THE REGISTRY

Newsletter of the NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry

Winter 2014
Vol. 22, No. 1

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 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 the scientific community about research on the pathology of the human auditory and vestibular systems.

Bacteria Within the Fibrous Matrices in Chronic Otitis Media

Authors: *Muzeyyen Yildirim-Baylan, M.D.[1,2], Patricia Schachern, B.S.[1], Vladimir Tsuprun, Ph.D.[1], Michael M. Paparella, M.D.[1,3], Sebahattin Cureoglu, M.D.[1]*

[1] Department of Otolaryngology, University of Minnesota, Minneapolis MN, USA
[2] Department of Otolaryngology, University of Dicle, Diyarbakir, Turkey
[3] Paparella Ear, Head and Neck Institute, Minneapolis MN, USA

Introduction

The management of chronic otitis media (COM) requires a complete understanding of the disease process. The nature and locations of the pathologic changes in the middle ear cleft are important considerations in medical and surgical decision-making. In many cases, COM with frequent reactivation may require extensive medical treatment and surgical cleaning. Despite substantial medical and surgical treatments, failure of management of COM is not uncommon.

To date, structural organization of bacteria in otitis media was mostly studied in association with bacterial communities in biofilms using small mucosal specimens removed during surgical biopsy[1,2] and chinchilla experimental models of otitis media.[3] In biofilms, structured communities of microorganisms adjacent to the tissue surface and embedded in a self-produced polysaccharide matrix are suggested to provide persistence of infection and resistance to antibiotic treatment.[1] The presence of host-derived fibrous material was also found in connection with biofilm in chinchilla middle ears.[3]

The purpose of this study was to evaluate the structure, occurrence, frequency, location, and extent of formations composed of bacteria within a network of fibrous matrix that were found in our collection of human temporal bones (HTBs) with COM. These formations were observed concurrently in the mastoid, middle ear, and inner ear and may play a role in reactivation of the disease. Since the detection of bacteria and bacterial communities in archival, celloidin-embedded HTBs may be technically challenging, a combination of different stains for bright-field light and fluorescent microscopy were used in this study to show the presence of bacteria within a fibrous matrix in these samples.

continued on page 2

THE REGISTRY

NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry

Massachusetts Eye and Ear
243 Charles Street
Boston, MA 02114

(800) 822-1327 Toll-Free Voice
(617) 573-3711 Voice
(617) 573-3838 Fax

Email: ***tbregistry@meei.harvard.edu***
Web: ***www.tbregistry.org***

Materials and Methods

One hundred and fifty cases with a clinical diagnosis of COM were screened and those with a history of immunosuppressive drugs, otological surgery, other sources of infection, or leukemia were excluded. COM was confirmed histologically by the presence of inflammation of the middle ear cleft that included at least one of the following types of tissue pathology: granulation tissue; cholesterol granuloma; cholesteatoma; bony changes; fibrosis; and tympanosclerosis. The study group including 20 cases (37 temporal bones) were from 5 females and 15 males with a mean age of 30.5 years and a range of 0-73 years.

Bacteria and their aggregates were detected by H-E. Additional sections were stained with gram Weigert stain to localize gram-positive bacteria by light microscopy or with 10 µg/ml propidium iodide (PI) (LIVE/DEAD Biofilm Viability Kit, Invitrogen) for 1 hour for red-fluorescent nucleic acid analysis to visualize bacteria and cell nuclei. PI is commonly used for DNA staining of bacteria cells with damaged membranes (DEAD stain) in biofilms. It has been used to stain cell nuclei in thick 100-µm celloidin-embedded sections of brain tissues.[4] Both gram Weigert and PI can be applied for staining a wide variety of both gram-positive and gram-negative bacteria, including bacteria in biofilms. Slides were examined using a conventional Nikon Eclipse 600 microscope with white light or epifluorescent illumination. To increase fluorescent staining, celloidin was removed from temporal bone sections using methanol saturated with sodium hydroxide, as described in previous studies.[5]

Results

Bacteria within the fibrous matrix composed mostly of rounded particles, approximately 0.5–1.2 µm in diameter and embedded within a matrix of amorphous extracellular material, were found adjacent to the mucosal surface in the middle ear cleft (Figure 1a). We observed bacteria within the fibrous matrix in 59% of the 37 temporal bones with histological evidence of COM, indicating these structures may correspond to some stage in the growth of biofilms in chronic human ears. The composition and role of the fibrous matrix in these structures is not clear; however, the network of the fibrous matrix may reduce the clearance of the bacteria by the host immune system. The bacteria within a fibrous matrix are likely to play potential role in reactivation of disease.

