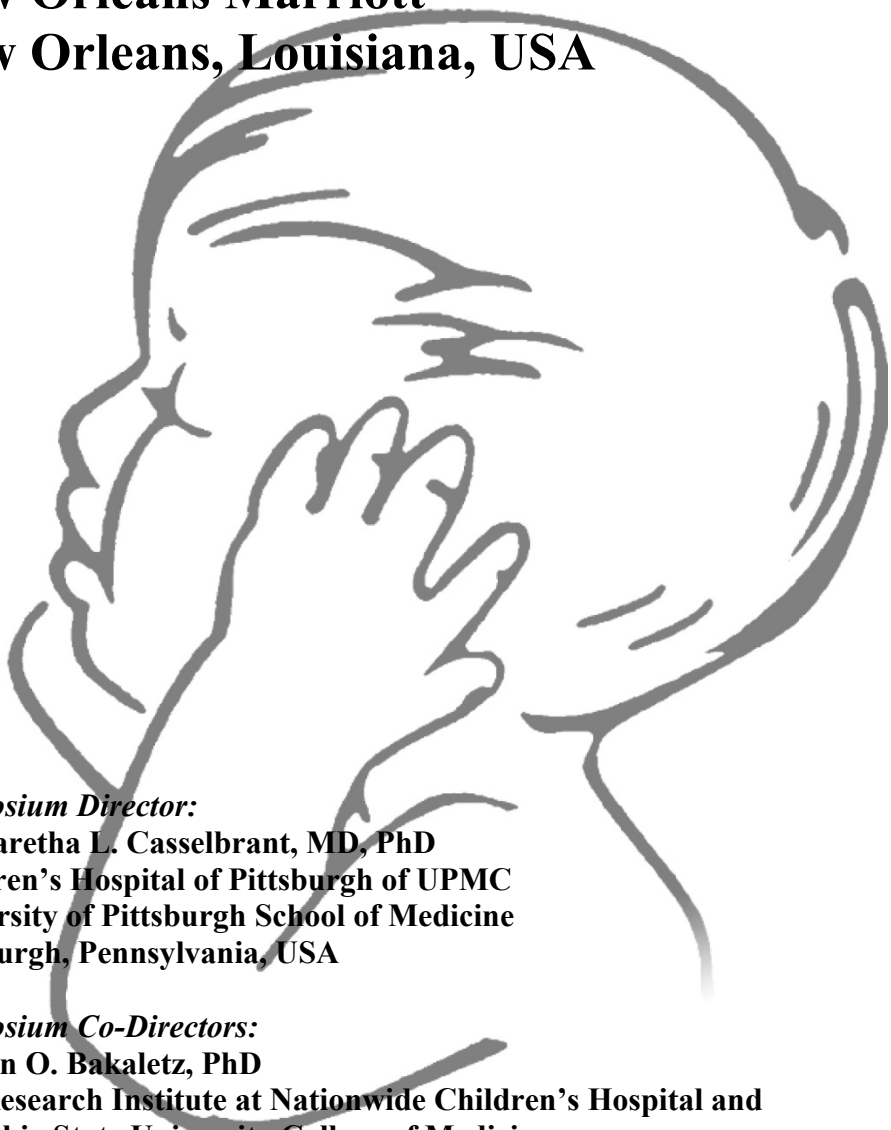
Proceedings of the 10th International Symposium on Recent Advances in

Otitis Media

June 5-9, 2011
New Orleans Marriott
New Orleans, Louisiana, USA



Symposium Director:
Margaretha L. Casselbrant, MD, PhD
Children's Hospital of Pittsburgh of UPMC
University of Pittsburgh School of Medicine
Pittsburgh, Pennsylvania, USA

Symposium Co-Directors:
Lauren O. Bakaletz, PhD
The Research Institute at Nationwide Children's Hospital and
The Ohio State University College of Medicine
Columbus, Ohio, USA

Richard M. Rosenfeld, MD, MPH
SUNY Downstate Medical Center and Long Island College Hospital
Brooklyn, New York, USA

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Recent Advances in Otitis Media

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RECENT ADVANCES IN OTITIS MEDIA

PREFACE

Dear All:

We want to express our sincere gratitude to all of you who attended the 10th International Symposium on Recent Advances in Otitis Media in New Orleans for making the meeting such a success. This was a very special meeting with a change in venue from Florida to New Orleans, Louisiana, but more importantly, also a change in leadership. Lauren, Richard and I are very honored to have been entrusted with the task of organizing this meeting by the three honorees, Drs. David Lim, Charles Bluestone and Jerome Klein. Dr. Lim and Dr. Bluestone were co-founders of the International Symposium on Recent Advances in Otitis Media, along with Dr. Ben Senturia, with the first meeting taking place in Columbus, Ohio, in 1975. Since then, they have successfully organized nine additional symposia (International Symposium on Recent Advances in Otitis Media) every four years with the help of Dr. Klein.

The 10th International Symposium was very well attended with 292 participating clinicians and scientists from 24 countries. In addition to the free papers (105 oral presentations and 107 posters), the program included highlights such as five plenary sessions with special invited lectures by leaders in their fields sharing their state-of-the art knowledge with us, as well as six mini-symposia, six panels, four workshops, and lunch-time presentations. Subjects that were covered during the symposium included areas of epidemiology, diagnosis, pathogenesis, as well as microbiology, immunology and biochemical aspects of middle-ear disease, genetics, molecular biology, animal models and management with special focus on prevention via vaccines as well as complications and sequelae.

Without great efforts on the part of so many individuals we could not have made this meeting the unqualified success that it was and therefore, we want to express our sincere gratitude to all. We especially want to thank the Program Committee, chaired by Joseph Kerschner, and his committee members, Allan Leiberthal, Adriano Arguedos, Kevin Mason, Diego Preciado, Terho Heikkinen and J. Douglas Swarts, for an outstanding job. We also want to express our gratitude to all the international advisors who actively contributed with recommendations for the preparation of the scientific program. We appreciate the efforts of the chair of the Vendor Relations Committee, Steven Pelton who, together with Kenny Chan and Joseph Dohar, reached

out to the sponsors to whom we also want to express our appreciation for their generous unrestricted financial support. Heather Glenn and Lisa Astorga from the Talley Management Company and Debi Buza from Division of Pediatric Otolaryngology, Pittsburgh, deserve all the praise for doing an outstanding job in organizing the meeting. It was truly a pleasure to work with them.

We especially want to thank Dr. James F. Battey, Director of the NIDCD, NIH, for taking time from his busy schedule to give the Special Remarks. We are also indebted to the NIDCD for their support of the Post-Symposium Research Conference.

We all take great pride in knowing that the International Symposium of Otitis Media, with its Post-Symposium Research Conference, and the Extraordinary Otitis Media Meeting have become a forum for research in otitis media and have promoted worldwide interest among young researchers in the field of otitis media, fostering collaboration among researchers from all over the world. What makes this meeting so very special is that over the span of a few days, we get to meet and learn from experts in all the various aspects of otitis media as well as get together with old and new friends from all over the world to discuss present research and also make plans for future collaborative research in otitis media.

We would like to inform you about the successful inauguration of the International Society of Otitis Media (ISOM). The primary mission of ISOM is to disseminate information about otitis media, enhance, support and coordinate communication and collaboration among society members, related disciplines and other societies as well as facilitate otitis media research by creating and exchanging knowledge. In other words, ISOM will continue the 40 year long tradition of promoting basic and clinical research in otitis media began by the founders of the International Symposia on Otitis Media. ISOM has been met with great enthusiasm and support. We hope that you will join us as a charter member by signing up at http://OtitisMediaSociety.org. Together we will change the future for children with otitis media.

We also want to remind you about the 18th International Symposium on Recent Advances in Otitis Media (http://OtitisMediaSociety.org) that will take place in Alexandria, Virginia, USA, June 6-11, 2015.

Looking forward to seeing you all there.

Warmest regards,
Margaretha L. Casselbrant Richard M. Rosenfeld Lauren O. Bakaletz

Symposium Director

Margaretha L. Casselbrant, MD, PhD
Eberly Professor of Pediatric Otolaryngology
University of Pittsburgh School of Medicine
Director, Department of Pediatric Otolaryngology
Children's Hospital of Pittsburgh
Pittsburgh, PA, USA

Symposium Co-Directors

Lauren O. Bakaletz, PhD
Professor of Pediatrics Department of Pediatrics
College of Medicine
The Ohio State University
College of Medicine
Columbus, OH, USA

Richard M. Rosenfeld, MD, MPH
SUNY Downstate Medical Center
and Long Island College Hospital
Brooklyn, New York, USA

Program Committee

Joseph Kerschner, MD - Chair
Children's Hospital of Wisconsin
Milwaukee, Wisconsin

Allan Lieberthal, MD
Kaiser-Permanente
Panorama City, California

Adriano Arguedas, MD
Universidad de Ciencias Medicas
San Jose, Costa Rica

Kevin Mason, PhD
Nationwide Children's Hospital
Columbus, Ohio

J. Douglas Swarts, PhD
Division of Pediatric Otolaryngology,
Children's Hospital of Pittsburgh of UPMC
Pittsburgh, Pennsylvania

Terho Heikkinen, MD
Department of Pediatrics,
Turku University Hospital
Turku, Finland

Diego Preciado, MD
Children's National Medical Center,
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Boston, Massachusetts

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The Children's Hospital
Denver, Colorado

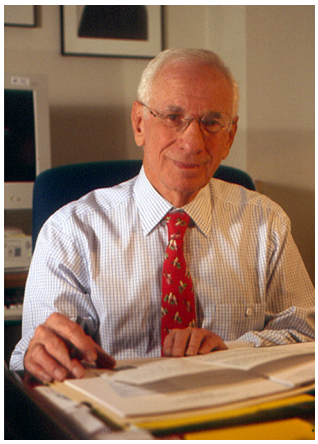
Joseph E. Dohar, MD
Division of Pediatric Otolaryngology,
Children's Hospital of Pittsburgh of UPMC
Pittsburgh, Pennsylvania

ADVISORS

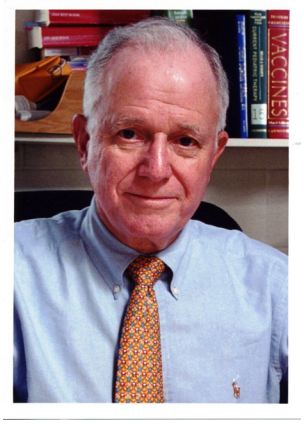
2011 HONOREES

David J. Lim, MD
Co-Founder
Distinguished Scientist and Emeritus EVP,
Research House Ear Institute
Adjunct Professor, Department of Cell and Neurobiology,
Keck School of Medicine, University of Southern California
Los Angeles, CA

Charles D. Bluestone, MD
Co-Founder
Distinguished Professor of Otolaryngology
University of Pittsburgh School of Medicine
Division of Pediatric Otolaryngology
Children's Hospital of Pittsburgh of UPMC
Pittsburgh, PA



Jerome O. Klein, MD
Professor of Pediatrics
Department of Pediatrics
Boston University School of Medicine
Boston, MA



FACULTY LISTING

Charles D. Bluestone, MD
Distinguished Professor of Otolaryngology
University of Pittsburgh School of Medicine
Division of Pediatric Otolaryngology
Children's Hospital of Pittsburgh of UPMC
Pittsburgh, PA

Paul W. Ewald, PhD
Professor, Department of Biology and the
Program on Disease Evolution
University of Louisville
Louisville, KY

Howard J. Jacob, PhD
Professor, Department of Physiology
Director, Human and Molecular Genetics
Center
Medical College of Wisconsin
Milwaukee, WI

Jerome O. Klein, MD
Professor of Pediatrics
Department of Pediatrics
Boston University School of Medicine
Boston, MA

David J. Lim, MD
Distinguished Scientist and Emeritus EVP,
Research House Ear Institute
Adjunct Professor, Department of Cell and
Neurobiology, Keck School of Medicine
University of Southern California
Los Angeles, CA

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Montefiore Medical Center
Bronx, NY

Jeffrey N. Weiser, PhD
Professor of Pediatrics and Microbiology
University of Pennsylvania School of Medicine
Philadelphia, PA

Robert C. Welliver, PhD
Professor of Pediatrics
Chief, Section on Infectious Diseases
Oklahoma University Health Sciences Center
Oklahoma City, OK

CHRONOLOGY OF THE SYMPOSIA

International Symposium on Recent Advances in Otitis Media with Effusion

1st Symposium	**May 29-31, 1975 in Columbus, Ohio**
Program Committee:	Ben H. Senturia, MD
	Charles D. Bluestone, MD
	David J. Lim, MD
Guest of Honor:	Ben H. Senturia, MD

2nd Symposium	**May 9-11, 1979 in Columbus, Ohio**
Program Committee:	Charles D. Bluestone, MD
	David J. Lim, MD
	Ben H. Senturia, MD
Guest of Honor:	Sven Inglested, MD

3rd Symposium	**May 17-20, 1983 in Bahia Mar, Ft. Lauderdale, Florida**
Program Committee:	Charles D. Bluestone, MD
	Jerome O. Klein, MD
	David J. Lim, MD
	John D. Nelson, MD
Guest of Honor:	Gunnar D. Proud, MD

1st Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

	January 13-15, 1985 in Kyoto, Japan
Symposium Directors:	Tadami Kumazawa, MD
	Kazutomo Kawamoto, MD
Program Committee	
Chairman:	Goro Mogi, MD
Co-Chairman:	Iwao Honjo, MD
	Tetsuo Ishii, MD

International Symposium on Recent Advances in Otitis Media

4th Symposium	**June 1-4, 1987 in Bal Harbour, Florida**
Program Committee:	David J. Lim, MD
	Charles D. Bluestone, MD
	Jerome O. Klein, MD
	John D. Nelson, MD

International Symposium on Recent Advances in Otitis Media

5th Symposium	**May 20-24, 1991 in Fort Lauderdale, Florida**
Program Committee:	David J. Lim, MD
	Charles D. Bluestone, MD
	Jerome O. Klein, MD
	John D. Nelson, MD
	Pearay L. Ogra, MD
Guest of Honor:	Tadami Kumazawa, MD

2nd Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

	March 31-April 3, 1993 in Oita, Japan
Symposium Director:	Goro Mogi, MD
Program Committee:	Iwao Honjo, MD
	Tetsuo Oshii, MD
	Tomonori Takasaka, MD
Guest of Honor:	David J. Lim, MD

International Symposium on Recent Advances in Otitis Media

6th Symposium	**June 4-8, 1995 in Ft. Lauderdale, Florida**
Program Committee:	David J. Lim, MD
	Charles D. Bluestone, MD
	Margaretha Casselbrant, MD, PhD
	Jerome O. Klein, MD
	Pearay L. Ogra, MD
Guest of Honor:	Goro Mogi, MD

3rd Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

	June 1-5, 1997 Copenhagen, Denmark
Symposium Director:	Mirko Tos, MD
Program Committee:	Viggo Balle, MD
	Jens Thomsen, MD
	Mirko Tos, MD
Guest of Honor:	Charles D. Bluestone, MD

International Symposium on Recent Advances in Otitis Media

7th Symposium	**June 1-5, 1999 in Ft. Lauderdale, Florida**
Symposium Director:	David J. Lim, MD
Symposium Co-Directors:	Charles D. Bluestone, MD
Program Chair	Margaretha Casselbrant, MD, PhD
Program Committee:	Lauren O. Bakaletz, PhD
	Thomas F. DeMaria, PhD
	William J. Doyle, PhD
	G. Scott Giebink, MD
	Jerome O. Klein, MD
	Pearay L. Ogra, MD
Guest of Honor:	Mirko Tos, MD, DMSc
Distinguished Service Award:	Marion P. Downs, DHS

4th Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

	April 16-20, 2001 Sendai, Japan
President:	Tomori Takosaka, MD, PhD
Vice President:	Ryo Yuasa, MD
Honorary Presidents:	Kazutomo Kawamoto, MD
	Tadami Kumazawa, MD
	Goro Mogi, MD
	Mirko Tos, MD, DMSc
Secretary General:	Koji Hozawa, MD
Guest of Honor:	Michael M. Paparella, MD

International Symposium on Recent Advances in Otitis Media

8th Symposium	**June 3-7, 2003 in Ft. Lauderdale, Florida**
Symposium Director:	David J. Lim, MD
Symposium Co- Directors:	Charles D. Bluestone, MD
	Margaretha Casselbrant, MD, PhD
Program Committee:	Lauren O. Bakaletz, PhD
	G. Scott Giebink, MD
	Jerome O. Klein, MD
	Pearay L. Ogra, MD

5th Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

	April 24-27, 2005 Amsterdam, the Netherlands
President:	Anne Schilder
Vice President:	Gerhard Zielhuis
Organizing Committee:	Frank van Balen
	Brechtje de Beer
	Jo Curfs
	Erwin Dunnebier
	Nelly van Eden
	Kees Graamans
	Niels van Heerbeek
	Gerrit Jan Hordijk
	Jan Kimpem
	Kees Langenhuijsen
	Jef Mulder Lieke Sanders
	Ad Snik
	Reinier Veenhoven
	Bert van Zanten
Secretary General:	Maroeska Rovers
Guest of Honor:	Professor Jerome O. Klein, MD

International Symposium on Recent Advances in Otitis Media

9th Symposium	**June 3 – 7, 2007 in St. Petersburg, Florida**
Symposium Director:	David J. Lim, MD
Symposium Co- Director:	Charles D. Bluestone, MD
	Margaretha Casselbrant, MD, PhD
Program Committee:	Lauren O. Bakaletz, PhD
	Jian-Dong Li, MD
	Jerome O. Klein, MD
	Pearay L. Ogra, MD

6th Extraordinary International Symposium on Recent Advances in Otitis Media with Effusion

May 6 – 10, 2009 in Seoul, Korea

President:	Keehyun Park
Vice President:	Hong-Joon Park
Organizing Committee:	Yun-Hoon Choung
	Min-Jung Cho
	Ho Seok Choi
	Seong Jun Choi
	Jun Ho Lee
	Jeong-Hoon Oh
	Hun Yi Park
	Moon Sung Park
Guest of Honor:	Steven K. Juhn

International Symposium on Recent Advances in Otitis Media

10th Symposium	**June 5 – 9, 2011 in New Orleans, Louisiana**
Symposium Director:	Margaretha L. Casselbrant, MD, PhD
Symposium Co- Director:	Lauren O. Bakaletz, PhD
	Richard M Rosenfeld, MD, MPH
Program Committee:	Joseph Kerschner, MD – Chair
	Allan Lieberthal, MD
	Adriano Arguedas, MD
	Kevin Mason, PhD
	J. Douglas Swarts, PhD
	Terho Heikkinen, MD
	Diego Preciado, MD
2011 Honorees:	David M. Lim, MD
	Charles D. Bluestone, MD
	Jerome O. Klein, MD

Contents

Section 1: Genetics/Epidemiology

Section 2: Eustachian Tube

Section 3: Pathogensis

Section 4: Treatment/Microbiology

Section 5: Vaccine/Microbology

Section 6: Epidemiology/Microbiology

Section 7: Animal Models/Middle Ear

Section 8: Biochemistry

Section 9: Diagnosis/Treatment

Section 10: Epidemiology

Section 11: Pathogenesis/Microbiology

Section 12: Physiology

Section 13: Animal Model

Section 14: Genetics

Section 15: Molecular Biology/Immunology

Section 16: Complications/Sequelae

Section 17: Immunology/Vaccine

Section 18: Senior Lecture/Plenary Session

Section 19: Workshops

Section 20: Mini-Symposia

Section 21: Panel Discussion

Genetics/Epidemiology

Acute Mastoiditis: The Role of Diagnostic Imaging in Identifying Intracranial Complications

Michal Luntz, MD[1]**,** Rabia Shihada, MD[1]**, Keren Bartal, MD**[2]**,** Alex Brodsky, MD[1]
[1]Otolaryngology, Bnai Zion MC, Technion - Israel Institute of Technology, Haifa, Israel, [2]Otolaryngology, Bnai Zion MC, Technion - Israel Institute of Technology, Haifa, Israel

Introduction
CT scan is performed in patients presenting with acute mastoiditis (AM) in order to identify intra-cranial complications (ICC). The necessity of CT scan in these patients has been questioned due to concern regarding the possible long-term effects of brain irradiation. Therefore clinicians try to base the decision to perform CT in AM patients on the clinical presentation.

Objectives
To compare the clinical presentation of AM patients with and without ICC.

Materials and methods
A prospective study for the period from 1997 to 2009 in an otologic referral center.

Results
71 patients were hospitalized with AM. 10 patients had ICC. AM patients with and without ICC did not differ in most clinical characteristics at presentation. There were however significantly more patients in the ICC group who had otorrhoea and sub-periosteal abscess, yet 60% of the ICC group did not have otorrhoea and 50% of the ICC group did not have a sub-periosteal abscess at presentation.

Conclusions
It is not possible to differentiate between AM patients with and without ICCs based on clinical characteristics at presentation.
The occurrence of otorrhea or sub-periosteal abscess at presentation can indicate that an AM patient is at high risk to develop ICC, but their absence in AM patient does not guarantee that the patient does not have an ICC.
Due to the possible life threatening outcome of delay in the diagnosis of otogenic ICCs, together with the relatively high incidence of ICC in AM patients, diagnostic imaging remains mandatory upon presentation of patients with AM.

Sequencing the Chinchilla Genome

Joseph Kerschner, MD[1]**, Garth Ehrlich, PhD**[2]**, Lauren Bakaletz, PhD**[3]**, Howard Jacob, PhD**[4]**, Jeremy Johnson, PhD**[5]**, Federica Di Palma, PhD**[6]**, Marcia Lara, PhD**[7]
[1]Pediatric Otolaryngology, Children's Hospital of Wisconsin/Medical College of Wisconsin, Milwaukee, WI, [2]Human Genetics, Center for Genomic Sciences/West Penn Allegheny Health System, Pittsburgh, PA, [3]Center for Microbial Pathogenesis, Nationwide Children's Hospital, Columbus, OH, [4]Human and Molecular Genetics Center, Medical College of Wisconsin, Milwaukee, WI, [5]Vertebrate Biology, [6]Genome Sequencing and Analysis, [7]Molecular and Biologic Production, Broad Institue, Cambridge, MA

Objective
To sequence the chinchilla genome in order to allow further development of this important animal model in the study of otitis media (OM).

Methods
An adult chinchilla (chinchilla lanigera), female, was euthanized with recovery of suitable genetic material for sequence development. Given that the chinchilla is an outbred animal without species variation; species selection was not required. Utilizing an Illumina Sequencer sixty-times coverage (60X) of the chinchilla genome was initiated. In addition, bacterial artificial chromosome (BAC) strategy was created and utilized to assist with assembly.

Results
Using 60X coverage methodology and Illumina technology greater than 60 million reads have been generated. Of these reads greater than 80% have been high quality providing excellent depth of coverage of the entire chinchilla genome, appropriate background and the template for the chinchilla genome draft sequence assembly.

Conclusions
The chinchilla has been the gold standard for OM animal research and has been a robust model for other respiratory illnesses as well. However, this model has been hampered in the development of reagents suitable for molecular research due to lack of access to chinchilla genomic sequence required for generating these tools. Sequencing of the chinchilla genome will have a transformational affect on OM research creating an era of exceptional opportunity for identifying genes and pathways underlying this disease. It will also provide unprecedented potential to utilize the chinchilla in important discoveries in areas such as vaccine development and antimicrobial development.

A Genome-Wide Association Study of Chronic Otitis Media with Effusion and Recurrent Otitis Media Identifies a Novel Susceptibility Locus on Chromosome 2

Michele Sale, PhD[1], Wei-Min Chen, PhD[1], Daniel Weeks, PhD[2], Fang Chen, PhD[1], Xuanlin Hou[1], E. Kaitlynn Allen[1], Jose Mattos[1], Josyf Mychaleckyj, PhD[1], Fernando Segade, PhD[3], Margaretha Casselbrant, MD, PhD[4], Ellen Mandel, MD[4], Robert Ferrell, PhD[2], Stephen Rich, PhD[1], Kathleen Daly, PhD[5]
[1]Center for Public Health Genomics, University of Virginia, Charlottesville, VA, [2]Dept Human Genetics, University of Pittsburgh, Pittsburgh, PA, [3]Department of Anatomy and Cell Biology, University of Pennsylvania, Philadelphia, PA, [4]Dept Otolaryngology, Children's Hospital of Pittsburgh, Pittsburgh, PA, [5]Dept Otolaryngology, University of Minnesota, Minneapolis, MN

Objectives
Chronic otitis media with effusion (COME) and recurrent otitis media (ROM) have been shown to be heritable, but candidate gene and linkage studies to date have been equivocal. Our aim was to identify genetic susceptibility factors using a genome-wide association study (GWAS).

Methods
We genotyped 605 subjects from 143 families with 381 COME/ROM subjects, using the Illumina HumanCNV370-Duo DNA BeadChip (324,748 SNPs). We performed imputation using MACH software. Forty-eight SNPs were selected for genotyping in an independent family-based sample: all SNPs with $P<10^{-4}$ (n=36), and 12 imputed SNPs with $P<10^{-4}$ on chromosome 15 (near our strongest signal). To date genotyping for 22 of these 48 SNPs has been completed.

Results
In primary analyses, the strongest association with COME/ROM was on chromosome 15 ($P=3.4 \times 10^{-7}$). For the 22 SNPs tested for replication, only SNP rs10497394, located on chromosome 2, was significantly associated after Bonferroni correction (uncorrected P=0.00026 in the replication data set). Two SNPs in adjacent genes (*C15orf42* and *KIF7*) at the initial chromosome 15 locus were marginally associated, with replication P-values <0.05.

Conclusion
We have performed the first GWAS of COME/ROM and have identified a SNP in a novel locus on chromosome 2 that significantly contributes to COME/ROM susceptibility. This SNP is within a 400 kb intergenic region, bordered by *CDCA7* and *SP3*, but is only 500 bp downstream of DNAse I hypersensitive ENCODE regions. The genomic and functional significance of this newly-identified locus in COME/ROM pathogenesis warrants additional investigation.

OM X Chromosome

Jose Mattos[1], Wei-Min Chen, PhD[1], Fang Chen[1], Xuanlin Hou[1], Emma Allen[1], Josyf Mychaleckyj, PhD[1], Stephen Rich, PhD[1], Kathleen Daly, PhD[2], MIchele Sale, PhD[1]
[1]Center for Public Health Genomics, University of Virginia, Charlottesville, VA, [2]Department of Otolaryngology, University of MInnesota, Minneapolis, MN

Objectives
Given evidence for a male sex bias for otitis media, we hypothesized that genetic markers on the X chromosome might be associated with chronic otitis media with effusion and/or recurrent otitis media (COME/ROM) susceptibility.

Methods
We genotyped the Illumina HumanCNV370-Duo DNA analysis BeadChip in families with COME/ROM subjects, resulting in a total of 9,727 SNPs genotyped on the X chromosome in 605 subjects from 143 families, including 381 affected individuals. SNPs were clustered using Illumina's GenomeStudio software. Association with COME/ROM was analyzed using the transmission disequilibrium test (TDT) from PLINK (version 1.07).

Results
The most significant results were with rs5933051 ($P=3.3 \times 10^{-5}$), located in an intergenic region, and rs5939926 ($P=7.2 \times 10^{-5}$), located within the gene encoding serine/threonine protein kinase MST4 which plays an essential role in epithelial cell brush border formation.

Conclusions
This study represents the first systematic examination of the X chromosome in relation to COME/ROM susceptibility. None of our results exceedchromosome-wide significance ($p<5 \times 10^{-6}$ after Bonferroni correction), suggesting that X chromosome loci are not major contributors to COME/ROM and do not explain the observed sex bias, however SNPs rs5933051 and rs5939926 warrant evaluation in a larger sample.

The Sh3pxd2b Gene Mutation Is Correlated with Eustachian Tube Dysfunction and Severe Otitis Media

Bin Yang, MD, Fengchan Han, PhD, Heping Yu, Cong Tian, Rami Azem, Qing Yin Zheng, MD
Department of Otolaryngology Head and Neck Surgery, Case Western Reserve University, Cleveland, OH

Craniofacial defects that occur through gene mutation during development can lead to an increased incidence of otitis media. These defects have been shown to increasevulnerability to eustachian tube dysfunction. We examined the effects of a mutation in the sh3pxd2b gene on the progression of otitis media and hearing impairment at various developmental stages. We used a mouse model to mirror craniofacial dysmorphology and otitis media in humans.Our findings showed that all mutant mice that had the sh3pxd2b mutation went on to develop craniofacial dysmorphologies and subsequently otitis media, by as early as 11 days age.We found bacterias in the exudates from middle ears in the mutant mice,and *Proteusmirabilis*are the dominant species. Hearing was tested by auditory-evoked brainstem response(ABR) and all mice were found to have hearing impairments, with lower frequency hearing impairment being more pronounced.The expressionofTNF-α, TLR-2, and IL-1, which correlate with inflammation in otitis media,were found to be up-regulated examined by immunostainingand Semi-quantitative RT-PCR in the mutant mice.
*Supported by NIH grants R01DC007392 and R01DC009246 to QYZ

Acute Otitis Media Severity: Association with Cytokine Gene Polymorphisms and Other Risk Factors

David McCormick, MD[1]**,** James J. Grady, MD, PhD[2]**,** Alejandro Diego, MD[1]**,** Reuben Matalon, MD, PhD[3]**,** Krystal Revai, MD[1]**,** Janak A. Patel, MD[4]**,** Yimei Han[5]**,** Tasnee Chonmaitree, MD[4]
[1]Pediatrics, [2]Epidemiology and Biostatistics, [3]Pediatric Cytogenetics, [4]Pediatric Infectious Disease and Immunology, University of Texas Medical Branch, Galveston, Texas, [5]Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, TX

Introduction
Genetic factors have been associated with increased susceptibility to AOM in children, as has been demonstrated in studies of twins, triplets and related family members[1-5]. Our studies have previously shown IL-1ß, IL-6 and TNFα in the nasopoharyngeal secretions during URI, and increased levels of IL-1ß correlate with transition from URI to AOM[6]. In addition, we have shown a relation between risk for AOM episodes complicating URI and $TNF\alpha^{-308}$ polymorphisms[7]. $TNF\alpha^{-308}$ and $IL\text{-}6^{-174}$ polymorphisms are associated with recurrent AOM[8]. It has not been known if genetic and environmental risk factors can be associated with severity of AOM. Such information is needed, as severity of AOM is considered an important variable in the current AOM treatment guidelines[9]. In the present study, we have evaluated the clinical severity of AOM in relation to cytokine gene polymorphisms and environmental risk factors[10].

Methods
Clinical evaluation: From January 2003 through March 2007 healthy children aged 6-35 months were recruited from the primary care pediatrics clinics at the University of Texas Medical Branch. Demographic and risk factor data were obtained by parent questionnaire. Each subject was followed for one year to study occurrences of URI and AOM. Subjects were seen by a study physician as soon as possible after the onset of URI symptoms (nasal congestion, rhinorrhea, cough, and/or sore throat, with or without fever) and followed up 3-7 days later. Study personnel provided 2 home visits and performed tympanometry during weeks 2-3 of URI. Parents were also advised to bring the child for examination whenever they suspected the child to have symptoms of AOM. Children diagnosed with AOM were observed or given antibiotic therapy consistent with standard of care.

Definition of AOM, severity of TM inflammation: AOM was defined by acute onset of symptoms, signs of tympanic membrane inflammation, and middle ear effusion. All children had URI symptoms. Signs of tympanic membrane inflammation included

erythema, opacification, and bulging, (OS-8)[11, 12]. The presence of middle ear effusion was documented by pneumatic otoscopy and/or tympanometry or the observation of an acutely draining ear due to perforation. Tympanic membrane appearance was categorized as non severe (erythema, with or without opacification, not bulging) or severe (erythema, full/bulging or acute perforation). TM inflammation was scored on a previously described scale (OS-8)[12].

AOM symptoms: Symptoms were quantified using a previously described five-item parent questionnaire (ETG-5) that assessed earache, fever, poor feeding, restless sleep, and irritability[11-13]. Each item was evaluated on a scale ranging from zero (no symptoms) to three (severe symptoms). ETG-5 total score was the sum of the items (range 0-15).

Cytokine gene polymorphism analysis: Deoxyribonucleic acid (DNA) was extracted from peripheral blood white cells or buccal epithelial cells of enrolled children, as previously described[8]. The study of polymorphisms yielded 3 genotypes for each cytokine: homozygous "normal" (low cytokine producing) and homozygous and heterozygous polymorphic (high cytokine-producing). For data analysis, children were considered to be polymorphic if they were either homozygous or heterozygous for the polymorphic genotypes.

Statistical analysis

The primary outcome variable was the total symptom score (ETG-5), which had a possible range of 0-15, and was approximately normally distributed in our models. Each child could have several URI episodes and hence subjects contributed varying amounts of correlated data to the study. The statistical approaches to data analysis accounted for multiple episodes of URI, which resulted in clusters of correlated data from each subject. We used a class of models called repeated measures general linear mixed models (GLMM) for parameter estimation and P values derived from the GENMOD procedure in SAS® (Cary, NC) specifying a normal distribution working correlation structure. The model provides a parameter estimate (see Table 2) which, for a given numeric variable such as "days" or "age in months", is the change in ETG-5 symptoms score attributable to a one unit change in that variable. If the variable is categorical, such as environmental smoke exposure "yes" or "no", then the parameter estimate is the simply the difference in ETG-5 score between the two groups, "smoke exposed" and "not smoke exposed". Associations between polymorphic gene status and risk factors were first analyzed for all episodes. A secondary analysis was then conducted only for episodes with moderate/severe inflammation of the TM (diagnosis of AOM with full or bulging TM). The 16 episodes in which a perforated TM was noted were included in the moderate/severe group.

Results

Clinical and demographic data: A total of 294 subjects had 1295 episodes of URI and 440 episodes of AOM. Included in this analysis were data from 128 children with 295 episodes of AOM that were evaluated by the study team prior to antibiotic treatment. Table 1 describes the subjects' demographic, genetic, and risk factor variables, including pneumococcal vaccine status.

AOM diagnosis, severity scores: AOM was diagnosed by the investigators most frequently between days 5 and 6 of the URI. All subjects had URI symptoms. Investigators described the TM inflammation as severe (full/bulging) in 70 percent (206/295) of AOM episodes and non severe (not bulging) in 30 percent (89/295).

Table -2 summarizes results of the analysis of symptom scores relative to age, gender, environmental risk factors and polymorphism. These results show statistically significant associations between AOM symptom severity and young age, a family member with a history of chronic/recurrent AOM, household tobacco smoke exposure, and early diagnosis following the onset of URI.

In a further analysis, looking for any correlation between signs of TM inflammation, clinical symptoms and risk factors, we included only episodes in which the worse ear was observed to have severe inflammation (full/bulging TM or acutely perforated TM with drainage, n=206 episodes in 104 subjects). In this analysis, IL-1 $ß^{+3953}$ polymorphism was associated with a higher symptom score (P = 0.02). Compared to a child 12 months older with no risk factors, a child with all risk factors, would be estimated to experience an ETG-5 score 1.0 point higher, which is equal to 0.38 of a standard deviation.

We also evaluated the relation between tympanic membrane inflammation scores (OS-8)[12] and risk factor variables in the model. Results did not show associations: age (P=0.57), gender (P=0.41), Race (P=0.49 to 0.76), family history of AOM (P=0.59), breast fed (P=0.14), tobacco smoke exposure (P=0.58), day of URI at time of diagnosis (P=0.95), heptavalent pneumococcal vaccine status (P=0.97), $TNFα^{-308}$ polymorphic (P=0.57), IL-1 $ß^{+3953}$ polymorphic (P=0.27), and IL- 6^{-174} polymorphic (P=0.12). These data were also analyzed using just the episodes with severe TM inflammation (n=206 episodes, n=104 subjects), and no significant associations between tympanic membrane inflammation scores and risk factor variables were noted.

Discussion

There have been recent reports on standardized assessment of AOM severity[12,14-16]. In addition to the number and timing of AOM episodes, AOM symptom severity can be an important factor when considering quality-of-life issues for parents and children. AOM symptom severity may predict office visits for AOM, since some parents may not seek medical attention for children with mild symptoms. Severity may also be related to antibiotic use, since children with non-severe AOM may become candidates for watchful waiting or a safety net antibiotic prescription[17-19].

AOM is a multifactorial disease, involving genetic, environmental factors, anatomical variations, pathogen, and host response. In a meta-analysis[20], the following factors increased the relative risk for an AOM event: a positive family history, day care attendance, pacifier use, tobacco smoke exposure. Breast feeding for at least three months reduced the risk of AOM. To our knowledge, the current study is the first to report associations between AOM clinical symptoms, environmental risk factors, and cytokine gene polymorphisms.

Our study provides further evidence for the importance of heredity in the development and clinical presentation of AOM. Prospective studies have shown that siblings of children with chronic/recurrent otitis media have an increased risk of otitis [1-3, 21, 22]. Progress has been made in identifying locations on specific chromosomes that may be involved in susceptibility to chronic or recurrent otitis media[4,5].

Polymorphisms of cytokine genes, proteins that regulate a large number of biological events, have been shown to influence susceptibility to OM in human and animal studies. It is known that cytokines such as IL-1ß, IL-6, and TNFα are actively induced in nasal secretions of children during viral URI, levels of which, in the nasal secretions, may be associated with the degree of inflammation and/or recovery from infection. We have previously reported[8] that TNFα^{-308} and IL-6^{-174} genotypes were associated with increased risk for recurrent otitis media and tympanostomy tube surgery. Similarly, Emonts el al[23] reported an association between polymorphisms in immunoresponsive genes TNFα, IL-6, IL-10, and TLR-4. In the same population as in the present study, we have shown an association between higher IL-1β concentrations in nasopharyngeal secretions of children with URI and AOM development after URI[6]. Our present data on the association between symptom severity and high-cytokine-producing IL-1ß$^{+3953}$ polymorphic genotype in a subset of children with more severe tympanic membrane involvement further support the significant role of IL-1ß in the inflammatory process during the transition from URI to AOM.

In conclusion, we have shown associations between AOM symptom severity and important risk factors: these include young age, early diagnosis of AOM during an episode of URI, family history of AOM, exposure to environmental tobacco smoke, and IL-1 ß$^{+3953}$ polymorphism. These results parallel previous studies that have shown associations between environmental risk factors, genotype, and AOM episodes. The results provide a basis on which to identify and target high risk children for new preventive and/or treatment regimens.

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Table 1. Demographic, genetic, and risk factor variables. N=128 subjects.

Age at time of enrollment (Months ± standard deviation)	Mean	17.2 (± 7.5)
		Number (percent)
Gender	Female	59 (46.1)
	Male	69 (53.9)
Race	Asian	4 (3.1)
	Black/African American	33 (25.8)
	White	75 (58.6)
	Other	16 (12.5)
Ethnicity	Hispanic/Latino	56 (43.8)
	Not Hispanic/Latino	72 (56.2)
Day care attendance	Home	81 (63.8)
	Home day care	10 (7.8)
	Day care center	36 (28.4)
Family history of AOM	No	50 (39.1)
	Yes	78 (60.9)
Breast fed	No	61 (47.7)
	Yes	67 (52.3)
Smoke exposure	No	87 (68.0)
	Yes	41 (32.0)
Heptavalent pneumococcal vaccine status at enrollment:	Number of doses	
	<3	33 (26.0)
	≥3	95 (74.0)
TNF α^{-308}	Polymorphic	34 (26.6)
	Wild	94 (73.0)
IL-1 β^{+3953}	Polymorphic	35 (27.3)
	Wild	93 (72.7)
IL-6^{-174}	Polymorphic	46 (35.9)
	Wild	82 (64.1)

Table 2. Relation of outcome variable, total symptom score (ETG-5), to variables in the model using general estimating equations: confidence limits and P values. 295 episodes (N = 128 subjects).

		Parameter estimate*	95% CI	P
Age at time of AOM (Months)		-0.01	-0.03 to -0.004	0.01
Gender	Female	0.03	-0.14 to 0.20	0.74
	Male (comparison)			
Race	Asian/Other	-0.23	-0.53 to -0.07	0.01
	Black	0.20	-0.01 to 0.42	0.06
	White/Hispanic (comparison)			
Family history of AOM	No	-0.31	-0.50 to -0.11	0.002
	Yes (comparison)			
Smoke exposure	Yes	0.24	0.06 to 0.42	0.008
	No (comparison)			
Day of URI at time of AOM diagnosis		-0.03	-0.06 to -0.006	0.02
Breast fed	No	-0.10	-0.27 to 0.07	0.25
	Yes (comparison)			
Heptavalent pneumococcal vaccine status	<3	0.04	-0.06 to 0.14	0.41
	≥3 (comparison)			
TNF α^{-308}	Polymorphic	-0.08	-0.28 to 0.12	0.41
	Wild (comparison)			
IL-1 β^{+3953}	Polymorphic	0.14	-0.05 to 0.33	0.15
	Wild (comparison)			
IL-6^{-174}	Polymorphic	-0.03	-0.22 to 0.16	0.77
	Wild (comparison)			

*The parameter estimate for a given variable is the change in ETG-5 symptom score attributable to that variable in the model (see methods). For example, a 12 month old child with no family history of AOM, diagnosed with AOM three days after onset of the URI, and not exposed to tobacco smoke in the home would have on average a statistically significantly lower total symptom score of 0.55 units, compared to a child aged 12 months with a positive family history, diagnosed at three days, and exposed to smoke in the home.

Transcriptional Profiles and Small-Molecule Inhibitors Identify VEGF Pathways Are Critical for Chronic Otitis Media in Junbo and Jeff Mouse Mutants

Michael Cheeseman, PhD, Hayley Tyrer**,** Debbie Williams, PhD**,** Mahmood Bhutta, MD**,** Paul Potter, PhD**,** Steve Brown, PhD
Mammalian Genetics Unit, Medical Research Council, Harwell, Oxfordshire

Introduction
Otitis media with effusion (OME) is the commonest cause of hearing loss in children, yet the underlying genetic pathways and mechanisms involved are incompletely understood. Ventilation of the middle ear with grommets is the commonest surgical procedure in children and the best treatment for chronic OME but the mechanism by which they work remains uncertain. As hypoxia is a common feature of inflamed microenvironments, moderation of hypoxia may be a significant contributory mechanism.

Methods
We investigated the occurrence of hypoxia and HIF mediated responses in *Junbo* and *Jeff* mouse mutant models which develop spontaneous chronic otitis media using *in vivo*hypoxia labeling with PIMO, gene transcription profiles and small-molecule inhibitors of VEGFR signaling.

Results
Mutant mice showed cellular hypoxia in middle ear mucosa and WBC in the middle ear lumen.Inflammatory gene networks were upregulated in the middle ear WBC including the cytokines Il-1β and Tnf-α that modulate HIF. Hif-1α gene expression was elevated in ear fluid WBC and there was upregulation of its target genes including Vegf, its receptorKdr and Vegf protein. Administration of PTK787, SU-11248and BAY 43-9006 significantly reduced hearing loss and modulated inflammatory changes in middle ear mucosa.

Conclusions
The effectiveness of VEGFR signaling inhibitors in suppressing OM in our genetic models implicate HIF mediated VEGF as playing a pivotal role in otitis media pathogenesis via its actions in angiogenesis, vascular leakiness and inflammatory cell chemotaxis. We conclude that targeting molecules in HIF-VEGF signaling pathways has therapeutic potential in the treatment of chronic otitis media.

Birth Characteristics and Acute Otitis Media in Early Life

Kari Kvaerner, MD, PhD[1]**, Yngvild Bentdal, MD, PhD**[2]**,** Siri E Håberg, MD, PhD[4]**, Gunnhild Karevold, MD, PhD**[5]**,** Hein Stigum, PhD[4]**,** Per Nafstad, MD, PhD[3]
[1]Research, Innovation and Education, Oslo University Hospital, Oslo, Norway, [2]Medical Faculty, [3]Epidemiology, University of Oslo, Oslo, Norway, [4]Epidemiology, National Insitute of Public Health, Oslo, Norway, [5]ENT, Lovisenberg Diakonale hospital, Oslo, Norway

Introduction
Associations between low birth weight and preterm birth and the risk for otitis media including acute otitis media (AOM), recurrent acute otitis media (rAOM) and otitis media with effusion (OME) have been assessed in several studies. So far, no clear consensus har emerged whether a relationship exists.

Objective
to assess whether preterm birth and low birth weight were associated with single and recurrent episodes of AOM the first 18 months of life.

Study design
The study is based on the Norwegian Mother and Child Cohort Study conducted by the Norwegian Institute of Public Health. The study population consisted of 33 192 children. Participating women received questionnaires during pregnancy and when the child was 6 and 18 months. Main outcome measures were maternal reports of AOM at ages 6, 11 and 18 months. Information on birth weight and gestational age was obtained from the Medival Registry of Norway. Regression analyses were performed controlling for a variety of potential confoounders.

Results
Preterm birth was slightly associated with both single and recurrent episodes of AOM the first 18 months of life. The adjusted relativ risk (aRR) for having any episode of AOM was 1.37, 95% CI (1.12-1.68) if born before week 33, and the aRR for having recurrent AOM was 1.34, 95% CI (1.01-1.77) if born in week 33 to 36 (reference group ³ 37 weeks). A corresponding tendency was not found for low birth weight.

