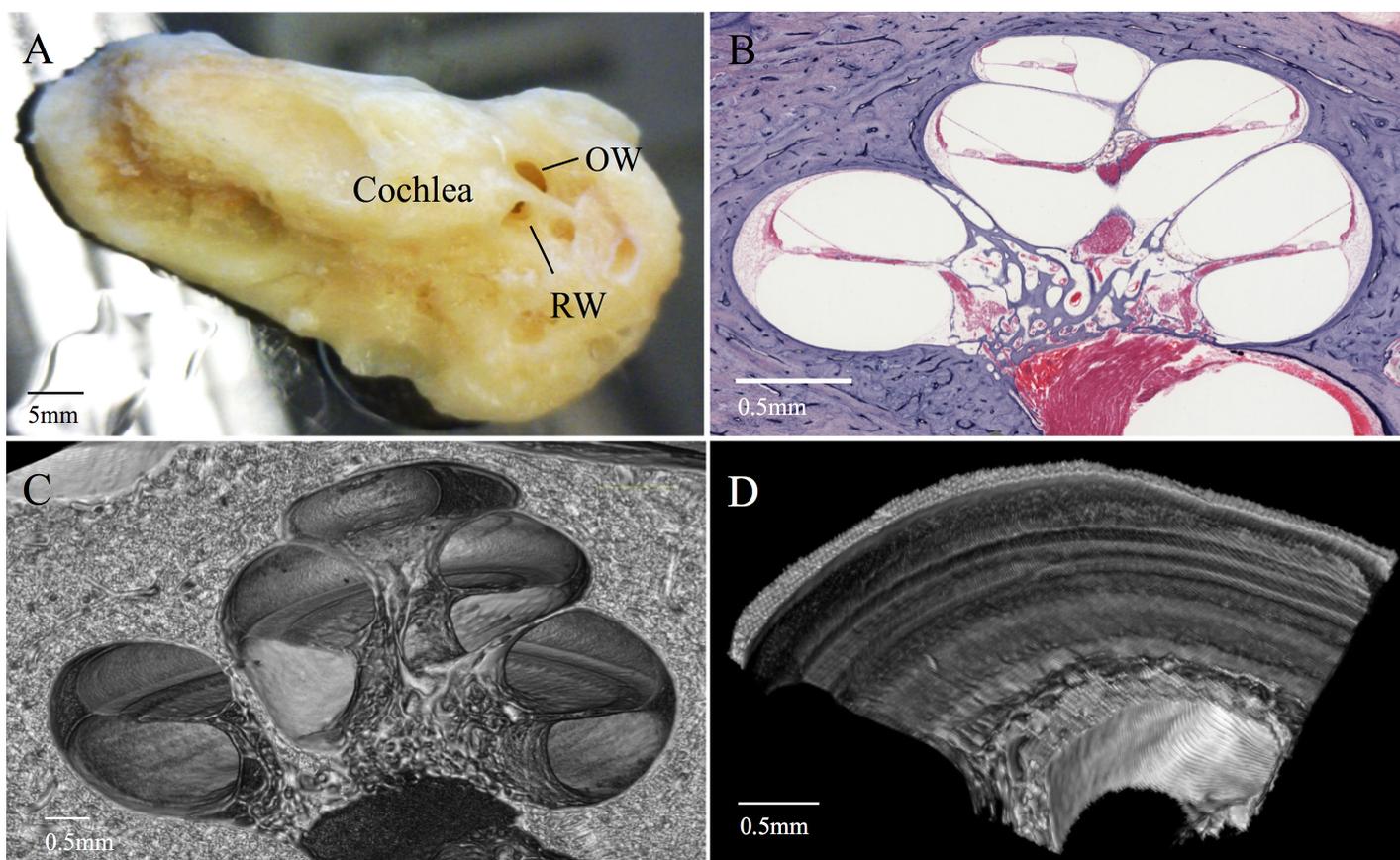
**Figure 3: Visualization of human intracochlear anatomy using standard histopathology and dissection technique versus synchrotron radiation phase contrast imaging (SR-PCI) and subsequent virtual sectioning using computer software. A)** Human cadaveric TB specimen, drilled to expose the round and oval windows (RW and OW, respectively). **B)** Mid-modiolar section through a hematoxylin and eosin-stained human cochlea. **C)** SR-PC image of a three-dimensionally intact, undecalcified, unstained TB, showing the spiraling nature of the organ of Corti. **D)** Virtual cochlear whole mount sectioned at the level of the organ of Corti, revealing rows of cells.

vectors.[35] While these encouraging results took place in animal models, similar approaches are now being implemented in the first clinical trial of gene therapy for deafness in humans.[36]

This review provides a comprehensive summary of results from human TB studies that have characterized inner ear histopathology in cases of genetic hearing loss. Additionally, we compare and contrast the available human histopathologic data to corresponding mouse models to highlight our continued reliance on human TB studies and the need to develop better tools to decipher cellular underpinnings of hearing loss in living people. Our findings strongly support the ongoing efforts to develop devices for cellular-level imaging and liquid biopsy of the living human inner ear to establish precise diagnosis and guide personalized therapies for deafness. In the meantime, the Latin saying "Mortui vivos docent" ("The dead teach the living") succinctly summarizes our approach, as human cadaveric TBs continue to guide the development and testing of new diagnostics for hearing loss. The maintenance and growth of human TB registries will be essential to accelerating progress in understanding and treating human hearing loss. ●

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# Otopathology Mini-Travel Fellowship Program

The NIDCD National Temporal Bone Registry's mini-travel fellowships provide funds for research technicians and young investigators to visit a temporal bone laboratory for a brief educational visit, lasting approximately one week. The emphasis is on the training of research assistants, technicians, and junior faculty.

These fellowships are available to:

- U.S. hospital departments who aspire to start a new temporal bone laboratory
- Inactive U.S. temporal bone laboratories who wish to reactivate their collections
- Active U.S. temporal bone laboratories who wish to learn new research techniques

Up to two fellowship awards will be made each year ($1,000 per fellowship). The funds may be used to defray travel and lodging expenses. Applications will be decided on merit.

Interested applicants should submit the following:

- An outline of the educational or training aspect of the proposed fellowship (1–2 pages)
- Applicant's curriculum vitae
- Letter of support from a temporal bone laboratory director or department chairman
- Letter from the host temporal bone laboratory indicating willingness to receive the traveling fellow

Applications should be submitted to:

**Felipe Santos, MD**
**NIDCD Temporal Bone Registry**
**Massachusetts Eye and Ear**
**243 Charles Street, Boston, MA 02114**
*felipe_santos@meei.harvard.edu*

**NIDCD National Temporal Bone,
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# Free Brochures for your Office or Clinic about Temporal Bone Research and Donation

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