Many rounded bacterial particles and their aggregates of different sizes were darkly stained with gram Weigert stain for gram-positive bacteria (Fig. 1b) and H-E staining (Fig. 1c). Bacteria within the fibrous matrix frequently occupied large areas of the middle ear (Fig. 1b, c). Fluorescent microscopy of celloidin sections of temporal bones stained for DNA with PI showed the inflammatory cells' nuclei and bacteria (Fig. 1d). All bacteria within the fibrous matrixes in the samples were attached to the mucosal surface and showed bacterial clusters of different sizes that were frequently seen in association with a fibrous meshwork. Some scattered polymorphonuclear leukocytes, monocytes, and other inflammatory cells were seen mostly on the periphery of these formations. Histologically they resemble the biofilms reported for *Streptococcus pneumoniae,* nontypeable *Haemophilus influenzae,* and *Pseudomonas aeruginosa* infections in chinchilla experimental models of otitis media. No fibrous matrix or bacteria was detected in temporal bones of patients without otitis media.

Although previous similar studies in humans were limited by the size of a biopsy specimen, the study presented in this paper allowed the evaluation of the entire temporal bone, and the identification of bacteria within a fibrous matrix in precise anatomic locations within the middle ear cleft. Temporal bones with COM showed multiple anatomical locations of these formations. The locations seemed to be related to those anatomical areas with poor drainage and poor aeration, which would provide suitable