Conclusions
The findings indicates a modest increased risk of having AOM in children born preterm, and preterm birth seems to be more important thhan low birth weight in determining risk of having AOM in early life.

Risk Factors and Long-Term Outcome for Chronic Suppurative Otitis Media in a High-Risk Population

Ramon Jensen, MD[1]**,** Preben Homoe, MD, PhD[1]**,** Anders Koch, MD, PhD[2]
[1]Dept. of Otolaryngology - Head & Neck Surgery, Rigshospitalet, University Hospital of Copenhagen, Copenhagen, [2]Dept. of Epidemiology Research, Statens Serum Institut, Copenhagen

Objective
Chronic suppurative otitis media (CSOM) is the leading cause of mild to moderate hearing impairment in children worldwide and a major public health problem in many indigenous populations. There is a lack of basic epidemiological facts and knowledge on the development of CSOM, as the disease primarily affects developing countries where research capacities often are limited. The purpose of this study was to determine the long-term outcome of CSOM in a high-risk population and to identify risk factors.

Methods
Follow-up study (2008) on a population-based cohort of 465 children in Greenland, initially examined (1996-8) between the ages 0-4 years. Follow-up was attempted among 307 children living in the two major towns. Binomial logistic regression analysis was made to identify risk factors for developing CSOM and for maintaining disease in to adolescence (odds ratios). Log linear binomial regression was used to estimate risk ratios and absolute risks.

Results
At follow-up 236 participated (77% of those available). The prevalence of CSOM was 32/236 (14%) at age group 0-4 years and 21/236 (9%) at age group 11-15 years. Thirteen had disease debut after the initial study. Of those with CSOM in the initial study 24/32 (75%) healed spontaneously. Risk factors for the development of CSOM at any time in childhood was the mother's history of CSOM OR 2.55 (95% CI 1.14-5.70; p=0.02), and mothers with low levels of schooling OR 1.57 (1.03-2.40; p=0.04). Once CSOM had developed boys were more likely to have persistent disease OR 5.46 (95% CI 1.47 – 20.37; p=0.01). The absolute risk of CSOM if the mother had both a history of CSOM and low schooling was for boys 45.4% (95% CI 26.5 – 77.7) and for girls 30.7% (95% CI 17.8 – 53.10). The cumulative risk of CSOM was 19% at follow-up.

Conclusions
Even though a large number of CSOM cases seemed to heal spontaneously, the prevalence of untreated CSOM among school-age children in Greenland remained high as new cases were found at follow-up. Increased focus on prevention and identification of children at special risk could reduce the high prevalence of CSOM.

Eustachian Tube Physiology/Pathogenesis

Eustachian Tube Function in 3-Year-Old Children with Histories of Recurrent Acute Otitis Media or Chronic Otitis Media with Effusion

Margaretha Casselbrant, MD, PhD, Ellen Mandel, MD, James Seroky**,** J. Douglas Swarts, PhD**,** William Doyle, PhD
Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction

Past studies suggest that poor Eustachian tube function (ETF) contributes to the pathogenesis of acute otitis media, (AOM) or otitis media with effusion (OME). these disease expressions with Eustachian tubes that passively open at relatively small nasopharyngeal pressures promoting the development of recurrent AOM (rAOM) and those with poor muscle-assisted Eustachian tube openings promoting the development of chronic OME (cOME)(1).

The Forced Response Test (FRT) was developed to provide measures of both the passive and active functions of the Eustachian tube in ears with a non-intact tympanic membrane. The passive functions include the ease of opening the Eustachian tube by middle ear over-pressure, the pressure at which the Eustachian tube passively closes and the intra-luminal airflow required to maintain a dilated Eustachian tube lumen. The active functions include the ability of the paratubal muscles to further dilate a pre-dilated Eustachian tube lumen as well as the extent of that dilation (2).

The purpose of this pilot study was to determine whether Eustachian tube (ETF) variables obtained by FTR in 3-year-old children with ventilation tubes placed for either cOME or rAOMdiscriminate between the two different clinical presentations of otitis media

Materials and Methods

The study was approved by the University of Pittsburgh Institutional Review Board. The parental obligations and procedures were explained to those parentsand, if in agreement, signed informed consent was obtained. In this report, we present FRT test results for subjects at 3 years of age tested 3 months after insertion of bilateral tympanostomy tubes. The FTR requires the presence of a patent ventilation tube (or a non-intact tympanic membrane) with no evidence of otorrhea. The diagnosis of recurrent AOM (history of $\geq$3 episodes of AOM in 6 months or $\geq$4 episodes in 12 months) and chronic OME (middle ear effusion for $\geq$3 months bilaterally or $\geq$6 months unilaterally) was confirmed by examination of the records from the child's primary care physician and/or from the Children's Hospital of Pittsburgh ENT clinic. Children classified as cOME could also have had AOM episodes. Children were excluded from the study if they had cleft palate or other craniofacial syndromes with a predisposition to otitis media, cholesteatoma, or other past ear surgery other than ventilation tube insertion..

On the day of testing , otoscopy and tympanometry was done to document ventilation tube patency. For FRT testing,a hermetically sealed plastic probe was introduced into the ear canal, which was coupled to a flow sensor, pressure transducer and, via a valve, to a variable-speed, constant flow pump(2). The constant flow pump was set to deliver ≈23 ml/min of air-flow to the system and middle ear. This application of air-flow increased middle ear pressure to a point where the Eustachian tube passively opened (Opening pressure-PO) and continued delivery of air-flow resulted in a semi-stable system pressure (PS) with the flow rate through the Eustachian tube being equal to that delivered by the pump (QS). The child was asked to swallow by drinking liquid from a cup causing activity of the tensor veli palatini and levator veli palatini muscles, which is associated with either further dilation or constriction of the pre-dilated Eustachian tube lumen, as measured by recording the pre-swallow system pressure (PA) and air-flow (QA) during the swallow. The pump was then turned off, allowing the Eustachian tube to passively close (PC). The FRT variables analyzed for this report are those representing the passive characteristics of the Eustachian tube (PO, PC and passive Eustachian tube resistance [RS=PS/QS]), and those representing the active, muscle-assisted function of the Eustachian tube (Eustachian tube constriction/dilation, active Eustachian tube resistance [RA=PA/QA] and Eustachian tube dilatory efficiency [DE=RS/RA]). We also calculated the active Eustachian tube resistance (RA*) and dilatory efficiency (DE*) for the subset of tests where the Eustachian tube was noted to dilate on swallowing as was described previously. Where possible, this test protocol was done bilaterally.

Each parameter of the FRT was compared between groups using a Students two-tailed t test evaluated at $p<0.05$. Also, the percent of tests evidencing an increase in airflow for the pre-dilated Eustachian tube lumen (Eustachian tube dilation) was compared between groups using a Chi-Square test evaluated at $p<0.05$.

Results

A total of 22rAOM and 24cOME ears were tested. For rAOM, these tests were based on 15children (5 left ears only, 3 right ears only, 7 both ears tested) and for cOME on 17 children (7 left ears only, 3 right ears, 7 both ears tested).Of the 24ears with cOME, 12 ears were from children who also had AOM episodes noted in their medical records. The average and standard deviation of the days between ventilation tube insertion and testing was 45.2$\pm$21.2 and 45.3$\pm$12.3 (t=-0.02, p=0.98) for the cOME and rAOM groups, respectively.

For the two groups, the summary data for each of the FRT variables are presented in the Figure along with the associated t-values and p-levels. Of the FRT parameters, only 2 were statistically different between groups, with RS and DE* being lower in the OME group when compared to the rAOM group. Of the 22 tests in the rAOM group, 9 (41%) evidenced tubal dilation with swallowing, as opposed to 11 (46%) of the 24 tests in the cOME group (ChiSquare=1.11, p=0.74)..

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Discussion

In this pilot study, the sample sizes were rather small allowing for the possibility of failure to identify significant between-group discriminators. However, we chose to limit our analyses to these two groups because the ears were well characterized with respect to disease assignment and the time between ventilation tube insertion and FRT testing.

We had expected that the passive function parameters would be significantly lower in the rAOM group. In contrast, we expected that the cOME group would be characterized by poor active function but relatively normal passive function. If we accept the validity of our results, these predictions were not realized but rather they were in direct opposition to these expectations.

In this study, the FRT was used as it is believed to be the most sensitive test for assessing the passive and active functions of the Eustachian tube (2). Based on the results, we question whether or not the FRT can discriminate between groupsof 3-year-old children with two different disease presentations presumably caused by differentpathogenic mechanisms. Moreover, given the high variability in all of the test parameters, we dismiss the possibility that an individual can be assigned to a particular disease expression based on the results of that test. The continued follow up of these children will elucidate the effect of growth on ETF in the two groups and the time course to resolution of these middle ear diseases.

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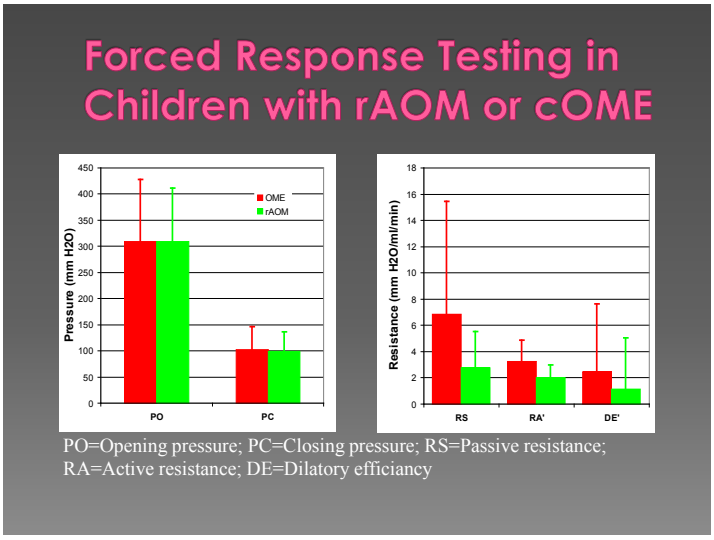

The Relationships Among Eustachian Tube, Cranial Base and Nasopharyngeal Dimensions in 4 Year-Old Children: a Cephalometric Study

Yusuf Kemaloglu, MD[1], J. Douglass Swarts, PhD[2], Margaretha L. Casselbrant, MD[2], Ellen M. Mandel, MD[2], Cuneyt M. Alper, MD[2], William J. Doyle, PhD[2]

[1]Department of Otolaryngology, Gazi University Faculty of Medicine, Ankara, Turkey, [2]Department of Otolaryngology, Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction

Craniofacial (CF) growth and development (GD) is a multistep process influenced by a large number of factors including regional changes and localized morphogenetic processes. The CB, nasomaxillary complex and mandible are the main developing components of the CF skeleton (CFS). These components are oriented to maintain specific functions (respiration, mastication, deglutition, and speech) and their development must be coordinated to allow for continuous function[1]. The ET and peritubal (PT) structures including the peritubal muscles (mET) may represent a separate functional unit with three major functions: 1) Middle Ear (ME) pressure-regulation, 2) protection of the ME from nasopharyngeal secretions and pathogens, and 3) drainage of secretions and fluids from the ME to the nasopharynx (NP). Impairment in the pressure-regulating function can cause otitis media with effusion (OME) while impairment of the other two functions may contribute to the pathogenesis of acute otitis media (AOM). Together, with the ME and mastoid air cells, the ET and PT structures can be referred to as the ME functional unit (MFU).

During the CF-GD process, the ET, mET and PT structures change in size, position and shape[2]. Past research has shown that most children have at least one episode of otitis media (OM) by age 3 years and other studies showed that the pressure-regulating function of the MFU continues to develop into late childhood and early adolescence[3].Therefore, it has been hypothesized that, in addition to other factors such as immunologic immaturity[4], the pathogenesis of OM may be related to anatomic deviations or differences in the CF structures related to the MFU which adversely affects function. Many studies focused on this issue and a number of CF variables measured in adults and children including head type, small mastoids and malocclusion were reported to be associated with OM in children[5-13]. Our purpose was to evaluate the relationship between the CB and NP parameters and the MFU structures in 4 year old children in an attempt to develop mathematical relationships among the various structures.

Methods and Material

This study included 34, 4-year old cases, 20 control children with no significant history of OM (Male:11, Female:9; mean age: 52.31±2.97 month) and 14 children with recurrent otitis media (rAOM; Male: 5, Female: 9; mean age: 50.41±3.54 month). As in our previous study[14], direct digital lateral cephalometric images (DDCIs) were acquired in a standardized manner using a direct digital cephalometer (PlanMeca ProMax) set at a ×1.13 magnification. DDCIs were stored in a jpeg format and uploaded to the CephX-Online Cephalometrics and Storage Service (CephX Inc, Las Vegas, Nevada, USA) via the internet. Then, as previously described[14], the first author traced 10 anatomical landmarks (Table 1), and calculated 18 linear and 9 angular variables (Table 2, Figures 1, 2a and 2b). Tracings and measurements in 10 cases were repeated twice for detection of the intra-researcher variability. For statistical analyses, first, intra-researcher variability was tested for each parameter by intra-class correlation coefficients (ICCs). Then, Pearson correlation analysis was performed between the variables with a significance level of $p < 0.01$ to account for multiple comparisons.

Results

Intra-researcher variability analysis showed that the ICCs were above 0.90 for all parameters.

There was no significant correlation between ACBL and PCBL, although TCBL was positively correlated with both (Table 3). ACBL, but not PCBL or TCBL, was correlated with 6 of the 8 SB lengths measured in this study. Also, ACBL was positively correlated with PFH and measures of nasopharyngeal depth (ND1 and ND2). PCBL and TCBL were each correlated with only one of the SB dimensions (VSBL2 and PISBL, respectively) (Table 3). Correlations for PCBL were highest for OCBL, and then for posterior facial heights (PSFH and PFH) and nasopharyngeal depths (ND1 and ND-2) (Table 3). Although TCBL was positively correlated with both ACBL and PCBL, there was no correlation between TCBL and ^CBA. TCBL was correlated with all of the measures of nasopharyngeal depth and further with posterior vertical dimensions (PFH, PSFH, PISBL and OCBL) (Table 3). In contrast to the linear CB dimensions, ^CBA was correlated with many of the angular variables. While it was positively correlated with ^SA, it was negatively correlated with the nasopharyngeal angles (^NA1 and ^NA2) and with posterior vertical dimensions (VSBL2, OCBL and PSFH) (Table 3).

Linear and angular parameters related to the ET and mET had a number of significant correlations (Table 4). The antero-posterior dimension of the region in which ET is located (ETL) was found to be associated only with the antero-posterior dimensions of the NP and TCBL, while the vertical length of the mTVP (vTVPL) was correlated only with PSFH (Table 4). The ET plane was located between mep and pt points (Tables 1, 2) and presented a number of significant correlations with the angular measures. Specifically, the position of the ET plane in relation to sep (^ETA-III) and to PCB (^ETA-IV) was significantly correlated, but the

position of the ET plane in relation to ACB (^ETA-V) was not. ^ETA-III was positively correlated with three SB dimensions (ASBL, SBbL, VSBL2) and was negatively correlated with PCBL and OCBL (Table .5). VSBL2 and OCBL were only correlated with ^ETA-IV, which was in a positive direction. The position of ET plane in relation to sella (s) (ETA-II) was negatively correlated with ACBL and VSBL2, while its position relative to pns (^ETA-I) was only related to VSBL2 in a negative direction. Further, ^ETA-III was positively correlated with ^CBA and negatively correlated with the nasopharyngeal angles (^NA1 and ^NA2). ^ETA-IV was negatively correlated with the nasopharyngeal angles. Linear dimensions related to the ET and mET (ETL, VTVPL) were not correlated and neither was correlated with the angular ET and mET variables measured in this study, but significant, positive correlations were noted among the angular variables. Specifically, there were significant correlations between the positions of the ET plane to sep (^ETA-III) and to PCB (^ETA-IV), and ^ETA-IV was correlated with ^ETA-II and ^ETA-V (Table 5).

Discussion

Cranial base angle (^CBA), a major determinant of dentofacial GD, is stable from very early ages with only minor changes with age. CBA is the result of brain enlargement and positioning of the eyes to provide straight vision during bipedality, and is shaped by local growth actions of the contributing bony structures underlying these major factors[15-19]. In 4 year old children, ACB and PCB present two distinct but related patterns of GD (Table .3). ACBL is mainly related to the dimensions of the SB. PCBL is mainly associated with the GD of the occipital bone[1,15-19]. On the other hand, the GD of PCB occurs with apposition on the anterior foramen magnum (basion, ba) and the anterior (nasopharyngeal) surface, with reposition in the posterior (cranial) surface and further by sutural growth in spheno-occcipital synchondrosis (SOS) [1,15-19]. Our data are in accordance with this growth pattern. In sum, our data show that, in 4 year old children, ACB is strongly associated with GD in the SB, and that PCB is related to the GD of either the occipital part of the SOS, basion, or both. Further, our data showed that that posterior facial heights and nasopharyngeal depths are associated with PCBL. NDs and PFH appeared to be the only shared communal associations for ACBL and PCBL in 4 year old children. By taking into account that TCBL is also mainly correlated with NDs and posterior facial heights, our data suggest that CF-GD until 4 years of age is mainly associated with nasopharyngeal development. The data related to ^CBA also support this hypothesis by showing that ^CBA changes in parallel to the GD of the SB and PCB until that age. In accordance with previous data[19], they show a decrease in the dimensions related to nasopharynx and posterior face.

During this period, the CB was associated with the size and position in which ET is located and the position of the ET Plane (Tables 4, 5). Linear dimensions related to the ET and mET were mainly associated with nasopharyngeal dimensions in 4 year old children. An antero-posteriorly larger NP is related to a larger ETL while a longer PSFH is related to a longer vTVPL. However, in these interpretations, it should be noted that the distance labeled ETL on the lateral x-ray extends between the TB on the lateral portion of the skull to ptm on the midline. Thus, ETL is a measurement of the inferiorly oblique distance on the lateral aspect. The position of this region (measured between mep and ptm in this study) relates to the SB and PCB, but not to ACB. It has been shown that this region takes a more vertical position to the SB (in relation to both s and sep, ^ETA-II and ^ETA-III, respectively), to the palate (to pns, ^ETA-I) and to the PCB (^ETA-IV) while vertical dimensions related to the SB and PCB increase. Further, although our data did not reveal any association between NDs and the position of the ET (probably because of small number of the subjects), we found that increase in the nasopharyngeal angles occured in conjunction with a steeper position of the ET plane relative to both the sep (^ETA-III) and the PCB (^ETA-IV). These data are in accordance with past studies that reported that during postnatal CF-GD, the ET takes a steeper position[2]. From our data, we suggest that this is also valid at very early ages, even before 4 years of age. Thus, GD of the ACB and PCB which were related mainly to nasopharyngeal GD in this age group is associated with a longer and steeper ET relative to the SB, the palate and PCB.

Interestingly, we found that longer ACBL is associated with a steeper ET in relation to the s point (^ETA-II) (but not to the other landmarks used, such as pns, sep or PCB). This is in accordance with other studies reporting that brachycephalic head-forms confer a higher OM risk[6,7,20]. However, the increase in antero-posterior dimensions of the SB (SBbL and ASBL) was found to be associated with a more parallel position of the ET plane in relation to the anterior portion of the SB (but not to the other points such as pns, s and PCB). Because the size of our study group was small, these data could be 'a shared-landmark related error', but, our data also showed that a larger ^CBA is related to a larger ^ETA-III. It is known that larger ^CBA is associated with dolichocephalic head-form. If these data are supported by other studies, we propose the following hypotheses. It is known that SB dimensions are mainly related to neural growth and that growth in antero-posterior dimension of the SB starts in the prenatal period and ends in the very early ages postnatally[1,15-19]. Therefore, we suggest that the balance between GD in the anterior cranial and mid-cranial fossae could be important to OM risk in early childhood. Early growth activity in the anterior cranial fossa partially determines the position of the ET, which makes it more horizontal. But, imbalances in these parts could be compensated by other factors, and changes in the vertical dimensions may cause a steeper ET angle even if it is horizontal due to, for example, a brachycephalic head-form. This study presents the mathematical associations between the variables measured on DDCIs and provides us a template to study postnatal development of the ET and mET. Since CF-GD is a multistep process in which regional imbalances are compensated for by other imbalances such that, ultimately, a functional balance is restored[1], evaluation of the relationships between ET/mET parameters and the CF at consecutive ages will provide us with a better understanding of the postnatal GD of the ET/mET, and if it is possible to describe variables that identify those children at high risk for OM.

Conclusions

1). Cephalometrics can be used to address associations of ET length and position with the growth of the CB and NP.

2. In 4 year old children, a number of CB and NP parameters are correlated with ET length and position relative to the SB and PCB. Specifically, the anterior size of the SB is associated with a more horizontal ET relative to the anterior portion of the SB, while ET length and a greater angle of decent are associated with posterior CB elongation (and infero-posterior lengths of the SB) which occurs in parallel to antero-posterior widening of the nasopharynx.

3. If there is an OM risk related to CF-GD during the childhood period, researchers should compare this set of parameters between normal and OM cases at different ages to define one parameter or a set of parameters that could underlie OM risk in any child.

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Table 1. Landmarks identified on Lateral Cephalograms.

ba	Basion; the most anterior inferior point on the margin of the foramen magnum, located on the most inferior of the basillar part of the occipital bone (on the intersection point of the nasopharyngeal and posterior surfaces of the basillar part of the occipital bone)
go	Gonion; the most postero-inferior point on the outline of the mandibular angle. Midpoint of the right and left sides' images was used.
mep	Middle ear point; The most antero-superior point of the middle ear image; Midpoint of the right and left sides' images was used.
n	Nasion; the intersection of the internasal and frontonasal sutures
pns	Posterior nasal spine; the most posterior point of the hard palate; the junction between the hard and soft palate was used.
ptm	Pterygo-maxillare; the most inferior point of the pterygo-maxillary fissure, located where the anterior and the posterior outline of the inverted teardrop merge with each other. Midpoint of the right and left sides' images was used.
s	Sella; geometric center of the sella turcica.
sb	Sphenoid bone point; The most anterior point of SB bony shadow on the axis between ptm and sos.
sep	Spheno-ethmoidal point; the most superior point of the intersection of the sphenoid and ethmoid bones.
sos	Spheno-occipital synchondrosis; the most cranial end of the spheno-occipital synchondrosis, located on the sphenoidal line of the synchondrosis.

Table 2. Calculated Linear and Angular Parameters for this Study.

Linear parameters		
s-n	Anterior Cranial Base Length	ACBL
s-sep	Anterior SB Length	ASBL
mep.ptm	This distance fits to the length of the region in which ET is located	ETL
ba-ptm	Nasopharyngel depth-1	ND1
ba-pns	Nasopharyngel depth-2	ND2
sos-ba	Occipital Cranial Base Length	OCBL
s-ba	Posterior Cranial Base Length	PCBL
s-sos	Posterior Sphenoid Bone Length	PSBL
s-pns	Postero-Superior Facial Height	PSFH
s-go	Posterior Facial Height	PFH
sos-ptm	Postero-inferior Sphenoid Bone Length	PISBL
sos-sep	Sphenoid Bone body Length-1	SBbL
n-ba	Total Cranial Base Length	TCBL
sep-ptm	Vertical Sphenoid Bone Length -1	VSBL1
s-ptm	Vertical Sphenoid Bone Length -2	VSBL2
sb-sos	Vertical Sphenoid Bone body Length-1	VSBbL1
sb-s	Vertical Sphenoid Bone body Length-2	VSBbL2
ptm-pns	This distance fits to vertical length of the Tensor Veli Palatini muscle	vTVPL
Angular parameters		
^n.s.ba	Cranial base angle	^CBA
^mep.ptm.pns	ET angle-I	^ETA-I
^mep.ptm.s	ET angle-II	^ETA-II
^mep.ptm.sep	ET angle-III	^ETA-III
^mep.ptm/s.ba	ET angle-IV between mep.ptm and s-ba axis	^ETA-IV
^mep.pt/s.n	ET angle-V between mep.ptm and s.n axis	^ETA-V
^ba.s.ptm	Nasopharyngeal angle-1	^NA1
^ba.s.pns	Nasopharyngeal angle-2	^NA2
^sos.ptm.sep	Sphenoidal angle	^SA

Table 3. The Pearson Correlation (r) and Associated Probability Level for the Cranial Base Variables.

CBA	ACBL	PCBL	TCBL
VSBL2 r:-0.61, *<0.001*	TCBL r: 0.77, *<0.001*	OCBL r: 0.88, *<0.001*	ND2 r: 0.86, *<0.001*
OCBL r:-0.52, *<0.002*	PISBL r: 0.61, *<0.001*	PFH r: 0.62,*<0.001*	ND1 r: 0.82, *<0.001*
PSFH r:-0.53, *<0.002*	ASBL r: 0.60, *<0.001*	TCBL r: 0.60, *<0.001*	PFH r: 0.59, *<0.001*
^NA2 r:-0.87, *<0,001*	ND1 r: 0.60, *<0.001*	ND1 r: 0.55, *<0.001*	PSFH r: 0.57, *<0.001*
^NA1 r:-0.84, *<0,001*	SBbL **r: 0.56, *<0.001***	PSFH r: 0.52, *<0.002*	PL r: -0.53,*<0.002*
^SA r: 0.49, *<0.005*	VSBbL1 r: 0.56, *<0.001*	VSBL2 r: 0.51, *<0.003*	PISBL r: 0.51, *<0.002*
	PFH r: 0.54, *<0.002*	ND2 r: 0.46, *<0.01*	OCBL r: 0.46, *<0.01*
	VSBL2 r: 0.53, *<0.002*		
	VSBbL2 r: 0.45, *<0.01*		
	ND2 r: 0.45, *<0.01*		

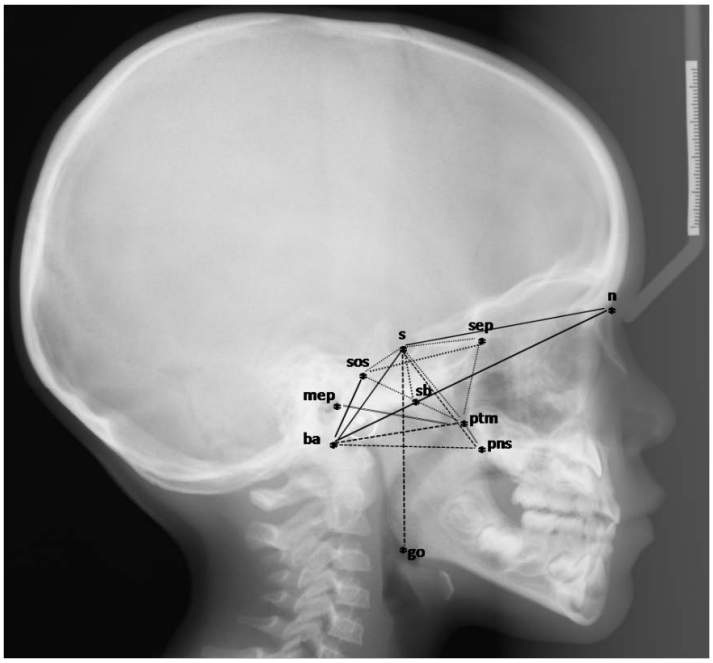
Table 4. The Pearson Correlation (r) and Associated Probability Level for the Linear and Angular ET and mET Variables.

ETL	vTVPL	ETA-I	ETA-II	ETA-III	ETA-IV
ND2 r: 0.72 *<0.001*	PSFH r: 0.45 *<0.01*	VSBL2 r:-0.53 *<0,003*	ACBL r:-0.54 *<0.002*	ASBL r: 0,65 <0.001	VSBL2 r:-0.60 <0.001
TCBL r: 0.57 *<0.001*			VSBL2 r:-0.45 <0,002	SBbL r: 0.62 <0.001	OCBL r:-0.50 <0.005
ND1 r: 0.59 *<0.001*				VSBL2 r:-0.54 <0,002	^NA2 r:-0.89 <0.001
				OCBL r:-0.54 <0,002	^NA1 r:-0.85 <0.001
				PCBL r:-0.52 <0.003	
				^NA2 r:-0.61 <0.0003	
				^NA1 r:-0.54, <0.002	
				^CBA r: 0.51, <0.005	

Table 5. Table 4. The Pearson Correlation (r) and Associated Probability Level Among the Linear and Angular ET and mET Variables.

	^ETA-II	^ETA-III	^ETA-IV
^ETA-IV	r:0.56 p< 0.002	r: 0.63 p< 0.001	
^ETA-V	r: 0.62 p< 0.001	-	(r: 0.45) p= 0.011

Figure 1. Landmarks and Linear Variables Measured on the Lateral Cephalograms (Continuous, dashed and dotted lines represent CB, NP and SB variables, respectively. The ETL and vTVPL were marked with double bar.)



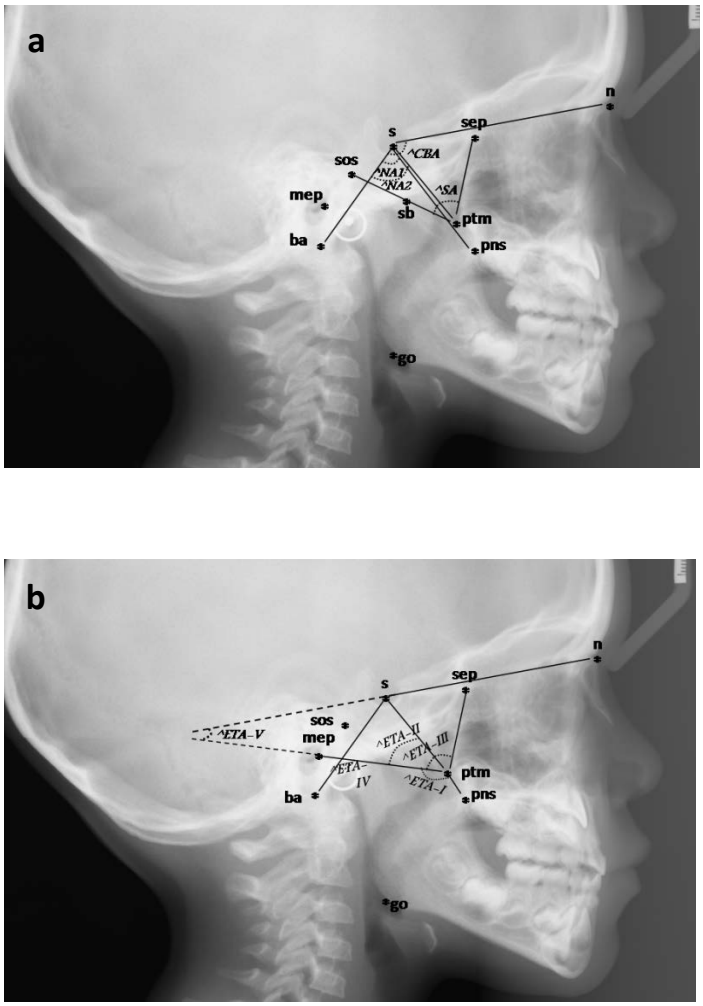


Figure 2. Landmarks and Angular Variables Measured on the Lateral Cephalograms (a) The angles related with the cranial base, sphenoid bone and nasopharynx. (b) The angles related with the Eustachian tube and peritubal muscles.

Assessing the Importance of Mucosal Adhesion on Eustachian Tube Function in Different Patient Populations

Samir Ghadiali, PhD[1], Francis Sheer[2]
[1]Biomedical Engineering, The Ohio State university, Columbus, OH, [2]Biomedical Engineering, The Ohio State University, Columbus, OH

The Eustachian Tube (ET) is a complex, three dimensional system whose function depends on many parameters such as tissue mechanics, anatomy, and muscle function. Adequate ET function also depends on the amount of adhesive force present in the ET lumen. These adhesion forces may be due to glycoproteins interactions in the mucus fluid and/or surface tension forces at the air-liquid interface. To investigate the importance of adhesion, we have created 3-dimensional, multi-scale computational models of ET function that account for both anatomical variations between patient populations and molecular scale adhesion forces. Models were created using histology from three different patient populations: healthy adults, normal children, and cleft palate infants. Using these models, the amount of adhesion energy present in the system was varied to determine the sensitivity of ET function to adhesion in each patient. In the healthy adult population, it was found that ET function was not sensitive to small changes in adhesion energy. However, in normal children and cleft palate infants, small increases in adhesion energy resulted in poor ET ventilation function. These results indicate that being able to quantify the adhesion status of a OM prone patients in the clinic might be an important way to determine the proper course of treatment. Supported by NIH R01DC007230 and P50DC007667.

Assessment of Eustachian Tube Function Using EMG of the Tensor Veli Palatini, Levator Veli Palatini and Submental Muscles, Videoendoscopy and Sonotubometry

Cuneyt M. Alper, MD, J. Douglas Swarts, PhD**,** Aloka Singla, MD**,** Julianne Banks**,** William J. Doyle, PhD
Department of Otolaryngology, Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction

The results of numerous studies showed that populations “at risk” for OMor with tympanostomy tubes inserted for OM have a dysfunctional ET as documented by an inability to reduce applied ME pressures and/or by a paradoxical constriction (instead of dilation) of a pre-dilated ET lumen.[1-3] This defect, termed functional ET obstruction by Bluestone and coworkers, has been related to structural variations in the ET and/or aberrant function of the mTVP, mLVP, or both.[4] Using simultaneous videoendoscopy of the ET lumen at the posterior nasopharynx and theforced response test of ET function, Takahashi and colleaguesreported that ET constriction could be associated in some cases with blockage of the tubal orifice by elevation of the soft palate during contraction of the mLVP.[1]

Electromyography (EMG)has been used to assess the possible role of the mTVP, mLVP and tensor tympani muscle in ET opening.In an early study, Kamerer documented synchrony of the EMGs for the mTVP and tensor tympani muscle during swallowing, but not other activities.[5]Other studies used EMG techniques to record the activities of the mTVP and mLVP individually in healthy and “diseased” human MEs, but the only study that recorded the activities of both muscles simultaneously was done in monkeys.[6-7]

Here, we conducteda multimodal study assessing ET function in normal adults with documented ET openings and no reported history of ME disease in childhood.Specifically, we simultaneously recorded the activities of the mTVP and mLVP using EMG, ET openings using sonotubometry and the movements and rotations of the soft palate and ET usingvideoendoscopy. This study is the first to combine these 3 assessment modalities and was performed to establish the feasibility of the methods and to establish normative data for later functional studies of MEs with concurrent or a history of OM.

Methods

Healthy adult (≥18 years) volunteers were recruited from the surrounding community by advertisement. After obtaining informed consent approved by the University of PittsburghInstitutional Review Board, subjects were screened for general health, past OM history, prior nasal and/or facial surgery, and a standard Ear Nose and Throat Examination was performed. Inclusion criteria included: 1) no reported past history of ME disease or nasal or facial reconstructive surgery; 2) no known allergic or previous reactions to Lidocaine and Oxymethazoline;3) an unobstructed nasal airway that allowedfor endoscope passage to the posterior nasopharynx; 4) a normal nasal and facial anatomy, and 5) at least one successful ET opening in a sequence of 3repeated sonotubometric tests.

For eligiblesubjects, a microphone for recording sonotubometric signals (i.e. microphone voltages during a swallow referenced to ambient) was placed in the external ear canal on the test side and covered with a standard ear protector. Submental surface electrodes (Noraxon Dual Electrodes, Noraxon USA Inc., Scottsdale, AZ) were placed along the anterior belly of the digastric muscle and a surface reference electrode (Meditrace 100, ECG Conductive Adhesive Electrodes, Tyco Healthcare Group LP, Mansfield, MA) was placed on the mastoid tip. Then, the nasal cavities were topically anesthetized and decongested first with a sprayer and then with cotton gauze (Medtronic Neuray® Neurosurgical Patties, ½ in. x 2 in., Medtronic Xomed, Inc., Jacksonville,

FL) both containing/soaked in asolution of 4% Lidocaine Hydrochloride (Roxane Laboratories Inc., Columbus, OH) and 0.05% OxymetazolineHydrochloride (Major® Soothing 12 Hour Nasal Decongestant Spray, Major Pharmaceuticals, Livonia, MI) mixed in 1:1 proportion. This procedure was repeated until satisfactory local anesthesia and decongestion was obtained. A 0° endoscope (Hopkins, 2.7 mm,,18 cm, Karl Storz Endoscopy, Germany) attached to a high speed digital video camera(DigiCam 2.0, JedMed Instrument Co., St. Louis, MO) was advanced through the most patent side of the nasal cavity to the level of the nasopharynx. The ipsilateral ET was visualized and the region of the tube and nasopharynx examined for pathology. The probe for the sonotubometry speaker was placed within the contra-lateral nostril. The images from the video recordings and the sonotubometry results were processed and saved on a microcomputer as described below. The endoscope was then withdrawn.
A 45° telescope (Hopkins, 2.7 mm, 18 cm, Karl Storz Endoscopy, Germany) with attached video camerawas than inserted into the test side. The movements of the ET system were captured by the video cameraswallow. Video imagesof these movements and were continuously recorded and routed to the processing programs of a microcomputer as described below.
Next, two disposable paired stainless steel insulated fine wire (27G, 0.02x8 in.)EMG electrodes (Chalgreen Enterprises Inc., Gilroy, CA) shielded and fittedwithin3.5 25G spinal needles for guidance(Spinocan®, B. Braun Medical Inc., Betlehem, PA) wereadvanced through the ipsilateral nasal cavity under endoscopic visualization. One pair of electrodeswas placed into the belly of the mTVP just anterior to the nasopharyngeal orifice of the ET and a second pair of electrodeswas placed into the belly of the mLVP just medial and inferior to the floor of the ET. The spinal needles were removed, and the sonotubometric sound source was placed in the contralateral nostril. Multimodal recoding of ET function was done by measuring the EMGsof the submental muscles, mTVP and mLVP,measuring the voltages from the external canal microphone and recording the movements of the soft palate and ET by videoendoscopy during swallowing. After the completion of these recordings, the 45° endoscope was withdrawn andthe electrodes wereremoved. A repeat endoscopy was performed with a 0° telescope to verify lack of bleeding before discharging the subject.
Throughout,the video images were routed to the memory of a microcomputer with reference marks to indicate timing. Microphone and EMG voltages were continuously and simultaneously recorded during swallows via a Multimodal Board (ADInstrument Octal Bio Amp Power Lab 8/30 Model ML138, ADInstruments Pvt Ltd, Australia) and those signals and the time-marked videoendoscopic images were displayed and analyzed using the PowerLab Data Acquisition Software (ADInstruments LabChart v. 7.2, ADInstruments Pvt Ltd, Australia).Data for sonotubometry, and mLVP, mTVP and submental surface EMGs are reported as amplitude, area and 50% peak width. The intervals between the sonotubometry peak and onset of the EMG activities of the three muscles were calculated.Video images were examined by an investigator blinded to the EMG data for the subject. Using the PowerLab software, the video images were reviewed frame by frame, by first identifying the still frame with the greatest opening of the nasopharyngeal orifice of the ET. This frame was labeled frame-0. All other frameswere labeled by number (positive or negative) in reference to frame-0. At each time frame, the elevation of the soft palate and the displacement and degree of axial rotation of the medial lamina of the ET cartilage representing mLVP contraction, the posterior displacement of the lateral wall of the ET lumen due to mTVP contraction and ET lumen opening (ETO) were recorded.The degree of displacement from baseline was rated on a scale of 0-4. The string of data for each of the anatomical structures that had a score greater than 0 represented the duration of the event during a swallow.

Results

Twenty subjects were enrolled. The electrodes and insertion method were modified after the first 3 subjects. In 15 subjects EMG data was collected from both TVP and LVP muscles, but in 2 subjects no EMG data was collected from one of the muscles. Video recording of the ET opening was consistent with the EMG activity and ET openings by sonotubometry. mTVP activity had a shorter duration but a greater amplitude than mLVP activity. mLVP activity occurs before that of mTVP and the submental muscle group.
Overall, mTVP activity had a shorter duration but greater amplitude than mLVP activity. mLVP activity occurred before that of the mTVP and the submental muscle group. Video recording of the ET openings were consistent temporally with the EMG activity of the paratubal muscles and with the ET openings as measured by the peak amplitude of the sonotubometry microphone.
Endoscopic findings during swallowingaveraged for all subjects are summarized asfollows: Maximum opening of the ET lumen occurred at frame-0. The maximum range of a swallow was captured in 21 frames, 10 frames prior to and 10 frames after frame-0. The first anatomical change observed during a swallow wasthe elevation of the soft palate as indicated in the figure bythe onset of mLVPcontraction. The posterior displacement and axial rotation of the medial lamina of the ET cartilage mirroredmLVP activity with a slight delay relative to the onset of a swallow. The average posterior displacement of the lateral wall of the ET lumen corresponded to mTVPcontraction and approximated the timing of maximum ET opening.

Discussion

A variety of methods to assess the pressure-regulating function of the ET has beendescribed but most require direct access to the ME airspace (either through a perforatedtympanic membrane or a patent tympanostomy tube) and therefore are usually limited toevaluations of pathological MEs. Alternatively, sonotubometry (with or without a pressurechamber) and videoendoscopy of the nasopharyngeal orifice of the ET were developed astechniques for assessing ET function in ears with an intact tympanic membrane, and EMG of the paratubal muscles was developed as a method to determine if ET dysfunction is associated with poor

muscle activity. Sonotubometry is a well described acoustic method for assessing the timing and duration oftubal openings during different activities such as swallowing, yawning and mandibularrepositioning.[3]
Videoendoscopy of the nasopharyngeal portion of the ET is a relatively old procedurewhose results remain difficult to interpret. Also, the effects of mLVP and mTVP constractions can be identified in those images and themovements they elicit can be assessed during differentmaneuvers such as swallowing and phonation.It was hypothesized that abnormal results on this test reflect abnormalactivities of the paratubal musclesand ET dysfunction.[2]
In the current study of healthy adults with no history of OM, we combined these three modalities in an attempt to describe the temporal relationships among ET opening as documented by sonotubometry, the EMG activities of the mTVP and mLVP and the videoendoscopic observations of soft palate and ET movements during contraction of the paratubal muscles. The results showed that mLVP activity preceded ET opening while mTVP activity was associated with maximum ET opening. The observed movements of the ET were related to elevation of the soft palate associated with contraction of the mLVP and to a later contraction of the mTVP. These videoendoscopic observations related well to the EMG activities of the two muscles. From these results, we achieved the primary goal of this study which was to demonstrate the feasibility of this methodology laying the foundation for future studies using these techniques in adults with a history of OM or ET dysfunction.

Conclusions
Multimodal assessment of ET openings by monitoring the EMG of the paratubal and submental muscles, videorecordings of the ET and sonotubometry in adult volunteers is a novel and promising method for expanding our knowledge of middle ear physiology and pathophysiology.

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Cephalometric Parameters in 4 Year-Old Children with or Without History of Recurrent Acute Otitis Media

Yusuf Kemaloglu, MD[1], J. Douglas Swarts, PhD[2], Margaretha L. Casselbrant, MD[2], Cuneyt M. Alper, MD[2], Ellen M. Mandel, MD[2], William J. Doyle, PhD[2]
[1]Department of Otolaryngology, Gazi University Faculty of Medicine, Ankara, Turkey, [2]Department of Otolaryngology, Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction
The impact of craniofacial (CF) growth and the development (GD) on otitis media (OM) risk has been suggested by many researchers[1-12]. As reported by Mew and Meredith (1992), Hippocrates, first, noted the relationship between head type, malocclusion and otorrhea in his sixth book of Epidemics (6). Recently, the evolutionary aspects of this relationship have been explored[13]. Specifically, flattening of the face in contemporary humans may be associated with an impaired function of Eustachian Tube (ET) by changing palatal anatomy and, thus, the orientation and function of the peritubal muscles (mET) [13]. Others showed that poor ET function plays a causal role in the pathogenesis of OM. However, it has been difficult to define a set of cephalometric or anthropometric parameters related to the cranial base (CB), nasopharynx (NP) or peritubal (PT) structures that affect ET function and/or the prevalence of OM. Such studies are limited by technical difficulties and by the structural changes associated with CF-GD. For example, in addition to the conventional problems associated with cephalometrics, the ET and mET landmarks are masked by bony and soft tissue shadows [6,14,15]. Moreover, as stated by Enlow and Hans (1990), CF-GD is multifactorial and multistep process[16] wherein regional changes and localized morphogenetic processes contribute to the final pattern of the CF skeleton (CFS). The ET and mET are located between the CB and maxilla, and hence it is logical to suggest

that various parameters describing the anatomy of these anatomical units (as well the mandible due to muscular function in deglutition and mastication) contribute to the functional changes in the ET and PT during CF-GD.