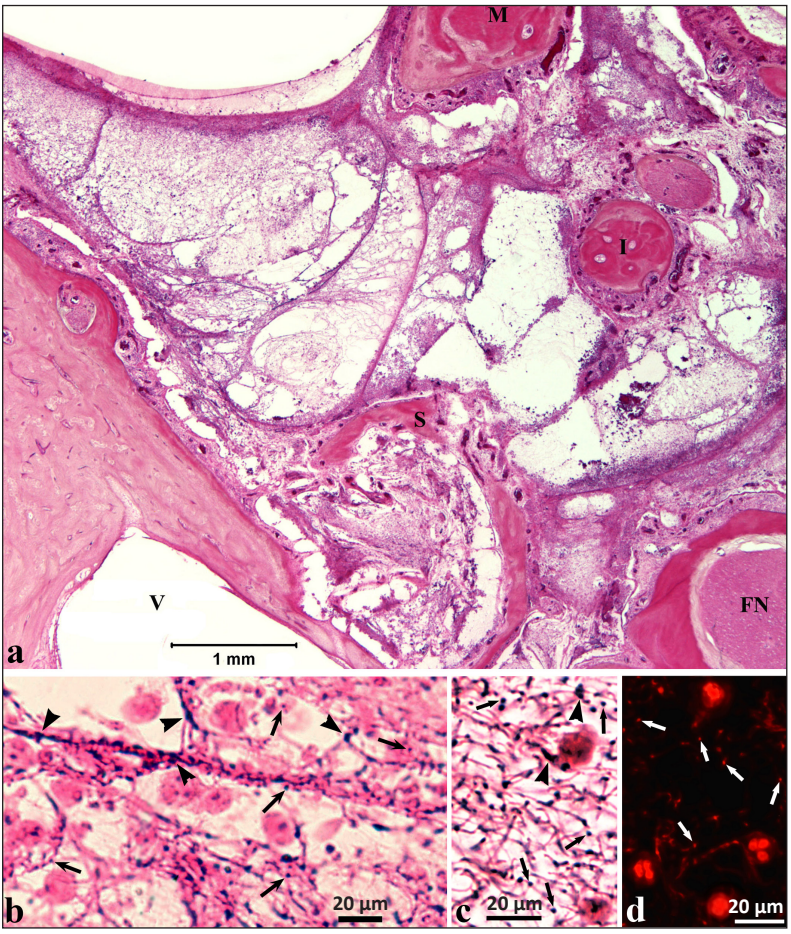

Figure 1

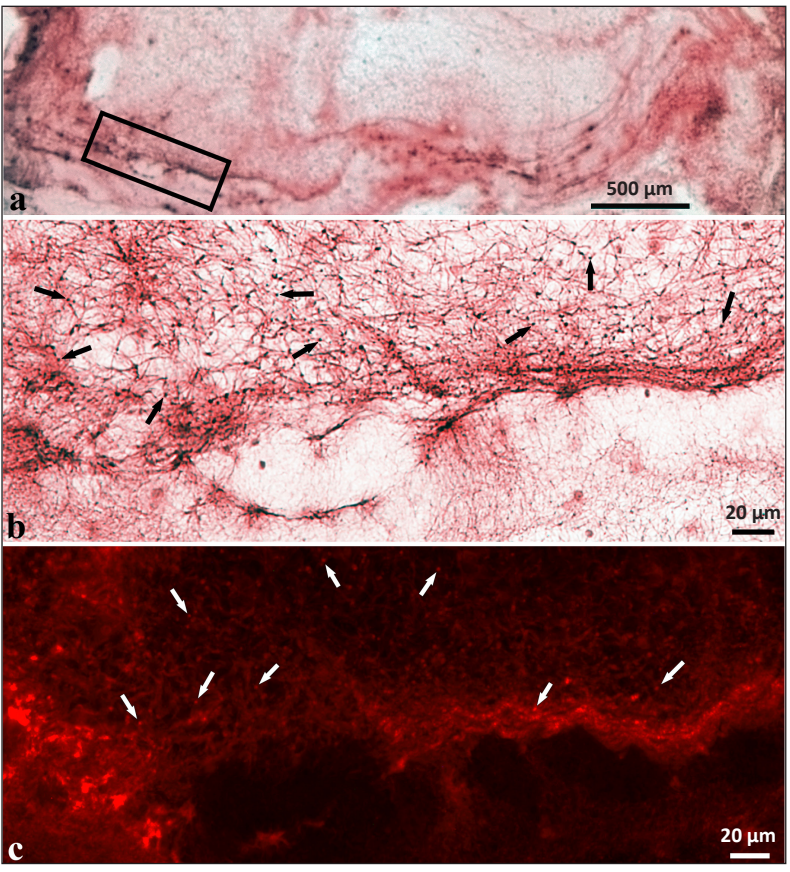

Figure 2

conditions for survival of bacteria within the fibrous matrix in the ear cavities. The facial recess was the most common location of these formations. Other concealed areas that might not lend themselves to drain readily, such as the epitympanum, sinus tympani, oval window, the area near the round window membrane, and stapes crus, were also common sites. Due to the bacteria within a fibrous matrix in otitis media being found in multiple locations throughout the ear along with having a complex structural organization, a unique therapeutic approach may be required. ●

CORRESPONDENCE

Sebahattin Cureoglu, M.D.
Department of Otolaryngology
University of Minnesota
2001 6th Street S.E.
Lions Research Building, Room 210
Minneapolis, MN 55455
E-mail: *cureo003@umn.edu*
Phone: 612-626-9883

ACKNOWLEDGMENTS

The authors thank Richard Chole, M.D., Lindburg Professor and Department Chairman of Otolaryngology–Head and Neck Surgery at Washington University School of Medicine in St. Louis and Director of the American Board of Otolaryngology for his critical review and comments. We also thank Suanur M. Cureoglu, Carolyn Sutherland, and Monika Schachern for their technical assistance.

FUNDING/SUPPORT

This study is supported by the NIDCD 3U24 DC008559-03S109, R01 DC006452, U24 DC011968-01, 5M Lions International, the International Hearing Foundation, and the Starkey Foundation.

REFERENCES

1. Hall-Stoodley L, Hu FZ, Gieseke A et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* 2006;296(2):202-11.

2. Chole RA, Faddis BT. Evidence for microbial biofilms in cholesteatomas. *Arch Otolaryngol Head Neck Surg* 2002;128(10):1129-33.

3. Ehrlich GD, Veeh R, Wang et al. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA.* 2002;287(13):1710-5.

4. Challa VR, Thore CR, Moody DM, Brown WR, Anstrom JA. A three-dimensional study of brain string vessels using celloidin sections stained with anti-collagen antibodies. *J Neurolog Sci* 2002;203-204:165-7.

5. O'Malley JO, Merchant SN, Burgess BJ et al. Effect of fixative and embedding medium on morphology and immunostaining of the cochlea. *Audiol Neurotol* 2009;14(2)78-87.

FIGURES

Figure 1. Characterization of bacteria within fibrous matrix in a human temporal bone with chronic otitis media using different stains. **(a)** Seropurulent effusion and the bacteria within fibrous matrix can be seen in the middle ear, facial recess, and between the stapes crura. Gram Weigert stain. FN: Facial nerve, M: Malleus, I: Incus, S: Stapes, V: Vestibule. **(b)** A higher magnification of (a) reveals gram-positive bacteria (arrows), bacterial aggregates (arrowheads), and inflammatory cells. **(c)** Hematoxylin-eosin (H-E) staining also demonstrates single bacteria (arrows), bacterial clusters (arrowheads), and inflammatory cells. **(d)** Fluorescent (red) staining for DNA with propidium iodide shows host cells nuclei and bacteria (arrows). Bars: 1 mm **(a)** and 20 µm **(b-d).**

Figure 2. H-E-stained section taken at lower **(a)** and higher **(b)** magnifications demonstrate large, darkly-stained bacterial aggregates. **(c)** Propidium iodide staining of the contiguous section verified the presence of large bacterial colonies. Arrows indicate bacteria. Bars: 500 µm **(a)** and 20 µm **(b,c).**

Changes Related to the Study of Temporal Bones: A Progress Report

Authors: ***Akira Ishiyama, M.D.[1], Fred H. Linthicum, Jr., M.D.[1], and Ivan Lopez, Ph.D.[1]***

[1]Neurotology and Temporal Bone Laboratory and House Temporal Bone Laboratory, University of California at Los Angeles, Los Angeles, CA, USA

The Temporal Bone Laboratory of the House Research Institute has moved to the campus of the University of California at Los Angeles to join UCLA's Neurotology and Temporal Bone Laboratory and is now known as the "House Temporal Bone Laboratory of UCLA." This gives us access to some of the more advanced methods of analysis in collaboration with other departments, such as proteomics and newer techniques of electron microscopy (as are discussed below).

Histopathologic evaluation of human temporal bones began in Europe more than a century ago. It came to the United States with the establishment of the temporal bone laboratory at Johns Hopkins University by Stacey Guild, circa 1927. Guild developed a method of quantitative analysis of the various structures in the peripheral aspects of the cochlea (Fig. 1).[1]

Decalcification of temporal bones using ethylenediamine-tetraacetic acid (EDTA) and embedding with celloidin (nitrocellulose) for microtome sectioning is tedious and takes several months. Various acids have been tried for the decalcification to hasten the process; but due to a distortion of the organ of Corti and interference with immunohistochemical staining, they have been abandoned. Different embedding materials have also been tried, but only celloidin is able to maintain the integrity of the intracochlear anatomy because of the large fluid spaces that are not found in other types of tissue.

Electron microscopy has been of limited use in the study of temporal bones removed in the usual way due to subtle postmortem artifacts not visible by light microscopy. Techniques have been developed for re-embedding the celloidin sections in plastic and sectioning for transmission electron microscopy.[2] Perfusion of the inner ear within a few hours of death can result in tissues satisfactory for transmission or scanning electron microscopy (Fig. 2).

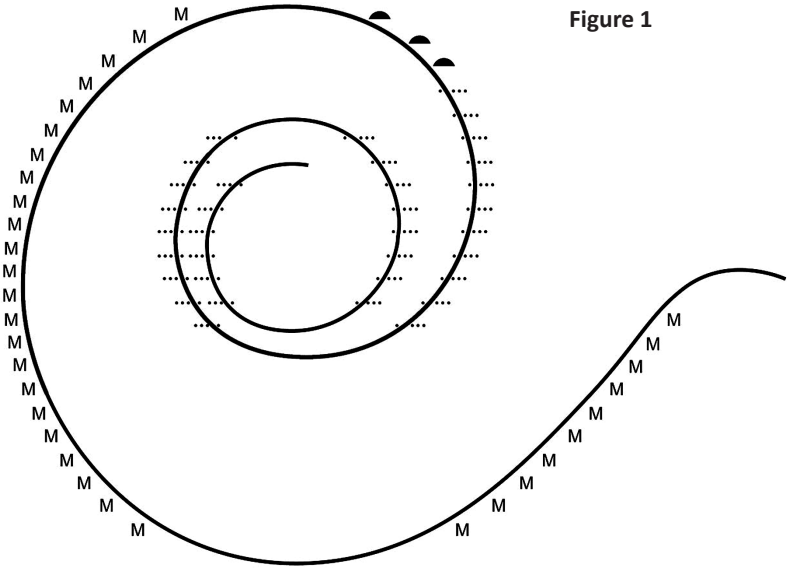

Figure 1

Immunohistochemistry is a method of identifying specific proteins within a cell or area. An antibody is linked to a specific chromogen or fluorescing dye and examined through a light, ultra-violet, or confocal microscope (Fig. 3 and 4).[3]