Past studies show that the function of the ET improves and the prevalence of OM decreases with age and these changes could be caused by the morphologic changes at different steps during CF-GD. Previous investigators reported various parameters that were different in OM when compared to control groups. These include: shorter anterior or posterior CB lengths (ACBL, PCBL), posterio-superior facial height (PSFH), palatal length (PL), antero-superior facial height (ASFH) and mandibular body lengthin OM cases[1-5,9,12]. An adult cephalometric study reported that GD of ETL is dependent on total cranial base length (TCBL), PL and PSFH[10]. Some or all of these differences alone or in combination could contribute to impaired ET function during childhood. However, these studies focused on widely different age groups, some of which extended beyond the period CF-GD and, thus, may have identified spurious associations. Moreover, the definition of OM was often made based on past history and the statistical methods employed were often inappropriate which could have introduced errors in developing the form-function associations. Therefore, we concentrated on 4 year old children which is the earliest age at which stable cephalometrics can be obtained and our population is well characterized with respect to disease presence/absence.

Material and Methods

This study included 32, 4-year old cases, 18 control children (M: 11, F:7; mean age: 53.93 ± 3.12 month) and 14 children with recurrent acute OM (rAOM; M: 5, F: 9; mean age: 50.41 ± 3.54 month). As in a previous study[15], direct digital lateral cephalometric images (DDCIs) were acquired in a standardized manner using a digital cephalometer (PlanMeca ProMax) set at ×1.13 magnification. DDCIs were stored in jpeg format and uploaded to the CephX-Online Cephalometrics and Storage Service (CephX Inc, Las Vegas, Nevada, USA) via the internet. In this study, the same anatomical landmarks and 19 linear and 9 angular variables described previously were used[17](Tables 1,2). Tracings and measurements in 10 cases were repeated twice for detection of intra-researcher variability. For statistical analyses, first, intra-researcher variability was tested for each parameter by intra-class correlation coefficients (ICCs). Then, differences in the various measures between the rAOM and control groups were evaluated using a Wilcoxon Signed Ranks Test with a significance level of $p < 0.05$.

Results

Intra-researcher variability analysis showed that the ICCs for all parameters were above 0,90.

The data for the between-group comparisons are presented in Tables 1 and 2. None of the angular parameters was different between the groups. Among the linear variables, only one, the vertical length of the SB body (VSBbL2) measured on a line from the axis between the spheno-occipital synchondrosis (sos) and apex of pterygoid fissure (ptm) to the sella (s), was significantly larger in control compared to rAOM children (17±0.9 and 15.5±1.7).

Discussion

OM is common disease in childhood of contemporary humans who, compared to other animal species, are characterized by a big brain, bipedality, small jaws and a correspondingly loss of prognatism[13]. Humans are one of the few animal species to spontaneously develop OM and epidemiologic studies show that OM is common in early childhood and decreases in prevalence with advancing age[18]. It has also been shown that OM prevalence is greater in preterm babies when compared with term babies[19]. Moreover, while OM occurs in almost all children by age 3, some children have repeated bouts of OM or persistent OM while others maintain relatively health MEs throughout life. It is interesting that this pattern for OM prevalence mirrors that for the pressure-regulating function of the ET which is also poor in infants and young children while improving with advancing age. Our main hypothesis is that there are anatomic differences reflecting CF-GD between control children and those with rAOM.

Until 4 years of age, neural growth (brain and eyes) is the main contributor to CF-GD. Although this effect of neural growth, particularly in the median and posterior cranial fossae, on CB-GD continues into later ages, dentofacial GD influences the shape and size of the CB in the later years. It has been shown that the external surface of the CB, particularly SB, (in which the ET and mET are located) is shaped by later dentofacial growth activities[20].

Nakamura et al (1972) reported that, at 4 years of age, ASBL and SBbL achieved about 91% and 86% of the dimensions at 16 years[20], and in a previous study we found that these dimensions were associated with a more horizontal ET position in relation to the anterior SB[17]. Therefore, in this study, we might have limited our data analysis to those parameters mainly related to neural growth which is much more influenced by genetic factors, head shape and familial characteristics. Because this is part of a longitudinal study, in future years, it will be possible to study the effects of the later steps associated with dentofacial GD. These are expected to be associated with epigenetic factors, such as nasal obstruction, poor masticatory habits or other acquired problems affecting dentition, mastication and deglutition, which are reported to be common in the industrialized societies[6].

Therefore, the purpose of this study was to define CB differences which might be attributed to PT structures and correspondingly to abnormal ET function in 4 year old children[17]. In this study, no difference was found in the linear and angular parameters related to the ET or mET. Although the results of some previous papers presented data that showed the total, bony or

cartilaginous ET lengths measured on adult cadavers were associated with OM risk[3,9], cephalometric data related to the dimension of the region in which whole ET is located (ETL in this study) did not support those results[8] and this negative finding was reproduced in our study. Most likely, this discrepancy is associated with the use of adults and an indirect measure of OM (mastoid pneumatization) in the cadaver study, while the OM phenotype was clearly defined in our study and disease presentation was concurrent with the time of measurement.

Previous papers reported that ACBL was shorter in children with OM, or that it was positively correlated with OM indicators[1,9,11,12]. However, we could not reproduce this result. This discrepancy could reflect the difference in age of the subjects at the time of study. ACBL is not only related to neural growth but also to the growth of the nasomaxillary complex, with the former more active in early life and the latter in older children. Thus, between-group comparisons at different ages may produce different results. However, some previous studies that included older children also reported no difference in this dimension[5]. A shorter ACBL is associated with a smaller anterior cranial fossa and with a brachycephalic head type[16] which has been suggested as predisposing to OM[2,4,13]. For this head type, a narrow CBA is also expected. However, as in our study, many past studies showed no difference in CBA between OM and control groups[1,9,11,12]. Alternatively, Di Francesco's reported a shorter CBA in adults with a history of OM[11] while Maw and colleagues reported a larger CBA in the OM group[5,7]. This issue cannot be resolved with the available data and must await the results of longitudinal studies of the effect of CF-GD on OM risk.

In this study, we found only one between-group difference, the vertical length of the SB body (VSBbL2, Figure 3) measured on a line from the axis between the spheno-occipital synchondrosis (sos) and apex of pterygoid fissure (ptm) to the sella (s) which was larger in the control group compared to the rAOM group. This measure is related to the vertical SB body length (to sb from s, VSBbL2). The sb point is not a routine landmark in cephalometrics and this SB dimension has not been reported in the literature. One measurethat is similar to our parameterwas reported by Maw and colleagues[5,7] who drew an axis through ba and the most superior point of the NP and measuredthe depth of the basisphenoid bone perpendicular to this axis from sella (this variable has been reproduced in Figure 3 by using their description[5,7]). This dimension was significantly shorter in children with OM (3 y 11 mo to 6 y 10 mo). It is not clear if and how this parameter relates to the morphology of the ET or mET. We speculate that it may reflect the lateral projection of the osseous-cartilaginous juncture of the ET or the most posterior-superior origin of the tensor veli palatini muscle.

Regarding CF determinants of VSBbL2, it should be noted that the basicranial region of the human CB exhibits a characteristic flexure. The axis about which this bending occurs passes transversely through the body of SB dividing the CB into pre- and postsella components. A change in the form or position of the SB complex will greatly influence the angular relations of the CB affecting the maturation of both the neural and facial skeleton[20,21]. Hence, it might be speculated that this parameter, at least in 4 years-old children, is associated with CB flexure that, in turn, is associated with a large brain, straight vision line and bipedality.

In summary, from the data for this study with small sample sizes, we suggest that an early tendency to rAOM could be associated with SB dimensions affecting the position of the cartilaginous ET and/or mET.

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Table 1. Landmarks identified on Lateral Cephalograms.

ba	Basion; the most anterior inferior point on the margin of the foramen magnum, located on the most inferior of the basillar part of the occipital bone (on the intersection point of the nasopharyngeal and posterior surfaces of the basillar part of the occipital bone)
go	Gonion; the most postero-inferior point on the outline of the mandibular angle. Midpoint of the right and left sides' images was used.
mep	Middle ear point; The most antero-superior point of the middle ear image; Midpoint of the right and left sides' images was used.
n	Nasion; the intersection of the internasal and frontonasal sutures
pns	Posterior nasal spine; the most posterior point of the hard palate; the junction between the hard and soft palate was used.
ptm	Pterygo-maxillare; the most inferior point of the pterygo-maxillary fissure, located where the anterior and the posterior outline of the inverted teardrop merge with each other. Midpoint of the right and left sides' images was used.
s	Sella; geometric center of the sella turcica.
sb	Sphenoid bone point; The most anterior point of SB bony shadow on the axis between ptm and sos.
sep	Spheno-ethmoidal point; the most superior point of intersection of the sphenoid and ethmoid bones.
sos	Spheno-occipital synchondrosis; the most cranial end of the spheno-occipital synchondrosis, located on the sphenoidal line of the synchondrosis.

Table .2. Calculated Linear Parameters for this Study, and Average and Standard Deviation (SD) in Control and Recurrent AOM (rAOM) groups.

Linear parameters			GROUPS	Mean	SD
s-n	Anterior Cranial Base Length	ACBL	Control	58,92	2,65
			rAOM	58,63	3,51
s-sep	Anterior SB Length	ASBL	Control	21,02	2,48
			rAOM	21,62	3,35
mep.ptm	This distance fits to the length of the region in which ET is located	ETL	Control	35,93	1,46
			rAOM	35,21	2,76
ba-ptm	Nasopharyngel depth-1	ND1	Control	40,77	2,51
			rAOM	40,93	3,65
ba-pns	Nasopharyngel depth-2	ND2	Control	36,48	1,90
			rAOM	35,87	2,66
sos-ba	Occipital Cranial Base Length	OCBL	Control	23,07	2,72
			rAOM	22,13	3,59
s-ba	Posterior Cranial Base Length	PCBL	Control	33,66	2,33
			rAOM	33,37	2,96
s-go	Posterior Facial Height	PFH	Control	55,50	3.25
			rAOM	54.05	3.80
sos-ptm	Postero-inferior Sphenoid Bone Length	PISBL	Control	30,40	2,53
			rAOM	31,13	2,46
s-sos	Posterior Sphenoid Bone Length	PSBL	Control	11,58	,79
			rAOM	12,30	1,66
s-pns	Postero-Superior Facial Height	PSFH	Control	36,96	2,34
			rAOM	38,34	2,58
sos-sep	Sphenoid Bone body Length	SBbL	Control	31,82	2,66
			rAOM	32,81	3,83
n-ba	Total Cranial Base Length	TCBL	Control	86,13	3,19
			rAOM	84,88	4,2
sep-ptm	Vertical Sphenoid Bone Length -1	VSBL1	Control	27,40	1,20
			rAOM	26,48	2,19
s-ptm	Vertical Sphenoid Bone Length -2	VSBL2	Control	28,09	2,29
			rAOM	28,74	2,39
sb-sos	Vertical Sphenoid Bone body Length-1	VSBbL1	Control	13,84	2,61
			rAOM	15,20	2,01
sb-s	Vertical Sphenoid Bone body Length-2	VSBbL2 **	Control	15,47	1,70
			rAOM	16,82	1,04
ptm-pns	This distance fits to vertical length of the Tensor Veli Palatini muscle	vTVPL	Control	9,00	1,72
			rAOM	9,73	1,62

**, p= 0.007, Wilcoxon Signed Ranks Test.

Table 3. Calculated Angular Parameters for this Study, and Average and Standard Deviation (SD) in Control and Recurrent AOM (rAOM) groups.

Angular parameters			GROUPS	Mean	SD
^n.s.ba	Cranial base angle	^CBA	Control	135,64	5,74
			rAOM	133,40	7,75
^mep.ptm.pns	ET angle-I	^ETA-I	Control	48,07	12,12
			rAOM	50,08	11,04
^mep.ptm.s	ET angle-II	^ETA-II	Control	43,63	3,73
			rAOM	46,15	3,31
^mep.ptm.sep	ET angle-III	^ETA-III	Control	88,18	6,86
			rAOM	91,95	7,27
^mep.ptm/s.ba	ET angle-IV between mep.ptm and s-ba axis	^ETA-IV	Control	115,78	6,55
			rAOM	116,55	9,20
^mep.pt/s.n	ET angle-V between mep.ptm and s.n axis	^ETA-V	Control	160,04	5,01
			rAOM	163,15	3,49
^ba.s.ptm	Nasopharyngeal angle-1	^NA1	Control	109,53	4,52
			rAOM	110,64	7,01
^ba.s.pns	Nasopharyngeal angle-2	^NA2	Control	108,05	4,51
			rAOM	109,61	7,73
^sos.ptm.sep	Sphenoidal angle	^SA	Control	66,74	8,11
			rAOM	68,93	7,30

Figure 1. Landmarks and Linear Variables Measured on the Lateral Cephalograms (Continuous, dashed and dotted lines represent CB, NP-posterior face and SB variables, respectively. The ETL and vTVPL were marked with double bar.)

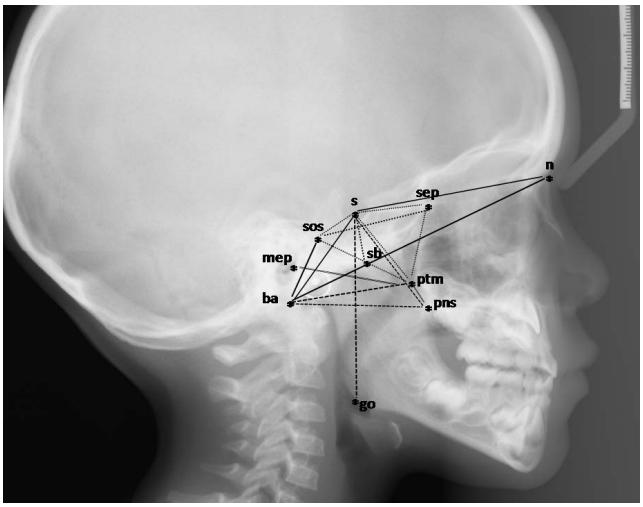
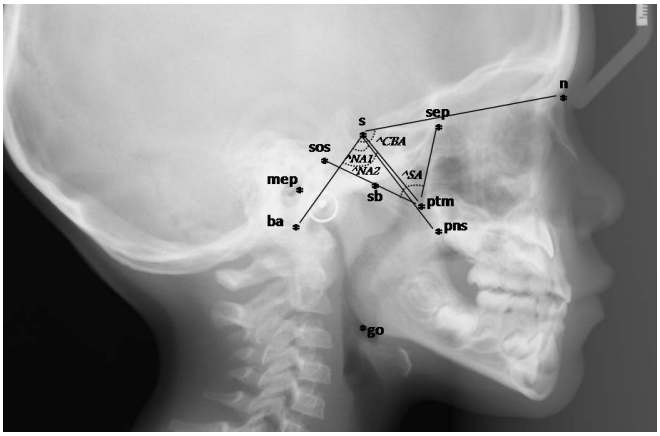


Figure 2. Landmarks and Angular Variables Measured on the Lateral Cephalograms (a) The angles related with the cranial base, sphenoid bone and nasopharynx. (b) the angles related with the Eustachian tube and peritubal muscles.

a



b

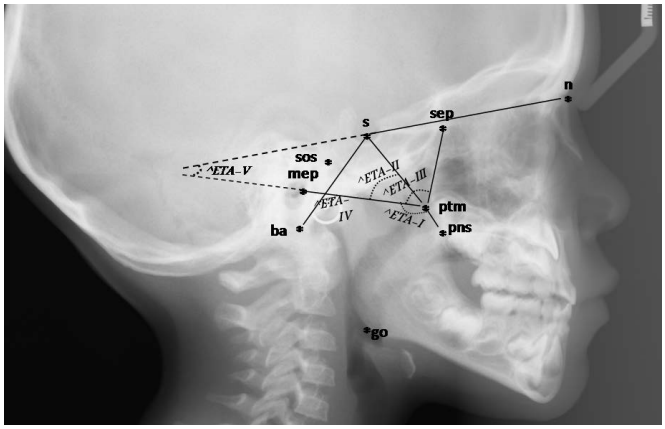
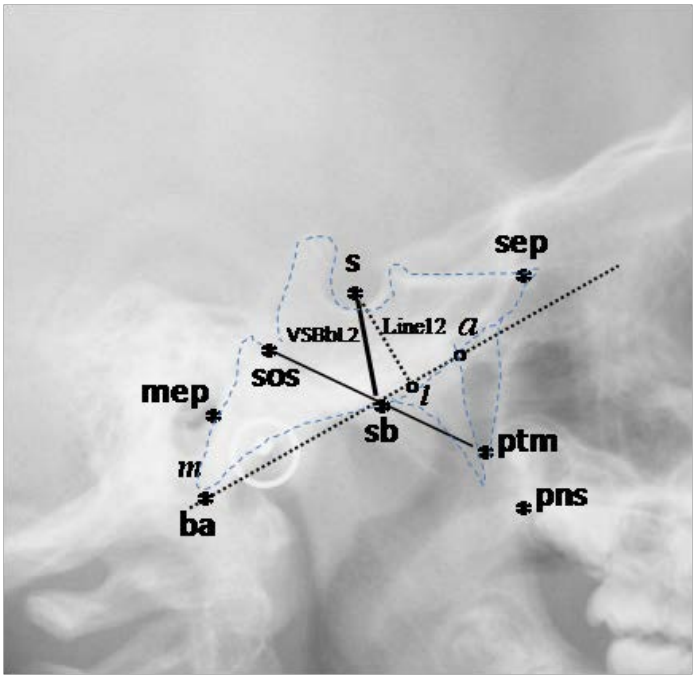


Figure 3. VSBbL2 (dark line between s and sb) which was found to be different in this study.
'Line12' (dark dotted line between s and *l*, which is perpendicular to the tangent line (dotted) between *m* and *a* points; *a, l and m* points and the dotted lines between them were drawn as described by Maw et al (5,7)).
(The spheno-occipital bone, pterygoids and posterior maxillary wall were outlined by dashed line.)



Pressure Chamber Assessment of Eustachian Tube Function Using Tympanometry and Sonometry

J. Douglas Swarts, PhD[1], Cuneyt Alper, MD[1], Juliane Banks[1], Ellen Mandel, MD[1], Richard Villardo, MD[1], William Doyle, PhD[2]
[1]Pediatric Otolaryngology, Children' s Hospital of Pittsburgh of UPMC, Pittsburgh, PA, [2]Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA

Objectives
To develop Eustachian tube (ET) testing protocols for subjects with intact tympanic membranes (TM) using a pressure chamber (PCh) as the source of ambient pressure differences. Compare the results of this testing modality to those derived from the forced response test (FRT) following unilateral myringotomy.

Methods
Twenty adults (19 to 48 years) without a recent OM history (5 had OM in childhood) were tested twice in a PCh to evaluate ET function in the context of an intact TM. For each experimental session a subject entered the PCh in which they were exposed to a series of pressure changes beginning at ambient and progressing through the sequence: -500, ambient, +400, +200, ambient -100 and-200 mmH2O and returning to ambient. At each pressure level a test sequence consisting of tympanomety, sonometry (2 measurements with sound probe in alternating nares) and tympanometry was performed. ET opening pressure was ascertained from the initial pressure excursion to -500 mmH2O. From each test sequence, data reflecting middle ear pressure (MEP), before and after sonometry, and the sonometric peak sound pressure and duration were available.

Results
At each session the MEP of the right and left sides were correlated ($r^2 = 0.5$), however this correlation decreased between sessions. Sonometry showed similar relationships. MEP changes suggested that 62% ± 19% of the pressure equilibration attempts in this study were successful. The value for sonometry was less (50% ± 20%). Comparisons of these results to our published data for the FRT of these subjects will be presented.

Conclusions
The PCh testing protocol was developed to evaluate ET function in subjects with intact tympanic membranes. While we can successfully assesses the passive and active ET function, this test protocol does not provide information as detailed and robust as the more invasive tests such as the FRT. Supported in part by grant DC007667 from the NIH.

Middle Ear Pressure Regulation – Complementary Action of the Mastoid and Eustachian Tube

Michael Gaihede, MD[1], Henrik Jacobsen, MD[1], Kjell Tveterås, MD[1], **Joris JJ Drickx, PhD**[2]
[1]Department of ORL, H&N Surgery, Aalborg Hospital, Aarhus University Hospital, Aalborg, [2]Laboratory of Biomedical Physics, University of Antwerp, Antwerp

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Introduction
In normal ears the middle ear (ME) pressure is maintained close to ambient pressure, so that the pressure difference across the tympanic membrane (TM) is close to zero. However, negative MEP is frequently found in diseased ears and considereda highly significant factor inME pathophysiology. The most frequent condition related to negative ME pressure isotitis media with effusion, whichaffects80 % of children before the age of 4 years (1).Otitis media with effusionis often treated by insertion of ventilation tubes into the TM, and in some countries 28 % of children are currently being treated with tubes before the age of 7 years (2). Thus, basic studies on ME pressure regulation is important to understand underpressure encountered in diseased ears.

The overall physiological regulation of ME pressure has been suggested to be based on a neural feedback control similar to respiratory control and related to similar centres in the nucleus of the solitary tract of the brain stem (3). This hypothesis has not gained much attention in research, but a range of sparse reports have pointed to the existence of such neural control (4). In the context of this idea, physiological studies on ME regulation demand an intact TM, because mechano-receptors in the TM hypotheticallyconstitute the afferent neural activity conveying information about ME pressure to the brain stem (3,4). Currently, clinical studies with direct measurements of ME pressure with an intact TM are very few; moreover, none of these have monitored changes in pressure over time. We have previously reported a new method for clinical studies of ME pressure via a catheter inserted into the mastoid of normal human subjects (5); here direct measurements of the counter-regulation of pressure in

response to experimental pressure changes is reported to illustrate the combined actions of the Eustachian tube (ET) and the mastoid (6).

Methods and Materials

Subjects, procedure, and instrument. Twelveadult subjects were recruited among patients referred to our department for parotidectomy. All patients were normal from an otological point of view (normal pure tone audiometry, tympanometry, otomicroscopy, andno history of previous middle ear disorders). The mastoid tip was exposed as a part of the routine procedure, and a small hole was drilled through the compact bone into the mastoid cells. Subsequently a catheter was inserted into the mastoid, connected to a transducer, and the wound was closed. The transducer was connected to a sampling unit which monitored the ME pressure at 10 Hz at an accuracy of ±1 Pa (range ±4 kPa). The study was approved our Ethical Committee (2005/50)(6).

Experiments. The day after surgery, a series of experiments were performed, where volume changes in the range of ±50, 100, and 200 µl were introduced via metal three-way stop-cock and a 500 µl gas syringe. The corresponding changes in ME pressure varied accordingly, and the subsequent counter-regulation of pressure was monitored over small time frames of 10 min. Thus, in each subject a series of six experiments was performed (6).

Results

From the group of 12 subjects, three subjects were excluded due to technical problems (leakage of fluid into the catheter, sclerotic mastoid). From the remaining group two distinct patterns of counter-regulations were found in response to the experimental pressure changes: 1) fast step-wise pressure responses against 0 Pa, and 2) slow gradual pressure responses in both positive and negative directions(6). These patterns of pressure responses showed large individual variation, but overall larger pressure deviations resulted in more step-wise responses, whereas smaller pressure deviations resulted in more gradual responses. Individual preferences also seemed to characterize each subject, so that only four subjects showed predominantly ET openings (≥10 openings during the total set of six experiments), while theother five subjects showed predominantly gradual responses with only few ET openings.

The distinct patterns of counter-regulation have been illustrated in Figs. 1-3. Fig. 1 illustrates an experimental underpressure and the subsequent pressure changes including both more step-wise events as well as gradual counter-regulation; this means that both patterns were found in the same subjects. This step-wise regulation could be related toET openings by observing the concomitant swallowing; since this pattern would be expected, we focus on the gradual responses. Fig. 2 illustrates twoexperimental overpressures and their subsequent pressure changes; these are exclusively gradual responses in the opposite (negative) direction. The lower curve even crosses 0 Pa progressing into negative pressures; this demonstrates that no leakage was present. Fig. 3 illustrates three experimental underpressures, where the intermediate curve showed a stable course with pressure around – 800 Pa, while the remaining two curves showed both a negative and positive slope merging towards the intermediate curve. This means that both directions of pressure changes could be found in the same subject and pressure area (underpressure). The gradual responses appeared linear and their slopes described the rates of pressure changes; the slopes were determined by regression analysis and appear in the legends (range = -63 to +25 Pa/min).

Discussion

The current approach has demonstrated physiological ME pressure changes in response to experimentally induced under- and overpressures. Two distinct patterns of counter-regulation were demonstrated, which were characterized by 1) fast step-wise changes against 0 Pa explained by ET openings, and 2) slow gradual changes which must be explained by the mastoid. The individual patterns varied considerably, but some preference for either pattern seemed to characterize the individual subject. It should be noted that the pressure changes created by the volumetric changes were influenced by the individual compliance of the TM as well as the volume of the mastoid including the ME cavity.

ME gas exchange is an important factor in ME pressure regulation which could explain the slow gradual changes, and the slope of the curves would express the net effect of the gas exchange. However, in normal awake subjects this should be limited to gas absorption exhibiting a negative direction of pressure change (7). Thus, since we found gradual changes in both negative and positive directions, gas exchange cannot explain our findings. An alternative hypothesis for pressure regulation has been suggested, where volumetric changes in the thickness of the ME mucosa causes changes in the pressure of the enclosed air volume (mastoid and ME cavity) (8). Such volumetric changes may be effected by changes in the congestion of the mucosa; this mechanism is suggested to be responsible for ME pressure regulation in diving mammals, where multiple submucosal cavernous venous structures are found in the ME cavity (9). It follows that this mechanism will be able to work in both negative and positive directions similar to the responses illustrated in Figs. 1-3.

Based on the average surface area and volume of the human mastoid (10), it has been calculated that a change in mucosal thickness of only 6 µm will result in a pressure change of 1 kPa, so that it would seem a rather effective mechanism (8). The mastoid mucosa has a rich capillary network, which may facilitate both changes in its congestion, but also gas exchange (11).

Moreover, the mastoid contains abundant air filled cells with mucosal lining which enhances its surface area significantly compared to the ME cavity itself (10). This implies that the mastoids contribution in ME pressure regulation whether by mucosal volumetric changes or gas exchange seems quantitatively to be more important than the ME cavity (10). Hence, the gradual responses must be attributed to the mastoid.

The congestion of the mucosa may be controlled by vaso-motor innervation of the mucosal blood vessels, and hence, any reflex response to environmental or experimental pressure changes may be an active mechanism (3). However, the pressure gradient between the mucosa blood and the air phase may also drive the congestion passively (9)

Whereas the presented method showed results with high accuracy and temporal resolution, it also has inherited limitations. We were only able to investigate normal adult subjects, since in diseased ears the occurrence of a sclerotic mastoid will pose a risk for injury to the facial nerve. Moreover, during the experiments the day after surgery, the insertion of the 3-way stop-cock and the gas syringe caused a small period, where admixture of atmospheric air was possible as well as in theory also the surface of the catheter may be a source of admixture. Such admixture of atmospheric air would disturb the distribution of the partial gas pressures inside the mastoid and the ME cavity, and possibly affect the pressure changes observed in Figs. 1-3 (7). However, the counter-regulation always seemed systematic against the induced pressure changes, which was not likely due to these sources of admixture.

In summary, we have demonstrated two distinct patterns of counter-regulation of experimental ME under- and overpressure. These patterns implied that both the ET openings as well as mastoid related factors act together in maintaining the pressure equilibrium of the mastoid and the ME cavity. The mastoid factors include gas exchange, but additionally the possible role of mucosal congestion must be considered to explain our findings. Future experiments need to focus further on the role of the mastoid in order to improve our understanding of the overall pressure regulation in healthy and diseased ears.

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Fig. 1. Experimental underpressure (volume displacement -100 µl; peak pressure = -496 Pa). The 1st ET opening (ETO) is found after 2 min and more times at the last part of the curve reaching 0 Pa at 10 min. A longer period is interposed with gradual pressure increase from 2 to 7.5 min with a slope of 25 Pa/min (inserted straight line) (Subject No. 2).

Fig. 2. Experimental overpressures (volume displacements +50 and +100 µl; peak pressures = 378 and 782 Pa). For both curves a gradual decreasing pressure is seen with slopes of -63 and -57 Pa/min, respectively (inserted straight lines). For the +50 µl displacement curve (lower) the pressure gradually decreases and crosses 0 Pa reaching negative pressures after around 5 min (Subject No. 5).

Fig. 3. Experimental underpressures (volume displacement -50, -100, and -200 µl; peak pressures = -378, -820, -1566 Pa, respectively. The rates of pressure change are 30, 0, and 12 Pa/min, respectively (inserted straight lines). This means that small a negative pressure results in further decreasing pressure, while moderate negative pressure remains stable around -820, and finally, high negative pressure results in increasing pressure (Subject No. 5).

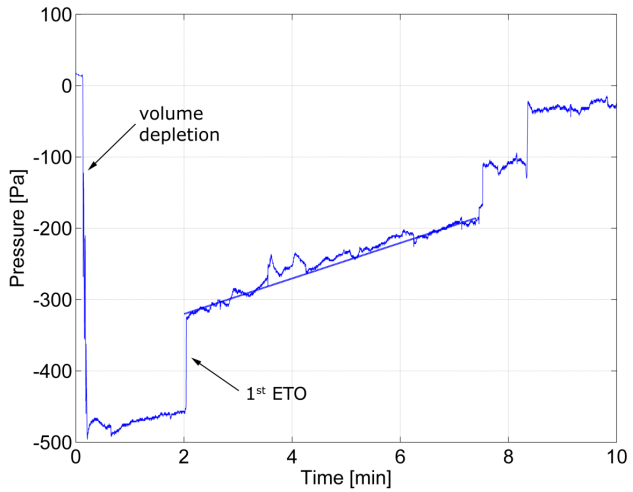

Fig. 1. Gaihede et al. MEP regulation

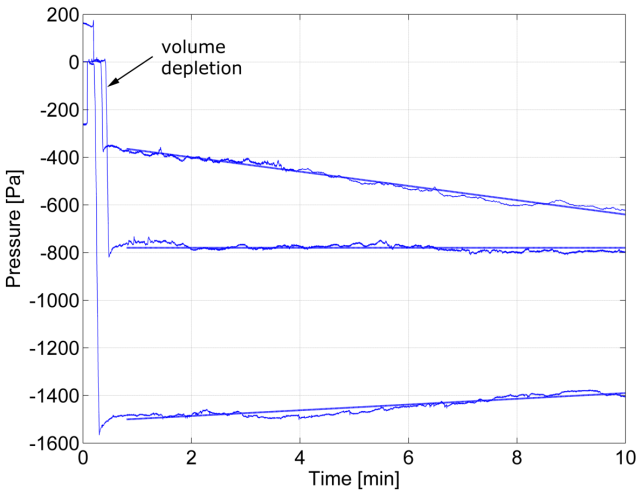

Fig. 3. Gaihede et al. MEP regulation

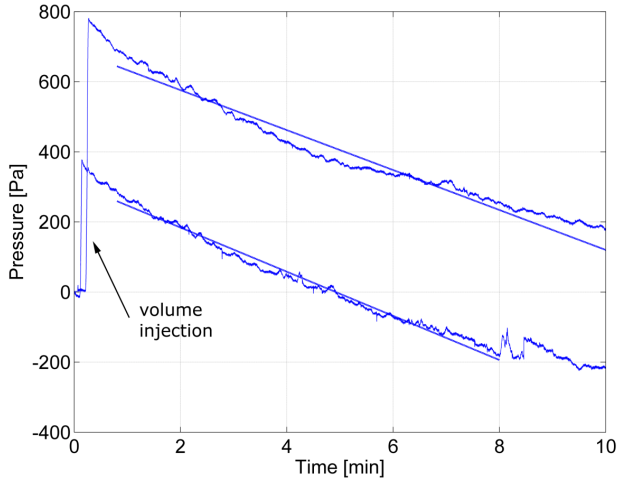

Fig. 2. Gaihede et al. MEP regulation

Eustachian Tube Function Testing as a Predictor of Persistent Middle Ear Disease After Tympanostomy Tubes

Ellen Mandel, MD, Margaretha Casselbrant, MD, PhD**,** J. Douglas Swarts, PhD**,** James Seroky**,** Beverly Richert, PhD, NP/PA**,** William Doyle, PhD
Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA

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Introduction

This is a preliminary look at some of the data from an ongoing study of Eustachian tube function and chronic otitis media with effusion (OME) in children.The purpose of this analysis is to determine whether Eustachian tube function testing, specifically the Forced Response Test (FRT), performed within 6 weeks after tympanostomy tube insertion for chronic OME, predicts recurrence of chronic OME after the tubes become permanently nonfunctional or extruded.

Methods

The study was approved by the University of Pittsburgh Institutional Review Board. Children were entered who were 3-6 years of age, were within 6 weeks of undergoing tympanostomy tube insertion for chronic OME, and had effusion at surgery or the visit before surgery. Chronic OME was defined as ≥3 consecutive months of bilateral OME, or ≥6 consecutive months of unilateral OME, or ≥3 episodes of OME in the previous year, or recurrence of OME after previous tubes for OME.Children with cleft palate or other syndromes predisposing to otitis media were excluded, as were children who had a history of only acute otitis media, a history of complications of otitis or its treatment, or who were unable to cooperate for testing.

ETF testing was performed within 6 weeks of tympanostomy tube insertion and then approximately every 3 months until the tube was permanently non-functional or extruded. Once this occurred, the children were examined monthly for up to 1 year, either in the clinic or at home visits, with pneumatic otoscopy and tympanometry to determine middle-ear status."Failure" was defined as 4 consecutive visits with unilateral otitis media (OM) or 3 consecutive visits with bilateral OM. "Success" was making it through this close follow-up for 1 year without meeting "failure" criteria. If one or both ears met failure criteria, the child was discharged from the study and referred back to their original ENT surgeon.

Results

Data from the first 26 subjects who completed the protocol were analyzed. There were 12 subjects (15 ears) who were considered "failures". Three subjects were failures in both ears and9 subjects were failures in one ear only.Of the 9 non-failure ears in these subjects, 4 were observed for <12 months and had not yet met failure criteria, 4 ears still had functional tubes, and 1 child had 1 ear that made it through 12 months without being a failure.

There were 14 children (27 ears)who were observed for 12 months without meeting failure criteria in either ear. One child still had a patent tube in 1 ear after 33 months but their other ear was observed for 12 months without chronic OME.

Outcome was examined by age at entry, race, sex, and number of sets of tympanostomy tubes prior to the set at entry (Table 1). All 3 year olds, by the time the tubes came out and they were followed, were "successes", not having chronic OME again. The older children did not fare as well.Interestingly, most of the African-American children met failure criteria, while more of the Caucasian populationwas in the success column. Sex was fairly evenly split between fail and success. Eight of the 26 (30.8%) subjects had not had any tubes prior to the set at entry.

The distribution of mean time (months) until the tube became non-functional is shown in Table 2. It appears that the longer the tube remained functional, the better the ear did without a tube: 22 of 27 (81.5%) ears in the success column had functional tubes for >1 year.

The results of the FRT are shown in Table 3. There were no statistically significant differences in the passive measures of Eustachian tube function (opening pressure, closing pressure, and passive resistance). Active resistance was also not different between the groups.

Presence of constriction or dilation on swallowing was available for 13 ears that were failures and 22 ears that did not have recurrence of chronic OME. Of the ears that constricted, about half went on to have chronic OME and half did not. Of those that dilated on swallowing, 83% did not have recurrence of chronic OME in the 12 months after the tube became non-functional;however, this is not statistically significant (p=0.07).

Conclusion

The forced response test measures of passive function (opening pressure, closing pressure, passive resistance) were not different between ears that had recurrence of chronic OME and those that did not. Although not statistically significant, ears that dilated

during swallowing were less likely to have recurrence of chronic OME. As more children complete the study protocol, we will see if these initial trends remain.

Supported in part by NIH grant DC005832

TABLE 1. OUTCOME BY SUBJECT CHARACTERISTICS

CHARACTERISTIC	FAIL (N=12)	SUCCESS (N=14)
Age at entry		
3yrs	0	4
4 yrs	3	3
5 yrs	4	1
6 yrs	5	6
Race		
Caucasian	4	12
African-American	8	2
Sex		
Male	9	8
Female	3	4
Number of previous tympanostomy tubes		
0	3	5
1	6	8
2	2	0
3	1	1

TABLE 2. TIME UNTIL TUBE EXTRUDED OR NON-FUNCTIONAL

TIME (MONTHS)	FAIL (N=15)	SUCCESS (N=27)
Mean	10±3.8	16±6.9
0-3	0	0
4-6	2	2
7-12	7	3
13-24	6	18
25+	0	4

TABLE 3. FORCED RESPONSE TEST

	FAIL	SUCCESS
Opening pressure	299 ± 156 (n=15)	344 ± 122 (n=24)
Closing pressure	110 ± 57 (n=13)	105 ± 96 (n=24)
Passive resistance	8.3 ± 5.3 (n=15)	8.5 ± 6.3 (n=23)
Active resistance	5.2 ± 6.1 (n=10)	4.1 ± 3.4 (n=20)
Constriction	11	12
Dilation	2	10

NTHi Induction of Cxcl2 and Middle Ear Mucosal Metaplasia in Mice

Diego A. Preciado, MD, PhD
Fellowship Program Director, Division of Pediatric Otolaryngology, Children's National Medical Center, Washington, DC

Introduction:
Otitis media (OM) is a ubiquitous condition of early childhood accounting for 16 million physician office visits a year at a national cost of $3 billion to $6 billion. Acute OM (AOM) is defined as acute onset of middle ear effusion along with signs and symptoms of middle ear inflammation and infection. Chronic OM (COM)is postulated toresult as a response to the middle ear infection, and is characterized by secretory epithelial metaplasia and persistence of middle ear effusion, most frequently mucoid. Non-typable *Hemophilus influenza* (NTHi), the most common AOM pathogen, is knownto activate inflammation and mucin expression*in vitro* and in animal models of OM. The goals of this study were to: a) examine a murine model of COM upon bacterial NTHi challenge, and b) determine the expression profile of middle ear epithelium over time after middle ear bacterial lysate inoculation. Methods: Weekly repeated inoculation of Balb/c mice middle ears with300 ug/ml of NTHi bacterial lysate over 4 weeks was performed. Middle ear histopathology with PAS staining then done. Expression microarray analysis of extracted middle ear RNA was performed to detect the transcriptome of middle ear tissue in response to NTHi exposure over time. Real time quantitative PCR was done to validate microarray findings in the independent animal samples and in a cultured murine middle ear epithelial cell (mMEEC) line.Results: Histopathologic analyses revealed chronic middle ear mucosal metaplasia after NTHi exposure. Microarray data was analyzed with the Plier algorithm. ANOVA analyses demonstrated that 3578 genes changed significantly with a p value of < 0.01. When exploring inflammatory response genes that changed significantly, *Cxcl-2* was found to have the largest fold-change with significantly increased expression at 1 and 7 days with NTHi injection compared to either saline or no-injection ($p<0.001$). Validation real-time PCR in the same set and in independent mouse middle ear tissues, revealed significantly increased relative mRNA levels for *Cxcl2*, similar to that seen on the microarray. Parallel to the animal model, NTHi was found directly significantly up-regulate the transcription of Cxcl-2 in mMEEC in a time and dose dependent manner ($p<0.05$).Conclusions: Middle ear NTHi challenge in mice leads to chronic epithelial mucosal metaplasia and over-expression of inflammatory mediators, most notably Cxcl-2. This finding is parallel to NTHi mediated pulmonary mucosal metaplasia where Cxcl-2 has been identified as an important inflammatory mediator.

Materials and Methods
Histopathological evaluation of mice middle ear upon NTHi inoculation: Briefly, NTHi clinical strain 12 was obtained from the lab of Dr. Xin-Xing Gu (NIDCD, Bethesda, MD). Bacteria were grown on chocolate agar at 37ºC in 5% CO2 overnight and inoculated in brain heart infusion (BHI) broth supplemented with 3.5 mg of nicotinamide adenine dinucleotide per ml. After overnight incubation, bacteria were subcultured into 5 ml of fresh BHI upon reaching log phase growth NTHi were then washed and suspended in phosphate-buffered saline (PBS) followed by sonification for lysis. Weekly repeated transtympanic inoculation of Balb/c mice middle ears with 300 ug/ml of NTHi bacterial lysate over 4 weeks was performed. Middle ear histopathology with PAS staining then done.

Expression array profiling upon acute middle ear NTHI inoculation: Total RNA was extracted from the middle ear of mice after 0, 1, and 7 days of saline or NTHi transtympanic inoculation. RNA extracted from both ears was pooled together for each animal. Three animals were done for each time point (biologic triplicates). Microarray analyses were performed in the Microarray Core at Children's National according to established protocols[1]. Briefly, total RNA was isolated by three separate 50 µl middle ear washes with Trizol(Invitrogen, Carlsbad, CA). After purification, quanitification, and purity/integrity control, 100 ng of total RNA was used to initiate the complementary DNA–complementary RNA (cDNA-cRNA) cycle. Biotin-labeled cRNA from each sample was fragmented and hybridized to Affymetrix Mouse 430_2 Arrays (Affymetrix, Santa Clara, CA) for 16 hours, followed by standard washing-scanning protocols on the Affymetrix Fluidics Station 400 (Affymetrix Inc) and incubation with phycoerythrin-streptavidin to detect bound cRNA. The signal intensity was amplified by means of biotin-labeled antistreptavidin antibody. Fluorescent images were captured by means of a gene array scanner (Hewlett-Packard G2500A; Palo Alto, CA). All data were analyzed by means of Affymetrix Microarray Analysis Software version 5.0 (MAS 5.0), dChip, and Plier algorithms. Only probe sets that were statistically significant with 1-way analysis of variance (ANOVA) and a $p< 0.01$ were used.

*NTHi effects on middle ear cell line Cxcl2 expression:*Time course experiments to determine the effects of NTHi lysate exposure on Cxcl2 gene expression in an immortalized mouse middle epithelial cell line (mMEEC) were perfromed. This cell line was immortalized with a temperature sensitive SV40 virus allowing for cell differentiation at 37˚C, and was provided to us by Dr. Jizhen Lin (University of Minnesota, Minneapolis, MN)[2]. RNA was extracted from mMEEC cells exposed to NTHi lysates at (0, 150, 300 µg/ml) or vehicle for 2, 8, and 24 hours using TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription reaction was then performed by using 1 µg of total RNA from each sample and the SuperScript III reverse transcriptase enzyme (Invitrogen, Carlsbad, CA), as previously described[3]. Generated cDNA was used for PCR using specific pairs of primers as follows: Cxcl2 forward primer, 5'-CCCAGACAGAAGTCATAGCCAC-3'; Cxcl2 reverse primer, 5'-GCCTTGCCTTTGTTCAGTATC-3'. β-actin was used as an internal control and primers for mouse β-actin were obtained from GeneLink (Hawthorne, NY). Real-time

RT-PCR was performed on the generated cDNA products in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) as described previously[3]. Relative quantification of Tnf-α mRNA in control and experimental samples was obtained using the ΔΔCt method.

Results

1. Murine OM model shows chronic mucosal metaplasia.
2. Identification of differentially expressed gene products in mouse middle ear epithelium exposed to NTHi.
3. Cxcl2 mRNA expression is increased in vitro in mouse MEEC exposed to NTHi.

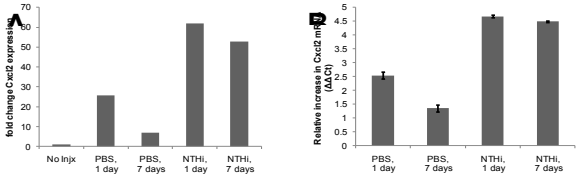

Figure 2. A. Fold change in Cxcl2 transcript expression from microarray data results (Table 1)

Table 1. Fold changes of top 10 inflammatory genes significantly changed with middle ear injection (p<0.01).

	NI	PBS, 1 day	PBS, 7 days	NTHi, 1 day	NTHi, 7 days	Common	Genbank
1449984_at	1	25.888079	6.951383	61.88025	52.705723	Cxcl2	NM_009140
1450678_at	1	2.4282444	2.1476412	3.6850636	10.557129	Itgb2	NM_008404
1437726_x_at	1	4.0626206	2.4800029	2.471357	9.408059		BB111335
1448620_at	1	2.1986768	2.1814113	3.4431455	9.255703	Fcgr2b	NM_010188
1434366_x_at	1	3.7023203	2.2580907	2.5791407	8.758987	C1qb	AW227993
1451713_a_at	1	6.699404	1.6755381	5.5819488	8.396786	Fcer2a	AY069981
1418747_at	1	1.6761173	1.6682166	2.6820774	6.014558	Sfpi1	NM_011355
1442233_at	1	1.8414448	1.9775968	4.2426195	5.504724	Fyb	BE853428
1422903_at	1	1.348012	1.3415849	1.9282683	4.926996	Ly86	NM_010745
1422260_x_at	1	0.9166759	0.6660496	3.4394472	4.3354917	Ccr5	X94151

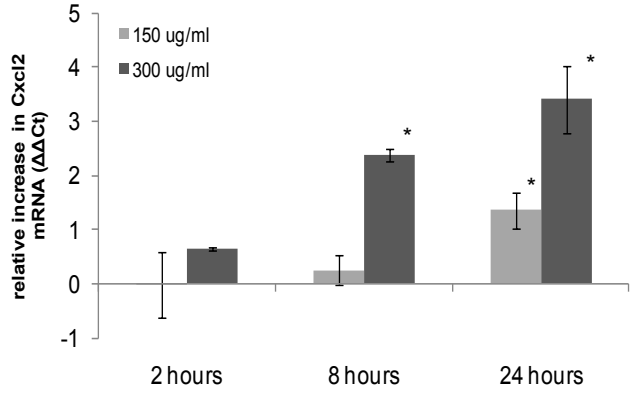

Figure 3.NTHi increases Cxcl2 expression in mMEEC. Cells were stimulated with 0, 150, or 300 µg/ml of NTHi lysates for the shown time points prior to RNA extraction and cDNA generation. Real time RT-PCR was performed to determine the relative amounts of Cxcl2 mRNA utilizing the ΔΔCt method with β-actin as an internal reference gene. Data show relative time and dose dependent increases in Cxcl2 transcript levels with NTHi lysate exposure in mMEEC. *p<0.05 compared to 0 ug/ml NTHi).