In situ hybridization has been used to locate a specific RNA or DNA sequence in a specimen.[4, 5]

Three-dimensional reconstruction of microscopic inner ear anatomy has made it possible to clarify the contours of small structures that are difficult to conceptualize when seen in just two dimensions. The endolymphatic sac was thought to be a sac with a rugose surface based on animal observations. Three-dimensional reconstruction of human sacs demonstrated that

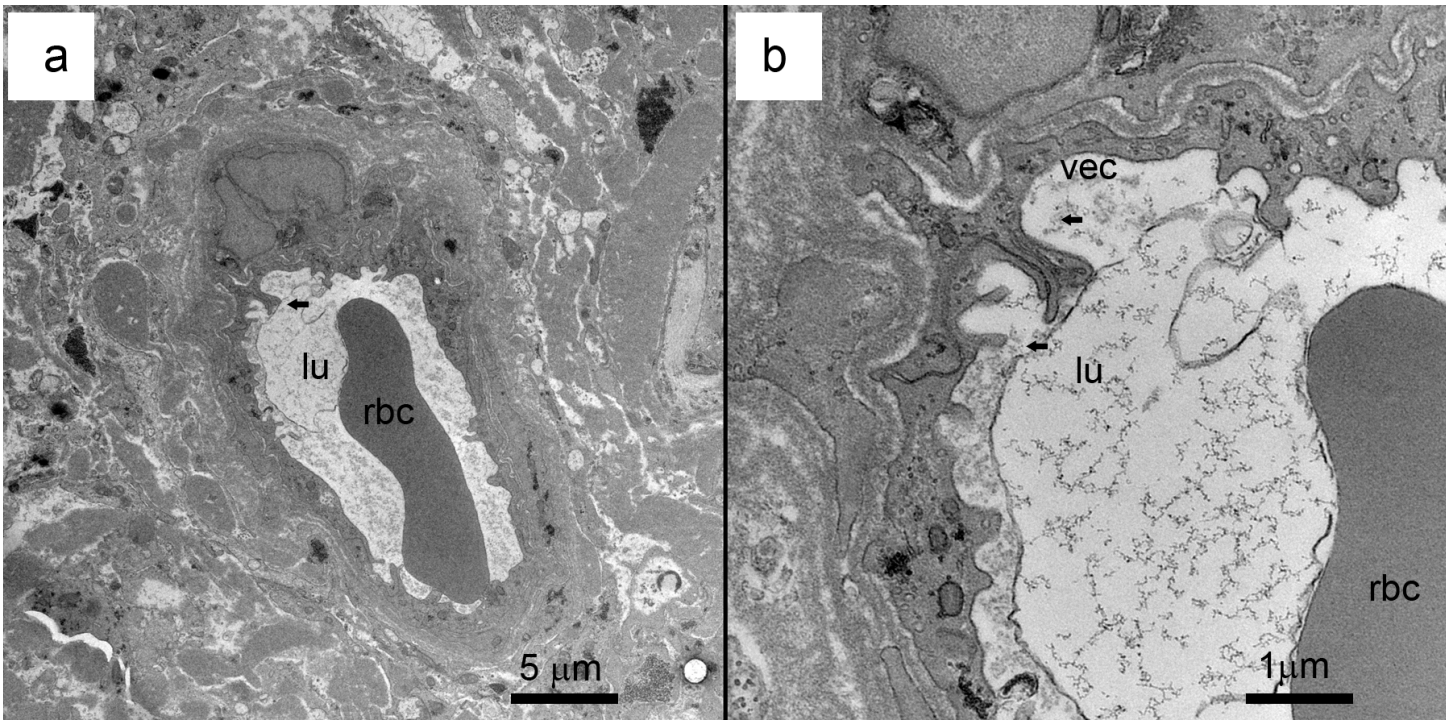

Figure 2

the interosseous and the proximal part of the intracranial sac were actually a group of tubules connecting the cisternal part of the sac to the intracranial portion (Fig. 5).[6]

Stereology makes it possible to determine the volume or surface areas of microscopic structures, such as the endolymphatic sac or cochlear structures. The measurement may be computer-aided,[7] or made with a grid overlying microphotographs or in the microscope eyepiece.

Polarized light is useful for observing the different types of bone structure in the ear.[8] It is particularly effective, especially with the addition of quartz light, for the examination of the different configurations of the bone structure in otosclerosis.

Proteomic studies are powerful tools that characterize the protein expression profiles of tissues, providing a platform upon which other studies may be conducted to better understand normal and disease states of specific organs. Proteomics has been applied to inner ear studies in mouse models uncovering key proteins involved in the integrity of the blood-labyrinth barrier and protein expression alterations in Usher Syndrome and cisplatin-mediated ototoxicity.

Translation to human applications, however, represents a clinical dilemma. While fresh tissue remains the gold standard for tissue source, procurement of fresh human inner ear tissue has several limitations including access and limited volume. Therefore, efforts have turned toward developing techniques for proteomic analysis using human archival temporal bone specimens. These specimens are available in the temporal bone bank and may be stored indefinitely, making it the ideal alternative tissue source. Recently developed techniques have demonstrated successful

continued on page 6

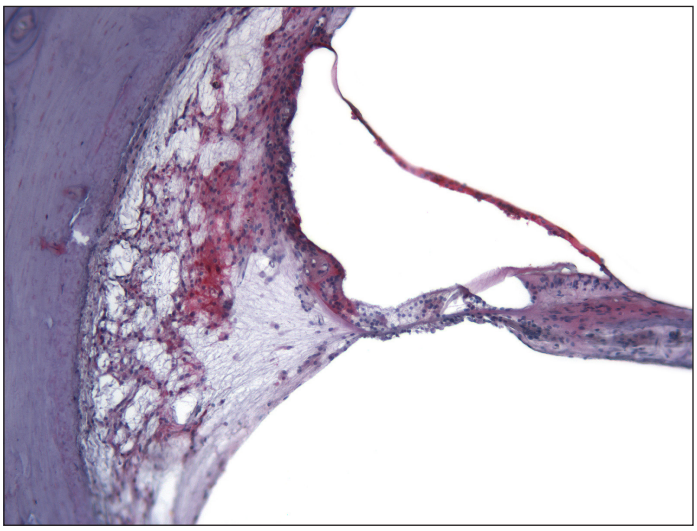

Figure 3

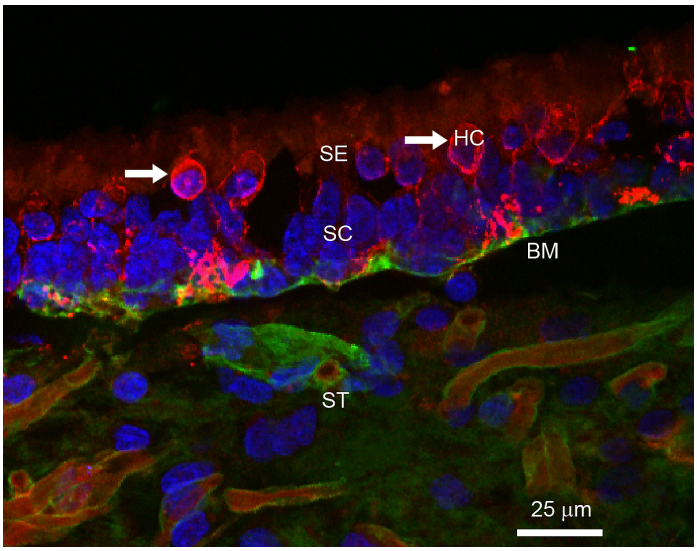

Figure 4

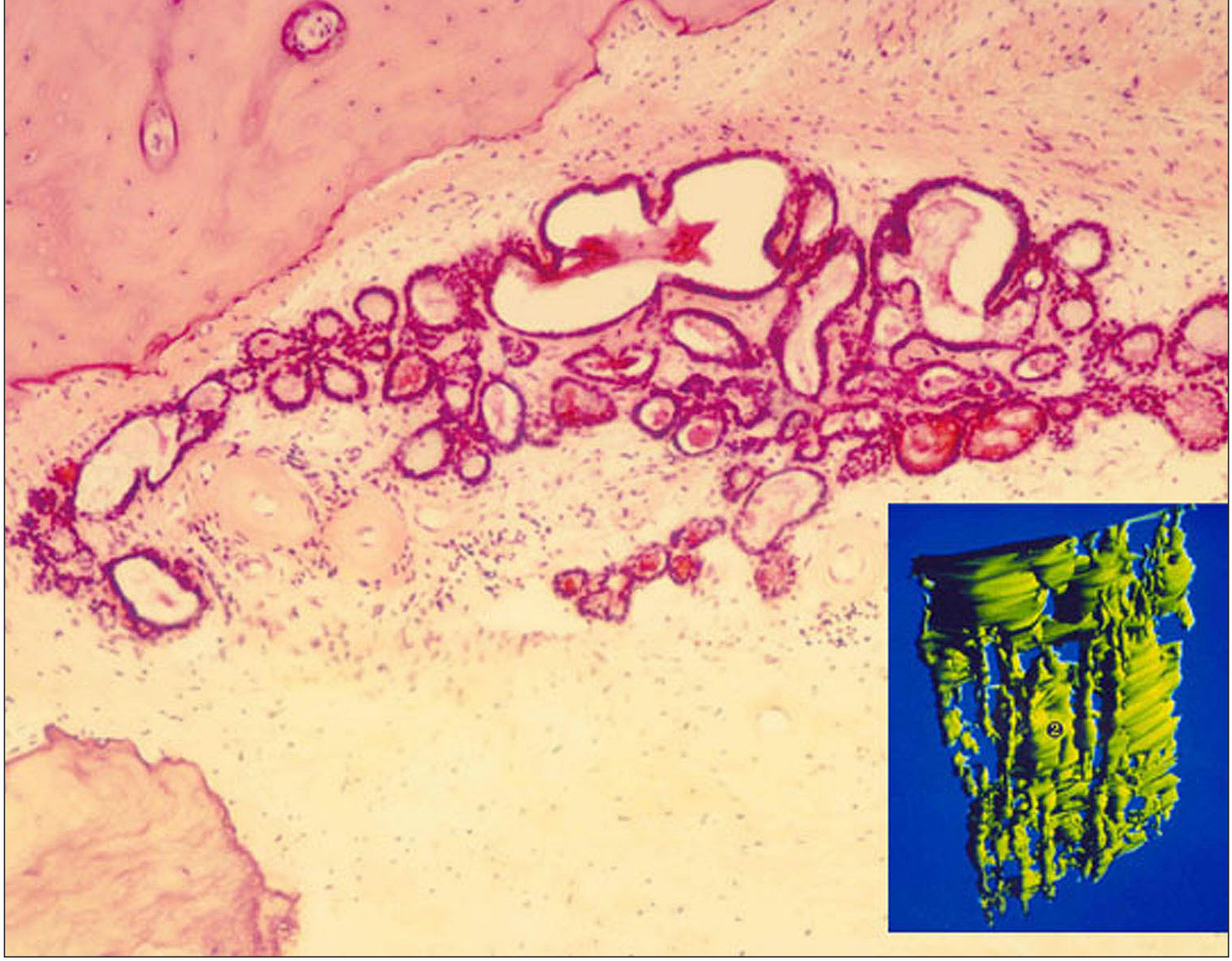

Figure 5

celloidin removal and protein extraction, making proteomic analysis of formalin-fixed, celloidin-embedded (FFCE) human temporal bones feasible.[9,10] We have been able to detect from a mid-modiolar cochlea FFCE section from a normal control patient a total of 385 proteins, of which 252 were identified with high confidence (CI>95%). From the 252 high confidence proteins, 190 corresponded to known proteins, while 62 were unnamed proteins.

Thin-sheet laser imaging microscopy (TSLIM) optical sectioning may be used to evaluate soft tissue morphology in small specimens from animals before celloidin sectioning. It has recently been used on human temporal bones.[11]

In summary, many new methods of analysis of human temporal bones have been added to the repertoire since the time of Guild, including electron microscopy to see greater detail; immunohistochemistry to identify proteins; in situ hybridization to locate a specific RNA or DNA sequence; 3D reconstruction to explore the contours of small structures; stereology to determine volume or surface areas; use of polarized light to observe bone structure; proteomics to characterize protein expression of inner ear tissues; and, recently, TSLIM to evaluate soft tissue morphology.

Undoubtedly, the future holds promise for more methods to maximize our knowledge of both normal and diseased temporal bones in order to better treat and even prevent otologic problems. ●

CORRESPONDENCE

Fred H Linthicum, M.D.
David Geffen School of Medicine
University of California, Los Angeles
Room 32-28 Rehabilitation Center
1000 Veteran Avenue
Los Angeles CA 90095
Email: *flinthicum@mednet.ucla.edu*

FUNDING/SUPPORT

This study is supported by the NIDCD U24 DC011962.