Conclusions

1. Repeated NTHi lysate exposure induces middle ear mucosal thickening in mice.
2. NTHi lysates induce significant mouse middle ear over-expression of CxCl-2 *in vivo* and *in vitro.*

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Pathogensis

Phylogenetic Relatedness and Diversity of Non-Typeable *Haemophilus influenzae* in the Nasopharynx and Middle Ear Fluid of Children with Acute Otitis Media

Ravinder Kaur, PhD[1], Arthur Chang[1], Qingfu Xu, PhD[1], Janet Casey, MD[2], Michael Pichichero, MD[1]
[1]Research Institute, Rochester General Hospital, Rochester, New York, [2]Pediatrics, Legacy Pediatrics, Rochester, New York

Introduction
*NTHi is c*ommonly carried in the nasopharynx and a major cause of
acute otitis media (AOM) in children. NT*Hi* can also cause sinusitis and AECB and pneumonia (in developing countries). There is need to characterize NTHi isolates if we want to understand disease transmission, track the spread of virulent and/or antibiotic-resistant strains, monitor the impact of vaccines on bacterial populations and probe the nature of virulence population and evolutionary biology of bacterial species.

Method
Multi-locus sequence typing (MLST) method was used to characterize NTHi strains.
MLST is a nucleotide sequence based approach for the unambiguous characterization of bacteria isolates via the internet.
We characterize NTHi isolates to answer the following:
Genetic relatedness among different strains of *NTHi* in 6-30 month old
children in the Rochester community 2) *NTHi* colonization at different time points with the same strain or different strains 3) Genetic relatedness among colonizing strains vs. strains that progress to AOM disease4) Genetic relatedness of *NTHi* strains distinguished based on β-lactamase testing 5)Characterization of *NTHi* strains present in the middle ear fluid vs. nasopharynx of children with AOM.

Result
NTHi were detected in 73 (43.5%) of 168 children at least once. *NTHi* colonization was observed more than once in 33 (45.2%) of 168 children. A total of 165 *NTHi* isolates were characterized by MLST. 70 different MLST sequence types were found among the *NTHi* isolated; 29 were novel.

Work supported by US NIH NIDCD R01DC08671

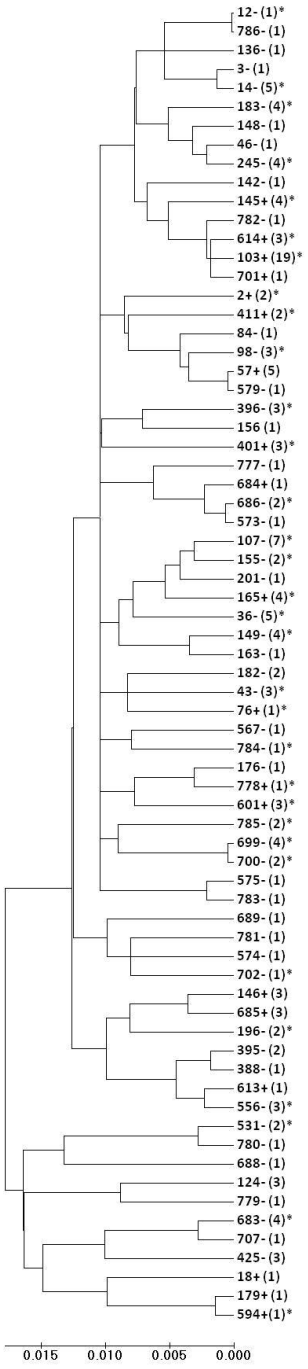

Figure 1. Phylogenetic tree from concatenated sequences of MLST of 70 *NTHi* strains isolated from Rochester NY children. The linkage distance is shown at the bottom of the dendrogram and is in the units of the number of base substitutions per site. The number of isolates cultured for each sequence type is shown in parentheses. The strains that were found in MEF of children with AOM are shown with *. β-lactamase results are also shown for each strain with + and – symbol after the strain number.

eBURST group from H.influenzae database	# of different ST types present in the eBURST group (% age)	# of isolates present in the eBURST group (% age)	ST number of *NTHi* isolates, isolated from chidren during NP colonization and Acute otitis media
Group 2	13 (18.5%)	46 (27.9)	136, 3, 14, 183, 148, 46, 245, 145, 782, 614, 103, 701, 777
Group 3	6 (8.6%)	9 (5.5%)	684, 686, 573, 176, 778, 601
Group 4	1(1.4%)	1 (0.6%)	18
Group 5	1(1.4%)	3 (1.8%)	124
Group 7	3 (4.3%)	10 (6.1%)	107, 155, 201
Group 8	2 (2.9%)	5 (3.0%)	149, 163
Group 9	2 (2.9%)	6 (3.6%)	396, 401
Group 10	1(1.4%)	2 (1.2%)	411
Group 12	2 (2.9%)	5 (3.0%)	683, 707
Group 13	1(1.4%)	1 (0.6%)	84
Group 15	1(1.4%)	1 (0.6%)	574
Group 16	1(1.4%)	1 (0.6%)	784
Group 18	4 (5.7%)	7 (4.2%)	395. 388, 613, 556
Group 19	2 (2.9%)	3 (1.8%)	531, 780
Group 20	3 (4.3%)	9 (5.5%)	98, 57, 579
Group 21	2 (2.9%)	2 (1.2%)	12, 786
Group 22	1(1.4%)	3 (1.8%)	425
Group 23	1(1.4%)	1 (0.6%)	156
Group 26	1(1.4%)	2 (1.2%)	785
Groups contain less than 5 ST	14 (20%)	29 (17.6%)	142, 165, 182, 43, 567, 699, 700, 575, 783, 146, 685, 196, 179, 594
Singletones	8 (11.4%)	13 (7.9%)	2, 36, 76, 689, 781, 702, 688, 779

Table 1: eBURST group analysis of the *H. influenzae* database showing where the *NTHi* ST belongs from this study. First column shows the eBURST group and the last column shows the ST found in that group in our study. The number of different STs and # of isolates found in each group are also shown. Note: Percentages in the parenthesis shown are from total number of 70 ST and total number of 165 *NTHi* isolates from this study population.

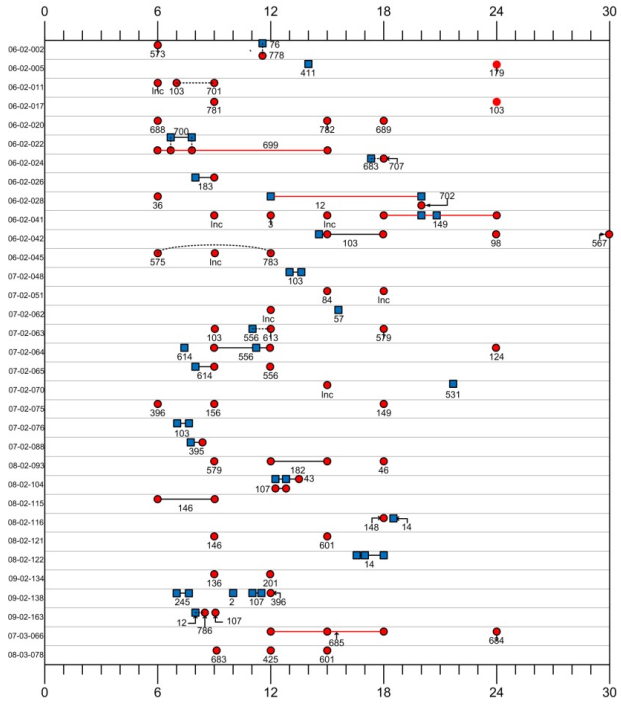

Figure 2: Comparison of MLST sequence types (ST) obtained from *NTHi* isolates at different time points from the same subjects. X axis represent the child's age in months and y axis indicates the child's identification number.

Note: Red circle (●) denotes when the NTHi strain was colonizing the NP and blue Square (■) denotes when the *NTHi* strain found in MEF. When two isolates of *NTHi* are connected by a line in a child they were same ST and connected by a dashed line if they were different STs, but the same clone (Single locus variant).

Inc means it was not possible to obtain a sequence for all seven genes for that isolate and therefore the sequence type was not obtained but partial analysis clearly allowed us to determine that strain was different from the other strains in that particular child.

Data shows that In ~85% of the nasopharyngeal (NP) colonizing *NTHi* strains cleared from the host within 3 months. 18.1 % of the children had prolonged colonization with different strains of *NTHi.* In ~36% of the AOM cases, *NTHi* did not clear from the NP of the child after antibiotic treatment.

WhenNTHi isolates cultured from MEF vs. NP samples of 37 children with AOM were compared, In 84% of the children the same MLST-STs was found in the MEF that was in the NP sample. In 8% of the cases a different MLST-STs, but the same clone was found in NP vs. MEF and 8 % of the children a different clone was detected in the MEF compared to the NP.

Patient #	Date of visits	Age in months	Allele number of house-keeping genes							ST
			adk	atpG	frd B	fucK	mdh	pgi	recA	
07-02-063 Triplet 1	11-20-2007	6.5	ND	51	16	ND	15	2	83	-
	2-13-2008	9.5	1	1	1	14	9	14	13	103
	5-13-2008	12.5	14	5	16	48	29	2	31	613
07-02-064 Triplet 2	1-18-2008	8.5	1	1	1	8	9	14	13	614
	2-13-2008	9.5	14	51	16	48	29	2	31	556
	4-24-2008	12	14	51	16	48	29	2	31	556
07-02-065 Triplet 3	1-29-2008	9	1	1	1	8	9	14	13	614
	2-13-2008	9.5	1	1	1	8	9	14	13	614
	5-13-2008	12.5	14	51	16	48	29	2	31	556
06-02-042 Sibling	1-7-2008	18.5	1	1	1	14	9	14	13	103
	1-23-2008	19	1	1	1	14	9	14	13	103
	4-23-2008	23	14	7	13	15	17	13	1	98

Table 2: Comparison of *NTHi* MLST sequence types (ST) obtained from one familythat included triplets and one of adoted sibling. Results are shown from different visits demonstrating the transmission of *NTHi* isolates.

Note: ND indicates that it was not possible to obtain a sequence for this locus from this isolate and therefore the sequence type was not obtained, indicated by – sign.

Conclusion

Understanding the dynamics of *NTHi* colonization in NP of children is critical if strategies to prevent infection or colonization are to be developed.*NTHi* isolates are very diverse, and their population structure is characterized by very frequent recombinations.

ERK/AP-1 Signaling Pathway Is Involved in NTHI-Induced Up-Regulation of Cxcl2 in SLFs

Sejo Oh, PhD, Jeong-Im Woo, PhD**,** David J. Lim, MD**,** Sung K. Moon, MD, PhD
Division of Clinical and Translational Research, House Ear Institute, Los Angeles, CA

Introduction

Otitis media (OM), one of the most common pediatric infectious diseases, induces inner earinflammation resulting in sensorineural hearing loss. [1]OM-induced inner ear complications in children are clinically important since they could result in a delay in the development of language.[1]It is believed that bacterial molecules in the middle ear cavity enter the inner ear through the round window membrane.[2] Considering that spiral ligament fibrocytes (SLFs) are one of the abundant cell types in the cochlea and that they secrete cytokines and chemokines after proinflammatory stimuli,[3] we hypothesized that the SLFs are major responders to such signals.[4] Previously, we reported that SLFs recognize nontypeable *Haemophilus influenza*(NTHi) via TLR2-dependent NF-κB activation. [5] Recently, we demonstrated that SLFs release monocyte-attracting molecules in response to NTHi.5 [6]However, we poorly understand the molecular mechanism related to the cochlear infiltration of the polymorphonuclear leukocytes (PMNs). Among PMN-attracting chemokines, we showed that Cxcl2, also known as macrophage inflammatory protein-2, is highly up-regulated in the SLFs in response to NTHi[4]. Cxcl2 is known to be associated with inflammatory diseases such as arthritis, glomerulonephritis, and sepsis.[6] In this study, we aim to further determine if SLFs up-regulate PMNs-attracting molecules suchas Cxcl2 in response to NTHi and investigate signaling pathways involved.[7-9]

Material and Methods

Live NTHi was transtympanically inoculated to C57BL/6 mice and histological analysis wasconducted after temporal bones were harvested. After the rat SLF cell line was exposed to the lysate of NTHi with and without pretreatment of the chemical inhibitors (ERK inhibitor, AP-1 inhibitor) and dominant negative constructs (ERK1/2, TAM67), the conditioned medium and total RNA were collected. After reverse transcription, real-time quantitative PCR (qPCR) was performed using the specific primers and probe to Cxcl2. Migration assay was performed using bone marrow cells from the Lys-EGFP mice[10]and the conditioned medium. Primary SLFs derived from TLR2-deficient and MyD88-deficient mice were exposed to the NTHi lysate and qPCR was performed. To determine NTHi-responsive elements in the promoter region of the Cxcl2, promoter analysis was performed using luciferaseexpressingconstructs with 5' flanking region of Cxcl2.To furtherdetermine transcription factors involved, chromatinimmunoprecipitation (ChIP) assays and transcriptional assays were performed.

Results

Transtympanic inoculation of live NTHi led to inner ear inflammation and induced Cxcl2 expression in the spiral ligament. Migration assay showed that NTHi-induced SLF-derived molecules attracted PMNs. qRT-PCR analysis showed that SLFs highly up-regulated Cxcl2 mRNA upon exposure to NTHi, which was inhibited by TLR2 and MyD88 deficiency. NTHi-induced Cxcl2 up-regulation was inhibitedby the ERK inhibitor and the AP-1 inhibitor, indicating ERK/AP-1-dependent signaling pathway is involved in the NTHi-induced up-regulation of Cxcl2 expression in the SLFs. Unexpectedly, NTHi-induced Cxcl2 up-regulation was notinhibited by the NF-κB inhibitors, suggesting the involvement of other transcription factors. Promoter analysisand ChIP assays showed that c-Jun binds to two AP-1 binding sites of the Cxcl2 promoter in response to NTHi, leading to Cxcl2 up-regulation in SLFs.

Conclusions

In this study, we showed SLFs release molecules attracting PMNs in response to NTHi. Among NTHi-induced SLF-derived molecule, we demonstrated that Cxcl2 is a major attractant of PMNs. We also found that MEK1-ERK signal pathway is required for NTHi-induced Cxcl2 up-regulationand c-Jun binding to the two AP-1 binding motif of Cxcl2 is involved in NTHi-induced Cxcl2 up-regulation. In summary, we suggest that NTHi-induced SLF-derived Cxcl2 contributes to inner ear inflammation through ERK/AP-1 pathway which mediated recruitment of PMNs. Our results may provide us with new therapeutic strategies for prevention of inner ear dysfunction secondary to chronic middle ear inflammation.

This work was supported in part by NIH grant DC5025, DC8696, and DC6276.

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Persistent Alternobaric Vertigo at Ground Level Caused by Chronic Closed-Nose Swallowing ("Toynbee Phenomenon")

Charles Bluestone, MD[1]**,** J. Douglas Swarts, PhD[1]**,** Joseph Furman, MD, PhD[2]**,** Robert Yellon, MD[2]
Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittburgh School of Medicine, Pittsburgh, PAPennsylvania, [2] Department of Otolaryngology, Eye and Ear Institute of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA.

Introduction
Lundgren was the first to describe alternobaric vertigo (AV) as a diving hazard and subsequently as also occurring in Swedish Air Force pilots.[1, 2] Classically AV occurs during ascent as the result of asymmetrical middle-ear pressures. The vertigo is usually transitory but can last for several minutes. However, we recently encountered an unusual case of AV occurring spontaneously at ground level that, to our knowledge, has not been previously reported.

Case History
A 15-year-old female with achondroplasia presented to our Emergency Department with acute onset of vertigo severe enough to cause falling which required admission. She had been vertiginous intermittently for several weeks, but the condition worsened and became persistent. She had had long-standing chronic nasal obstruction and had been receiving treatment for allergic rhinitis. Her past otologic history was significant in that she had bilateral myringotomies with placement of tympanostomy tubes on several occasions for recurrent acute otitis media and chronic otitis media with effusion. Three years prior to her admission for vertigo, she had insertion of long-term tympanostomy tubes.

The physical examination revealed that she had gross mid-face hypoplasia associated with mouth breathing. Nystagmus was not present. Examination of the ears revealed the bilateral long-term tympanostomy tubes were in the tympanic membranes. There was no middle-ear effusion visualized in either ear. Examination of the nose revealed moderately severe edema with grossly enlarged turbinates and serous drainage.

Audiometric testing revealed normal hearing bilaterally. Tympanometry showed a patent left tympanostomy tube but the right tube was obstructed with +200 mm H_2O middle-ear pressure. Eustachian tube function testing, which could only be performed on the left side with the patent tube, revealed abnormal function characterized by a semi-patulous Eustachian tube which constricted upon swallowing. Computed tomographic scans of the temporal bones and maxillofacial area showed dysplastic bones consistent with achondroplasia, with no evidence of inner ear (no superior semi-circular canal dehiscence), middle-ear or mastoid disease; the adenoids were enlarged and there was moderate to severe nasal obstruction due primarily to enlarged turbinates. Vestibular system testing revealed severely reduced caloric responses and mildly reduced rotational responses with evidence of an ongoing vestibular-ocular reflex asymmetry.

We made a diagnosis of AV which was the result of the right obstructed tympanostomy tube, chronic nasal obstruction, and the Toynbee phenomenon (i.e., closed-nose swallowing), which caused repeated insufflation of positive pressure into her right middle ear during swallowing, resulting in asymmetric middle-ear pressures and vertigo.

She was taken to the operating room at which time she had the right tympanostomy tube replaced with a new long-term tympanostomy tube, an adenoidectomy, and bilateral reduction of her inferior turbinates. Following surgery, her vertigo was totally absent and her nasal obstruction and mouth breathing was relieved.

Eustachian tube function testing following the surgery showed the left abnormal function was unchanged and the function in the right ear was abnormal similar to that of the left ear. Postoperative vestibular testing indicated a significant left reduced vestibular

response and normal rotational testing. These results suggested that the child preoperatively had a vestibular imbalance with central suppression of peripheral responses. However, postoperatively, as central suppression was no longer present, a reduced vestibular response from the left ear, with the open tympanostomy tube, was manifested. The absence of a directional preponderance on rotational testing despite asymmetric caloric responses indicates central nervous system compensation for a unilateral peripheral vestibular reduction in the left ear. Compensation followed surgical placement of the new tympanostomy tube in the right ear and relief of the chronic nasal obstruction.

Discussion

Achondroplasia patients are prone to otitis media. A recent study found 68% of 22 patients with achondroplasia had an otitis media diagnosis and two-thirds had had tympanostomy tube insertions.[3] To our knowledge, our patient is the first patient with achondroplasia to have Eustachian tube function testing, and these tests revealed abnormal function.

Alternobaric vertigo has been reported in as many as 27% of sport divers,[4] and in Portuguese Air Force pilots the prevalence was 29%.[5] The typical diver or flyer who develops AV has problems equilibrating middle-ear pressures during barometric pressure changes, a history of otitis media, or Eustachian tube dysfunction.[6] The vertigo can occur during periods of an upper respiratory infection, which presumably adversely affects their Eustachian tube function. Our patient had abnormal vestibular function during the persistent vertiginous period which resolved following postoperative elimination of the high positive middle-ear pressures. We propose that the unilateral high positive middle-ear pressure adversely affected her vestibular system. In an experiment in guinea pigs positive middle-ear pressure resulted in an objective vestibular response.[7]

Swallowing when the nose is pinched off can result in first positive and then negative pressure within the nasopharynx that can be transmitted through the Eustachian tube into the middle ear, which is the Toynbee test of tubal function. Bluestone and colleagues suggested that any type of nasal obstruction (i.e., pathophysiology) could create the "Toynbee phenomenon" during swallowing.[8] The Toynbee phenomenon has been studied by Finkelstein et al in adults who had intranasal packing.[9] They recorded nasopharyngeal pressures which ranged from a maximum positive pressure of +450 mm H_2O to a negative pressure as low as -320 mm H_2O. The middle-ear pressure tympanometric recordings reached a maximum of + 190 mm H_2O. In the ferret, Buchman et al studied the effects of nasal obstruction on Eustachian tube function and middle-ear status.[10] Unilateral nasal obstruction had no effect on middle-ear pressures, but bilateral obstruction resulted in middle-ear pressures of approximately + 170 mm H_2O. The high positive pressures persisted for the 6- to 8-weeks post-obstruction follow-up period, but none of the animals developed a middle-ear effusion. Our patient had tympanometric recordings of + 200 mm H_2O pressures when her nose and nasopharynx were obstructed, which is consistent with both the clinical and laboratory studies described above.

Summary and Conclusions

This unique case history describes a child who developed the Toynbee phenomenon due to nasal obstruction and a unilateral obstructed tympanostomy tube which resulted in a persistent, high positive middle-ear pressure in that ear, and due to its asymmetry, stimulated the vestibular system causing AV. When confronted with patients who have vertigo, the possibility that nasal obstruction and the Toynbee phenomenon are involved should be considered, especially in those patients who have Eustachian tube dysfunction.

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NOD2 Contributes to TLR2-Independent Up-Regulation of DEFB4 in Response to Internalized NTHI in Human Middle Ear Epithelial Cells

Sung K. Moon, MD, PhD[1], Jeong-Im Woo, PhD[1], Paul Webster, PhD[2], Sejo Oh, PhD[1], David J. Lim, MD[1]
[1]Clinical and Translational Research, [2]Advenced Electron Microscopy and Imaging, House Ear Institute, Los Angeles, California

Nontypeable *H. influenzae* (NTHi), a common otitis media pathogen,selectively adheres and internalizes to therespiratory epithelial cells.[1, 2] We found that *Listeria monocytogenes*escapes from the phagosome to the cytoplasm.[3] However, it is unclear if internalized NTHi can exist freely in the cytoplasmand which cytoplasmic pathogen recognition receptor is involved in its recognition. Epithelial cells secrete antimicrobial molecules to defend against invading pathogens. DEFB4, a cationic antimicrobial peptide, is an inducible defensin that responds to proinflammatory signals such as cytokines and bacterial molecules.[4,5] Recently, we demonstrated that NTHi up-regulates DEFB4 through a TLR2-dependent signaling pathway.[6] However, inhibition of TLR2 did not completely block this up-regulation, indicating the existence of an alternative TLR2-independent signaling pathway. To define the nature of such a pathway, we studied the role of cytoplasmic pathogen-associated pattern recognition receptors such as nucleotide-binding oligomerization domain 1 (NOD1) and NOD2, which are known to be involved in DEFB4 up-regulation induced by muramyl dipeptide (MDP) and *Helicobacter pylori*, respectively.[7,8] In this study, weaimed to determine that (1) NTHi can escape from the endosome to the cytoplasm; (2) membrane modification affects NTHiinduced DEFB4 up-regulation; and (3) NOD2is involved in recognition of internalized NTHi, leading to DEFB4 up-regulation.

Materials and Methods

NTHi strain 12, originally a clinical isolate from the middle ear fluid of a child with acute otitis media, was used in this study.[9] As a model epithelial cell, we used the human middle ear epithelial cell line (HMEEC), which was immortalized withthe E6/E7 genes of human papilloma virus type 16.[10] Transmission electron microscopy (TEM) was performed after cellswere exposed to live NTHi to determine NTHi internalization.Real time quantitative PCR analysis with FAM^{TM}-conjugated probe for DEFB4 and luciferase assays with 5'-flanking region (-2625 to +1) of the DEFB4 gene were performed to determine NTHi-induced DEFB4 up-regulation. To determine the involvement of NODs, expression of NOD1 or NOD2 was silenced using the gene-specific siRNAs. We also performed bacterial internalization assays and endocytosis assays after pretreatment with cytochalasin D and hemolysin.

Results

TEManalysis demonstrated that NTHi exists either surrounded by an enclosing membrane or freely in the cytoplasm after rupturing the enclosing membrane, which indicate the requirement of the cytoplasmic receptors of the human epithelial cells for recognizing the internalized NTHi.We confirmed that NTHi-induced DEFB4 up-regulation was preserved despite of TLR2 deficiency (~2 fold increased) compared to the wild type control (~7 fold increased), indicating the involvement of the TLR2-independent signaling pathways. Silencing ofNOD2 was found to inhibit NTHi-induced DEFB4 up-regulationmore thanthat of NOD1.Silencing of both TLR2 and NOD2 appeared to completely suppress NTHi-induced DEFB4 up-regulation, indicating that TLR2 and NOD2 are mainly required for recognizing NTHi in the HMEECs. We demonstrated the involvement of NOD2-assoicated signaling molecules such as CARD12 and RICK in NTHi-induced DEFB4 up-regulation. NTHi-induced DEFB4 up-regulation was enhanced by silencing of Card12, but was inhibited by silencing of RICK. Bacterial internalization assays and endocytosis assays showed that NTHi internalization was inhibited by cytochalasin D, but was enhanced by hemolysin.Silencing of NOD2 inhibited hemolysin-mediated enhancement of NTHi-induced DEFB4 up-regulation, suggesting pore formation enhances NOD2-mediated DEFB4 up-regulation in response to NTHi.

Conclusion

It is suggested that internalized NTHi exists freely in the cytoplasm of the HMEECsand membrane modification affects NTHi-induced NOD2-mediated DEFB4 up-regulation. NOD2 is suggested tobe required for the recognition of internalized NTHIin the HMEECs, leading to DEFB4 up-regulation. These results may enable us to understand the regulatory mechanism of the receptor molecules in host innate immune and provide us with new therapeutic strategies for infectious diseases.

This work was supported in part by NIH grant DC5025, DC8696, and DC6276.

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The Haemophilus Sap Transporter Is Required for Commensal Establishment and Pathogenesis

Forrest Raffel, Kevin Mason, PhD
College of Medicine, The Ohio State University, Columbus, Ohio

Commensal bacterial colonize host environments, and in turn provide nutrients and limit pathogencolonization at these sites. Mechanismsused to establishcommensalism can likewise equip these bacteria intransition to an opportunistic pathogen.Nontypeable *Haemophilus influenzae* (NTHI) is a commensal of the human nasopharynx, yet mediates diseases of the airway such as otitis media. Commensalism requires adaptation to host nutrition and innate immune mechanisms, therefore coordination of adherence, metabolism and bactericidal resistance mechanisms are necessary.We previously demonstrated that the NTHI Sap transporter is aninner membrane multifunctional complex that mediates resistance to host antimicrobial peptidesand is required for heme homeostasis. We hypothesized, therefore, that NTHI commensal behaviorrequired Sap transporter functions. Thus, SapA-deficient NTHI were cultured on the surface of respiratory epithelial cells to examine NTHI-host cell membrane interactions.SapA-deficientNTHI were less adherent to bronchial, middle ear and nasopharyngeal epithelial cellsyet demonstrateddramatic alterations in NTHI-host cell homeostasisas characterized by epithelial cell membrane ruffling,increased actin polymerization and enhanced invasion of epithelial cell layers.Additionally, cytokine profiles indicated altered inflammation induced by SapA-deficient NTHI. Our preliminary data support the hypothesis that the Sap transporter is required for commensalism; however we seek to better understand the outer membrane changes in SapA deficient NTHI and the epithelial host cell response to these changes.This study supports the essential role for the Sap transporter in the commensal and pathogenic behavior of NTHI. Thus, targeted therapies to block Sap transporter function could significantly reduce the clinical and economic burden of NTHImediated diseases.

Import and Degradation of Antimicrobial Peptides as a Mechanism of Innate Immune Evasion and Nutritional Foraging in Nontypeable *Haemophilus influenzae*

Sara Johnson[1]**,** Catherine Shelton[2]**,** Wandy Beatty, PhD[3]**,** Forrest Raffel[1]**,** Kevin Mason, PhD[1]
[1]College of Medicine, The Ohio State University, Columbus, Ohio, [2]Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio, [3]School of Medicine, Washington University, St. Louis, Missouri

Nontypeable *Haemophilus influenzae* (NTHI) is a gram negative inhabitant of the human nasopharynx yet can cause opportunistic diseases of the upper and lower respiratory tracts including the medically important disease otitis media (OM). NTHI must acquire nutrients and resist insult by the host innate immune response to colonize and establish infection. Host antimicrobial peptides (AMPs) are innate immune molecules that promote sterility of environments such as the middle ear. Previously, we demonstrated that the Sap ABC-transporter binding protein, SapA, binds AMPs and equips NTHI to resist AMP lethality. A SapA mutant is attenuated for survival in the middle ear, demonstrating that Sap transporter function is essential for pathogenesis. Thus, we hypothesize that Sap-dependent transport and cytoplasmic degradation of AMPs is vital for removal of AMP accumulation at the cell surface and thus provides a mechanism of acquiring nutrients. Toward this end, we demonstrated that AMPs localize to the periplasm and cytoplasm of whole cells when exposed to sublethal concentrations of AMPs. We showed that trafficking of AMPs to the bacterial cytoplasm was dependent upon a functional SapBC permease complex. SapBC-deficient NTHI lacked the ability to transport AMPs as demonstrated by a striking accumulation of AMPs in the periplasm. Importantly, we observed increased growth of NTHI when exposed to sublethal concentrations of AMPs, supporting our hypothesis that NTHI transport

AMPs for degradation and nutritional benefits. Incubation of AMPs with NTHI cytoplasm resulted in their degradation, which was inhibited in the presence of protease inhibitors. This novel mechanism of innate immune evasion linked with amino acid recycling would provide a metabolic benefit for NTHI survival. Blockade of the functional Sap transporter could hinder this mechanism thus rendering NTHI more susceptible to the host innate immune response, promoting clearance of the bacteria and treating OM.

Haemophilus influenzae Quorum Signals and Sensing

W. Edward Swords, PhD
Microbiology and Immunology, Wake Forest University Health Sciences, Winston-Salem, NC

Introduction
Quorum signaling is a means for coordination of community behaviours in bacteria via diffusible signaling molecules. For Haemophilus influenzae, we have demonstrated that dihydroxypentanedione (DPD) production by means of luxS is a determinant of biofilm maturation and bacterial persistence in vivo. In this study, we identify the determinant of DPD quorum signal uptake by H. influenzae, and show that this uptake is essential to quorum sensing, production of luxS-mediated virulence determinants, and persistence in the chinchilla model for otitis media.

Methods
Molecular biology and genetic experiments were performed using standard methods. Biofilm assays were performed using established in vitro static and continuous-flow assays. Otitis media infections were performed using the chinchilla model for otitis media.

Results
The NTHI0623 open reading frame is essential to H. influenzae uptake and sensing of DPD, as a null mutant lacked the ability to take up and respond to DPD. This mutant was attenuated in the chinchilla infection model, to a degree that was comparable to the luxS mutant we have previously described.

Conclusions
Sensing of DPD by H. influenzae requires active uptake of signal mediated by the product of the NTHI0623 open reading frame. Thus, like other quorum signaling systems, H. influenzae has a dedicated mechanism for taking up quorum signal.

Nontypeable *Haemophilus influenzae* Outer Membrane Vesicles Contain Virulence-Associated Proteins and Adversely Affect Epithelial Cells

Samantha Sharpe[1]**,** Meta Kuehn, PhD[2]**,** Kevin Mason, PhD[3]
[1]Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio, [2]Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, [3]College of Medicine, The Ohio State University, Columbus, Ohio

Outer membrane vesicles (OMVs) are an integral component of bacterial growth and host interaction, produced by all Gram negative microorganisms studied to date. The contribution of OMVs to biological processes is diverse; mediation of bacterial stress response, selective packaging and secretion of virulence determinants, modulation of host immune response, and contribution to biofilm formation, growth, and stability. Nontypeable *Haemophilus influenzae* (NTHI), a causative agent of otitis media, produces OMVs *in vivo* yet there is a paucity of information regarding the composition and role of OMVs during NTHI colonization and pathogenesis. Thus, we isolated and purified NTHI OMVs, characterized vesicle-associated proteins, and examined OMV interaction with host epithelial cells. We demonstrated that NTHI vesicles are 50-200nm in size and contain numerous outer membrane proteins, including OMP P6 and adhesins P2 and P5-fimbriae, both current targets in NTHI vaccine efforts. OMVs also package periplasmic IgA endopeptidase, serine protease, and heme utilization protein, supporting a multifaceted role in virulence. NTHI OMVs bind and aggregate on the cell surface then are internalized within 2 hours. Host cell internalization is temperature dependent as OMVs remained cell surface associated when cultured at 4° C. Further, OMV-host cell interaction stimulated significant release of the immunostimulatory cytokine IL-8 by both human and chinchilla epithelial cells. Thus, we demonstrated that NTHI OMVs containing virulence-associated proteins dynamically interact with host epithelial cells. We seek to understand how OMV protein composition is affected by *in vivo* stressors such as nutrient limitation and innate immune molecules as we continue to investigate the role of NTHI vesiculation in critical functions of pathogenesis; host interaction, bacterial stress response, and biofilm maintenance.

Treatment/Microbiology

A Randomised Controlled Trial of Swimming Versus No Swimming on Resolution of Ear Discharge Associated with Chronic Suppurative Otitis Media (CSOM)

Anna Stephen, Amanda Leach, PhD**,** Peter Morris, MD, PhD**,** Louise Boyle**,** Kim Hare**,** Vanya Hampton
Child Health Division, Menzies School of Health Research, Darwin, NT

Objective
To measure the impact of one month of daily swimming in a chlorinated pool compared to no swimming, on the proportion of children with i) ear discharge ii) nasopharyngeal colonization and ear discharge microbiology.

Methods
Australian Aboriginal children 5 -12 years of age with CSOM from remote Northern Territory communities were randomised to four weeks of either daily swimming classes or swimming restriction (alternative activities). Ear examinations at baseline and end of therapy were standardised using tympanometry and video-otoscopy. Swabs were taken from the nasopharynx and the ear discharge.

Results
In an intention to treat analysis, 59% (24/41) had ear discharge at end of therapy in the swimming group versus 67% (32/48) in the restricted swimming group (Risk Difference = -8%, [95% CI: -29 to 12]). 12 children were lost-to-followup. Among children with dry TMPs, new discharge was found in 40% (6/15) of swimmers and 44% (7/16) restricted swimmers (RD = -4% [-37 to 30]). Healed perforations were identified in 7% (3/41) of swimmers and 8% (4/48) restricted swimmers (RD = -1%, [-14 to 12]). Mean perforation size at followup for the swimmers was 21% and 26% for the restricted swimmers. [RD = –5%, [-17 to 7]. (Microbiological results will be finalised by 1/3/11)

Conclusions
We found no statistically significant or clinical relevant benefit of swimming over non-swimming for resolution of ear discharge or TM healing. Importantly, our study indicated that swimming does not cause substantial harm for children with CSOM. Larger RCTs are needed to confirm our findings.

Causative Treatment of Retraction Cholesteatomas by Obliteration of the Mastoid

Jacob Tauris, MD, PhD, Suzan Al Kole, MD, MD**,** Kjell Tveteraas, MD**,** Michael Gaihede, MD
Department of Otolaryngology, Head and Neck Surgery, Aalborg Hospital, Aarhus University Hospital, Aalborg

Introduction
Formation of middle ear cholesteatoma is primarily based on retraction pockets of the tympanic membrane due to a negative middle ear pressure[1-3]. Cholesteatomascausedestruction of middle ear structures and display high recurrence ratesafter surgical removal[3, 4]. The goals of cholesteatoma surgery are complete eradication of pathology with preservation or improvement of hearing, restoration of hygiene status, and prevention of recurrent disease.
Surgical procedures with canal wall up (CWU) techniques generally provide a better functional outcome than canal wall-down (CWD) techniques[5-7], but entail a higher risk of residual as well as recurrent disease[4, 5, 8, 9].Recurrence after CWU procedures most commonly result from redevelopment of retraction pockets, due to negative middle ear pressure caused by Eustachian tube and/or mastoid dysfunction[1-3].
The mastoid plays a significant role in gas-exchange and a negative middle ear pressure may result from increased gas-absorption from diseased mastoid mucosa[10, 11].Therefore, combining CWU surgery with mastoid obliteration can be expected to reduce the incidence of recurrent disease by eliminating the source of negative middle ear pressure, and removing the space for retraction pocket formation.However, mastoid obliteration carries a potential risk of residual disease, which is higher in combination with CWU procedures[12-20].

Objective
We set out to determine the frequency of residual and recurrent cholesteatoma in a group of patients treated with mastoidobliteration.

Methods
The study was a retrospective study of 180 patients who underwent cholesteatoma surgery withCWUmastoidectomy and bony obliteration technique (BOT) during a 13-year period from 1997 to 2007.

The inclusion criterion was cholesteatoma with attico-antral involvement, either primary acquired (n=131) or recidivistic (n=32).The exclusion criteria were a follow-up time shorter than one year or incomplete registration of data.Follow-up investigation was possible in 164 cases, giving a one year follow-up rate of 91%.Of the included 164 patients, 77% (n=126) were adults whereas 23% (n=38) were children, giving a mean age of 34 years (34.0±19.6 (range 2-78)).
The mean follow-up time was 42 months. The outcome measures were residual or recurrent cholesteatoma, hearing results and postoperative complications.

All patients included in the study were operated using a combined approach CWU cortical mastoidectomy with BOT.Briefly, bone chips were collected using a flat chisel and bone pate was collected with a cutting bur during cortical mastoidectomy. Subsequently, the cholesteatoma was removed using a combined approach and followed by reconstruction of the ossicular chain. Next, the epitympanon was closed with cartilage and bone pate and finally, the mastoid was obliterated.

Results
The overall frequency of recidivistic cholesteatoma was 8.5% (n= 14/164). The total incidenceof residual cholesteatoma was 1.2% (n=2/164), whereas the total incidence of recurrent cholesteatoma was 7.3% (n=12/164). Both rates were higher in children compared to adults(*Table 1*).

Table 1 Incidence of residual and recurrent cholesteatomas (n=164).

	Total cases (%)	Residual (%)	Recurrent (%)
Children*	38 (23.2)	2 (5.2)	5 (13.1)
Adult	126 (76.8)	0 (0)	7 (5.5)
Total	164 (100)	2 (1.2)	12 (7.3)

* Children: <16 years of age.
Hearing results showed a significant improvement after surgery with regards to ABG-closure and PTA. Mean air bone gap closure was 9.2dB (9.2±2.9) and the difference between mean preoperative (29.6dB) and postoperative (20.5dB) ABG was significant (p<0.01). SRT did not change significantly (*Table 2*).

Table 2 Pre- and postoperative hearing results (dB HL).

	Preoperative mean (SE)	Postoperative mean (SE)	Gain mean	p-value
ABG (dB)	29.6 (1.1)	20.5 (1.0)	9.2	<0.01
PTA (dB)	40.9 (1.6)	36.1 (1.6)	4.8	0.03
SRT (dB)	35.2 (1.7)	31.1 (1.6)	4.1	0.07

ABG = Air-bone gap; PTA = Pure tone average (500, 1000, 2000 and 4000Hz); SRT = Speech recognition threshold
No major complications were observed.Minor postoperative wound infections occurred in 6% (n=10) of cases and one patient developed a transient facial nerve palsy, which later recovered completely (*Table 3*).

Table 3 Incidence of postoperative complications (n=164).

	n	(%)
Major complications:		
(anacusis, sinus thrombosis, meningitis, brain abscess)	0	(0)
Minor complications:		
Postoperative wound infection	10	(6)
Facial nerve palsy (transient)	1	(0.6)
Total	164	(100)

Discussion
Mastoid obliteration carries a potential risk of residual disease, which is further enhanced when combined with CWU procedures. The present study revealed a low overall residual rate of 1.2%, indicating that CWU with bony obliteration is a safe method to eradicate cholesteatoma yet avoiding large cavity problems, although less good in children (5.2%).Furthermore, we found a total recurrence rate of 7.3%, which is far lower than recurrence rates reported for CWU surgery without obliteration[3, 4].

The hearing results are comparable with previous studies (*Table 4*). The improvements may not be impressive, but it is important to emphasize that many of the patients had a functional intact ossicular chain and a normal or subnormal hearing at the time of surgery, during which a type II or type III tympanoplasty was performed, partly to ensure complete eradication of cholesteatoma and also because obliteration cannot be performed with preservation of an intact ossicular chain.

Table 4 Functional results pre-and postoperatively after mastoid obliteration.

		Preoperative		Postoperative		Gain		*p*-values	
Previous studies	n	PTA	ABG	PTA	ABG	PTA	ABG	PTA	ABG
Vartiainen et al.[21] (1987)*	126	40±17	29±11	31±20	20±15	8.8	8.7	NS	NS
Grantz et al.[20] (2005)*	130	-	28±13	-	22±11	-	6	-	-
Abdel-Rahman et al.[13](2008)*	70	47±5	27±7	47±9	26±6	0	2	-	>0.05
Lee et al.[14] (2009)**	18	57±3	35±2.2	49±4	18±3	8.2	17.6	-	-
Sun et al.[19] (2009)**	48	-	3±8	-	17±5	-	9.28	-	<0.01
Kang et al.[6] (2009)	100	-	32±12	-	25±12	-	6.0	-	<0.01
Present study*	164	41±21	30±14	36±20	20±12	5	9.15	0.03	<0.01

PTA = Pure tone average; ABG = Air-bone gap; Gain = Preoperative minus postoperative PTA or ABG.
* Mean± SD; ** Mean± SE

In our study, children had a higher incidens of recurrence (13%) compared to adults (5.5%). This may be explained by persistent Eustachian tube dysfunction and incomplete obliteration (i.e. leakage).Alternatively, air cells different from mastoid tract cells could be responsible for the continuous negative pressure and hence formation of new retractions.

The obliteration technique with bone chips and pâté was originally described by Palva in 1979[22]. This technique has been discredited by some authors because of high rates of postoperative infection[14, 23-25]. In the present study, infection only occurred in 6% of cases - most likely due to impregnation of bone chips with ciprofloxacin. Furthermore, bone chips have the advantage of retaining their bulk in the obliterated cavity[12, 20, 25, 26].

Conclusions

The present study clearly indicates that CWU mastoidectomy with bony obliteration is a safe method for treating primary or recurrent cholesteatoma, and a useful technique to eliminate cavity problems while preserving auditory function.Exenteration of the gas-absorbing epithelium followed by sealing of the posteriorepitympanon and mastoid obliteration, effectively prevent long-term postoperative reformation of retraction pockets.

Obliteration of the mastoid cavity cannot prevent recurrence of cholesteatoma. Nevertheless, recurrence rates are significantly lower compared to conventional CWU surgery and therefore, obliteration of the mastoid cavity can be considered as a causative treatment of retraction cholesteatomas.

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Otitis Media and Its Control in Children with Cochlear Implants – a Long Term Prospective Study

Michal Luntz, MD, Jawad Khalaila, MD
Otolaryngology, Bnai Zion MC, Technion - Israel Institute of Technology, Haifa, Israel

Introduction
The age at which cochlear implantation (CI) is performed in children corresponds to the age of highest prevalence of otitis media (OM). Since the risks of problematic middle ear infection in implanted children are relatively high, these children represent a unique group in whom close OM related follow-up is absolutely indicated.

Objectives
To report the current results of an on going prospective study aimed at assessing the short and the long term risks for OM after CI in children who were treated according to a structured protocol designed to control OM prior to - and after CI.

Patients and Methods
Of 164 children referred for CI during the study period (1998-2008), implanted under the age of 7 years, and continue their follow-up in our center, 99 were classified as OM-prone and 65 as non-OM-prone. All patients were seen at the outpatient clinic every 6 months and whenever OM was suspected. Post-implantation follow-up ranged from 6-114 months (average 48.2 month)

Results
Mean age at referral was significantly lower in the OM-prone group as compared to the healthy group (21.7m Vs 34.2m, $p<0.001$). Mean age at implantation was significantly lower in the OM-prone group as compared to the healthy group (31.2m Vs 40.7m, $p<0.01$). 33% of the 99 OM- prone children required more than one VT for control of OM prior to implantation. Children who needed repeated insertions were older at referral than those who needed only one VT, 25m (range: 7-57m) Vs 19 m (range: 4-61m). Rate of perforations was 5% for the whole group and 3% after one VT. 47% of the OM-prone children developed OM after CI Vs 12.3% in the non-OM prone. Challenging OM occurred in 3% of the non-otitis prone children and in 11% of the otitis prone children. In the first year after implantation most pathogens isolated from the otorrhea of the challenging otitis cases were typical for OM. In the second post implantation year most pathogens isolated were not typical for otitis media.

Conclusions
With a strict protocol for OM control early referral can lead to early implantation, even in children susceptible to OM. A significant number of OM-prone children may need more than one VT to reach OM control. When OM exists in a relatively older child the chance for rapid resolution is low. Rate of perforations after one VT is 3%. In the first year after CI, otorrhea via a VT is usually the result of AOM, later, otorrhea is likely to be the result of the VT.

The Role of Palatoplasty Technique on Otitis Media, Ventilation Tube Placement and Hearing Loss in Cleft Palate Population in 4 Year Follow-Up

Allison Tobey, MD[1], Joseph E. Losee, MD[2], **Cuneyt M. Alper, MD[2],** William J. Doyle, PhD[2]
[1]Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA, [2]Department of Otolaryngology, Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction

Nonsyndromic clefts of the lip and/or palate (CL/P) are among the most common birth defects, affecting approximately 1 in 595 newborns in the United States.[1] Problem with otitis media (OM)[2-4] are common in the cleft palate population.

Cleft palate patients have multiple anatomic abnormalities that may result in the increased prevalence of OM seen in these patients. For example, cleft palate patients have reduced sphenopalatine angles,[5] immature, collapsible Eustachian tube (ET) cartilage,[6] abnormal ET angle[7] and abnormal insertion and function of the tensor veli palatini and levator veli palatini muscles.[6, 8]

Objectives

Classical objectives of palatoplasty are palate closure with restoration of normal eating, drinking and speech. Multiple studies have also shown palate closure may improve ET function as well.[9, 10] However, the effects of palatoplasty on OM and hearing have been mixed[11-14]

The goal of this study was to compare the numbers of ENT visits and ventilation tubes, the degree of hearing loss and the duration of speech therapy between Furlow (FP) and straight line (SLP) palatoplasties in cleft palate (CP) patients.
Classical goals of palatoplasty are palate closure with restoration of normal eating, drinking and speech. Studies have also shown palate closure improve the Eustachian tube (ET) function as well. However, the impact of palatoplasty on OM and hearing have been controversial, especially regarding the effects of specific palatoplasty techniques. Objective of this study is to evaluate otitis media, the number of VTs, and the degree of hearing loss in cleft palate (CP) patients over a 6 year period after Furlow (FP) and straight line palatoplasty (SLP).

Goal was to determine if there is a difference between Furlow (FP) and straight line (SLP) palatoplasties in cleft palate (CP) patients in the: numbers of ENT visits, numbers of ventilation tubes, degree of hearing loss, duration of speech therapy.

Methods

A retrospective study of 118 CP patients without known syndromes or chromosomal abnormalities and followed at a tertiary CP center. Data from operative notes, otoscopic exams and tympanometric and audiometric tests were analyzed. This was a retrospective, IRB approved study. Descriptive analysis was performed. Inclusion criteria was being non-syndromic, having obtained parental consent, was followed for 5 yrs at University of Pittsburgh Cleft Palate and Craniofacial Center (UPCCC), seen by age 1, also seen within 12 months of 5 yr birthday, and the surgical care was performed at UPCCC. The endpoints were #of ENT visits in 5 years, # of ventilation tubes in 5 years, degree of hearing loss @ 5 years, and duration of speech therapy in 5 years.

A total of 237 total patients born between 1/2000-12/2004 seen at the UPCCC. 91 were eliminated because of being syndromic, having not been seen within 12 months of 5yr birth day, and not been seen within first year of life. Total of 146 were initially included and 23 eliminated because of early T-tube placement, and 4 eliminated missing initial OP note. 119 were included in the analysis.

Demographic information included the following: Sex, Males/Female: 60 /59; Race: Caucasian: 108 (91%), African American: 10 (8%), Asian: 1 (1%); Birth Information Term: 38.4 ±2.5 weeks, Weight: 3.2±0.6 kg, NICU stay: 7.2±10.7 days; Maternal Age: 27.8±6.5 years; Prenatal smoking: 10 (n=10); Post natal second hand exposure: 31 (n=38)

Results

The average number of otolaryngologic visits after palatoplasty was 3.4 and 4.8 in the FP and SLP groups respectively (p=0.03). After primary palatoplasty, the mean number of tympanostomy tubes placed in the FP group was 0.93+/-1.2 as compared to 2.0+/-2.5 for the SLP group (p= 0.002). The cumulative time subjects had at least one tube in place was 29.0 and 35.0 months for the FP and SLP groups, respectively (p=0.03). Of those with audiograms within 12 months of their 5th birthday, 28% in the SLP group and 13% in the FP group had a >25db hearing loss in at least 1 ear (p=0.12). None in the FP group had persistent hearing loss, whereas 8 (12%) in the SLP group did. The average duration of speech therapy was shorter in the FP group when compared to the SLP group (11.6+/-15.9 vs. 8.7=/-25.4 Months, p=0.07), whereas the percent of patients with developmental language delay was similar for the two groups (15 and 17%, respectively).

Table 1. Cleft type

Cleft Type	n	%
Veau I	9	7.5%
Veau II	41	35.0%
Veau III	46	38.5%
Veau IV	23	19.0%

Table 2. Repair Type

Repair Type	N	%
Furlow	43	36%
V-Y pushback	23	19%
Von Langenbeck	20	17%
Bardach	33	28%

Discussion

Reduction in ENT visits & OME

Within our data set there was a reduction in overall ENT visits as well as visits where OME was diagnosed. These date however, may be effect by patient follow up. Many of the patients followed at the UPPCC do not live locally and the distance required to travel may influence there follow and thus the number of ENT visits. Guneren et al. did not detect a difference in ear disease[12] however the sample size was small and the mean age at which the palatoplasties were performed much greater than the mean age observed in our study.

Reduction in Tympanostomy Tubes

Similar to Smith et al.,[13] we detected a reduction in the number of tympanostomy tubes placed in Furlow group compared to the SLP group. We also showed a reduction in the length of time Furlow patients had tubes. However, multiple ENT surgeons were involved in the care of these patients, thus introducing possible bias as to timing of tube placement . Moroever 19 (16%) patients had T-tubes placed at some point within the five years and the long term nature of these tubes may influence the number and duration of tubes.

Less Hearing Loss with Furlow

We detected less hearing loss in the Furlow group (11%) versus the SLP group (28%), however our data was limited by the fact that not all patients obtained audiograms between ages of 4-6 years of age.

Furlow & Speech

In our study we were not concerned with articulation or phonological delays that would not be related to speech development and hearing. When speech delay was defined as only that effected by hearing, we showed no difference in speech development between Furlow and SLP. The reduction in duration of speech therapy seen in our study is consistent with other studies [15, 16] however, we were unable to separate speech therapy that was for speech development that related to hearing versus articulation, VPI, etc.

Conclusion

Compared to Straight line palatoplasty, patients who had Furlow palatoplasties had fewer ENT office visits, fewer tubes, total and after palate repair, possibly less hearing loss @ 5 years (not statistically significant), possible reduction in speech therapy duration (not statistically significant), no measureable difference in degree of developmental language delay.

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Effect of Topical Ciprofloxacin 0.3% and Dexamethasone 0.1% (Ciprodex) Vs. Ciprofloxacin 0.2% and Hydrocortisone 1% (CiproHC) on LPS-Induced Experimental Otitis Media

Sang Gyoon Kim, MD, Mary Ann Nyc**, G Michael Wall, MD,** Timothy Jung, MD, PhD
VA Loma Linda Healthcare System, Dr. Jung's otology lab, Loma Linda, CA

Introduction

Temporal trends in otitis media (OM) diagnosis and reporting have changed in recent years. Historically, OM is the most common and prevalent diagnoses in children, which exhibited a 150% increase from 1975 to 1990.[1] With the advent of heptavalent pneumococcal conjugate vaccines and other treatment advances, OM has seen a slight decline since 2000. However, current epidemiological reports suggest that while the overall incidence of otitis media (OM) has decreased over the past decade, OM incidence and prevalence has increased in low socioeconomic populations, particularly in high risk individuals such as those <2years.[2] Concomitantly, antibiotic prescription rates used to treat these conditions have also increased, leading to greater incurred medical costs and additional risks of adverse effects from antibiotic treatments, such as diarrhea and development of multi-drug resistant bacteria in the naso-pharynx .[3, 6] To improve upon the aforementioned, investigations have reported the advantages of using combination antibiotics and topical steroids for treating OM, thereby reducing the need for extended antibiotic treatments and decreasing the chance for OM recurrence.[4] Reduced OM duration and recurrence is key to good clinical treatment measures because long inflammatory processes, can lead to chronic OM or acute OM, can impact inner ear mechanisms and can cause permanent damage—not only to otic physiology but to hearing and learning development.

Given that early eradication of pathogens found in middle ear fluid results in improved outcomes, it reasons that reducing the fluid in which these pathogens subsist will likewise lead to improved outcomes.[5]Combination treatments work well, but it is unclear which combinations work best. Previous analyses of scientific literature have examined types of systemic antibiotics with inconclusive determination of superior antibiotic types.[7] Fewer investigations have conducted comparable reviews or experiments among topical otic treatments. Therefore, the goal of this study was to provide evidence to help determine which of two commonly used topical glucocorticoids would best reduce middle ear fluid rapidly and deter onset of inflammation caused by OM.

Methods

Animal Model

28 adult female chinchillas weighing approximately 600-700 grams were obtained from Bowen's Chinchillas, LLC and housed at the Veterinary Medical Unit of the Jerry L. Pettis VA Medical Center for approximately one week prior to study start. All chinchillas were screened for absence of OM by anesthetizing them with isoflurane (induction 3.5%, maintenance 2.5%) inhalation and evaluating each ear canal with a micro-otoscope. All chinchillas were determined to be healthy, and were then randomly assigned to one of three treatment groups: vehicle control, Ciprodex 0.1%, or CiproHC 1%.

Treatment Groups

Vehicle control was composed of the sterile, preserved suspension used to make Ciprodex and Cipro HC and was obtained by Alcon, Inc. This was selected as a control solution over other potential control substances such as saline because it remains as similar to the treatment groups, but without antibiotics or steroids present. The composition for the vehicle control includes benzalkonium chloride as a preservative, boric acid, sodium chloride, hydroxyethyl cellulose, tyloxapol, acetic acid, sodium acetate, edetate disodium, and purified water.

Ciprodex (ciprofloxacin 0.3% and dexamethasone 0.1%; NDC#: 0065-8533-02) and Cipro HC (ciprofloxacin 0.2% HCl and hydrocortisone 1%; NDC#: 0065-8531-10) were obtained as sterile otic suspensions. Both are combination antibiotic (quinolone), steroid treatments commonly used by clinicians to treat acute OM externa and acute OM following tympanostomy tube placement. Ciprodex and Cipro HC are registered trademarks of Bayer AG licensed to Alcon, Inc and are available by prescription only. Their compositions are akin to the underlying difference being the anti-inflammatory corticosteroid: Dex vs. HC.

Experimental Procedure

Each treatment group solution was administered to chinchilla middle ear cavities by a 0.2ml transbullar injection. Two hours later, each chinchilla was inoculated with LPS *S. pneumoniae* (prepared at 1mg/ml). Treatments for each group were then administered via 0.2ml transbullar injection into the inferior bullae cavity at 24, 48 and 72hrs post inoculation. At 120hrs post-inoculation, all chinchillas were euthanized via ketamine (Bedford Laboratories, Bedford, OH, USA) IM injection (40mg/kg) and decapitated. Middle ear fluid was collected via needle and syringe through the superior bullae and inferior bullae; volumes were measured in mL using a cuvette. Temporal bones were harvested in 10% formalin further analyses. Additionally, each chinchilla was examined daily by otoscopy (from time 0 through 120hrs) and notable erythema changes were documented.

Results

Otoscopy

Otoscopy differences between groups became apparent as early as 24 hrs post-inoculation, and became significantly different at 72 hrs post-inoculation. The difference became more pronounced and remained significantly different at 120 hrs post-inoculation. Specifically, 72 hr and 120 hr otoscopieswere significantly different between the control group and both treatment groups. While Cipro HC® treated subjects exhibited slightly decreased signs of erythema compared to those treated with Ciprodex®, the difference was not significant.

Middle Ear Effusion (MEE)

Graphical review of MEE data showed non-parametric characteristics. Therefore, Kruskal-Wallis analyses were conducted and revealed statistical difference across treatment groups (χ^2=15.16, df=2, p-value=0.0005). The overall trend of MEE collected at 120 hrs post LPS-innoculation revealed a decline with treatment but was significantly lowered by Ciprodex. Post-hoc ANOVA testing using ranked values of MEE were conducted in conjunction with Tukey's Studentized Range (HSD) Test. Results showed that the magnitude by which DEX reduced MEE when compared to the HC treatment and the Control group was statistically significant with $\alpha = 0.05$ and 95% confidence .

Mucosal Thickness (MT)

MT was evaluated using both radiographic and histology methods in order to gain better understanding of imaging strategies for future otitis media animal studies. T2-weighted MRI was used to measure MT in ex-vivo samples, which produced measurements in 2-dimensional space. CT imaging was also used to measure MT, but produced 3-dimensional measurements. Histology was also conducted to confirm MT findings. All calculations of measurements were standardized and represented in millimeters. The findings indicate that DEX treatment reduced MT significantly compared to HC treatment and the Control group.

Discussion

Untreated chinchillas in the current study showed significant progression of otitis media, with greatest amounts of MEE and MT. Treated chinchillas had improved ear conditions. Both Cipro HC and Ciprodex when used alone are effective treatments for otitis media. This study shows, however, that important differences were noted in the improved capacity of Ciprodex to clear middle ear effusion and to reduce mucosa in the middle ear cavity. This evidence could assist intervention strategies for patients suffering from chronic otitis media since the safety and benefits of topical otic drugs are well documented in the literature and preferred to systemic antibiotic treatments[8]. In this study, we evaluated the effect of Ciprodex using three methods: MEE, MT and otoscopy analyses. All three types of examinations arrived at the same conclusion, namely that Ciprodex reduced inflammation permanently and quickly.

Previous reportshave also documented the effectiveness of Ciprodex in treating otitis media in the chinchilla model [(9-11)]. The present studywas designed to evaluate Ciprodex as commercially available, and without altering concentrations, inorder to compare its use with another common glucocorticoid treatment, Cipro HC. Additionally, this study evaluated otitis media from different angles using a variety of tools, including radiographic analyses.

Our investigations show that Ciprodex may be the more potent drug of choice.

Conclusion

Topical Ciprodex is superior to Cipro HC as a treatment for otitis media.

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Novel Delivery Approach for the Treatment of Middle Ear Effusion at Time of Tympanostomy

Fabrice Piu, PhD[1]**,** Xiaobo Wang, MD[1]**,** Rayne Fernandez[1]**,** Luis Dellamary[2]**,** Anne Harrop[1]**,** Qiang Ye, PhD[2]**,** Elizabeth Keithley, PhD[1]**,** Jay Lichter, PhD[1]**,** Carl LeBel, PhD[1]
[1]Preclinical Research, [2]Formulation, Otonomy Inc, San Diego, CA

Objectives
Recurrent AOM and persistent OME are effectively treated with surgical placement of a tympanostomy tube. To reduce the incidence of post-operative otorrhea and tube occlusion, surgeons routinely administer antibiotic eardrops (e.g., Floxin, CIPRODEX®) during surgery and prescribe continued use for several days following surgery. However, these products are not FDA approved for this use and rely on caregiver compliance for efficacy. A sustained release antibiotic gel administered during surgery would eliminate compliance concerns and minimize post-operative complications.

Methods
Guinea pigs received a single intratympanic injection of ciprofloxacin (+/- dexamethasone) in a poloxamer hydrogel. Drug pharmacokinetic profile, histological evaluation of otic tissues, and bactericidal activity were monitored, and compared to that of topical administration of CIPRODEX® Otic (bid, 7 days). Efficacy studies were conducted in the chinchilla model of otitis media (S. pneumoniae and H. influenzae).

Results
A single administration of a sustained release ciprofloxacin (+/- dexamethasone) formulation yielded therapeutic middle ear drug exposure levels comparable or superior to that CIPRODEX® Otic treatment. Preliminary toxicology assessment, including inner ear and systemic drug exposure as well as histology of otic tissues, revealed a safety profile comparable to CIPRODEX® Otic. Furthermore efficacy in vivo as an anti-microbial against pathogens present in the middle ear was noted. Supporting data from studies in the chinchilla model of otitis media will be presented.

Conclusions
A sustained release antibiotic gel designed for intra-operative administration represents an attractive approach for the treatment of patients with middle ear effusion at the time of tympanostomy.

Nasopharyngeal Biofilms Are a Reservoir for Recurrent Acute Otitis Media

Anthony Sheyn, MD[1]**,** James Coticchia, MD[2]
[1]Department of Otolaryngology, Wayne State University, Detroit, MI, [2]Department of Otolaryngology, Wayne State University School of Medicine, Detroit, MI

Hypothesis
Polymicrobial nasopharyngeal biofilms play a role in the pathogenesis of recurrent acute otitis media (RAOM) in children.

Introduction
There is increasing evidence that nasopharyngeal biofilms play a significant role in the pathogenesis of otitis media in children. Over sixty-five percent of human infections have been linked to the biofilm phenotype. In addition, in addition the biofilm paradigm provides a paradigm for the chronic and recurrent nature of otitis media.

Biofilms are a unique life style of microorganisms defined as an assemblage of microbial cells enclosed in an exopolysaccharide(EPS) matrix. Biofilms are also 100 – 1000 times more resistant to antimicrobial therapy and the identification

of biofilms in both the middle ear and nasopharynx of otitis media suggests that these microbial ecosystems may play a dominant role in the pathogenesis of acute otitis media.

We attempted to prove this theory by answering several questions: What role do nasopharyngeal biofilms play in the pathogenesis of RAOM?; Are biofilms present in the nasopharyngeal mucosa of otitis prone children?; Do these nasopharyngeal biofilms contain middle ear pathogens?; Do these nasopharyngeal biofilms act as a significant reservoir for otitis prone children.

Methods and Materials

Several experiments in our laboratory were performed to help answer these questions utilizing three levels of evaluation: 1. Scanning electron microscopy; 2. Fluorescence in-situ hybridization; and 3. Real Time Polymerase Chair Reaction. Additionally an animal model was utilized to demonstrate how an ascending infection from nasopharyngeal biofilms can contribute to the development of middle ear disease.

Scanning Electron Microscopy:

The biofilm density of adenoids removed from children with RAOM to that of adenoids removed from children with a diagnosis of obstructive sleep apnea (OSA). A comparative microanatomic study of adenoid mucosa was performed using scanning electron microscopy in patients with diagnoses of RAOM and OSA.

Fluorescence in-situ Hybridization

Adenoid tissue was removed from the nasopharynx of children with diagnosed RAOM at the time of adenoidectomy. The tissue subsequently underwent hybridization with probes for the most common middle ear pathogens: *H. influenzae, S. pneumoniae, M. catarrhalis,* as well as various *Streptococcal* and *Staphyloccal* species.

Real-Time Polymerase Chain Reaction

Adenoid tissue was removed from patients undergoing adenoidectomy for RAOM or OSA. The adenoid tissue then underwent RT-PCR utilizing probes for *H. influenzae, S. pneumoniae,* and *M. catarrhalis* to quantify the amount of bacterial DNA present in the nasopharyngeal tissue of patients with RAOM versus those of patients with OSA.

Demonstration of Nasopharyngeal Biofilms in the Animal Model

Chinchillas were inoculated initially with the influenza A virus and subsequently with *S. pneumoniae.* Over a two week period each animal underwent tympanometry to determine the status of the middle ear. At the end of the two week time period the animals were sacrificed and underwent SEM to evaluate the presence and density of nasopharyngeal biofilms.

Results

Scanning Electron Microscopy:

After undergoing scanning electron microscopy it was found that the adenoid tissue obtained from patients with RAOM had on average 93.53% of the adenoid surface covered with biofilm (Figures 1a and 1b). In contrast, the adenoid tissue of patients with OSA had only 1.01% biofilm coverage on average (Figures 2a and 2b). Figure 3 shows that all specimens obtained from patients with RAOM had a biogilm coverage of at least 80%, whereas those with OSA had a biofilm coverage of no more than 10%. A Wilcoxian two sample test demonstrated that the results were statistically significant ($p<.0001$)

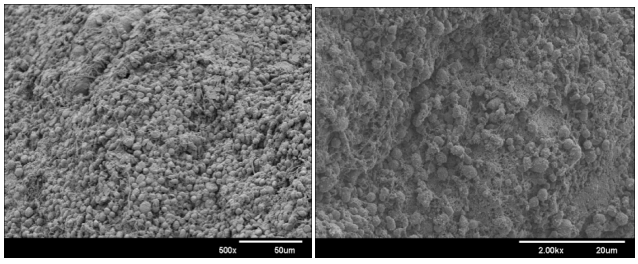

Fig. 1a and b: 500x and 2000x SEM images of biofilm coverage of adenoid tissue from a patient with RAOM

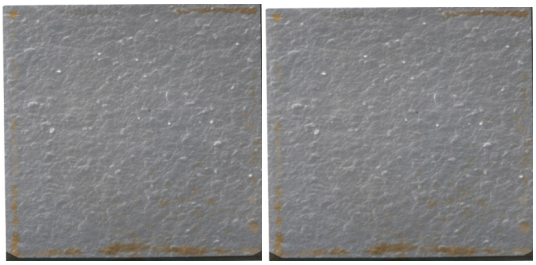

Fig.2a and b: 500x and 2000x SEM images of biofilm coverage of adenoid tissue from a patient with OSA

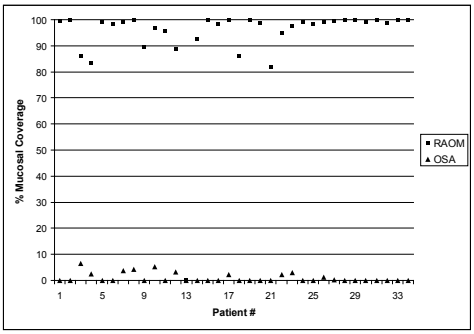

Fig. 3: %Mucosal coverage of adenoid by polymicrobial biofilms in patients with RAOM vs. OSA

Fluorescence in-situ Hybridization

We found that 3 of the adenoid specimens contained multiple middle ear pathogens. The majority of the pathogens were *M. catarrhalis* and *S. pneumoniae*. Figures 4a and b demonstrate the presence of Staphylococcus and Streptococcus, respectively, on adenoid specimens of two different patients with RAOM.

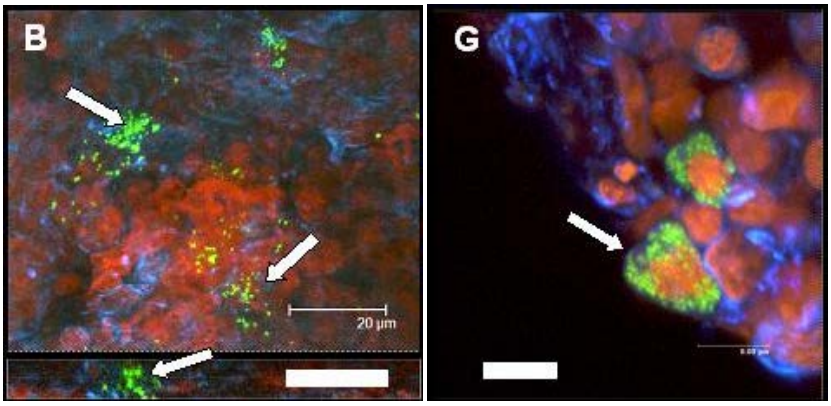

Fig. 4a: Extracellular biofilms with flourophores labeling Staphylococcus Fig. 4b: Biofilm pods containing S. pneumoniae cells species.

Real Time – Polymerase Chain Reaction

Results demonstrated that there was on average a 2059.57 increase in the amount of all bacterial species that were found in the adenoid tissue of patients with RAOM versus those with OSA. The greatest concentration of bacterial DNA was found to be that of *S. pneumoniae*. Using a paired t-test the results were found to be statistically significant (p=.045). The results are summarized in Figure 5.

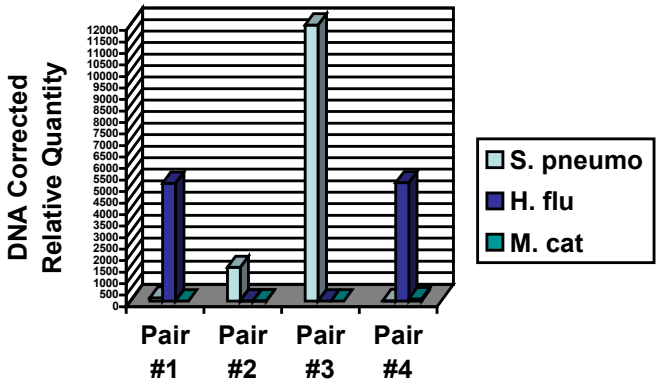

Fig. 5: Amount of Bacterial DNA in paired adenoid specimens (RAOM vs. OSA).

Demonstration of Nasopharyngeal Biofilms in the Animal Model

Over a two week time period 77.7% of the animals clinically significant middle ear infection and/or inflammation. Of these, 78.5% had nasopharyngeal biofilms identified and 64.2% had middle ear biofilms identified. No middle ear changes were noted in the control group, nor did these animals demonstrate biofilm formation in either the middle ear or the nasopharynx.

Additionally, the tympanometry results demonstrated that the peak of the middle ear disease, as measured by the negative middle ear pressure, peaked around day 7 for both left and right ears and began to resolve by day 14 (Figures 6a and 6b). No changes in middle ear pressure were seen in the experimental animals ($p<0.05$). Figure 7 demonstrates the amount of biofilm coverage on the nasopharyngeal tissue of the experimental animals based on tympanometry type. The greatest coverage was found in those animals with a Type B tympanogram ($p<0.05$).

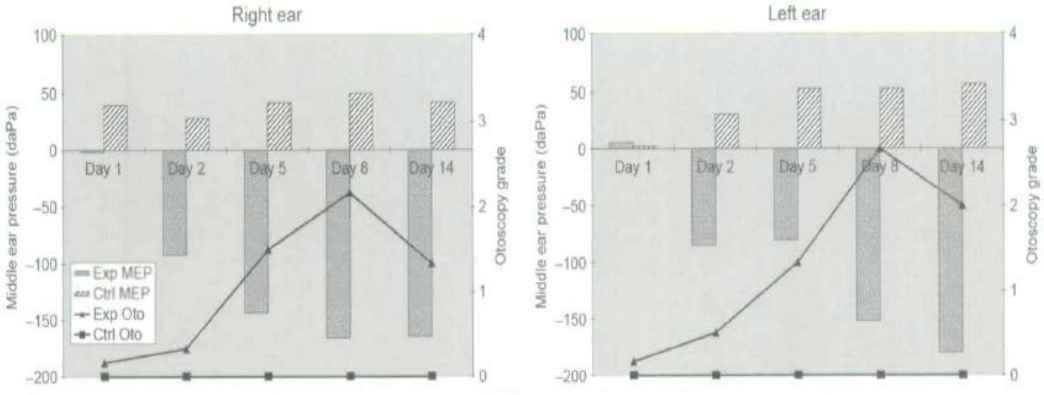

Fig. 6: Averaged experimental middle ear pressures over a 14 day period.

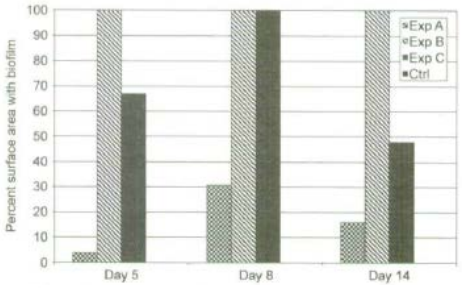

Fig. 7: Nasopharyngeal biofilm involvement in experimental animals with type A, B, and C tympanograms.

Discussion

There is increasing evidence that nasopharyngeal biofilms play a significant role in the pathogenesis of otitis media in children. Over sixty-five percent of human infections have been linked to the biofilm phenotype. In addition, in addition the biofilm paradigm provides a paradigm for the chronic and recurrent nature of otitis media.

Biofilms can exist attached to inert abiotic or biotic surfaces and are the predominant phenotypes expressed by 98% of bacteria within the environment. Biofilms form when individual cells adhere and coalesce to various surfaces. The proximity of the cells to a surface stimulates the production of the EPS matrix, which provides many survival advantages. The EPS matrix provides protection from the environment and the hosts mechanisms of opsonization and phagocytosis. Microchannels form connections among the microbes and affect the diffusion of antimicrobials, nutrient exchange, and removal of toxic metabolites. Hence, known mechanisms of altered sensitivity such as efflux pumps, modifying enzymes, and target mutations do not play a role in the resistance imparted by biofilms. These microchannels also allow for environmental and intercellular signals to be communicated throughout the EPS matrix.

Biofilms are also 100 – 1000 times more resistant to antimicrobial therapy and the identification of biofilms in both the middle ear and nasopharynx of otitis media suggests that these microbial ecosystems may play a dominant role in the pathogenesis of acute otitis media. The importance of bacterial biofilms in otitis media has been demonstrated by work by Post et al showing formation of biofilms within 24 hours in an animal model of acute otitis media. Additionally, our lab has shown that intranasal inoculation with influenza A followed by intranasal inoculation with *S. pneumoniae* of a chinchilla, which mimics the typical clinical presentation of AOM which is usually preceded by a viral URI, will produce acute middle ear disease with the associated finding of a nasopharyngeal biofilm.

In human studies, our laboratory has also identified nasopharyngeal biofilms in children with RAOM. Using SEM, adenoid mucosal surfaces removed from children with RAOM were found to have dense biofilm formation versus scant biofilm formation from control subjects. Additionally, half of the specimens that were collected for the SEM study were sent for fluorescent in-situ

hybridization to determine the genotypes of the bacteria that were present in the biofilm architecture. The majority of the bacterial DNA that was found in these specimens were those of the common middle ear pathogens, specifically *H. influenzae, S. pneumoniae, M. catarrhalis*. Real-time polymerase chain reaction was also used to demonstrate that the bacterial DNA lode was significantly greater in the adenoids of patients with RAOM versus those with OSA.
Based on these and other studies we believe that nasopharyngeal biofilms contribute to acute otitis media and recurrent otitis media in an ascending fashion. A middle ear pathogen initially enters the nasopharynx and begins colonization of the tissue. The biofilm phenotype is eventually expressed in the nasopharynx with periodic shedding of middle ear pathogens resulting in Eustachian tube dysfunction and entry of the pathogen into the tubotympanum, which, ultimately, leads to acute infections. Periodic treatments of the acute infections lead to development of antibiotic resistance of the nasopharyngeal biofilms which makes later infections much more difficult to treat. This theory is supported by the antibiotic resistance of patients who have had numerous ear infections and the fact that a significant fraction of these patients improve with a combination of antibiotic treatment and mechanical debridement, either myringotomy or adenoidectomy or both.

Conclusion
We found that all patients with RAOM had high densities of biofilm on their adenoids compared to those of patients with OSA. With PCR and FiSH we have demonstrated that the common middle ear pathogens make up a significant portion of the nasopharyngeal biofilms. We have also found that resistant biofilms act as a reservoir for recurrent infections of the middle ear. Based on the results of our studies, as well as those of others, we believe that the biofilm phenotype plays a major role in the pathogenesis of AOM and RAOM.

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Polymicrobial Infection Alters Bacterial Adhesion to Nasopharynx and Does Not Correlate with Cytokine Production

Ajay Krishnamurthy, PhD[1], Jenifer Alsemgeest[1], Jessica Browne[1], Allan Cripps[2], Jennelle Kyd, PhD[1]
[1]Capricornia Centre for Mucosal Immunology, CQUniversity, Rockhampton, Queensland, [2]Griffith Health, Griffith University, Gold Coast, Queensland

Introduction
Bacterial adherence to various mucosal and epithelial surfaces is an important step in its colonisation. Bacteria such as Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis cause respiratory tract infections, with respiratory viruses recognised as major triggers in enhancing bacterial adherence and colonisation. The innate immune system recognises the pathogen-associated molecular patterns through various pattern recognition receptors (PRR's). We hypothesised that in a polymicrobial infection the nature of microbe-host interactions will be altered, suppressing immune system and increasing bacterial adherence to respiratory epithelia.

Methods
In vitro human nasopharyngeal cells (Detroit 562 cells) were infected with and without adenovirus 5, along with the above mentioned bacteria. Following infection with single bacteria and appropriate combinations, bacterial adherence and induction of pro-inflammatory cytokines IL-6 and IL-8 was measured. The expression of PRR's was analysed in response to adenovirus infection by staining with antibodies against PRR's using FACS.

Results
The results showed that pre-viral infection enhanced M. catarrhalis adhesion to nasopharyngeal cells ($p<0.001$), characterised by reduced IL-6 and IL-8 levels at 12hrs post-infection. IL-8 levels at 24hrs were elevated during co-infection (S. pneumoniae+M. catarrhalis) in presence of virus, with a decrease in M. catarrhalis adherence. The expression of PRR's was dependent on the culture time and not much on the adenovirus infection.

Conclusion
Pre-viral infection affects the nature of the microbe-host interactions, suppresses the immune response while increasing bacterial adhesion. This finding could suggest a mechanism for colonising bacteria to progress from healthy colonisation in the nasopharynx to enhanced colonisation and inflammation during the disease state.

Bacterial Biofilm and Intracellular Infection in Otitis Media in Indigenous Australian Children

Ruth Thornton[1], Selma Wiertsema, PhD[1], Paul Rigby, PhD[2], Harvey Coates[3], Karen Prosser[4], Shyan Vijayasekaran[3], Anthony Keil[5], Peter Richmond[1]
[1]School of Paediatrics and Child Health, University of Western Australia, Subiaco, Western Australia, [2]Centre for Microscopy, Characterisation and Analysis, [3]Department of Otolaryngology, Head and Neck Surgery, University of Western Australia, Perth, Western Australia, [4]Vaccine Trials Group, Telethon Institute for Child Health Research, Perth, Western Australia, [5]Department of Microbiology, PathWest Laboratory Medicine WA, Perth, Western Australia

Introduction
Indigenous Australian children experience disproportionately high rates of otitis media and high rates of treatment failures. The reasons behind this are unclear, though evidence suggests that bacteria are able to persist in biofilm and/or intracellularly in the middle ear.

Objective
To investigate the presence and distribution of otopathogenic species on the middle ear mucosa of children from high risk populations.

Methods
Middle ear mucosal biopsies from 17 Australian Indigenous children undergoing surgery for ventilation tube insertion or tympanoplasty were examined for the presence of otopathogens. Species-specific fluorescence in situ hybridisation (FISH) was conducted and biopsies were imaged using confocal laser scanning microscopy. Biopsies were each hybridised with 1 universal bacterial probe and 2 species-specific FISH probes.

Results
At least one known otopathogen was identified in each sample with 82% of biopsies positive for biofilm presence and 94% positive for intracellular pathogens. Biofilm and intracellular bacteria were observed together in 82% of samples. Five of 8

biopsies were positive for Streptococcus pneumoniae, 6 of 10 for Haemophilus influenzae, and 7 of 11 samples tested for Moraxella catarrhalis were positive. Two samples examined for Pseudomonas aeruginosa were positive and 2 of 3 samples examined for Staphylococcus aureus were positive. All bacterial species were observed in biofilm structures and/or located intracellularly at similar rates.

Conclusions

Our results demonstrate that otopathogens reside intracellularly and/or in biofilms on the middle ear mucosa of high-risk Indigenous Australian children with persistent ear disease. This may help to explain the ineffectiveness of current otitis media treatments in this population.

Vaccine/Microbology

Cost Effectiveness of Pneumococcal Conjugate Vaccination Against Acute Otitis Media in Children: a Review

Maroeska Rovers, PhD[1], Chantal Boonacker[2], Pieter Broos[2], Elisabeth Sanders, PhD, MD[3], Anne Schilder, PhD, MD[4]
[1]Julius Center, University Medical Center Utrecht, Utrecht, Utrecht, [2]Julius Center, [3]Children, [4]Pediatric Otorhinolaryngology, UMC Utrecht, Utrecht, Utrecht

Introduction
So far the relationship between the costs and benefits of available vaccines for OM remains a controversial topic.

Objective
To systematically review the literature on the cost effectiveness of pneumococcal conjugate vaccination against AOM in children.

Methods
We searched PubMed, EMBASE, Cochrane and DARE from inception until 18 February 2010. We used the following keywords with their synonyms: 'Otitis Media', 'Children', 'Cost-effectiveness', 'Costs' and 'vaccine'. Costs per AOM episode averted were calculated based on the information in this literature.

Results
A total of 21 studies evaluating the cost effectiveness of pneumococcal conjugate vaccines were included. The cost per AOM episode averted varied from €168 to €4214, and assumed incidence rates varied from 20 952 to 118 000 per 100 000 children aged 0–10 years. Assumptions regarding direct and indirect costs varied between studies. The assumed vaccine efficacy of the 7-valent pneumococcal CRM197-conjugate vaccine was mainly adopted from two trials, which reported 6–8% efficacy. However, some studies assumed additional effects such as herd immunity or only took into account AOM episodes caused by serotypes included in the vaccine, which resulted in efficacy rates varying from 12% to 57%. Costs per AOM episode averted were inversely related to the assumed incidence rates of AOM and positively related to the estimated costs per AOM episode. The costs per AOM episode averted tended to be lower in industry-sponsored studies.

Conclusion
Key assumptions regarding the incidence and costs of AOM episodes have major implications for the estimated cost effectiveness of pneumococcal conjugate vaccination against AOM. Uniform methods for estimating direct and indirect costs of AOM should be agreed upon to reliably compare the cost effectiveness of available and future pneumococcal vaccines against AOM.

Prevention of Experimental Otitis Media (EOM) Due to Non-Vaccine Pneumococcal Serotypes

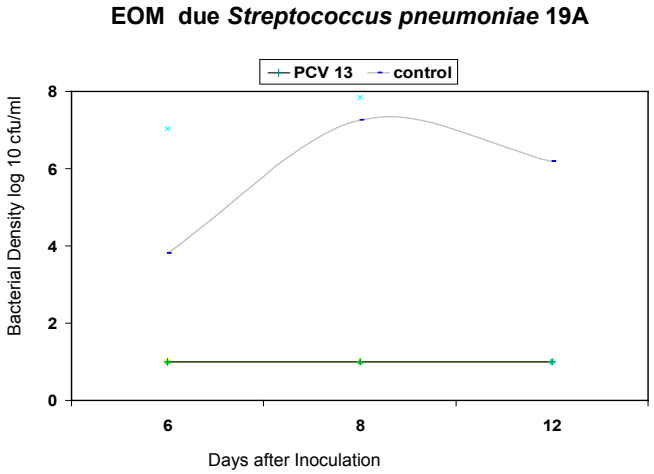
Marisol Figueira, MD, Vishakha Sabharwal, MD**,** Abbie Stevenson**,** Loc Truong**,** Stephen Pelton, MD
Pediatrics Infectious Diseases, Boston Medical Center, Boston, MA

Introduction
Following introduction of PCV 7 in the community nasopharyngeal colonization with non-vaccine serotypes (NVS) has increased. Recent studies of acute otitis media (AOM) report that NVS, specifically 19A is commonly isolate from the middle ear (ME). We evaluated PCV 13 in a chinchilla EOM model to determine its potential for prevention of EOM due to *Sp* 19A, 3 and 23A.

Methods
To date we have evaluated the efficacy of PCV 13 against EOM due to serotype 19A and 19F. Animals are immunized with a 3 dose regimen. Two weeks after 3rd dose, colonization is established by intranasal challenge, 4 days later barotraumas (BT) is performed and animals are followed for the development of EOM. When EOM is observed or by day 6-7 after inoculation,ME cultures are obtained and the proportion and density of ME infection were compared in immunized and control animals. Serotype specific immune response is evaluated by ELISA.

Results
To date studies of serotype 19A and 19F have shown complete protection against EOM compared to controls. Studies of serotypes 3 and 23A are in progress.

Conclusion
PCV13 demonstrated protection against EOM due to *Sp* 19A. As serotype 19A is the most common cause of pneumococcal otitis in the post PCV era it is likely that universal immunizationwith PCV 13 will further reduce the burden of AOM in children.



Antibody Response to *Streptococcus pneumoniae* Vaccine Targets PhtD, LytB, PcpA, PhtE and PlyD1 After Nasopharyngeal Colonization and Acute Otitis Media in Children

Michael Pichichero, MD[1], Ravinder Kaur, PhD[1], Janet Casey, MD[2], Qingfu Xu, PhD[1], Anthony Almudevar, PhD[3], Martina Ochs, PhD[4]
[1]Research Institute, Rochester General Hospital, Rochester, New York, [2]Pediatrics, Legacy Pediatrics, Rochester, New York, [3]Biostatistics, University of Rochester, Rochester, New York, [4]Vaccine Research, Sanofi Pasteur, Ontario, Canada

Introduction
The objective of this prospective study was to compare the natural antibodies elicited to 5 *S. pn*eumoniae (Spn) proteins simultaneously in a cohort of children 6-30 months of age during NP colonization and AOM. The results include: 1. Changes in the levels of PhtD, LytB, PcpA, PhtE and Ply- specific IgG antibodies in children as they increased in age from 6 to 30 months of age; 2. Changes in antibody levels following detected colonization of the NP with *Spn* and AOM infection by *Spn*; 3. Characterization of IgG and IgM in acute and convalescent serum following *Spn* AOM; and 4. Variations in individual antibody repertoire and responses in the AOM vaccine target age of children.

Methods
Five *Spn* target protein candidates were studied: PhtD and PhtE: pneumococcal histidine triad (Pht) proteins D and E with possible complement binding/degrading/interaction activity. LytB: a pneumococcal choline binding protein that is a cell wall hydrolases. PcpA: a choline binding surface protein that elicits protection against pneumococcal infection in an animal model. Pneumolysin (Ply): cytotoxin that has a wide range of cytotoxic and inhibitory effects on host tissue and immune cells. We used the pneumolysin derivative, PlyD1, which has three point mutations that do not interfere with anti-pneumolysin antibody responses.

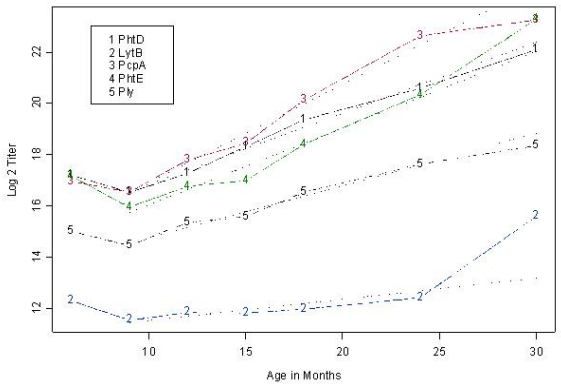
Results
S. pneumoniae (*Spn*) NP/OP (Nasopharyngeal and/or oropharyngeal)
colonization &*Spn* AOM episodes in different visits of children (Table 1).

S. pneumoniae (*Spn*) NP/OP (Nasopharyngeal and/or oropharyngeal) colonization & *Spn* AOM episodes in different visits of children

Total children enrolled in 3.5 years	**168**
Total # of visits	**737**
Total # of visits having *Spn* OP/NP colonization episodes	**259**
Total # children with *Spn* OP/NP colonization episodes	**102 (61%)**
Spn culture-positive at one sampling visit	**31 (18.5%)**
Spn culture-positive at two sampling visit	**25 (15%)**
Spn culture-positive at three sampling visit	**20 (12%)**
Spn culture-positive at four or more sampling visit	**27 (16%)**
No *Spn* at any of the seven visits	**66 (39%)**
Total # children with *Spn* AOM episodes	**32 (19%)**
# of children experienced one AOM due to *Spn*	**24 (14%)**
# of children experienced two AOM due to *Spn*	**8 (5%)**
# of children experienced three AOM due to *Spn*	**2 (1.1%)**

Tahle 1

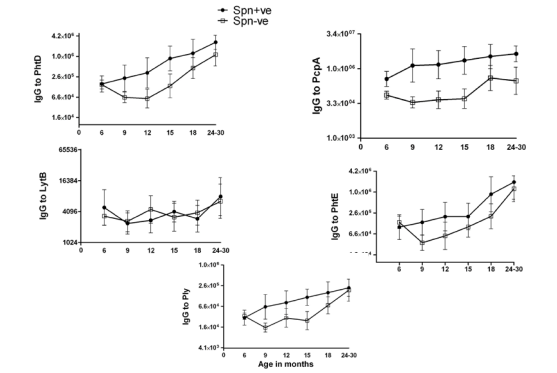
Natural acquisition of serum IgG antibodies to *Spn* proteins with age



Plots of the geometric mean titers (end point titer) during 7 sampling visits at 6, 9, 12, 15, 18, 24 and 30 months of age. The numbers of sera included at each time point were 107, 88, 65, 61, 55, 44, and 6.

Figure 1.

Comparison of serum IgG antibody GMTs to *Spn* proteins
NP colonized (·*Spn*+ve) and non-colonized (□ *Spn*-ve) healthy children



Comparison of convalescent serum IgG GMTs to *Spn* proteins PhtD, LytB, PcpA, PhtE and Ply following an AOM or an NP

Figure 2.