REFERENCES

1. Guild SR. A graphic reconstruction method for the study of the organ of Corti. *Anat Rec.* 1921;22:141-157.

2. Portmann D, Fayad J, Wackym PA, Shiroishi H, Linthicum FH Jr, Rask-Andersen H. A technique for reembedding celloidin sections for electron microscopy. *Laryngoscope.* 1990;100

3. Arnold W. Possibilities of immunohistochemical investigation on human temporal bone. *Acta Otolaryngol.* 1987;436 (Suppl):62-8.

4. Wackym PA, Popper P, Ward PH, Micevych PE. In situ hybridization for the study of gene expression in neuro-otologic research. *Otolaryngol Head Neck Surg.* 1990;103:519-26.

5. Nguyen KD, Mowlds D, Lopez IA, Hosokawa S, Ishiyama A, Ishiyama G. Mu-Opiod receptor (MOR) expression in the human spiral ganglia. *Brain Res.* 2014. dx.doi.org/10/1016/j.brainres.2014.09.051.

6. Antunez JC, Galey FR, Linthicum FH, McCann GD. Computer-aided and graphic reconstruction of the human endolymphatic duct and sac: a method for comparing Menière's and non-Menière's disease cases. *Ann Otol Rhinol Laryngol.* 1980;89(Suppl 6):23-32.

7. Tang Y, Lopez I, Ishiyama A. Application of unbiased stereology in archival human temporal bone. *Laryngoscope.* 2002;112:526-33.

8. Doden E, Halves R. On the functional morphology of the human petrous bone. *Am J Anat.* 1984;169:451-62.

9. Aarnisalo, AA, Green KM, O'Malley J, Makary C, Adams J, Merchant SN, et al. A method for MSE differential proteomic analysis of archival formalin-fixed celloidin-embedded human inner ear tissue. *Hearing Research.* 2010; 270:15-20.

10. Markaryan A, Nelson EG, Helseth LD, Hinojosa R. Proteomic analysis of formalin-fixed celloidin-embedded whole cochlear and laser microdissected spiral ganglion tissues. *Acta Otolaryngol.* 2010;130:984-89.

11. Johnson SB, Cureoglu S, O'Malley JT, Santi PA. Comparison of traditional histology and TSLIM optical sectioning of human temporal bones. *Otol Neurotol.* 2014;35:1145-9.

FIGURES

Figure 1. Example of two-dimensional representation of peripheral cochlear structures as developed by Stacey Guild.[1] Dots indicated surviving hair cells. Small mounds [ ] indicate surviving supporting cells with no hair cells present. M indicates total loss of organ of Corti.

Figure 2. Transmission electron micrograph of a human blood vessel underneath the vestibular sensory epithelia. a) low magnification view (3500x), b) high magnification view. Magnification bar in (a) is 5 µm, in b is 1 µm. Arrow points to two vascular endothelial cells (vec). Lu: capillary lumen, rbc: red blood cell. Ultrathin section 100 nm thick, counterstained with uranyl acetate and lead citrate. A T12 (120 Kv) FEI, transmission electron microscope was used to capture the image.

Figure 3. Immunohistochemical labelling of carbonic anhydrase (red) in spiral ligament.

Figure 4. Laser confocal micrograph. Co-localization of two basement membrane proteins in the human utricular sensory epithelia. Tenascin (red) immunofluorescence is located around the calyceal terminals throughout the neuroepithelium. Tenascin is present uniformly throughout the basement membrane as well. There is little to no staining within the underlying stroma. Collagen IV is present only the basement membrane (green). All nuclei are stained with DAPI (blue). 630x magnification. SE: sensory epithelium, SC: supporting cells, HC: Hair Cell, BM: Basement membrane, ST: stroma. Arrows point to calyceal terminals. Magnification bar is 25 µm. A Leica laser confocal microscope was used to acquire the micrographs.

Figure 5. Tubular part of the intraosseous sac with 3D reconstruction (inset).

Otopathology Mini-Travel Fellowship Program

The NIDCD National Temporal Bone Registry is pleased to announce the availability of mini-travel fellowships. The fellowships provide travel 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 that wish to reactivate their collections.
- Active U.S. temporal bone laboratories that 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 temporal bone laboratory director or department chairman.
- Letter from the host temporal bone laboratory, indicating willingness to receive the traveling fellow.

Applications should be submited to:

Michael J. McKenna, M.D.
NIDCD Temporal Bone Registry
Massachusetts Eye and Ear Infirmary
243 Charles Street
Boston, MA 02114
michael_mckenna@meei.harvard.edu