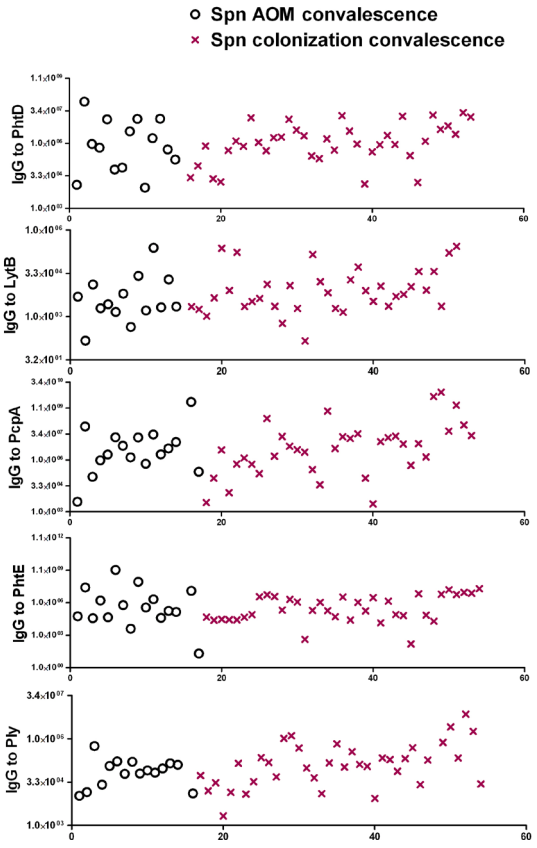


Figure 3.

IgG, IgM and IgA antibody levels to *Spn* proteins PhtD, LytB, PcpA, PhtE and Ply in paired acute and convalescent sera of children with *Spn* AOM

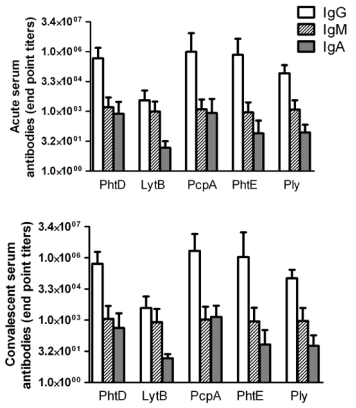


Figure 4.

Individual IgG, IgM and IgA antibody levels to *Spn* proteins PhtD, LytB, PcpA, PhtE and PlyD1 in acute and convalescent sera of children with *Spn* AOM

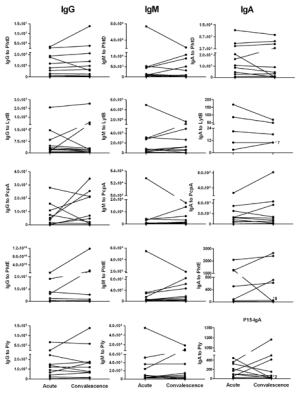


Figure 5.

Conclusions

This is the first study to compare antibody levels to 5 *Spn* proteins in children following asymptomatic NP colonization and episodes of AOM. We found increasing levels of PhtD, PcpA, PhtE and Ply-specific IgG antibodies in children as they increased in age from 6 to 30 months of age. Increased antibody levels were specifically measured following detected colonization of the NP. Evaluation of acute and convalescent sera from children with AOM revealed variations in response and kinetics of the response

Distribution and Dynamics of Streptococcus Pneumoniae Serotypes Causing Acute Otitis Media in Children in Southern Israel During the 10 Year-Period Before the Introduction of the 7-Valent Pneumococcal

Eugene Leibovitz, MD[1]**,** David Greenberg, MD[2]**,** Alberto Leiberman, MD[2]**,** Ron Dagan, MD[2]

[1]Pediatric Infectious Disease Unit, Soroka University Medical Center, Beer-Sheva, [2]Pediatric Infectious Diseases Unit, Soroka University Medical Center, Beer Sheva

Extended Abstract

Introduction

S. pneumoniae (SP) is a major pathogen isolated from the middle ear fluid of children with acute otitis media (AOM), being identified in 30-60% of AOM cases worldwide.After the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7), a decline in AOM cases, in particular of those caused by vaccine SP serotypes (VT), was observed.[1-6] At the same time, a shift in the proportion of pathogens causing AOM, with an increase in the proportion of nonvaccine SP serotypes (NVT) and of non-typable *Haemophilus influenzae,* was observed.[7,8]Of NVT, serotype 19A became a leading factor in nasopharyngeal colonization, invasive pneumococcal disease and AOM. In addition, serotype 19A has become increasingly resistant to antibiotics.[9,10] In southern Israel, a prospective epidemiologic study was initiated in 1999 to determine the dynamics, relative importance of VT and NVT, and antibiotic-resistance patterns of SP causing AOM in 2 distinct populations of children (Jews and Bedouins) differing in lifestyle and having minimal contact with each other.

Objectives

To determine the dynamics of serotype prevalence, potential coverage by pneumococcal conjugate vaccines (PCV) and antibiotic resistance patterns of SP causing AOM in southern Israel before PCV7 introduction in the routine national immunization program.

Methods

This prospective study was conducted from January 1st, 1999, to the 31st of December, 2008. The Negev region is a heterogeneously populated area, with >800,000 inhabitants (of whom >200,000 are children) belonging to 2 major ethnic groups, Jewish and Moslem Bedouin. Both ethnic groups have access to the same medical services. All children in the area are born in one hospital, where they also receive all emergency and inpatient services. No change in the recommendations concerning tympanocentesis in children with AOM occurred during the study period. During most of the study period, PCV7 had not yet been introduced in Israel. All SP isolates from middle ear fluid from children <5 years with AOM were included.. Demographic and clinical information was prospectively obtained from children with cultures positive for *S. pneumoniae*. For each episode, we

collected information on the patient's age, sex and ethnicity; the method of acquisition of the culture specimen (ie, by tympanocentesis or from pus draining after spontaneous perforation of the tympanic membrane); and the patient's recent history of antibiotic treatment.Data were obtained from the medical records, the child's physician, or the child's parents

Results

A total of 14,911 tympanocenteses yielded 5281(35%) SP (in 3060, 20.5%, as single pathogen, in 1859, 12.5% together with non typeable *Haemophilus influenzae* and in362 (2.4%) together with *Streptococcus pyogenes* or *Moraxella catarrhalis*. 2993 (57%) SP-AOM episodes occurred in Bedouin children. Mixed infections with SP occurred in 1407/2993 (47%) Bedouin chuildren *vs*. 814/2288 (36%) in Jewish children (P<0.001). Proportion of SP-AOM did not vary significantly (overall 35%, from 33% in 2007 to 38% in 2002 and 2003); 91% SP-AOM occurred in patients <2 years. Most frequent serotypes were 19F, 14, 23F and 19A in both Jewish and Bedouin children (Table 1). Serotype 14 (18%) was the most common serotype isolated in Jewish children and serotype 19F (15%) was the most common in Bedouin children. No significant changes were recorded in yearly proportions of serotypes 23F, 19F, 19A, 14 and 6A in both populations. Serotypes 6A and 19A were 6% and 10%, respectively, of isolates, with no increase with time among both populations.Non-PCV13 serotypes were more common in Bedouin than in Jewish children (25.5*vs*. 12.1%, *P*<0.001).The 4 most frquent non-PCV13 serotypes were (in descending order) 21, 35B, 15B/C and 33F (1% each). Serotypes included in PCV7, PCV10 and PCV13 represented 60%, 64%, 85% in Jewish children *vs*. 49%, 55% and 74%, respectively, in Bedouin children (*P*<0.001). TMP/SMX nonsuceptibility decreased significantly; nonsusceptibility to erythromycin (ERY) and clindamycin and multidrug resistant (MDR) rates increased significantly. No changes were found in proportion of SP with penicillin (PEN) MIC ≥1.0 µg/ml. Proportion of PEN and ERY-nonsusceptible and of MDR serotypes 6A and 19A increased significantly in Bedouins. No changes in susceptibility to PEN, ERY and in MDR rates were recorded among non-PCV13 isolates.

Conclusions

1) No significant changes were recorded in the yearly proportions of serotypes 23F, 19F, 19A, 14 and 6A in both ethnic populations; 2) Potential coverage of the 3 PCVs was higher in Jewish than in Bedouin children; 3) The high coverage of macrolide and multidrug-resistant SP by PCV13 and lack of changes in PEN and ERY susceptibility and in MDR isolates among the non-PCV13 isolates were encouraging.

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Serotype	All episodes n (%)	Jewish children n (%)	Bedouin children n (%)	*P* value***
19F**	795 (15)	355 (16)	440 (15)	0.522
14**	752 (14)	411 (18)	341 (12)	<0.001
23F**	528 (10)	341 (15)	287 (10)	0.424
19A	523 (10)	223 (10)	300 (10)	0.970
6B**	385 (7)	229 (10)	156 (5)	<0.001
6A	308 (6)	159 (7)	149 (5)	0.021
3	197 (4)	99 (4)	98 (3)	0.033
9V**	184 (4)	63 (3)	121 (4)	0.028
5	151 (3)	46 (2)	105 (4)	0.006
18C**	107 (2)	38 (1)	69 (2)	0.712
1	94 (2)	30 (1)	64 (2)	0.023
4**	58 (1)	16 (1)	42 (1)	0.034
7F	53 (1)	24 (1)	29 (1)	0.717
Other	1101 (21)	333 (15)	768 (26)	<0.001
Total	5236	2267	2969	

* Serotypes not available for 45 *S. pneumoniae* isolates

** Serotypes included in PCV7

*** Adjusted for year, age (months), season, antibiotic treatment last month and previous AOM history

Table 1. *S. pneumoniae* Serotype Distribution* (in Decreasing Frequency) in 5,236 AOM Episodes from 1999 through 2008

Dominance of H. Influenzae, but *Not S. pneumoniae* or *M. catarrhalis*, in Ear Discharge Compared to the Nasopharynx in Paired Swabs

Michael Binks, Peter Christensen, Robyn Marsh, Peter Morris, PhD, Amanda Leach, PhD, **Heidi Smith-Vaughan, PhD**
Child Health Division, Menzies school of health research, Darwin, Northern Territory

Introduction
Nasopharyngeal respiratory pathogen load and total bacterial load predict presence and severity of ear disease. Indigenous Australian children are at high risk of acute otitis media with perforation (AOMwiP).

Objective
To determine the relationship between the respiratory pathogen load in the nasopharynx and ear discharge of children with AOMwiP.

Methods
Culture and quantitative real time PCR estimation of pneumococcus, H.influenzae, M.catarrhalis and total bacterial load were performed on 32 matched nasopharyngeal (NP) and middle ear discharge (MED) swabs from 25 children with AOMwiP.

Results
Culture of NP swabs detected the three pathogens in >72% of swabs, which was marginally increased by real-time PCR detection. Culture of MED detected H.influenzae in 66% swabs, pneumococcus in 34% swabs, and M.catarrhalis in 6.3% of swabs; real-time PCR increased these detection rates to 94%, 66%, and 12.5%, respectively. By PCR, the mean load was significantly higher in the MED than the NP for H.influenzae (p=0.045) only. As a proportion of total bacterial load in the MED, H.influenzae ranged from 0-61% (mean 11%); whereas pneumococcus and M.catarrhalis were consistently <4% of the total bacterial load. NP and MED loads correlated negatively for H.influenzae, and positively for pneumococcus and M.catarrhalis.

Conclusions
Nontypeable H. influenzae is a major causative pathogen of AOMwiP. This study demonstrated significantly higher loads of H. influenzae in MED compared with loads in the NP, and confirmed that H. influenzae has a predilection for the middle ear. Quantitative methods are needed to measure the impact of interventions for AOMwiP.

Bacterial Resistance in Nasopharyngeal Samples from Otitis-Prone Children in Sickness and in Health

Ann Hermansson, MD, PhD[1], Marie Gisselsson-Solén, MD[1], Åsa Melhus, PhD, MD[2]
[1]ENT, University of Lund, Lund, Skåne, [2]Medical microbiology, Uppsala Universitet, Uppsala

Objectives
The occurrence of bacteria with resistance or reduced sensitivity to antibiotics is a growing problem especially in small otitis-prone children. In Sweden treatment with beta-lactam antibiotics have been standard so far.

Methods
In a study of small otitis-prone children were the effect of pnemococcal vaccination have been explored nasopharyngeal swabs have been obtained both at infections and in health. The occurance rates of pneumococci with a reduced susceptibility/resistance

to penicillin, erytromycin or tetracyclin, of betalactamase-producing *H. influenzae* and of *H. influenzae* with chromosomal resistance were analyzed.

Results
H. influenzae with chromosomal resistance seemed to be more frequent in the vaccinated cohort which is interesting since they have been scarce in Sweden so far.
Pneumococci with some sort of reduced susceptibility to antibiotics were found in 58 of the totally 1150 nasopharyngeal cultures. In 30 of these cases, the pneumococci showed a decreased susceptibility to penicillin, and in the remaining 28 cases, they were resistant to erytromycin (13 cases), tetracyclin (6 cases), trimetoprimsulfametazole (15 cases) and clindamycin (1 case). Since the patients had cultures taken quite often, we examined how many episodes with not entirely sensitive pneumococci we could find. Most of the 24 patients required a resistant strain only once, but three of them aquired resistant strains twice. Betalacamase-producing *H. influenzae* was found in 19 cultures from 15 patients (6 vaccinated and 9 controls).

Concusions
Although the occurrence of resistant bacteria still is quite moderate in Sweden the problems are increasing. Important mechanisms are studied and explored.

Microbiology of Spontaneous Otorrhea in Italian Children with Acute Otitis Media: A Ten-Year Study

Paola Marchisio, MD[1], Sara Torretta, MD[2], Miriam Fattizzo, MD[3], Giada Albertario, MD[3], Lorenzo Pignataro, MD[2], Susanna Esposito, MD[1], Nicola Principi, MD[1]
[1]Department of Maternal and Pediatric Sciences, [2]Department of Specialistic Surgical Sciences, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Milan, [3]Department of Maternal and Pediatric Sciences, University of Milan, Milan, Milan

Introduction
Acute otitis media (AOM) is the most common disease occurring in infants and children and has major medical, social and economic effects.[1] As far as the bacterial etiology of AOM is concerned, it is known that *Streptococcus pneumoniae* (*S.pneumoniae*), nontypable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis* (*M.catarrhalis*) and *Streptococcus pyogenes* (*S.pyogenes*) are the most common causes of AOM in almost any geographical area and age range[2].

S. pneumoniae is the most relevant and frequent cause of severe disease, although it is isolated less frequently in areas where the heptavalent conjugated pneumococcal vaccine (PCV-7) is used. PCV-7 has also modified the frequency of pneumococcal serotypes as major etiological agents. Before the use of PCV-7, the most frequently serotypes encountered in Europe and the United States were 23F, 19F, 19A, 14, 6A and 6B; the introduction of the vaccine has reduced the frequency of vaccine serotypes and led to a limited increase the incidence of diseases caused by non-vaccine serotypes that does not counterbalance the reduction in vaccine serotypes. Among the non-vaccine serotypes, serotype 19A (an invasive strain often resistant to the most commonly used antibiotics) has gained particular relevance, mainly in cases of mastoiditis[3-5]. NTHi currently causes one-third of the cases of AOM, and its absolute frequency has increasedin USA since the introduction of PCV-7 because of the reduction in *S. pneumoniae*. *M. catarrhalis* is often present in mixed infections and is frequently found in young children with a positive clinical outcome of spontaneous recovery and a lower risk of spontaneous membrane rupture or mastoiditis.*S.pyogenes*, which was the most commonly found otopathogen during the preantibiotic era, is deemed responsible for a limited percentage of AOM cases. It significantly correlates with more severe acute episodes in terms of spontaneous otorrhea and a higher risk of intracranial complications including mastoiditis.[6-7].
Since the early '90s the Milan Otitis Media Outpatient Unit has recorded the prevalence of microrganisms isolated from middle ear effusion in children with acute otitis media. The Unit is based in the Pediatric Department of the University of Milan and is the reference point for children with middle ear diseases in Milan. The information obtained from children presenting to the Unit for acute otitis media has been systematically entered a microbiology database and subsequently used to determine trends in bacterial distribution. In 2000 the use of tympanocentesis was interrupted because it is a difficult and invasive procedure that should be performed in special cases of presumed uncommon etiology[8]. On the contrary, the early collection of middle ear fluid in case of spontaneous rupture of the tympanic membrane was favoured.
We aimed to describe the microbiological characteristics and changes of AOM with spontaneous otorrhea over a ten-year period.

Patients and Methods
This retrospective study included all available otopathogens isolated from middle fluid specimens derived from spontaneous otorrhea between January 1, 2001, and June 30, 2010 in the Otitis Media Outpatient Unit inMilan. Infants and children aged 6 month to 6 years and who met strict criteria for AOM were enrolled. All the children were otherwise generally healthy.
The diagnosis of acute otitis media was based on (a) evidence of middle ear effusion (demonstrated by otoscopy which showed either opacification not attributable to scarring or an air-fluid level or absent mobility of the tympanic membrane as demonstrated

by pneumatic otoscopy) accompanied by definite abnormalities of the eardrum that indicate acute inflammation (including bulgingof the tympanic membrane, accompanied by marked discoloration such as hyperemia , white or yellow) and by acute symptoms (fever, irritability, or earache) and (b) otorrhea within 12 hours of spontaneous rupture of the tympanic membrane .
We excluded children with tubes, craniofacial abnormalities, chronic middle ear conditions (including chronic perforation), those who had received antibiotics in the previous 2 weeks, and those being given topical treatments.
Middle ear fluid was obtained by aspiration: the tympanic membrane was visualized after the otorrhea fluid was swabbed, and the ear canal was cleansed with a dry cotton swab. After the otorrhea fluid was removed, the remaining middle ear effusion was aspirated with a syringe and a 45-degree-bent 18-gauge spinal needle through the perforation. In case of bilateral disease only one ear was tapped or aspirated.
The middle ear specimens were inoculated into Stuart transport medium tubes and processed within 2 hours. Initial isolation procedures were performed on blood and chocolate agar plates. All isolates were identified by standard laboratory procedures and in vitro susceptibility testing was performed. Microbiologic processing was the same throughout the study period. A standard antibiogram was determined using the Kirby-Bauer disk diffusion method and then MIC were determined.
As PCV-7 was progressively introduced in our community in 2005, the study was divided in 3 periods: 2001-2004, 2005-2007 and 2008-2010.

Results
Six hundred one specimens were obtained from 388 children. Most were males (221,57.0%) and about 75% were younger than 36 months (Table 1). Otopathogens were identified in 71% of the episodes. The average distribution of the pathogens in the 10-year period was: *S. pneumoniae* 15.6%, NTHi 34.4%, *S.pyogenes* 13.0%, *M. catarrhalis* 0.8%. The frequency of *S.pneumoniae* declined (18.7%, 15,6%, 13.7%) whereas NTHi increased (32.5%, 33.5%, 36.2%) in periods 1 to 3. A decline with increasing ages was noted for *S. pneumoniae*, NTHi remained stable and then declined , whereas *S.pyogenes* increased with increasing age (Figure 1). The number of previous recurrent episodes of AOMwas associated with an increased prevalence of NTHi (figure 2). No substantial difference was seen in the distribution of otopathogens between children who had received PCV-7 and those who had not (*S.pneumoniae*18.9% versus 14.3%, NTHi 35.5 % versus 34.0%, *S.pyogenes* 13.0% versus 12.9% in vaccinated versus non vaccinated children, respectively).
Resistance to penicillin/ampicillin and to macrolides of *S.pneumoniae* and NTHiwas limitedwhereas resistance of S.pyogenes to macrolides reached 20%(Table 2).

Discussion
Our study shows that , in Italian children with AOM,NTHiis the predominant otopathogen, followed by *S.pneumoniae* and *S.pyogenes*. The findings differ from other studies,[9-10] but they are in line with those described in Italy by our group in the '90s.[11]
The microbiology of AOM has slowly changed over the 10-year period: *S.pneumoniae* decreased over time, where the role of NTHi increased. The trend over time can be, at least in part, be attributable to the introduction of PCV-7 in 2005. The decreasing role of *S.pneumoniae* is in agreement with the studies is USA and in France which described, in the first years after PCV-7 universal introduction, a shift in the predominant otopathogen from *S.pneumoniae* to NTHi.[4,5,12] However, our data stress the importance of considering both the impact of PCV-7 vaccination per se and herd immunity, as if we consider only children vaccinated versus the ones who were non vaccinated, the decrease in the role of *S.pneumoniae* becomes less evident. In addition, it must be underlined that the decrease in *S.pneumoniae* prevalence began before the introduction of PCV-7, suggesting that secular trends can have a role.
NTHiwas the main otopathogen in our population throughout the study period. Its predominance may be only in part be explained by the fact that about 50% of the children included in our series had had a history of recurrent AOM, as it is known that recurrent AOM is associated with a major role of H.influenzae.[13]
The role of *S.pyogenes* in causing AOM was relevant and predominant in children older than 3 years of age with spontaneously draining ear.This finding is in line with data from Israel[7]and might have an impact on clinical practice. Macrolides are considered among the possible choices for AOM treatment, and especially new macrolides such as azithromycin are deemed to be effective in very simple treatment regimens.[14] So far, resistance among strains of *S.pneumoniae* has been considered the main reason to limit widespread use of macrolides. Our findings which enlighten the role of GAS give further support to a cautious use of this class of antimicrobials in treating acute otitis media in areas in which, an in Italy, resistance to macrolides is relevant[15].
The antibiotic resistance of strains of *S.pneumoniae* and NTHi isolated from middle ear fluid was limited. Unlike in other countries, resistance to *S.pneumoniae* penicillin in Italy is still about 20%, with only half of the resistant strains having MIC values of more than 4 mcg/mL, whereas there is 40% resistance to macrolides and azalides.[15] In addition,β-lactamases production has been reported in up to 40% of the strains of H.influenzaeand 80 % of *M.catarrhalis*. Our findings are therefore interesting because they suggest that, as regards AOM, one should not rely on data derived from microbiological surveys including data from various infectious sites, diseases and patients but, whenever possible, address the issue of choosing the right antibiotic on the basis of the real antibiotic resistance of only otopathogens.

Our study has some limitations. Tympanocentesis was not used because of its difficulty and invasiveness. However it is unlikely that this could impact in our findings, as it has been demonstrated that culturing otorrhea is accurate, as the culture of the middle ear fluid is obtained through the tympanic membrane perforation.[16] The study was retrospective, but it is unlikely that this could have impacted ion the results, given the consistency of diagnostic inclusion, the adoption of the same inclusion criteria, the fact that the same investigator was in charge of examining the children and culturing the middle ear fluid. Finally no attempt was made to ascertain the serotypes or the resistance of *S. pneumoniae* isolates as this was considered beyond the aim of the study.
In conclusion, our data support continuous monitoring of the changes of AOM microbiology in a specific country and open new questions, considering the role of NTHi, regarding the role of vaccines in the prevention of AOM.

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Table 1 – Characteristics of the study population

	Children n° 388 (%)	Specimens n° 601 (%)
Sex (males)	221 (57)	--
Age (group)		
< 18 months	153 (39.4)	214 (35.6)
19-36 months	131 (33.8)	212 (35.3)
37-72 months	104 (26.8)	175 (29.1)
Period		
2001-2004	124 (31.9)	166 (27.6)
2005-2007	110 (28.4)	173 (28.8)
2008-2010	154 (39.7)	262 (43.6)
N° AOM in the previous 6 months	47 (12.1)	47 (7.8)
None	147 (37.9)	218 (36.3)
1 – 2	194 (50.0)	336 (55.9)
3 or more	86 (22.2)	169 (28.1)
Previous PCV-7 vaccine		

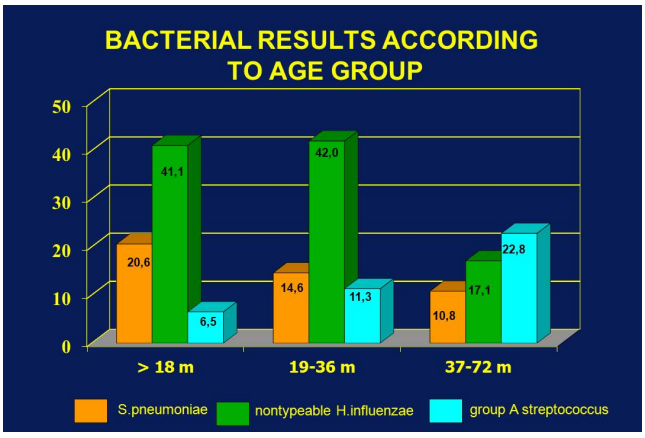

Figure 1 – Bacterial results according to age group

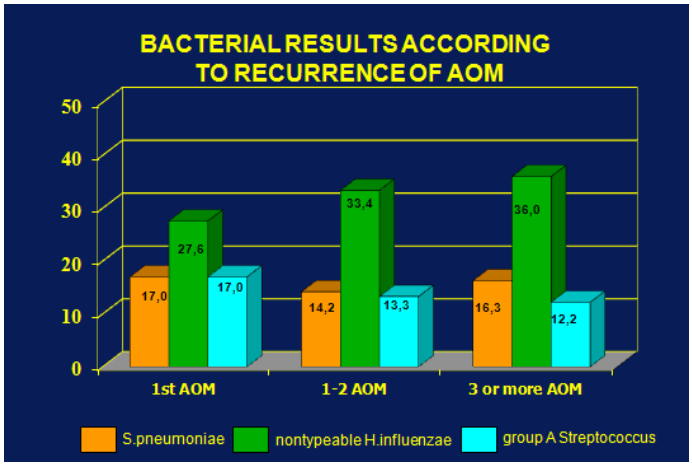

Figure 2 – Bacterial results according to recurrence of acute otitis media

Table 2- Resistance of otopathogens to penicillin/amoxicillin and to macrolides: comparison of resistance in isolates from pediatric AOM and isolates from overall population

pathogen	antibiotic	% R in AOM episodes (average, 2001-2010)	% R in Italy (overall population, 2008)
S.pneumoniae	penicillin	8	23
S.pneumoniae	macrolides	18	40
nontypeable H.influenzae	amoxicillin	8	22
nontypeable H.influenzae	macrolides	4	-
S.pyogenes	macrolides	20	25
M.catarrhalis	amoxicillin	90	90

Expression of Critical Nontypeable *Haemophilus influenzae* Virulence Determinants Is Altered in an In Vitro Model of the Polymicrobial Disease Otitis Media

Michael Mullins, PhD**,** Zachary Jordan**,** Lauren Bakaletz, PhD
The Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital & The Ohio State University College of Med., Columbus, Ohio

Otitis media (OM) is a polymicrobial disease of the uppermost airway wherein commensal bacteria of the pediatric nasopharynx gain access to the middle ear as a result of prior or concurrent upper respiratory tract viral infection. In an experimental OM model, we previously demonstrated that respiratory syncytial virus (RSV) or adenovirus (AV) infection resulted in an increased relative bacterial load of nontypeable Haemophilus influenzae (NTHI) in the nasopharynx, likely due to altered expression of host cell receptors in concert with bacterial virulence determinants. We therefore hypothesized that NTHI modulates expression of these important determinants in response to changes in the host microenvironment caused by viral co-infection.
To better characterize molecular changes that occur during co-infection, we utilized NTHI luciferase reporter constructs to monitor in vitro expression of two critical NTHI adhesins, outer membrane protein (OMP) P5 and the type IV pilus (Tfp), as well as the NTHI porin, OMP P2. Each exhibited identical growth kinetics as measured by optical density, however the time required for maximal luciferase expression and overall luminescence varied amongst these reporters upon inoculation of NTHI either into cell culture medium or onto normal human bronchial epithelial cells. Overall, the OMP P2 promoter construct exhibited the greatest luminescence, followed by the OMP P5 reporter, then that of the major Tfp subunit. RSV and AV are known to induce expression of specific receptors and TLR4 on host epithelial cells to which NTHI can bind, and we observed greater expression of Tfp and OMP P5 when inoculated onto virus-infected respiratory tract epithelial cells, as measured by detection of luciferase and to be confirmed by qRT-PCR.
These data demonstrated that NTHI modulated expression of important virulence determinants in response to microenvironmental changes induced by viral co-infection. Understanding the mechanisms that underlie polymicrobial infections will facilitate development of more effective therapeutic or preventative strategies for diseases like OM.

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Nontypeable *Haemophilus influenzae* Type IV Pili: Biogenesis of a Critical Colonization Factor

Michael Carruthers, PhD, Robert Munson, Jr., PhD**,** Lauren Bakaletz, PhD
Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital, Columbus, OH

Nontypeable *Haemophilus influenzae* (NTHI) is a causative agent of multiple respiratory tract diseases such as otitis media. While we have demonstrated that NTHI type IV pili (Tfp) play a role in NTHI-induced experimental OM pathogenesis and that vaccine candidates directed towards the NTHI Tfp pilin have demonstrated efficacy in prevention of NTHI-induced experimental OM, little is known about the biogenesis of NTHI Tfp. Herein, we characterized the interactions between proteins in the NTHI Tfp biogenesis complex.

In order to study interactions between members of the NTHI strain 86-028NP *com* locus was expressed in *E. coli*. Expression of the *com* locus allowed for detection of a heat/SDS resistant high molecular mass complex via Western blot with an antibody against ComE, which was indicative of a secretin or pore through which Tfp pass through the outer-membrane. Mutational analysis performed on the *com* locus in *E. coli* to identify the Com proteins necessary for ComE secretin formation demonstrated that ComD was required for ComE secretin formation, which indicated a ComD-ComE interaction. To determine if the Com proteins interact, a reversible cross-linker was used to cross-link potential complexes. Western blot analysis of cross-linked samples with primary antibodies against individual Com proteins demonstrated a drastic reduction in each of the monomeric forms of the Com proteins and an appearance of a high molecular mass band. These data suggested that Com proteins physically interact to form a complex that may be involved in formation of NTHI Tfp.

Our data are first to demonstrate that NTHI ComD is required for secretin formation and that NTHI Com proteins form a complex. Elucidation of the biogenesis of adhesins critical for NTHI colonization, such as Tfp will aid in the design of therapeutics or the preventive means against NTHI-induced disease.

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Epidemiology/Microbiology

Hearing Loss in Adolescents with Abnormal Tympanograms, History of Frequent Ear Infections, and Loud Noise Exposure: the U.S. National Health and Nutrition Examination Survey (NHANES), 2005-2008

Howard J. Hoffman[1], May S. Chiu[1], Katalin G. Losonczy[1], Christa L. Themann[2]
[1]Epidemiology and Statistics, NIDCD, NIH, Bethesda, Maryland, [2]Hearing Loss Prevention Section, NIOSH, CDC, Cincinnati, Ohio

Introduction

This report analyzes tympanograms and reported clinical or epidemiological risk factors relating to hearing health, as potential predictors of hearing loss in adolescents. Tympanograms and hearing thresholds have been collected as a standard part of the National Health and Nutrition Examination Survey (NHANES) since 1988, when NHANES III began testing the hearing of school-aged children. Only one report from NHANES III has been published based on the tympanometric results,[1] while the hearing results were described in several papers.[2,3]

Compared to hearing thresholds, scant attention has been given to tympanometryin the public health field, apart from scattered clinical studies stretching back over the past four decades.[4-6]The terminology and choice of critical parameters to describe impedance audiometry (includes tympanometry) is unfamiliar to most epidemiologists or public health researchers and has been a subject of controversy even among experts in the field.[7] The basic vocabulary isadapted from physics from which the study of acoustics originates. An important concept, acoustic immittance, is a general term referring to how well energy flows through theouter ear and middle ear system. The "obstruction" to the flow of energy is termed impedance. In recording measurements, it is more convenient to plot the inverse (reciprocal) of impedance, admittance, which isthe standard term describing the response of the middle and outer ear when pressure is applied through the ear canal after sealing off air flow from outside.

Systematic studies of tympanometry have been reported in the clinical literature, particularly for young pre-school and school-age children for whom abnormal tympanograms often signify pathological dysfunction of the middle ear (e.g., otitis media).[8-11]However, the majority ofstudies on unselected or population-based sampleshave useda single stimulus frequency (220 Hz or 226 Hz) to assesstympanometryand then describe the results for screening purposes by characterizing normal ranges of peak compliance (admittance) and middle ear pressure in relation to age, sex, race or ethnic groups, and children with normal hearing or severe-to-profound sensorineural hearing loss.[12-17]Whilesome clinical studies have documented the association of abnormal tympanograms with the subject's hearing level, the focus has been mostly on the transitory versus longer-term nature of abnormal tympanograms, particularly in young children, and not the attendant hearing loss.[8,11,18,19]In this paper, we adopt the view that abnormal tympanogramsshould be assessed in terms of hearing threshold levels, since that is the metric most often used to assess impact of hearing disorders.[20]

The age range of our available sample, adolescents 12 to 19 years old, has received much less attention in the past for the application of impedance audiometry and, hence, this report will supplement the available literature. Our objective in this report is to determine to what extent tympanogram type, history of ear infections, and noise exposure separately or jointly predict adolescent hearing loss. We have used multivariatestatistical methods that allow us to assess the strength of associations while simultaneously adjusting for the effects of other potential contributors to hearing loss.

Materials and Methods

The NHANESis a stratified, multistage probability sample of the civilian, non-institutionalized population of the United States.Since 1999, NHANES has been a continuously operating, general purpose survey including many different health examson about 5,000 subjects annually. The sample consists of randomly-selected children and adults across the lifespan (age 1 month through 85+ years). From the beginning of the U.S. national health examination survey in 1959, hearing tests have always been included, although the age range for hearing exams has varied. Tympanometry was added to the survey in the third cycle (1988-1994), when the test technology had been sufficiently developed to make it feasible in a field study environment.

The data analyzed in this report are based on data fromNHANES 2005–2008, when a full sample of 3,526adolescents, aged 12 to 19 years, representing 33.5 million in the U.S. population, were invited to participate in the heath exams. The adolescents selected for the study were first identified during home interviews. If the child was less than 16 years old, parents/caregivers provided answers to questions about their children's hearing and on many other health-related topics. These questions were asked directly of the adolescents aged 16 to 19 years. Of the adolescents selected for the NHANES exams, 3,169 (93.8%) participated in the hearing exam (Table 1). The hearing protocol includedotoscopic inspection of both ears, impedance

audiometry(tympanometry and acoustic reflex screening)in each ear, andpure-tone, air-conduction audiometry measuring thresholds at frequencies of 0.5, 1, 2, 3, 4, 6, and 8 kHz in each ear. While both tympanometry and acoustic reflex screening were performed on each ear, only the tympanograms are currently available in public use files for analysis.

The hearing exams were conducted in a specially-designed, dedicatedspace for audiometry located in the mobile examination center (MEC).A sound booth manufactured by Acoustic Systems has been built into a room in one of the four trailers that comprise the MEC. Background noise levels in the sound booth met or were below the maximum permissible ambient noise levels for ears-covered hearing testing from 500-8000 Hz, as specified by ANSI S3.1-1999 (R2008). Hearing thresholds were measured with Interacoustics Model AD226 audiometers using standard TDH-39P headphones with acoustically-transparent disposable hygienic covers. EARtone™ 3A insert earphones using disposable foam tips were used when the subject had collapsing ear canals or large intra-aural differences in hearing thresholds.

Micro Audiometrics Earscan™ acoustic impedance tympanometers were used in NHANES 2005–2008. Testing was performed by a trained examiner (health technician) in the audiometry room of the NHANES MEC. Tympanometry is an objective test of middle ear function, assessing the mobility of the eardrum, from which the function of the middle ear system can be inferred. The equipment is automated, performing the test and recording the results without any need for response or feedback from the examinee. The equipment ran an automatic protocol after a hermetic seal was obtained by the health technician. The test is conducted by sealing off the entrance to the ear canal with a rubber cuff, changing the air pressure within the ear canal, and recording the flexibility of the eardrum in response to the changing pressure. The tympanometer produced a graphical output, which was reviewed by the health technician for smoothness and symmetry; the test was re-run if the first results were noisy or flat.

The automated tympanometer utilizes an electromagnetic impedance unit incorporating a pressure transducer to indicate changes in compliance of the tympanic membrane by varying the air pressure in the sealed ear canal. This is accomplished by measuring the sound pressure level (SPL) of a probe tone introduced into the ear canal (226 Hz at 85 dB SPL), while the air pressure in the ear canal is changing in a positive to negative sweep from +200 to –312 decaPascals (daPa). The pump speed, 150 daPa/second, was set at the normal slow speed; the fast speed setting was not used.The output derived from the procedure includes numeric values for maximum middle ear compliance(peak-compensated static acoustic admittance), the middle ear pressure corresponding to maximum "peak" compliance, a measure of the shape of the tympanometric curve(tympanometric curve *width* at 50% of the peak height),[21]and the physical volume of the outer ear canal. The physical volume calibration of the tympanometer was checked at the start and end of each stand and daily throughout testing. Air pressure was calibrated automatically by the unit each time it was turned on. The tympanometer also received an exhaustive NIST-traceable calibration check annually.

The normal eardrum is most flexible when the air pressure in the ear canal is near zero (i.e., equal to the ambient air pressure). Peak mobility (maximum compliance) occurring at extreme positive or negative pressures is an indication of amiddle ear disorder.[22-26]If the eardrum shows little mobility at any pressure, it indicates that something in the middle ear system—such as fluid from an infection or fixation of the ossicles—is preventing the eardrum from vibrating properly in response to sound.[27-29]Tympanometry can also indicate that the eardrum is too flexible or that the eardrum is perforated.[12,28-30] While tympanometry can point out problems with how the ear is functioning—which can affect hearing sensitivity as a conductive loss—it does not directly indicate how well a person can hear. It is important, however, forgauging whether an individual with hearing loss may have either a conductive or sensorineural hearing loss or perhaps both, i.e., mixed types of hearing loss.[31-33]

The tympanometry portion of the NHANES data file includes equivalent volume compliance data for pressure values sweeping from +198 daPa down to -300 daPa. Each tympanogram "curve", one for each ear, was plotted on a standardized graph and stored electronically. One co-author (MSC) was trainedto code the tympanogram curvesinto a modified Jerger classification scheme: Types A, A_s, A_d, B, C, or "other".[8,12,30] The large majority, 73%, were coded as Type A tympanograms, withnormal-appearing,symmetrical peakshavingmaximum compliance at approximately 0 daPa (range –100 to +100). The Type A_s("s" for shallow or stiff) tympanogramshave the same normal range, but are distinguished by having peak heightsat maximum compliance values between 0.3 and 0.4 ml. Conversely, Type A_d("d" for deep) tympanograms have peak heights exceeding 1.5 ml, whilethe middle ear pressure corresponding to maximum compliance still occurs within the normal range ($\pm$ 100 daPa). These A_d tympanograms are often associated with scarred or flaccid ear drums that can result from tympanostomy tube insertions and healed eardrum perforations.[30]

Some tympanogram maximum compliance peaks and middle ear pressuresoccur outside the normal range, such as Type C tympanograms with peak compliances between -100 daPa and -300 daPa. These tympanograms are associated with an early or resolving infection such as an upper respiratory infection (URI) or an episode of acute otitis media (AOM).[9,11,26]Type C tympanograms may also indicate the presence of middle ear fluid and Eustachian tube dysfunction.[23,34,35]

In addition, tympanograms may appearflat, with no discernible peak. These Type B tympanograms are of particular interest since they usually signify effusion (fluid or pus) in the middle ear. A long-term, persisting flat tympanogram isa hallmark of chronic

otitis media with effusion (COME).[27]However, somecases of apparent Type B tympanograms within the NHANES sample may have inadvertently includedsubjectswho had impacted cerumen in their ear canal. Other casesmay have resulted fromthe probe tip pointing into the ear canal wall, from perforated eardrums, or from the presence of open (patent) pressure equalization (PE) tympanostomy tubes. During the coding of tympanogram types, these potential,extrinsic reasonsfor Type B tympanograms were checked against the ear canal volume. If the ear volume was less than 0.2 ml and possible impacted cerumen was commented onduring otoscopy, the tympanogram type was re-coded as "other". Also, if the ear canal volume was unusually large, this increasedthe likelihood of a perforated eardrum or patent PE tube in the eardrum; these cases were also reclassified as "other". Still other tympanograms were classified as "other", if the curve was very irregular or asymmetric. The number of aberrant tympanograms classified as "other" in this NHANES sample was,however,quite small, less than 1%.Importantly, the health technicians performing tympanometry were instructed to rerun the measurement when the first result appeared to be flat, in order to try to obtain a better test and/or to verify the flat result. If the results were clear and consistent, abnormality was not a reason to reject a tympanogram.

Hearing loss in either ear is defined by a four frequency,pure-tone average (PTA) of air-conduction thresholds at 0.5, 1, 2, and 4 kHz greater thanor equal to 15 dB hearing level (HL) for the children in this study. The cut point at 15 dB HL, to differentiate normal hearing (below 15 dB HL) in children from mild hearing loss (15–30 dB HL) was selected, following the recommendationof Northern and Downs, to distinguish children for whom a mild auditory dysfunction may lead to difficulties understanding language.[36]A screening questionnaire was administered just before the auditory exam to recordany recent factors that might affectthe hearing exam thresholds or tympanometry results, such as recent loud noise exposure or earache.

Analyses of data were performed with SAS™ [37]programs, and adjustments to take into account the NHANES complex national sample design for variance estimation were accomplished using SUDAAN™.[38]Odds ratios (OR) as estimates of relative riskfor hearing loss were calculated using multivariable logistic regression models while controlling for age, sex, race/ethnicity, and education of head of household (HH). Potential explanatory variables included in the analyses were: (a) history of 3 or more ear infections, (b) history of PE tympanostomy tubes having been inserted in one or both ears, (c) prior use of firearms for target shooting, hunting, or other purpose, (d) job-related exposure to loud noise, (e) non-job related (leisure time) exposure to loud noise or music for more than 5 hours/week, (f) a cold, sinus problem, or earache in the past 24 hours, and (g) exposure to loud noise or listening to music with headphones in the past 24 hours.

Results

The numbers of adolescents who had exams conducted in the NHANES MEC is shown in Table 1 by age, sex, race/ethnicity, and education of HH. To evaluate any potential bias in the sample of adolescents who had audiometry exams in the NHANES MEC, these percentages were examined for each of the above socio-demographicgroups. Overall, 93.8% of the 3,380 adolescents who came to the MEC for a health examination had hearing threshold measurements and 93.5% had tympanograms. Variation in the percent having audiometry exams across socio-demographic groups was minor; each category exceeded 92%, except when educational level of HH was "missing" or "refused". Even in this group (n=162), the participation rate was 88%.

Based on the definition of hearing loss as a four frequencyPTA greater than or equal to 15 dB HL in either ear, 7.2% of the adolescents in NHANES had hearing loss in their right ear and 7.8% in their left ear. With regard to the modified Jerger classification of tympanograms, 72.9%–72.8% (right ear–left ear) had typical, normal-appearing Type A tympanograms, 15.1%–13.4% had Type A_s,4.5%–4.8%Type A_d, 3.1%–5.1% Type B, and 3.7%–2.9% Type C tympanograms in the right–left ears, respectively. History of 3 or more ear infections earlier in childhood was reported for 38.6%; 11.2% had ever had tympanostomy tubes placed in one or both ears. Non-job related exposure to loud noise or music for 5 or more hours/week was reported by 26.0% of the adolescents.

The prevalence distributions of Types A/A_s (combined), A_d, B, and C tympanograms are displayed by sex and age groups (upper panel) and by race/ethnicity (lower panel) in Figure 1. The Type A/A_s in the figure corresponds to any combination of Type A or Type A_s in the left and right ears. An adolescent with at least one ear classified as Type B is classified in the figure as Type B. Otherwise, an adolescent with at least one ear classified as Type C is classified as Type C. Type A_d corresponds to adolescents with at least one ear classified as Type A_d, while the other ear is neither Type B nor Type C. The pattern that emerges is of a significantly increased prevalence of females with Type B tympanograms, 7.6% (population weighted %) with a standard error of $\pm$ 0.9% compared to males (5.8% $\pm$ 0.8%).Younger adolescents 12–14 years had a significantly increased prevalence of Type B (8.9% $\pm$ 1.2%) compared to adolescents 15–17 years (5.5% $\pm$ 0.9%) or 18–19 years (5.1 $\pm$ 1.0%). Non-Hispanic black adolescents had a decreased prevalenceof Type B (4.6% $\pm$ 1.0%), compared to the other race or ethnic groups. The changes in prevalence of Type C tympanograms by adolescent age groups are similar to the Type B pattern. However, the pattern for Type A_d is reversed; when compared to the Type B pattern, the percentages are significantly higher for males than females (2.9% $\pm$ 0.6% versus 1.6% $\pm$ 0.4%) and for older adolescents, ages 15–17 years (2.4% $\pm$ 0.7%) and 18–19 years (3.1% $\pm$ 0.9%), compared to younger adolescents 12–14 years (1.6% $\pm$ 0.5%).

Aural acoustic admittance measures of middle ear (ME) compliance, peak pressure, tympanogram curve width, and ear canal volume are shown in Table 2by each of the modified Jerger tympanogram types, separately for right and left ear. For each measure, the number subjects, mean and standard deviation (SD), and 90% range (5^{th} and 95^{th} percentiles) are provided. The acoustic admittance measures are tabulated for comparison to earlier studies with normative values and to documentthe concordance between the present coding of tympanogram types with accepted conventions for scoring tympanograms.
The outcomes of primary interest in Table 2are the four frequency PTA hearing threshold levels by tympanogram types, which are shown using the same summary statistics as for the compliance measures. There is a consistent pattern of lowest thresholds,a PTA of approximately 5.9 dB HL,for adolescents with Type A tympanograms. The PTA hearing levels wereslightly elevated by 0.4 dB HL for Type A_s, followed by a much larger increase of nearly 3.0 dB HL for Type A_d tympanograms, and an even greater increase of 6.7 dB HL for Type C tympanograms. For Type B the increase in mean was 6.1 dB HL for both ears combined (7.6 dB HL in the right ear and 4.5 dB HL in the left ear), as compared to the mean PTA for adolescents with Type A tympanograms. Also noteworthy were the 95^{th} percentiles for adolescents with Type B tympanograms: 45 dB HL for right ears and 35 dB HL for left ears. These values greatly exceed the 95^{th} percentiles for Type A or A_s tympanograms and, also, exceed the 95^{th} percentiles for Type A_d and C tympanograms.

Results of multivariable logistic regression analysis are provided in Table 3. The likelihood of hearing loss (left ear) increased for male sex (OR=1.6; 95% confidence interval [CI]: 1.1-2.3), older adolescents 15–17 years (OR=1.6; CI: 1.1-2.2)and 18–19 years (OR=1.7; CI: 1.1-2.6), education of HH less than high school (OR=2.1; CI: 1.3-3.4), ever had ear tubes (OR=1.9; CI: 1.2-3.1), tympanogram Type B (OR=5.5, CI: 3.0-10.3), Type C (OR=5.3, CI: 2.9-9.7), and Type A_d (OR=3.1, CI: 1.7-5.6). The history of 3 or more ear infections and non-job related (leisure time) exposure to loud sounds or music were non-significant. If history of ear tubes is omitted from the model, then a history of 3 or more ear infections becomes significant, albeit with a smaller OR. Many different noise exposure variables were examined in specifying different multivariable logistic regression models, however, none of them achieved statistical significance either when used singly or in combination.

Discussion
Only a few prior studies have systematically addressed the averagehearing level associated, in general, with different tympanogram types, and most such studies have been focused on the clinically important condition of otitis media with effusion (OME).While the two studies by Fria et al. and Sabo et al. were not population-based, they were both based on samples of approximately 1,000 children, selected from large clinical populations to participate in clinical trials with longitudinal assessmentsand repeated pure-tone audiometry and tympanometry.[39,40] The earlier study by Fria et al. found thatwhile children with OME, ages 7 months to 12 years, did not always demonstrate a mild to moderate conductive hearing loss, the speech frequency PTA (.5, 1, and 2 kHz) mean was 24.5 dB HL and 20% of the OME children had hearing loss greater than 35 dB HL. They found no association between duration of OME and hearing levels.[39]A more recent study by Sabo et al. reported on infants and young children enrolled by 2 months of age, with exams conducted at least monthly, to detect middle ear effusion (MEE) and perform auditory and tympanometric exams.[40] The youngest children were assessed with visual reinforcement audiometry and older ones with conventional audiometry. Their conclusions were: (a) the lowest thresholds were in children with normal middle ear status, (b) thresholds were significantly higher in children with unilateral MEE, and (c) thresholds were the highest in children with bilateral MEE. The latter group with bilateral MEE had hearing threshold levels 10 to 15 dB higher than the normative values for the corresponding age group.[40]The PTA hearing levels for children with bilateral MEE were similar to the earlier study by Fria, et al. The children with unilateral MEE had somewhat better hearing (compared to those with bilateral MEE), but their thresholds were still significantly elevated above the normative values. The PTA hearing levels for the NHANES sample shown in Table 2, Type B or Type C tympanograms, indicate results that are consistent with these two clinical studies in terms of the elevation of mean hearing levels and distribution of hearing thresholds about the mean,as characterized by the standard deviation and the 90% range.

Based on this recent U.S. NHANES nationally-representative sample of more than 3,100adolescents12 to 19 years of age with audiometry and tympanometry, several factors have been shown to be associated with hearing loss: (1) male sex, (2)older age of adolescent, (3) lower HH education, (4) ever use of ear tubes, (5) Type B tympanogram,(6) Type C tympanogram, and(7) Type A_d tympanogram. As shown in the multivariate model in Table 2, a history of 3 or more ear infections and non-job related exposure to loud sounds or music for 5 or more hours/week, did not have a significant influence on hearing loss when examined jointly with the otherpredictor or explanatory variables. The most significant effects were associated with Types B, C, and A_d tympanograms, each with large, highly significant odds ratios indicating 3 to 5.5 fold increasedrisk of hearing loss. Most knowledgeable experts will probably not be surprised by the increased risk for hearing loss associated with Types B and C tympanograms; however, the increased risk associated with Type A_d tympanograms, which have often been ignored or left undifferentiated from Type A tympanograms, is an important finding that merits further consideration in future epidemiologic and clinical research studies.

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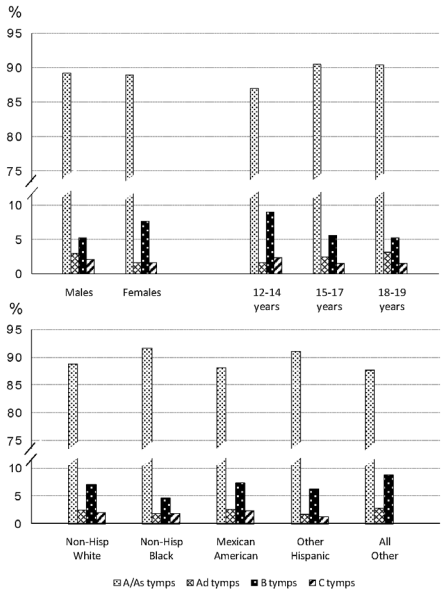

Figure 1

*Prevalence (population weighted %) distributions of Types A/A_s (combined), A_d, B, and C tympanogram classifications by sex and age (top panel) and race/ethnicity (lower panel) for U.S. adolescents in NHANES, 2005–2008. Type A/A_s corresponds to any combination of Type A or Type A_s in the right or left ear. An adolescent with at least one ear classified as Type B, is classified in the figure as Type B. Otherwise, an adolescent with at least one ear classified as Type C, is classified as Type C. Type A_d corresponds to adolescents with at least one ear classified as Type A_d, while the other ear is neither Type B nor Type C. In order to visualize the lower prevalences of non-Type A/A_s tympanograms, the vertical scale is shown with a break or gap above 10% which extends to just below 75%.

Table 1. Number of U.S. adolescents with health examinations, including the number and percent (%) with hearing threshold exams and tympanograms, in the mobile exam centers (MEC) for the 2005–2008 National Health and Nutrition Examination Survey (NHANES)

	MEC health examinations No.	Hearing thresholds* No. (%)	Tympanograms available† No. (%)
Age in years			
12–14	1,255	1,185 (94.4)	1,188 (94.7)
15–17	1,289	1,210 (93.9)	1,203 (93.3)
18–19	836	774 (92.6)	768 (91.9)
Sex			
Female	1,653	1,556 (94.1)	1,546 (93.5)
Male	1,727	1,613 (93.4)	1,613 (93.4)
Race/ethnicity			
Non-Hispanic white	929	882 (94.9)	875 (94.2)
Non-Hispanic black	1,066	991 (93.0)	989 (92.8)
Mexican-American	997	928 (93.1)	928 (93.1)
Other Hispanic	227	217 (95.6)	217 (95.6)
All other (Asian, AI/AN‡,etc.)	161	151 (93.8)	150 (93.2)
Education of head of household(HH)			
<12 years	1,020	948 (92.9)	940 (92.2)
=12 years (high school graduate)	766	729 (95.2)	729 (95.2)
13-15 years (some college)	931	888 (95.4)	882 (94.7)
16+ years (college plus)	501	462 (92.2)	465 (92.8)
Missing/refused	162	142 (87.7)	143 (88.3)
Total	3,380	3,169 (93.8)	3,159 (93.5)

Not all children who were examined in the MEC had audiometry performed; this column shows the number and percent of children with hearing threshold exams.

† This column shows the number and percent of children with tympanogram curves available for coding with the modified Jerger classification scheme.

‡ AI/AN is an abbreviation for the American Indian/Alaskan Native population.

Table 2.Auralacoustic admittance measures ofmiddle ear (ME) compliance, peak pressure, tympanogram curve width, and ear canal volume,plus the four frequency pure-tone average (PTA) of air-conduction hearing levels for the modified Jerger classification typesoflow frequency (226 Hz probe tone at 85 dB SPL) tympanograms, right and left ears separately, NHANES 2005–2008

		Modified Jerger Classification of Tympanograms				
		Type A	Type A_s	Type A_d	Type B	Type C
ME Compliance (ml)						
Right ear	N	2,309	463	143	110	115
Mean±SD		0.80±0.27	0.37±0.05	2.03±0.59	0.16±0.08	0.82±0.45
90% Range: 5th, 95th		0.5, 1.4	0.3, 0.4	1.6, 3.0	0.0, 0.3	0.3, 1.8
Left ear	N	2,290	435	150	145	110
Mean±SD		0.80+0.27	0.36±0.06	2.00±0.47	0.13±0.09	0.77±0.44
90% Range: 5th, 95th		0.5, 1.3	0.3, 0.4	1.6, 3.0	0.0, 0.2	0.3, 1.5
ME Pressure (daPa) at peak compliance						
Right ear	N	2,309	463	143	110	115
Mean±SD		-8.1±21.7	-12.9±23.2	-2.9±23.2	—*	-161.0±50.9
90% Range: 5th, 95th		-54, 12	-48, 12	-54, 24	—*	-270, -102
Left ear	N	2,290	434	150	145	110
Mean±SD		-8.0±20.8	-12.6±20.8	-1.2±22.5	—*	-168.1±53.4
90% Range: 5th, 95th		-48, 12	-54, 12	-42, 24	—*	-282, -108
Width (daPa) at 50%of peakcompliance						
Right ear	N	2,309	463	143	110	115
Mean±SD		90.9±24.5	111.8±30.8	61.4±14.7	—*	102.3±35.9
90% Range: 5th, 95th		60, 132	72, 168	42, 84	—*	55, 168
Left ear	N	2,290	435	150	145	110
Mean±SD		89.4±21.6	110.2±34.3	63.5±20.0	—*	103.8±44.1
90% Range: 5th, 95th		60, 126	72, 162	36, 90	—*	60, 160
Physical volume (ml) of outer ear canal						
Right ear	N	2,309	463	143	109	115
Mean±SD		1.30±0.36	1.18±0.31	1.41±0.39	1.11±0.44	1.23±0.36
90% Range: 5th, 95th		0.8, 1.9	0.8, 1.7	0.9, 2.2	0.5, 1.9	0.8, 1.9
Left ear	N	2,290	435	149	151	110
Mean±SD		1.25±0.35	1.13±0.33	1.42±0.46	1.11±0.47	1.15±0.35
90% Range: 5th, 95th		0.8, 1.9	0.7, 1.6	0.8, 2.2	0.4, 2.0	0.7, 1.7
Pure tone average of hearing thresholds for 0.5, 1, 2, and 4 kHz						
Right ear	N	2,279	459	143	105	114
Mean±SD		5.9±6.0	6.3±5.3	8.9±8.1	13.5±14.1	12.4±8.2
90% Range: 5th, 95th		-1.5, 15.0	0.0, 13.8	0.0, 20.0	0.0, 45.0	3.8, 22.5
Left ear	N	2,268	430	150	142	105
Mean±SD		5.8+6.5	6.1±6.3	8.6±8.1	10.3±11.9	12.7±9.6
90% Range: 5th, 95th		-1.3, 15.0	-1.3, 15.0	0.0, 20.0	0.0, 35.0	3.8, 27.5

* Undefined or indeterminate values; for example, several values may have corresponded to the “maximum” compliance, since the tympanometric curves were essentially flat.

Table 3. Prevalence (population weighted %) and odds ratio estimates of relative risk with 95% confidence intervals predicting hearing loss, *viz*, the four frequency pure-tone average (PTA) of thresholds across 0.5,1, 2, and 4 kHz ≥ 15 dB HL in the left ear, based on sociodemographic variables, history of ear infections, ever had tympanostomy tubes, loud noise exposure, and abnormal tympanograms, NHANES 2005–2008

Selected characteristics	Left Ear (n = 3,114)		
	Population weighted %	Odds ratio	95% Confidence interval
Sex			
Female	48.5	1.00	—
Male	51.5	**1.57†**	**1.08–2.28†**
Age, in years			
12–14	37.4	1.00	—
15–17	39.5	1.60	1.14–2.24
18–19	23.1	1.68	1.09–2.57
Race			
Non-Hispanic white	61.6	1.00	—
Non-Hispanic black	15.0	0.95	0.60–1.50
Mexican-American	11.7	0.84	0.53–1.34
Other Hispanic	5.6	0.51	0.21–1.22
All Other (Asian, AI/AN,* etc.)	6.1	0.47	0.17–1.31
Education, head of household			
<12 years	18.0	2.10	1.30–3.41
=12 years (high school grad)	24.4	1.10	0.59–2.06
13–15 years (some college)	31.0	1.08	0.53–2.20
16+ years (college plus)	21.6	1.00	—
Ever had 3 or more ear infections			
No	60.5	1.00	—
Yes	38.6	1.11	0.70–1.76
Ever had tubes placed in ear(s)			
No	88.0	1.00	—
Yes	11.2	**1.90**	**1.17–3.08**
Non-job related exposure to loud noise or music for 5+ hours/week			
No	74.0	1.00	—
Yes	26.0	1.39	0.95–2.05
Have had cold, sinus, or earache last 24 hours			
No	87.1	1.00	—
Yes, cold	7.5	1.53	0.83–2.81
Yes, sinus	3.8	1.04	0.42–2.55
Yes, earache	1.4	1.01	0.31–3.30
Tympanogram classification			
Type A	72.8	1.00	—
Type A_s	13.4	1.20	0.58–2.46
Type A_d	4.8	**3.10**	**1.71–5.63**
Type B	5.1	**5.54**	**2.98–10.3**
Type C	2.9	**5.32**	**2.91–9.73**

* AI/AN is an abbreviation for the American Indian/Alaskan Native population.

† Bolded numbers are used to draw attention to statistically significant odds ratios, those for which 95% confidence intervals exclude 1.0.

Otitis Media Outcomes Database

Joseph Kerschner, MD[1]**,** Laura Cassidy, PhD[2]**,** Mallory O'Neil[1]**,** T. Roxanne Link, NP/PA[1]
[1]Pediatric Otolaryngology, Children's Hospital of Wisconsin/Medical College of Wisconsin, Milwaukee, WI, [2]Surgery, Children's Hospital of Wisconsin, Milwaukee, Wisconsin

Objective

To implement a database for children with a diagnosis of otitis media (OM) to enable comparative outcomes and long-term prospective follow-up.

Methods
A customized database was constructed to universally consent and enroll all patients seen in consultation with a diagnosis of OM in a web-based format. Unique database fields include patient history, demographics, physical exam findings, risk factors, quality of life (QOL) assessment, intervention, surgical outcomes and long-term outcomes. Major surgical complications measured include: hearing loss, tympanic membrane perforation and cholesteatoma formation.

Results
459 unique patients have been prospectively enrolled. Patient follow-up has extended to 22 months. Initial capture rates exceeded 75%, with follow-up entry below 50%; prompting data recapture efforts. Outcomes demonstrate high prevalence of OM risk factors associated with surgical patients including: 54% in daycare and 23% with a sibling requiring tympanostomy tubes. Substantial improvement in QOL is associated with surgery and there is a low long-term surgical complication rate at less than 2%.

Conclusions
Long-term, outcome driven investigations assessing the surgical management of OM are needed given the prevalence of this disease and the frequency of surgical intervention required. The current database represents the largest prospective cohort of patients enrolled and followed in this fashion and has generated data demonstrating a procedure associated with significant improvement in patient QOL in the short-term with low complication rates in the long-term. This ongoing prospective investigation is providing data which have the potential to be important in treatment algorithms, procedure justification and risk factor modification.

Epidemiology of Pediatric Otitis Media in Denmark Based on the Danish National Birth Cohort

Tanja Todberg[1], Mikael Andersson[1], Jørgen Lous, MD[2], Sjurdur Olsen, MD, PhD[1], Anders Koch, MD, PhD[1], Preben Homøe, MD, PhD[3]
[1]Department of Epidemiology Research, Statens Serum Institute, Copenhagen South, [2]Researh Unit for General Practice, Institute of Public Health, University of Southern Denmark, Odense, [3]Department of Otolaryngology, Head & Neck Surgery, Rigshospitalet, University Hospital of Copenhagen, Denmark, Copenhagen East

Objective
Otitis media (OM) is a common disease in childhood. In Denmark welfare has increased resulting in societal changes, reduction in smoking habits, and prolonged maternity leave, which may influence the incidence of OM. We have examined the age-specific incidence of OM in a cohort of unselected children aged 0-7 years born in Denmark in 1996-2003.

Methods
The Danish National Birth Cohort consists of 101.042 pregnant women and their offspring. Data were collected through five maternal interviews. Age-specific incidence of OM was calculated based on interviews at 6-months, 18-months, and 7-years and was validated against two registries containing information of insertion of ventilation tubes. The study was approved by the national data and ethical committees.

Results
Validation showed high sensitivity (96.4%), specificity (98.2%), positive (94.8%) and negative predictive value (98.8%). At 7 years of age 60.6% reported their children to have ≥ 1 episodes of OM. For 16.6% of children with OM the first episode occurred before 7 months of age, for 44.3% between 7-18 months of age, and for 39.5% between 19 months and 7 years of age. Four or more episodes before 7 years of age were reported by 39.4% and ≥ 4 episodes of OM was reported by 64.0% of those who had their OM debut between 0-6 months of age, 48.2% with debut between 7-18 months of age and 28.7% with debut between 19 months-7 years of age ($p<0,01$). At 7 years of age 26.1% reported their children have had ventilation tube insertion at least once.

Conclusion OM affects nearly 2/3 of preschool children in Denmark despite reduction in previously identified risk factors and early first episode is associated with repeated OM episodes.

Prevalence of Eustachian Tube Dysfunction in Infants with Cleft Palate

Cuneyt M. Alper, MD[1], J. Douglas Swarts, PhD, PhD[1], Joseph E. Losee, MD[2], Ellen M. Mandel, MD[1], Allison Tobey, MD[3], James T. Seroky[1], William J. Doyle, PhD[1]
[1]Department of Otolaryngology, Division of Pediatric Otolaryngology, [2]Department of Plastic Surgery, Division of Pediatric Plastic Surgery, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA, [3]Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction

Nonsyndromic clefts of the lip and/or palate (CLP/CP) are among the most common birth defects, affecting approximately 1 in 595 newborns in the United States[1]. Otitis media (OM) is a known complication of CLP/CP [2,3] and early studies reported OM to be a nearly universal condition in young CLP/CP infants [2,4,5]. The high prevalence of OM in the cleft palate population is thought to be secondary to Eustachian tube dysfunction. Multiple anatomic and tissue abnormalities contribute to Eustachian tube dysfunction within the cleft palate population such as midline soft palate diastasis with abnormal levator veli palatine insertion, abnormal tubal insertion of the tensor veli palatine and abnormal cartilage super structure [6-9]. The goal of palatoplasty is to recreate a functional soft and hard palate and thus restore speech and swallow function, recreate an oral/nasal barrier, and re-establish Eustachian tube function: mastoid aeration and drainage and middle/inner ear protection.

Cleft palate is associated with abnormal craniofacial growth, abnormal dental development, velopharyngeal (VP) insufficiency, poor speech, Eustachian tube (ET) dysfunction, otitis media with effusion (OME), hearing loss. OME in CP±CL patients is consequent to ET dysfunction. Following ETF abnormalities are reported in non-repaired CP: inability to equilibrate the applied me pressure with inflation-deflation test (IDT). and in the force response test (FRT): elevated opening pressure, reduced active pressure, reduced flow, constriction.

Using IDT and the FRT, ET function was evaluated in 56 ears of 41 children and adolescents with CL/P aged 3 months to 18 years (2 pre-palatoplasty, median age 7 years) with tympanostomy tubes inserted for pOME [10]. ET could not reduce applied positive or negative middle ear pressures by swallowing. Poor muscle-assisted ET openings 74% of the FRT tests showed decreased trans-ET airflow → a phenomenon termed "tubal constriction".

A study was conducted to assess the ET function, and degree and type of ET dysfunction prior to the cleft palate repair.

Material And Methods

Infants with non-syndromic cleft palate being followed in the Cleft Palate Craniofacial Center (CPCC) of Children's Hospital of Pittsburgh of UPMC were enrolled in research study. Routine appropriate medical and surgical management of the children continued as per CPCC and otolaryngology management protocols. The testing protocol included an otoscopic exam to verify the presence and patency of tympanostomy tubes (TT), tympanometric confirmation of the patency of the TTs and force response test (FRT) conducted prior to cleft palate repair.

Results

A total of 75 subjects were enrolled. Four subjects dropped out of the study before testing, and 3 subjects were not cooperative with the testing. Fourteen subjects could not be tested prior to the palatoplasty surgery. To date a total of 53 subjects were tested. Bilateral testing was performed in 43 subjects, however, in 10 only unilateral testing was possible, due to tube otorrhea in 6 and blocked tube in 4 in the other ear. In a subset of subjects, opening pressures were 380±162 and 349±147mmH_2O for the left (N=29) and right (N=26) ears respectively. Closing pressures were 187±137 and 156±79 mmH_2O for the left (N=26) and right (N=20) ears respectively. Analysis of swallow morphology in a subset of 24 demonstrated ET constriction in 46% of the subjects. The compete results of the dataset is seen in Tables 1-3.

Table 1.Study variables and results

	Valid N	Mean	Std.Dev.	Minimum	Maximum
Age At Palatoplasty (months)	88	13.2	1.9	10.3	20.7
ETF Testing Rel Palatoplasty (days)	88	-137	60	-223	-6
Opening Pressure (mmH2O)	70	352	149	134	892
Steady State Pressure (mm H2O)	62	238	135	102	844
Steady State Flow (ml/min)	62	24.4	4.1	12.7	33.7
Closing Pressure (mm H2O)	53	186.1	108.1	9.0	720.0
Swallow Pressure (mm H2O)	46	223	118	97	646
Swallow Flow (ml/min)	46	43.4	67.3	0.7	200.0
Passive Resistance (mmH2O/ml/min)	62	10.0	5.9	3.9	35.7
Active Resistance (mmH2O/ml/min)	46	27.5	30.8	0.5	154.8
Dilatory Efficiency	45	2.50	5.62	0.01	30.48

Table 2. Dilatory status

Distribution of Ears by Dilatory status		
Dilatory Efficiency	n	%
Dilation	13	29.5
Constriction	32	72.7
TOTAL	43	100

Table 3. Ear specific dilatory status

Distribution of Right and Left Ears Regarding Dilatory Status					
		RIGHT			
		Dilation	Constriction	NA	TOTAL
LEFT	Dilation	3	1	2	6
	Constriction	2	9	6	17
	NA	2	5	14	21
TOTAL		7	15	22	44

Discussion

The results showed that the ET of the pre-repair CP children could not reduce applied positive or negative middle ear pressures by swallowing. These results indicated poor muscle-assisted ET openings. In 72.7% of the ears FRT tests demonstrated a decreased trans-ET airflow ET during swallowing. Results confirm high prevalence of the phenomenon of "tubal constriction" in children with CP prior to palatal repair

Conclusions

Prevalence of ET dysfunction is high in children with CP. The high prevalence of the constriction phenomenon during the FRT is in part responsible for the ET dysfunction and resulting OM. Children with CP have a specific ETF abnormality i.e. "tubal constriction" instead of normal tubal dilation during swallowing. This abnormal function results in the inability to equilibrate the ME pressure with ambient pressure, leading to development and persistence of OME. Further studies should focus on theprevalence of OME in subpopulations that are constriction(+) and constriction (-), change in prevalence of constriction with CP repair, and the role of constriction in persistence or recurrence of OME after the palatal repair.

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Low Growth and Frequent Ear Infections: the Early Childhood Longitudinal Study

Kathleen Bainbridge, PhD[1], Howard Hoffman[2]
[1]Epidemiology and Statistics, National Institute on Deafness and Other Communication Disorders, Bethesda, MD, [2]Epidemiology and Statistics, National Institute on Deafness and Other Communications Disorders, Bethesda, MD

Introduction
Very low birthweight children have increased risk of frequent ear infections (FEI). Children who grow slowly may also experience frequent ear infections.

Objective
The objective was to determine whether FEI is associated with beinglow weight-for-age or low height-for-age.

Methods
As part of the Early Childhood Longitudinal Study Birth Cohort, 8126 children assessed at age 6-22 months were followed up after an average of 14 months, at which timeheight and weight were measured. Low weight-for-age and lowheight-for-age were defined as being less than two standard deviations below the sex- and age-specific means of the weight and height, respectively, specified in the 2000 CDC Growth Charts. FEI was defined as parent report ofat least 3 ear infections since the prior assessment. Date of birth and birthweight were ascertained from birth certificates. Number of siblings, breastfeeding duration, child care arrangements, parental smoking status, and history of pediatric illness were obtained during interviews.Compared to larger children, we estimated the odds of FEI among children who are low weight-for-age and those who are low height-for-age.

Results
Low weight-for-age children had 80% increased odds of FEI [OR=1.80 (1.08, 2.99)]. Children who were lowheight-for-age had a 40% increased odds of FEI [OR=1.40 (1.01, 1.94)]. Associationswere independent of sex, race, birthweight, parental smoking, type of child care, and pediatric illness.

Conclusions
Childrenwith slower growthhave a greater likelihood of experiencing frequent ear infections.

Detection of Respiratory Virus in Pediatric Acute Otitis Media

Muneki Hotomi, MD, PhD[1], Levent Beder, MD[1], Masashi Ogami, MD, PhD[1], Yuki Tatsumi[1], Shunji Tamagawa, MD[1], Noboru Yamanaka, MD, PhD[1]
[1]Otolaryngology, Wakayama Medical University, Wakayama-shi, Wakayama

Introduction
Recent studies have documented the close association between acute otitis media (AOM) and viral upper respiratory tract infection. Contribution of viruses and their co-infection with bacterial agents has been emphasized in the pathogenesis of AOM. In this study, we investigated the prevalence of respiratory virus in children with AOM.

Methods
We have conducted a surveillance study organized by nationwide study group; Advanced Treatments for Otitis Media Study (ATOMS) since 2006. We employed RT-PCR to identify RS virus, influenza virus (type A and B), adenovirus, human boca virus (hBoV) and human metapneumovirus (hMPV) in nasopharyngeal swabs (NPS) and middle ear fluids (MEF).

Results
The respiratory viruses were identified in 47 (25.4 %) out of 185 patients; 20 (12.6 %) in MEF and 48 (27.2 %) in NPS. The pathogenic viruses were 2 (10.5 %) influenza virus, 5 (26.3 %) RS virus, and 12 (63.2 %) hMPV. hBoV was found in NPS of 14 children (6.3 %) and in the MEF of 6 children (2.7 %). The resolution time of AOM was significantly longer in hBoV-positive group. Positive correlation was found between detection of hBoV and Streptococcus pneumoniae in the MEF.

Conclusion
AOM, which is generally considered a bacterial disease, is more likely co-infection of virus and bacteria or the secondary bacterial infections after virus upper respiratory infections. However, a virus agent alone will cause signs and symptoms of AOM. A better understanding of the mechanism of viral and bacterial interaction in AOM will lead new strategies for more effective treatment.

Replication of Respiratory Syncytial Virus, a Viral Co-Pathogen of Otitis Media, Is Inhibited by the Novel Host Defense Molecule Viperin

Glen McGillivary, PhD[1], Zachary Jordan[1], Lauren Bakaletz, PhD[1]
[1]Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital and The Ohio State University School of Med, Columbus, Ohio

Respiratory syncytial virus (RSV) is an important viral co-pathogen of otitis media (OM). An enhanced understanding of the host response induced by RSV will provide crucial information needed to develop novel preventative or therapeutic strategies against this virus as well as the polymicrobial diseases in which it plays a key role.

To begin to determine the global host response to RSV in the uppermost airway, we used an oligonucleotide microarray to analyze gene expression in chinchilla respiratory tract mucosa after intranasal challenge with RSV. We showed that expression of several genes involved in the host interferon response to virus were significantly upregulated in the airway, one of which included the gene that encodes the anti-viral protein Viperin. We showed that *viperin* was upregulated 3-fold four days after viral challenge and 4.2-fold after seven days, compared to mock challenged animals. We overexpressed human Viperin in epithelial cells *in vitro* with subsequent RSV infection and observed a statistically significant reduction in the percentage of RSV-infected epithelial cells, compared to controls. In addition, we provided evidence that overexpression of Viperin resulted in diminished production of filamentous RSV virions on the epithelial cell surface, which suggested that Viperin inhibited the budding ability of this virus. Current efforts focus on transduction of the chinchilla upper airway with a self-complementary recombinant adeno-associated vector which encodes human Viperin to study the anti-RSV effects of Viperin *in vivo*.

Collectively, we provided evidence that Viperin plays an important role in the innate immune defense against RSV. In addition, our data suggests that the anti-RSV activity of Viperin can be harnessed for enhanced host protection against this clinically important viral pathogen of the airway.

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Use of a Multi-Template Control to Assess the Usefulness of T-RFLP for Investigating Polymicrobial Otitis Media

Robyn Marsh, Mirjam Kaestli, PhD**,** Peter Christensen**,** Linda Ward**,** Michael Binks**,** Amanda Leach, PhD**,** Heidi Smith-Vaughan, PhD
Child Health Division, Menzies School of Health Research, Institute of Advanced Studies, Charles Darwin University, Darwin, NT

Introduction
Otitis media (OM) is commonly a chronic and polymicrobial infection in high-risk populations. While culture-based methods target common otopathogens, they limit polymicrobial investigations. Terminal-fragment restriction length polymorphism (T-RFLP) analysis can be used to better characterise bacterial populations.

Objective
The aim of this study was to optimise T-RFLP for OM samples.

Methods
T-RFLP was optimised using a multi-template control containing equal amounts of 13 common respiratory bacteria. Optimisation included assessment of primers; enzymes; reaction conditions; and peak-calling methods. Nasopharyngeal and ear discharge swabs from Indigenous Australian children with acute OM were then tested.

Results
Using the control, differential PCR amplification was noted for some species which may bias quantitative analyses. Two peaks were detected for *Moraxella catarrhalis* and this needs to be considered when assessing diversity in clinical samples. Optimal peak-calling was obtained using a constant baseline or constant percentage threshold method. The control was also used to show specific enzymes could differentiate common respiratory pathogens from closely-related commensal species.

Clinical samples showed variation in PCR yield with some samples providing insufficient product for T-RFLP. While primer choice did not affect yield from the control, it was important when amplifying from some clinical samples. Low PCR yield was more common for nasopharyngeal than ear discharge swabs, most likely reflecting smaller sample volume.

Conclusion
T-RFLP can be a useful tool in screening bacterial communities in upper respiratory swabs with sufficient sample volume. The multi-template control allowed identification of PCR amplification biases; profiling characteristics of important species; and assessment of peak calling methods.

Surveillance of Causative Pathogens of Pediatric Acute Otitis Media in Japan

Atsuko Masuno, MD, Muneki Hotomi, MD, PhD**,** Masaki Hayashi, MD, PhD**,** Masashi Ogami, MD, PhD**,** Shunji Tamagawa, MD**,** Yuki Tatsumi**,** Akihisa Togawa, MD, PhD**,** Shinji Tamura, MD, PhD**,** Noboru Yamanaka, MD, PhD
Department of Otolaryngology, Wakayama Medical University, Wakayama City, Wakayama

Introduction
Streptococcus pneumoniae and Haemophilus influenzae had long been susceptible to beta-lactams. However, penicillin resistant S. pneumoniae (PRSP), become the major causes of intractable clinical course of AOM. The antimicrobial resistance in H. influenzae has also evolved significantly. In this study, we reported the current status of antimicrobial resistant pathogens responsible for AOM studied by multicenter surveillance group (Advanced treatment for otitis media study group; ATOMS) in Japan.

Methods
S. pneumoniae and H. influenzae were collected from children with AOM during 2007 to 2010. Antimicrobial resistances were determined genotypically by polymerase chain reaction (PCR) and phenotypically depending on minimal inhibitory concentration (MIC).

Results
Penicillin-susceptible S. pneumoniae isolates with no abnormal pbp genes were identified at only 7%. 31% pneumococcal strains had mutation in their penicillin binding protein (PBP) 1a, 2b and 2x and 62 % pneumococcal strains had mutation in either PBP1a, 2b, or 2x gene in Japan. The resistant rates of S. pneumoniae to macrolide were 70-80 % in Japan. 43% of H. influenzae were gBLNAR: genetically beta-lactamase nonproducing ampicillin resistant strains with mutations in ftsI gene without producing β-lactamase in Japan.

Conclusion
PRSP are still prevalent in pediatric AOM. There is an alarming increase in Japan of the occurrence of BLNAR strains with mutations of their ftsI gene. Consequently, we need to continue the careful surveillance for BLNAR strains of H. influenzae in patient populations and continue our efforts to find out what is behind the high prevalence of antibiotic-resistant pathogens in AOM.

Animal Models/Middle Ear

Ossicular Bone Modeling in Acute Otitis Media

Rasmus Salomonsen, MD[1], Per Cayé-Thomasen, MD, PhD[2], Ann Hermansson, PhD[3]
[1]Department of Oto-rhino-laryngology, Head and Neck Surgery, Copenhagen University Hospital Gentofte, Hellerup, [2]Department of Oto-rhino-laryngology, Head and Neck Surgery, Copenhagen University Hospital Gentofte, Hellerup, Denmark, [3]2Department of Oto-rhino-laryngology, Head and Neck Surgery, Lund University Hospital, Lund, Sweden

Introduction
A number of middle ear diseases are associated with pathologic bone modeling, either formative or resorptive. As such, the pathogenesis of a sclerotic mastoid has been controversial for decades.
For obvious reasons of lacking tissue, pathological middle ear bone tissue modelling in human acute otitis media has been scarcely reported. Experimental studies have shown, that the bone tissue structures surrounding the middle ear cavity is involved early in the course of acute infection, as initial resorption is followed by progressive osteoneogenesis. [1,2] However, focusing on other issues, only a few studies have touched briefly upon acute changes of the ossicular chain,[1,3,4] which leaves detailed qualitative and quantitative information on ossicular bone modelling dynamics in acute otitis media undocumented.
This study presents such data by longitudinal temporal bone studies in a rat model, in which the bone modeling dynamics of the ossicular chain is examined and compared to the modeling dynamics of the bone surrounding the middle ear cavity. The rat temporal bones were harvested from day 1 through 28 after middle ear inoculation of *Streptococcus pneumoniae* type 3.

Material and Methods
The histopathology of the middle ear bone tissue structures was studied longitudinally in a rat model of acute pneumococcal otitis media, from day 1 through day 28 post-inoculation. The rats had been randomly selected and sacrificed from day 1 through day 28 post-inoculation. All rats had been examined by otomicroscopy on day 4, to ensure established infection (rats decapitated before day 4 were examined immediately before sacrifice). Cyto- and histomorphological features of the ossicular bone and the bone tissue structures surrounding the middle ear cavity, resorption and new bone formation (osteoneogenesis), were registered and semi-quantitated as follows: no changes (0), slight changes (1), moderate changes (2) and extensive changes (3).

Results
The first evidence of ossicular bone modeling following bacterial inoculation was seen as resorption pits and new bone formation on malleus and incus surfaces on day 4 post-inoculation, which was followed by continued resorptive and formative activity at all ossicles until day 14. The temporal and quantitative pattern of the ossicular bone modeling differed from that of the bony wall of the middle ear cavity, for which the earliest changes of bone histomorphology occurred already day one post-inoculation, seen as initial osteoresorption. Subsequently, progressive and massive osteoneogenesis continued throughout the 28-day period of observation. Thus, the bone modeling of the ossicles occurred postponed and to a much lesser extent, compared to the changes of the bone tissue structures surrounding the middle ear cavity, as accounted for and seen in Table I and II and Figure I.

Conclusion
A single incident of acute otitis media changes the osseous structures of the middle ear, evidenced by the initial appearance of resorption pits, subsequently proliferating osteoblasts and new bone formation on malleus, incus and stapes surfaces, which may alter properties of ossicular chain conduction. Modelling of ossicular bone occurs postponed compared to the bone tissue surrounding the middle ear cavity, and to a much lesser extent. The differentiated modeling pattern suggests that ossicular bone tissue possess biological properties in resistance of morphological changes.

The biological mechanism that control bone metabolism at the local level has been recognized to be the cytokine system receptor activator of nuclear factor kappa B (RANK), RANK ligand (RANKL) and osteoprogeterin (OPG), where the ratio of OPG to RANKL is critical in determining the kinetics of local bone turnover. Apparently, OPG is produced constitutively at high levels in the cochlea, which may explain the extremely slow turnover of peri-labyrinthine bone tissue structures.[5] The ossicular bone in OPG knockout mice exhibited diffuse abnormal bone modeling.[6] By hypothesis, a higher ossicular bone ratio of OPG to RANKL could explain our finding of a delayed and attenuated ossicular bone modeling during acute infection, compared to the bone tissue structures surrounding the middle ear cavity.

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Table I: Semi-quantitation of osteoresorptive activity in the rat middle ear on various days following inoculation of *Streptococcus pneumoniae*.

Day	No.	Ossicles			Bony MEC wall
		Malleus	*Incus*	*Stapes*	
1	3				1
2	4	0	0	0	2
3	5				3
4	5	1	1	-	3
5	3				2
6	5	1	*1*	-	2
7	4	1	1	1	1
8	4				1
9	4				0
10	4	2	2	-	0
11	4	2	2	1	0
12	4				0
13	1				0
14	4	1	-	-	0
28	4	0	-	-	0

0 None; 1 Slight; 2 Moderate; 3 Severe; - not sufficient osseous material for evaluation.
MEC: middle ear cavity. See Materials and Methods for further details.

Table II: Semi-quantitation of osteoneogenetic activity in the rat middle ear on various days following inoculation of *Streptococcus pneumoniae*.

Day	No.	Ossicles			Bony MEC wall
		Malleus	*Incus*	*Stapes*	
1	3				0
2	4	0	0	0	1
3	5				1
4	5	1	1	-	2
5	3				2
6	5	1	*1*	-	3
7	4	1	1	1	3
8	4				3
9	4				3
10	4	1	1	-	3
11	4	1	1	1	3
12	4				3
13	1				3
14	4	1	-	-	3
28	4	0	-	-	3

0 None; 1 Slight; 2 Moderate; 3 Severe; - not sufficient osseous material for evaluation.
MEC: middle ear cavity. See Materials and Methods for further details.

Figure 1

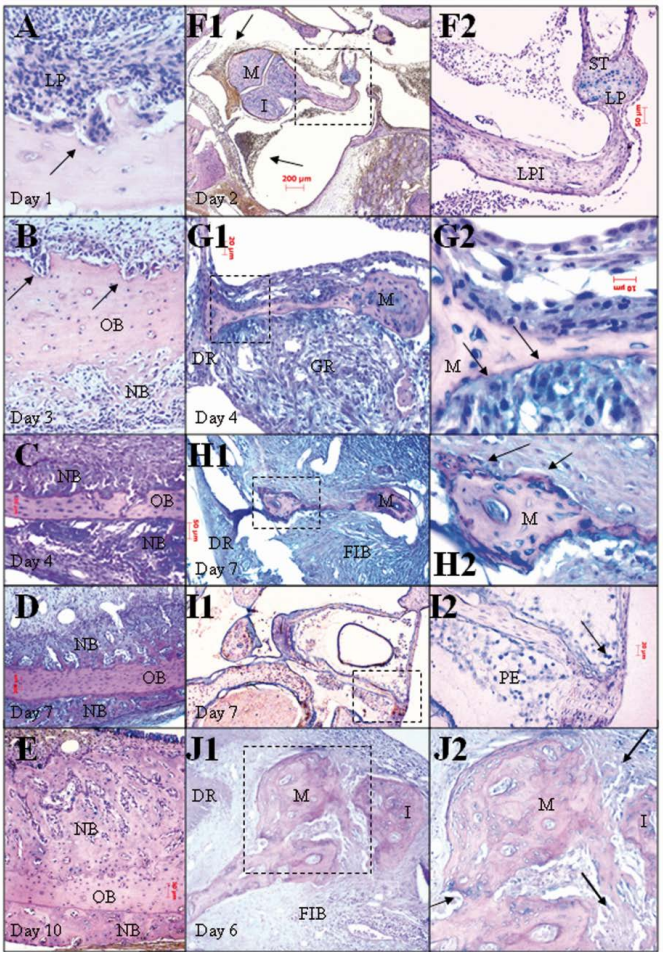

FIGURE
Fig. 1. Comparison of middle ear cavity wall and ossicular bone modeling in experimental acute otitis media.
The left column of micrographs represents images of the bony middle ear cavity (MEC) wall from day 1 through 10 post-inoculation. The middle column is overview micrographs of ossicular bone modeling from day 2 through 7 post-inoculation. High-power micrographs of the framed areas in the middle column are displayed in the right column (e.g. F2 is high-power of the framed area in F1). A comparison reveals that the modelling of ossicular bone occurs postponed compared to the bone tissue surrounding the middle ear cavity, and to a much lesser extent.
A. Bony MEC wall day one postinoculation, with heavily infiltrated lamina propria (LP) and a large, osteoresorbing, multinucleated osteoclast (arrow) at the inner periosteum, embedded in an erosion pit (i.e. Howships lacuna).

B. Bony MEC wall day three with resorbing osteoclasts (arrows) at the inner periosteum and new bone formation (NB) at the outer periosteum. OB = original bone.
C. Bony MEC wall day four. New bone (NB) has been formed at the outer, as well as the inner periosteum.
D. New bone formation at both sides of the original bone has more than doubled the thickness of the bony MEC wall day seven.
E. Massive new bone formation is evident on day ten postinoculation.
F1 and F2. No bone modeling occurs in the ossicular chain on day two, although surrounded by purulent effusion (arrows). F2 is magnification of the framed area in F1. M: malleus; I: incus; LPI: long process of the incus; LP: lenticular process; ST: stapes head.
G1. Malleus (M) embedded in granulation tissue (GR) on day four. High-power of the framed area is seen in G2, showing proliferating osteoblasts aligning the malleus surface (arrows). DR: drum.
H1 and H2. New bone formation (arrows) at the malleus surface on day seven. Fibrosis (FIB) has developed in parts of the MEC granulation tissue.
I1 and I2. Osteoresorption at the angle between the footplate and the posterior crus of the stapes (arrow) on day seven. Purulent effusion (PE) surrounds the ossicle.
J1 and J2. Resorption and new bone formation (arrows) at the malleo-incudial joint on day six.
Staining is PAS/alcian blue in all micrographs.

Effect of In Vivo Over-Expression of KGF by Electroporatively Transfected KGF CDNA on the Histology of Middle Ear Cholesteatoma

Tomomi Yamamoto-Fukuda, MD, PhD[1], Takehiko Koji, PhD[2], Yasuaki Shibata, PhD[3], Tohru Ikeda, PhD[3], Yoshitaka Hishikawa, MD[2], Haruo Takahashi, MD[1]
[1]Department of Otolaryngology-Head and Neck Surgery, Department of @Translational Medical Science, [2]Department of Histology and Cell Biology, [3]Department of Oral Pathology and Bone Metabolism, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Nagasaki

Introduction
Middle ear cholesteatoma is characterized by the enhanced proliferation of epithelial cells with aberrant morphologic characteristics.[1, 2] In our previous study, we indicated that keratinocyte growth factor (KGF)/fibroblast growth factor-7 plays an important role in cholesteatoma formation.[3]KGF is a mesenchymal-cell-derived paracrine growth factor that specifically stimulates epithelial cell growth. It is supposed to be secreted from fibroblasts mainly in stroma and binds to the KGF receptor, which has only been detected on the surface of epithelial cells.[4, 5]IL-1α, a pro-inflammatory cytokine, was known to stimulate the expression of KGF upon binding to a promoter lesion of KGF mRNA.[6]The cholesteatoma matrix is known to produce IL-1α and other cytokines, which seemed to induce hyper-proliferation of keratinocytes in previous studies.[7, 8]In this study, we investigated the effect of over-expressed KGF or over-expressed IL-1α*in vivo* on ear tissues, by the electroporatic transfection [9] of KGF-expressed vector or IL-1α-expressed vector into the external auditory canals (EACs) of Sprague-Dawley (SD) rats.

Methods
Male SD rats weighing 200-300g with normal tympanic membranes (TMs)were used. Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation, Nagasaki University, with approval of the Institute of Animal Care and Use Committee. After anesthetizing 15 male SD rats with pentobarbital, Flag-hKGF DNA plasmid (50μg) driven by a CMV promoter[10] or Flag-hIL-1αDNA plasmid (50μg) was transfected into an epithelial lesion on one side of the EAC. Electric pulses were given using a CUY21 Electroporator (Nepa Gene Co., LTD), while Flag-plasmid driven by a CMV14 promoter was injected on the other side of the EACas a control.

At one, four and seven days after injection, the otomicroscopical status of the TMs and EACs of five rats at each time-point were evaluated and immediately sacrificed. EAC tissues were removed,fixed with 4% paraformaldehyde in phosphate buffered salineat room temperature overnight and embedded in paraffin. Serial sections were cut to a 5μm -thicknessand then placed onto 3-aminopropyltriethoxysilane-coated glass slides. The expression of KGFwas examined by immunohistochemistry using anti-KGF antibody (Sigma, MO, USA) according to the protocol by our previous study.[3]To visualize signals, we performed immunoperoxidase staining as a chromogen. The stainings were graded as positive or negative, compared to the background staining with normal goat IgG. To assess proliferation activity, Ki-67 staining was performed by using anti-Rat Ki-67 antibody (MIB-5) (DakoCytomation, Denmark) according to the protocol described by Ehara et al.[11]To analyze the results quantitatively, more than 1,000 cells were counted in random fields at 400x magnification and the number of Ki-67 positive cells expressed as a percentage of positive cells per counted cells (Ki-67 labeling index [LI]; mean ± SD). Data for Ki-67 LI in the vector and control vector were compared with an unpaired Student's *t*-test. An A *P*-value of less than 0.05 was defined as the level of signification.

Results

As a result, we found chronic inflammation in two of three ears and crusts without inflammation in one of three ears at Day 1;keratin accumulations in theEAC were shown in two of three ears at Day 4. Chronic inflammation was shown in all of the ears, but keratin accumulations in theEAC were shown in one of three ears at Day 7 following KGF cDNA transfection (Table 1).In the IL-1αvector-transfected ears, we found chronic inflammation in three out of three ears at Day 1, one of three ears at Day 4,one of three ears on Day 7. No keratin debris was detected on any day (Table 1). In the control ear however, although an inflammatorychange occurred in one of three rats at Day 1, no inflammation or keratin debris wasdetected at Days 4 and 7(Table 1).Immunohistochemical results revealed that KGF was positive at Days 1, 4 and 7 in KGF vector-injected specimens(Fig. 1) and positive at Days 1 and 4in IL-1α vector-injected specimens, while no staining was found in control specimens on any dayexceptDay 1.Moreover, in contrast to the control specimens and IL-1αvector-transfected specimens, larger numbers of Ki-67-positive cells were detected in KGF cDNA-transfected specimens at Day 4 and Day 7 (<0.0001).

Discussion

In this study, we analyzed the effects of KGF protein during middle ear cholesteatoma formation using a new animal model. According to our results, a chronic inflammatory response and the proliferation of epithelial cells were induced by a single injection of KGF cDNA-expressive vector, continued from Day 1 to Day 7 after injection, with keratin debris finally produced in the ears.On the other hand, IL-1α, a pro-inflammatory cytokine, inducedan inflammatory reaction and stimulatedKGF expression in stromal cells,but did not induce epithelial cell proliferation with keratin accumulations.

These results indicate that the strong expression of KGF may trigger the proliferation of epithelial cells, suggesting a possibility that the KGF cDNA augment of KGF transfection might inducecholesteatoma formation in SD rats in the literature.

Conclusion

Our study indicated that the paracrine action of stromal KGF may play a major role in the pathogenesis of cholesteatoma formation in SD rats.

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, and Culture (no. 23791906 to T.Yamamoto-Fukuda) and by a grant from The Naito foundation (to T.Yamamoto-Fukuda).

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Figure Legend
Figure 1. Immunohistochemical detection of KGF in paraffin sections of KGFvector- transfected ears. Intense staining for KGF was detected in stromal cells and hair follicles of at Day 1 (a). KGF-positive cells were detected in the epithelium and stromal cells at Day 4 (b) and Day 7 (c).KGF positive cells were stained in brown.Arrows: positive cells.

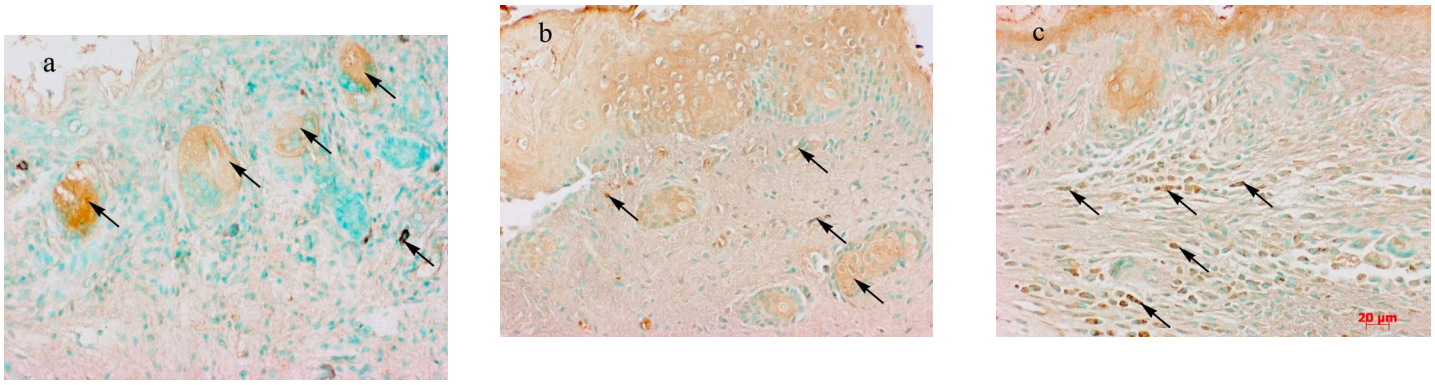

Table 1. Number of chronic inflammatory-changed EACs with/without debris of KGFvector-transfected ears, IL-1αvector-transfected ears and controlvector-transfected ears.

	Day 1		Day 4		Day 7	
	Inflammation	Debris	Inflammation	Debris	Inflammation	Debris
KGFvector (n/3)	3	0	1	2	0	1
IL-1α vector (n/3)	3	0	1	0	1	0
Controlvector (n/3)	1	0	0	0	0	0

Regulating Osteoclasts for the Maintenance of Auditory Ossicular Morphology, the Middle Ear and Hearing

Sho Kanzaki, MD, PhD[1], **Yasunari Takada, PhD**[2], **Kaoru Ogawa, MD, PhD**[3], Koichi Matsuo, MD, PhD[2]
[1]Otolaryngology, Keio University Medical School, Shinjuku, Tokyo, [2]Laboratory of Cell and Tissue Biology, [3]Department of Otolaryngology Head and Neck Surgery, School of Medicine, Keio University, Shinjuku, Tokyo

Introduction
Currently little is known about whether and how osteoclasts play a role in the vibration of auditory ossicles. This review focuses on morphological change of auditory ossicles and hearing function when osteoclasts increase or decrease.
Prevention of bone resorption may be implicated with clinical application of several middle ear diseases.

Bone remodeling may be important for auditory ossicular structure. Osteoclasts are specialized multinuclear macrophages that secrete HCL and hydrolases causing bone resorption. Osteoclast are upregulated after inflammation, and ossicles are eroded by repeated otitis media. Therefore, regulating of the number of osteoclastsis critical for maintaining the shape and size of auditory ossicles.. For bone remodeling to occur,receptor activatorof nuclear factor-κB (NF-κB) ligand (RANKL) must bind withits receptor (RANK), located on osteoclasts.The potentially continuous bone loss is mitigated by the decoy receptor osteoprotegerin (OPG) which competitivelybinds RANKL and blocks the interaction of RANKL–RANK. The osteoclastic bone resorption in adults isbalanced byosteoblastic bone formation through “coupling” mechanisms, which maintain bone integrity (bone remodeling)(Zhao et al., 2006). In this paper we reviewed the role of osteoclasts in the maintenance of the ossicular structure and how hearing function is impaired knock out micewith increased or decreased osteoclast numbers.

Auditory ossicles in osteopetrotic mouse
Conductive and/or sensorineural hearing loss is frequently seen in humanwith ostepetrosis(Benichou et al., 2000; Hawke et al., 1981; Maranda et al., 2008; Stocks et al., 1998).We analyzed hearing function and the morphology of the auditory ossicles inosteopetrotic mice, which lack osteoclasts due to either a c-Fos or RANKL deficiency.The auditory brainstem response showed that mice of both genotypes hadhearing loss, and that themobility ofthe malleus was dramatically reduced as revealed by laser Doppler vibrometry.(Kanzaki et al.).Althoughinvolvement of the nervous system cannot be excluded, impaired vibration ofthe malleus is the most plausible explanation forthe hearing loss of osteopetrotic mice(Kanzaki et al.2011). The impaired vibration was apparently due to contact ofthe malleus with the promontory, the reduced volume of the tympanic cavity andthe increased volume of auditory ossicles in comparion to control animals. [3,4,5]

Excessive numbers of osteoclasts in ossicles –

However, too much of a “good thing” also leads to hearing loss. The shape of ossicles and hearing weree also degraded when the numbers of osteoclasts were increased. Mice lacking OPG (*Opg*-/- mice), also known asa model for juvenile Paget's disease, exhibited excessive numbers of osteoclasts resulting inabnormal boneremodeling of the otic capsule.[4,9] Auditory ossicles in *Opg*-/-mice are massively resorbed by the abundant osteoclasts which may also result in impaired hearingfunction.[4,9] In *Opg*-/- mice, the ligament at the junction of the stapes and the otic capsuleis lost by bony ankylosis (fusion).[4,9]
Bisphosphonate Therapy in otosclerosis model

Opg-/- mice were intraperitoneally injected with risedronate, one of thewidely-used bisphosphonates, for5 days/week over 9 weeks. The treatment significantly inhibited bone loss in auditory ossicles as well as in long bones of *Opg*-/- mice compared to untreated control mice.[3] Thinning of malleus handle and bony fusion of the junction between the stapes and the otic capsule were reduced by the treatment. In addition, hearing loss in *Opg*-/- mice was significantly reduced by risedronate treatment.[3]

Conclusion
We have shown that both osteopetrosis and osteoporosis impacts the structure of the middle ear ossicles and impairs hearingfunction. The modification of the ossicles provides an explanation for the impaired vibration of auditory ossicles seen in bothosteoporotic and osteopetrotic mouse models.

This work was supported by Grants-in-Aid for Young Scientists B (17791198, 21791643to S.K.), for Scientific Research (23592495 to S.K.) C, and Grants-in-Aid for Scientific Research B (17390420 and21390425 to K.M.) from JSPS; and a Keio UniversitySpecial Grant-in-Aid for Innovative CollaborativeResearch Projects.

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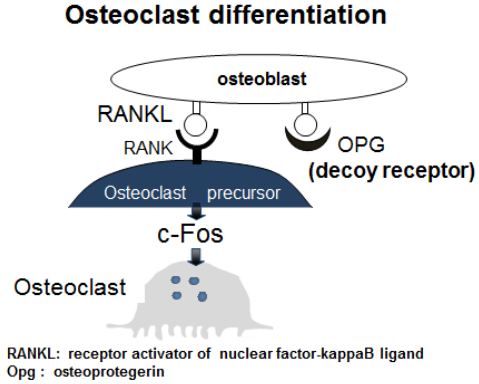
Figure 1 Summary of osteoclast differentiation



Regulation of Osteoclasts Is Required to Maintain Morphology and Function of Ossicles in Middle Ear

Sho Kanzaki, MD, PhD[1], Yasunari Takada, PhD[2], Kaoru Ogawa, PhD, MD[1], Koichi Matsuo, MD, PhD[2]
[1]Otolarynogology Head and Neck Surgery, School of Medicine,Keio University, Shinjuku, Tokyo, [2]Laboratory of Cell and Tissue Biology, School of Medicine, Keio University, Shinjuku, Tokyo

Currently, little is known about whether and how bone-resorbing osteoclasts play a role in the vibration of auditory ossicles. Osteoclasts are specialized multinuclear macrophages that resorb bone. Once bones develop through endochondral and intramembranous ossification (bone modeling), osteoclastic bone resorption in adults is usually followed and balanced by osteoblastic bone formation through ″coupling″ mechanisms, which maintain bone integrity (bone remodeling). Turnover of temporal bones including the otic capsule and auditory ossicles is much slower than that of the long bones because the former contain high levels of osteoprotegerin (*Opg*), which inhibits osteoclast formation.

We analyzed hearing function and morphology of auditory ossicles in both osteoporotic and osteopetrotic mice. Auditory ossicles in Opg deficient ($Opg^{-/-}$) mice are massively resorbed by abundant osteoclasts, resulting in impaired hearing function. In $Opg^{-/-}$ mice, the ligament at the junction of the stapes and the otic capsule is lost by bony ankylosis. In addition, administration of the anti-resorptive drug bisphosphonate prevents not only erosion of auditory ossicles but also progression of hearing loss, suggesting that excessive bone resorption underlies impaired hearing in $Opg^{-/-}$mice. Conversely, osteopetrotic mice, which lack osteoclasts due to either c-Fos or RANKL deficiency, show a smaller volume of the tympanic cavity but larger auditory ossicles compared to controls. The malleal processus brevis thus touches the medial wall of the tympanic in osteopetrotic mice. These data demonstrate that regulation of osteoclastic bone resorption is required to maintain morphology of auditory ossicles and normal hearing function.

Oto-Endoscopy: A New Tool for Phenotyping Otitis Media in the Mouse

Mahmood Bhutta, MD[1], Elizabeth Hedge[2], Andrew Parker[3], Michael Cheeseman, PhD[3], Steve Brown, PhD[3]
[1]Nuffield Department of Surgical Sciences, University of Oxford, Oxford, Oxfordshire, [2]School of Medicine, Kings College London, London, [3]Deafness Group, MRC Harwell, Harwell, Oxfordshire

Objectives
The mouse is widely used to investigate the pathophysiological and genetic bases of otitis media (OM). However, straightforward and robust phenotyping tools for identifying murine OM are lacking, which has precluded for example the identification of mice with OM in genetic screens without resorting to time-consuming histopathology. We set out to develop a phenotyping platform for the detection of OM in mice utilizing oto-endoscopy.

Methods
We undertook oto-endoscopy in a cohort of mice (72 ears) genetically susceptible to chronic otitis media, but with variable penetrance. We asked three blind assessors to independently rate the presence or absence of OM. We compared the results with histological evidence of inflammation, including mucoperiosteal thickness and presence of effusion.

Results
Oto-endoscopy was quick and with a low rate of complications. The technique was reliable with 95.3% agreement between assessors and a kappa coefficient of 0.91. Ears rated as OM present had significantly greater mucoperiosteal thickening on histology ($p<0.001$). Rating of OM status on endoscopy also demonstrated 93% accuracy in predicting the presence of a middle ear effusion.

Conclusions
Oto-endoscopy is a safe, reliable and valid method for detecting otitis media in the mouse and should have great utility in first-tier phenotyping screens to identify novel genes involved with susceptibility to OM. It may also have utility in monitoring progression of disease, or the effects of therapeutic intervention.

Progenitor Cells and Stem Cells in the Healthy Human Tympanic Membrane

Johan Knutsson, MD, PhD[1], Magnus von Unge, MD, PhD[2], Helge Rask-Andersen, MD, PhD[3]
[1]ENT-dept, Center for Clinical Research, Västerås, Sweden, [2]ENT-dept, Akershus University Hospital & Medical Faculty Division of University of Oslo, Norway, Oslo, Norway, [3]Department of Otolaryngology, Uppsala University Hospital, Uppsala, Sweden

Objectives
The closure of a tympanic membrane (TM) perforation is initiated in the outer epithelium. Embryonic stem cells have been used in experimental settings to enhance the healing capacity of induced perforations. Increased knowledge about the TM cell proliferation and migration is needed for a better understanding of the healing process of the TM. This includes the identification of regenerative zones within the outer epithelial layer of the TM where progenitor cells or stem cells may be present.

Methods
Healthy human TMs were harvested in the procedure of translabyrinthine surgery and were investigated using immunohistochemistry and immunofluorescence to detect the epithelial progenitor/stem cell markers α6 integrin, β1 integrin and cytokeratin 19 (CK19).

Results
α6 integrin was detected in the umbo, in the annular region and along the malleus but not in the intermediate portion of the pars tensa. α6 integrin was found in the basal layer of the keratinizing epithelium. β1 integrin and CK19 were found in the same locations, not only in the basal layer but also in the suprabasal layers of the keratinizing epithelium.

Conclusions
Possible progenitor cells are found in the umbo, the annular region and along the malleus. Further studies are needed to identify the source of these cells.

Tissue Remodeling in the Chronic Otitis Media Mouse Model

Nathan Sautter, MD[1], Dennis Trune, PhD[2], Katherine Delaney[2]
[1]Department of Otolaryngology - Head and Neck Surgery, [2]Oregon Hearing Research Center, Oregon Health and Science University, Portland, OR

Introduction
Chronic otitis media (COM) leads to fibrosis, scarring and osteogenesis within the middle ear space, which may contribute to hearing loss and increase difficulty of treatment. The Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF) and Matrix Metalloproteinase (MMP) families are known to contribute to tissue remodeling in other pathologic processes.

Methods
The C3H/HeJ mouse model of spontaneous COM was used. Mice were sacrificed at 2 weeks and 1, 2, 3 and 6 months following onset of COM (n = 8 each time point). Middle ears were analyzed using standard RT-PCR techniques. Change in regulation of mRNA expression of members of the BMP, FGF and MMP families were quantified and compared to untreated controls (n = 8).

Results
Statistically significant upregulation of BMP1 by a factor of 1.8 – 2.9 was noted at all time points ($p<0.05$). FGF7 was upregulated by a factor of 1.9 – 4.0 at all time points ($p<0.05$). MMP2 (factor of 1.8 – 2.9; $p<0.05$), MMP3 (factor of 34.1 – 218.5; $p<0.05$), MMP7 (factor of 2.3 – 15.8; $p<0.05$) MMP9 (factor of 2.4 – 5.9; $p<0.05$), MMP12 (factor of 2.1 – 3.5; $p<0.05$) and MMP14 (factor of 1.3 – 1.6; $p<0.05$) were upregulated at all time points.

Conclusions
Persistent upregulation of BMP1, FGF7 and several members of the MMP family occurs in the COM mouse model. These cytokines may play a role in orchestrating the tissue changes seen in COM. Dramatic upregulation of MMP3 suggests this molecule plays a particularly integral role in tissue remodeling.

Changes of Structure of the Tympanic Membrane During Its Transformation to Retraction Pocket in Children

Ivo Slapak, MD[1]**, Milan Urik, MD**[2]**,** Josef Machac, MD[2]**, Miroslava Sedlackova**[3]
[1]Pediatric ENT, Masaryk University, Brno, czech, [2]Pediatric ENT, Masaryk University, brno, czech, [3]Department of Histology and Embryology, Masaryk University, Brno, Czech Republic

Objective
Analysis of microscopic structure of retraction pocket of tympanic membrane and identification of morphological signs of its transformation into cholesteatoma in child.

Methods
In the project will be used tympanic membranes taken during operations at Paediatric ENT Dept.Brno with the diagnosis of retraction pocket. Material will be fixated immediately after taking and then standardly processed for light and microscopy. Evaluated will be the highest quality cuts. Each cut will be examined and assessed by several parameters for pathological TM and abnormalities.

Results
We found that almost all eardrums removed from patients with reasonable retraction pocket suspicion exhibited a significant morphological TM conversion. in our set we identified ingrowing tissues from the epidermis as plugs of the epithelium extending variously deep into the middle layer of these external TM. Furthermore, we found in two cases that the ingrowth spread even deeply into the tympanic cavity. We found that hypervascularisation, inflammatory infiltrates, and many haemorrhages were present in the middle layer of all the TM samples associated with retraction pockets. The inner layer we discovered structural conversion of the simple cubical epithelium to the squamous epithelium in 9 out of 10 cases. Notably, cholesteatoma derives at this location frequently from squamous epithelium cells.

Conclusion
Proven ingrowth of epidermis cells from the external TM layer to the middle layer, and in some cases even to the tympanic cavity is an essential pathological process to induce the development of the retraction pocket and the cholesteatoma. Both, the retraction pocket and the cholesteatoma can significantly damage the hearing in children. One of many reasons why the epidermis ingrows to the tympanic cavity is that myringotomy is use to treat otitis media. A myringotomy is a surgical incision of the TM, after which a slight injury remains. This tiny pore could potentially create the connection between epidermis and the tympanic cavity. Thus, further questions for otorhinolaryngologists and other specialists are initiated, regarding new treatment strategies to prevent, to diagnostic, and to treat otitis media and the retraction pocket of TM in children.

The Permeability Barrier in Cholesteatoma Matrx - Structure, Biochemical Analysis and Biophysical Microenvironment

Viggo Svane-Knudsen, MD, MD[1]**,** Maria Bloksgaard Mølgaard, PhD[2]**,** Jonathan Brewer, PhD[2]**,** Luis Bagatolli, PhD[2]
[1]Dept. of ENT surg, Odense University Hospital, Odense C, [2]MEMPHYS, University of Southern Denmark, Odense M

The configuration of cholesteatoma stratum corneum was demonstrated by the use of multiphoton fluorescence microscopy, the composition of the barrier lipids was determined by high performance thin layer chromatography (HPTLC)andthe biophysical behaviour of the lipids was achieved by calculation of the generalized polarization (GP) values.

The results demonstrate that the stratum corneum of cholesteatoma matrix is remarkably organized through more than 100 mm which indicates that the permeability barrier is much more substantial than it was formerly thought. The GP values of the intercellular lipids in cholesteatoma matrix demonstrated a tighter packing of the lipids and probably a higher tension, which together could explain the small defects in the intercellular membranes, which was demonstrated previously. HPTLC showed the existence of all the skin permeability barrier lipid classes. However one of the ceremides seems to be missing.This study demonstrate the presence of a permeability and defensive barrier abnormality in cholesteatoma matrix and opens up for the possibility that external stimuli influence cholesteatoma growth .

Biochemistry

Structural Tympanic Membrane Changes in Secretory Otitis Media and Cholesteatoma

Johan Knutsson, MD, PhD[1], Magnus von Unge, MD, PhD[2]
[1]ENT-dept, Karolinska Institute, Stockolm and Center for clinical research, Västerås, Sweden, [2]ENT-dept, Akershus University Hospital & Medical Faculty Division of University of Oslo, Oslo, Norway

Objective
Otitis media in childhood may predispose for retraction pathology. A weakening of the collagen fibers bundles in the lamina propria of the tympanic membrane (TM) is a prerequisite for the formation of a retraction pocket. Various collagen types have different tensile strength. The collagen type distribution in the TM during otitis media and cholesteatoma has not been reported before.

Materials and Methods
TM biopsies from patients with long-standing secretory otitis media and pars tensa cholesteatomas were analyzed with immunohistochemical staining for collagen types I-IV. The histology was also investigated using transmission electron microscopy.

Results
The biopsies showed an intact lamina propria with positive immunohistochemical stainings for collagen types I-III and showed normal collagen fiber bundles on electron microscopy. The outer epithelium of the cholesteatomas showed marked thickness variations and signs of edema. Normal collagen fiber bundles were present in smaller parts of the cholesteatomas, positive for collagen type I-II. In other parts only scattered collagen fibers were found.

Conclusions
Collagen types I-III are present in the lamina propria in long-standing otitis media and no ultrastructural changes of the collagen fiber bundles are seen. Collagen is found in cholesteatomas in the remnants of the lamina propria, with positive staining for collagen types I and II, whereas type III seems to be lacking.

Mucin Gene Expression Expression in Human Middle Ear Epithelium of Otitis Media Patients

Joseph Kerschner, MD[1], Pawjai Khampang[2], Christy Erbe[2], Wenzhou Hong, PhD[2], Blake Papsin, MD[3]
[1]Pediatric Otolaryngology, Children's Hospital of Wisconsin/Medical College of Wisconsin, Milwaukee, WI, [2]Otolaryngology, Medical College of Wisconsin, Milwaukee, WI, [3]Otolaryngology, Hospital for Sick Kids, Toronto, ON

Objective
To assess the expression of gel-forming mucins (GFM), MUC2, MUC5AC, MUC5B and MUC19, in human middle ear epithelium (MEE) of otitis media (OM) patients compared to control patients.

Methods
Children undergoing routine ventilation tube insertion (VTI) for recurrent otitis media (RecOM) or chronic otitis media with effusion (OME) were compared to control patients without a history of otitis media undergoing cochlear implantation. During routine VTI or cochlear implantation, a 1-mm biopsy of the ME epithelium was obtained. RNA was extracted, and real time RT-PCR was used to quantify levels of GFM expression.

Results
Seventy-four OM patients were analyzed using 31 controls. Mean age was not different between groups. The degree of increased expression in patients with OME was greatest for MUC5ACand MUC5B with mean expression increased 155.4 and 138.5 times greater than controls, respectively. The degree of increased expression in patients with RecOM was greatest for MUC5B and MUC5AC with mean expression increased 137.7 and 25.9 times great than controls, respectively.

Conclusions

Levels of GFM expression in human MEE are significantly increased in both OME and RecOM patients compared to controls. MUC5B andMUC5AC demonstrated the greatest response in this patient series. These two GFM have been the primary focus of previous non-clinical studies of GFM in otitis media. This translational study demonstrating this degree of up-regulation in OM patients would suggest an ongoing need for further understanding of the mechanisms involved in the control of these GFM in MEE in order to develop novel treatments for this disease.

Diagnosis/Treatment

Clinical Evaluation of Enzyme-Linked Immunosorbent Assay (ELISA) (ODK-0901) for Detection of *Streptococcus pneumoniae* Antigen in Nasopharyngeal Secretiona and Middle Ear Fluids

Yuki Tatsumi, PhD[1], Muneki Hotomi, MD, PhD[1], Rinya Sugita, MD, PhD[2], Gen Sugita, MD, PhD[1], Masamitsu Kono, MD[1], Akihisa Togawa, MD, PhD[1], Masaki Hayashi, MD, PhD[1], Shin Takei, MD, PhD[1], Yorihiko Ikeda, MD, PhD[1], Noboru Yamanaka, MD, PhD[1]
[1]Otolaryngology, Wakayama Medical University, Wakayama-shi, Wakayama, [2]Sugita ENT clinic, Sugita ENT clinic, Wakayama-shi, Wakayama

Introduction
Streptococcus pneumoniae is one of the major causative pathogens responsible for acute otitis media (AOM) and acute rhinosinusitis (ARS). There has been an alarming increase in penicillin resistant S. pneumoniae worldwide with special emphasis on AOM. For treatments of AOM, it is important to determine S. pneumoniae as a causative pathogen accurately and rapidly. We developed a novel immunochromatographic antigen detection kit (ODK-0901) for detecting C-polysaccharide antigen of S. pneumoniae in middle ear fluids (MEFs) and nasopharyngeal swabs from AOM and ARS.

Method
A total 523 samples, 257 MEFs and 265 nasopharyngeal swabs were obtained from patients with AOM and used in this study. We identified H. influenzae in both specimens by immunochromatographic antigen detection, real-time PCR and conventional bacterial culture. Informed consent was obtained by patients themselves, or their guardians in case of infant patients.

Results
The sensitivity and specificity of ODK-0901 were 81.4 % (48/59) and 80.5% (165/205) in the middle ear fluid or otorrhea of AOM patients, and 75.2 % (121/161) and 88.8 % (95/107) in the nasopharyngeal secretion of ARS patients, respectively.

Conclusion
ODK-0901 was shown to be a highly sensitive detection method of S. pneumoniae, and a prominent tool for identifying the pathogen as S. pneumoniae in AOM and ARS. ODK-0901 was shown to be a prominent tool for identifying the pathogen as S. pneumoniae in MEFs and nasopharyngeal secretions. The rapid immunochromatographic test showed high specificity and attractive for identifying S. pneumoniae in MEFs and nasopharyngeal swabs in the era of antimicrobial resistance.

The Range of Tympanometric Curves in Different Otoscopic Findings

Kjell Helenius, MD, Paula TÃ¤htinen, MD**,** Miia Laine, MD**,** Aino Ruohola, MD, PhD
Department of Pediatrics, Turku University Hospital, Turku, Finland

Objectives
Previously, studies on tympanometry have not analyzed the distribution of tympanometric curves in different otoscopic findings. The objective of our study was to observe the range of tympanometric curves in different categories of otoscopic findings in an outpatient setting.

Methods
The 569 patients aged from 6 to 35 months were followed up at 2857 visits, with tympanometry and pneumatic otoscopy performed at every visit. (BEST-Fin, ClinicalTrials.gov number, NCT00299455).

The tympanometric curves were divided as follows: A: tympanometric peak pressure (TPP) over -100 daPa, C: TPP less than -100 daPa, or B: no peak. Otoscopic findings were divided into 5 categories: healthy, middle ear fluid (MEF) with visible air-fluid level (a/f-MEF), MEF, acute otitis media (AOM) with visible air-fluid level (a/f-AOM), and AOM.

Results
Tympanograms were obtained from 3242 ears. Of these 1729 were otoscopically verified as healthy, 475 as a/f-MEF, 443 as MEF, 143 as a/f-AOM and 452 as AOM. Of the tympanograms, 1298 were type A, 952 type C and 992 type B.
The tympanograms were distributed as follows:
Healthy: A 63 %, C 33 %, B 4 %. A/f-MEF: A 18 %, C 45 %, B 37 %. MEF: A 10 %, C 17 %, B 73 %. A/f-AOM: A 20 %, C 30 %, B 50 %. AOM: A 9 %, C 11 %, B 80 %.

Conclusions
The range of tympanometric curves varies considerably depending on the category of otoscopic finding.

Symptoms of Children During Unilateral and Bilateral Acute Otitis Media

Johanna Uitti, MD[1], Paula Tähtinen, MD[2], Miia Laine, MD[2], Aino Ruohola, MD, PhD[2]
[1]Department of Pediatrics, Turku University hospital, Turku, [2]Department of Pediatrics, Turku University Hospital, Turku

Introduction
It has been suggested that bilateral acute otitis media (AOM) causes more severe symptoms compared with unilateral AOM. Still there are only a few studies concerning the issue. Our aim was to find out is it possible to differentiate unilateral and bilateral AOM based on symptoms.

Methods
232 children aged 6-35 months came for an outpatient visit due to parental suspicion of AOM, their symptoms were surveyed by structured questionnaire, and diagnosis of unilateral or bilateral AOM was done by pneumatic otoscopy added with tympanometry. Children were included in unilateral group, if they had AOM and the other ear was healthy or had middle ear fluid. The prevalences of symptoms were compared by using x^2 test.

Results
98 children had bilateral and 134 had unilateral AOM. 13% of bilateral AOM group were over two years old compared to 25% of unilateral AOM group (P=0.032). Possibly due to younger age, bilateral AOM group had less verbally self-expressed ear pain (bilateral AOM 11% vs. unilateral AOM 25%; P=0.010). Bilateral AOM group had fever (>38 ^{0} C) more often than unilateral AOM group (54% vs. 36%; P=0.006). No difference were been seen between unilateral and bilateral AOM groups in the occurrence of ear rubbing (71% vs. 68%), irritability (87% vs. 87%), excessive crying (91% vs. 84%), restless sleep (85% vs. 87%), rhinitis (93% vs. 94%), cough (85% vs. 75%), mucus vomiting (15% vs. 6%), and conjunctivitis (19% vs. 19%), respectively.

Conclusion
It seems to be impossible to differentiate unilateral and bilateral AOM solely based on symptoms.

Prior Detection of Nasopharyngeal Colonization Is Associated with Less Frequent Acute Otitis Media in Children Caused by *Haemophilus influenzae* and *Streptococcus pneumoniae*

Michael Pichichero, MD[1], Arthur Chang[1], Ravinder Kaur, PhD[1], Ryan Gallagher[1], Linlin Chen, PhD[2], Janet Casey, MD[3]
[1]Research Institute, Rochester General Hospital, Rochester, New York, [2]Biostatistics, Rochester Institute of Technology, Rochester, New York, [3]Pediatrics, Legacy Pediatrics, Rochester, New York

Introduction
Colonization by otopathogens in the nasopharynx (NP) precedes AOM infection and colonization by otopathogens has been shown to increase the risk of AOM infection.
NP Colonization by otopathogens may elicit a capsular specific serum antibody response to *Streptococcus pneumoniae* (Spn)and nontypeable *Haemophilus influenzae* (NTHi) and/or tosurface exposed antigen response to both Spn and NTHi.

Methods
Study Population. Prospective Enrollment: 3.5-year time span June, 2006 to December, 2009. Healthy children without previous episodes of AOM from a middle class, suburban socio-demographic pediatric practice in Rochester, NY (Legacy Pediatrics). Serum, NP and oropharyngeal (OP) cultures and NP wash samples obtained seven times, every 3-6 months, between 6 and 30 months of age (at age 6, 9, 12, 15, 18, 24, and 30 months). If a child developed symptoms compatible with AOM, a tympanocentesis was performed to confirm the diagnosis. At the time of the acute AOM diagnosis and three weeks later acute and convalescent serum, NP and OP cultures and NP wash samples were obtained, cultured.

Antigens
Protein D,P6 and OMP26- specific antibody titers were determined by ELISA using purified recombinant protein D (provided as a gift from GlaxoSmithKline Biologicals, Rixensart Belgium), P6 (provided as a gift by Dr. Tim Murphy, University of Buffalo) and OMP26 (provided as a gift by Jennelle Kyd, University of Canberra, Australia). PhtD, LytB, PcpA, PhtE and Ply- specific antibody titers were determined by ELISA using purified recombinant proteins (provided by sanofi pasteur CA, Toronto, Canada).

Results

Description of the Study Population

168 children enrolled, 737 visits were included in the analysis. Mean time until development of AOM was 5.1 months after enrollment (mean age= 11.1 months). Majority were white race (82.7%), did not attend day care (67.3%), was breast fed (64.9%), and had no tobacco smoke exposure (91.7%).

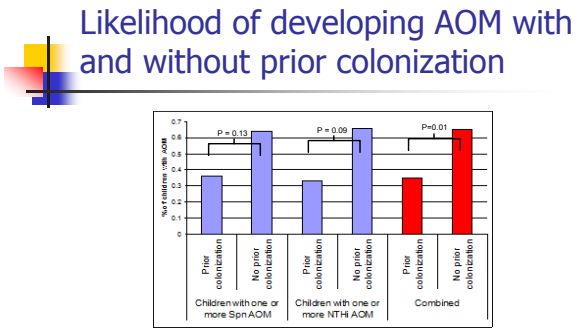
NP colonization and AOM events due to NTHi.

130 NTHi OP/NP colonization episodes were documented in 63 (37.5%) children at one or more of the seven sampling visits. There were 26 NTHi AOM episodes in 18 children. Six (33%) of the 18 children experienced an AOM due to NTHi following a detected NTHi NP colonization. Twelve (66%) of the 18 children experienced an AOM due to NTHi without a preceding NP colonization with NTHi detected, a higher frequency than among children who had NP colonization before an AOM (p=0.09).

NP colonization and AOM events due to Spn

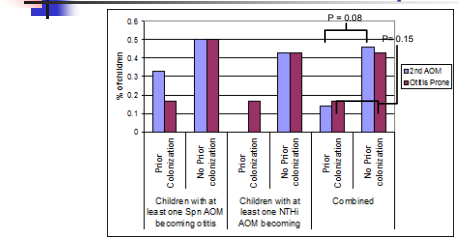
259 Spn OP/NP colonization episodes were documented in 102 (61%) children at one or more of the seven sampling visits. There were 29 Spn AOM episodes in 22 children. Eight (36%) of 22 children experienced an AOM due to Spn following a detected Spn NP colonization. 14 (64%) of 22 children experienced an AOM due to Spn without a detected preceding NP colonization, a higher frequency than among children who had NP colonization before an AOM (p=0.13).

Figure 1.



Organism-specific analysis showed trends that prior colonization reduces the likelihood of developing an AOM. When data for Spn and NTHi are combined the difference becomes statistically significant (P = 0.01)

Figure 2.



Organism-specific analysis of the difference in proportion of children progressing from one AOM to a second AOM for NTHi and Spn lacked the sample size and statistical power to identify a difference. However when both species were combined, there were trends for significance for both comparisons.

Figure 3.

Spn Acute / Post NP Colonization without Spn AOM

	Spn Colonization without AOM	
	Acute	Post
PhtD	**4.0** LCI: 2.0 UCI: 8.0 n=39	**14.8** LCI: 6.5 UCI: 33.9 n=39
LytB	**0.02** LCI: 0.01 UCI: 0.04 n=36	**0.04** LCI: 0.02 UCI: 0.07 n=36
PcpA	**16.4** LCI:6.1 UCI: 43.9 n=37	**80.4** LCI: 27.0 UCI: 239.5 n=37
PhtE	**1.7** LCI: 0.8 UCI: 3.7 n=40	**2.8** LCI: 1.2 UCI: 6.4 n=40
PlyD1	**0.8** LCI: .5 UCI: 1.3 n=40	**1.5** LCI: 1.0 UCI: 2.5 n=40

4 of the 5 antigens showed a statistically significant increase (P < 0.05) in serum antibody titers after colonization.

Figure 4.

NTHi Acute/post NP Colonization without NTHi AOM

	Protein D		P6		OMP26	
	Acute	Post	Acute	Post	Acute	Post
NTHi colonization without AOM	**.88** LCI: .54 UCI: 1.45 n=25	**1.29** LCI: .92 UCI: 1.83 n=25	**.96** LCI: .51 UCI: 1.80 n=25	**1.2** LCI: .80 UCI: 1.81 n=25	**.70** LCI: .46 UCI: 1.07 n=24	**.93** LCI: .64 UCI: 1.36 n=24

NTHi colonization without AOM when combining all three antigens showed a significant increase in serum antibody titers after first detectable colonization (P<0.05)

Conclusions
An asymptomatic episode of NP colonization with NTHI produced serum antibody responses to vaccine candidate proteins D, P6 and OMP26 (p=0.04) and those with colonization due to Spn produced antibody responses to vaccine candidates PhtD, LytB, PcpA, PhtE and Ply (p<0.05 – 0.01). Consequently AOM occurred less frequently in children with prior NP colonization (p=0.01), and children with prior NP colonization who developed one AOM were less likely to experience a second AOM with the same otopathogen (p=0.08) or to become otitis prone (p=0.15). Children who experience NP colonization due to NTHi or Spn develop AOM less frequently than children who have no prior colonization and this difference is associated with higher serum antibody levels to potential vaccine candidate proteins.

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National Institute for Clinical Excellence Guidelines on the Surgical Management of Otitis Media with Effusion: Are They Being Followed and Have They Changed Practice?

Mat Daniel[1], Tawakir Kamani[3], Suliman El-Shunnar[4], Marie-Claire Jaberoo[5], Anna Harrison[3], Seema Yalamanchili[5], Laura Harrison[6], WS Cho[1], Neil Fergie[7], Roger Bayston[2], John Birchall[1]
[1]Otorhinolaryngology Head & Neck Surgery, [2]Biomaterials Related Infection Group, The University of Nottingham, Nottingham, Notts, [3]Otorhinolaryngology Head & Neck Surgery, Royal Derby Hospital, Derby, Derbyshire, [4]Otorhinolaryngology Head & Neck Surgery, Lincoln County Hospital, Lincoln, Lincs, [5]Otorhinolaryngology Head & Neck Surgery, Royal Free Hampstead Hospital London, London, London, [6]Otorhinolaryngology Head & Neck Surgery, King's Mill Hospital, Sutton in Ashfield, Notts, [7]Otorhinolaryngology Head & Neck Surgery, Nottingham University Hospital, Nottingham, Notts

Objectives
UK National Institute for Clinical Excellence (NICE) guidelineson surgical management of OME in children call for an initial 3 month period of observation, with ventilation tubes (VTs) considered for children with persistent bilateral OME with a hearing level in better ear of 25–30dBHL or worse ("core criteria"), or for children not meeting those audiologic criteria but when OME has significant impact on developmental, social or educational status (extenuating circumstances). We aimed to establish whether VTs are being inserted in accordance with NICE guidelines, and whether guidelines have changed practice.

Methods
Retrospective case-notes review in five centres, analysing practice in all children having first VT insertion before (Jul-Dec06) and after (Jul-Dec08) guidelines' introduction.

Results
Records of 316 children were analysed, 172 before and 144 after guidelines' introduction. There were no significant differences in practice before and afterguidelines with respect to having 2 audiograms 3 months apart (57.8v54.8%), OME persisting ≥3 months (93.6v91.1%), or fulfilment of 25dB audiometric criteria (68.2v61.0%). Whereas 43.9% of VT insertions met the "core criteria" prior to guidelines' introduction, this fell to 32.2% after (p=0.032); however, if VTs inserted for extenuating circumstances were included there was no significant difference (85.5v87.0%) because the proportion of VTs inserted for extenuating circumstances increased after guidelines.

Conclusions
Most children have VTs inserted in accordance with NICE guidelines providing the extenuating cases are included, but fewer than a third meet the "core criteria". Guidelines do not appear to have changed practice.

Effect of Immediate Versus Delayed Initiation of Antimicrobial Treatment on Symptoms During Acute Otitis Media

Paula A Tähtinen, MD[1], Miia K Laine, MD[1], **Aino Ruohola, MD, PhD**[1]
[1]Department of Pediatrics, Turku University Hospital, Turku, Finland

Objectives
Several guidelines suggest watchful waiting for the management of acute otitis media (AOM). Nevertheless, the effect of delayed initiation of antimicrobial treatment on recovery of AOM is yet uncertain. We compared times to resolution of symptoms in patients who received either immediate or delayed antimicrobial treatment for AOM.

Methods
In our randomized, double-blind trial, patients (6-35 months) with AOM received amoxicillin-clavulanate (Immediate antimicrobial treatment group, n=161) or placebo (n=158) for 7 days. Rescue treatment was initiated in 34% of placebo group (Delayed antimicrobial treatment group, n=53).(BEST-Fin, ClinicalTrials.gov number, NCT00299455)

Based on diary, time to resolution of symptoms was analyzed and medians provided using Kaplan-Meier method with log-rank test.

Results
Immediate and delayed antimicrobial treatment groups were similar according to age (mean 1.3 years), gender, and mean number of previous AOM.

Delayed as compared to immediate antimicrobial treatment significantly elongated time to resolution of fever (median time to resolution 3.0 and 1.3 days, respectively, P<0.001), parentally reported ear pain (medians 3.5 vs. 2.0, P=0.039), poor appetite (medians 4.5 vs. 2.5, P=0.017), and decreased activity (medians 3.5 vs. 2.0, P=0.002), but not verbally self-expressed ear pain (medians 3.5 vs. 1.8, P=0.154), ear rubbing (medians 2.5 vs. 3.0, P=0.434), restless sleep (medians 3.5 vs. 2.5, P=0,089), irritability (medians 3.5 vs. 2.5, P=0.615), or excessive crying (medians 3.5 vs. 3.0, P=0.445).

Conclusions
Delayed initiation of antimicrobial treatment elongates time to resolution of symptoms during AOM episode. This should be taken into account when choosing between watchful waiting and immediate antimicrobial treatment of AOM.

A Placebo-Controlled Trial of Antimicrobial Treatment for Acute Otitis Media

Paula A Tähtinen, MD[1], Miia K Laine, MD[1], Pentti Huovinen, MD, PhD[2], Jari Jalava, PhD[3], Olli Ruuskanen, MD, PhD[1], **Aino Ruohola, MD, PhD**[1]
[1]Department of Pediatrics, Turku University Hospital, Turku, [2]Department of Medical Microbiology and Immunology, University of Turku, Turku, [3]Division of Health Protection, National Institute for Health and Welfare, Turku

Objective
The efficacy of antimicrobial treatment in acute otitis media remains controversial.

Methods
In this randomized, double-blind trial, patients, 6–35 months of age, with strictly diagnosed acute otitis media received amoxicillin-clavulanate (N=161) or placebo (N=158) for 7 days (BEST-Fin, ClinicalTrials.gov number, NCT00299455). Primary outcome was time to treatment failure from the first dose until end of treatment visit on day 8. Treatment failure was based on symptomatic condition (including adverse events) and otoscopic signs.

Results/Conclusions
The results are currently confidential due to submission in a peer-review journal. However, the results will be presented in the meeting and a complete abstract will be submitted.

Mobile Phones for Enhanced Case Management of Chronic Suppurative Otitis Media (CSOM) in Remote Australian Indigenous Communities: MOP-UP, a Pilot Randomized Controlled Trial

James Philips, Christine Wigger**,** Anna Steven**,** Peter Morris, PhD**,** Amanda Leach, PhD
[1]Child Health Division, Menzies School of Health Research, Darwin, Northern Territory

Introduction
Chronic suppurative otitis media (CSOM or 'runny ears') affects around 40% of Indigenous children in remote communities during the first 18 months of life. Evidence from RCTs indicates resolution of ear discharge may be achieved withlong term daily cleaning and application of antibiotic drops. Adherence is challenging.Availability of mobile phone (MOP) networks in some regions provided the opportunity to explore their use in enhanced case management. Potential limitations included poor literacy and English as a second language.

Methods
Children less than 13 years of age with a tympanic membrane perforation (TMP) and living in a family with access to mobile phone were eligible for randomisation to MOPmessages or no messages. Video caricature messages in the local Indigenous language were sent every 4 days for 6 weeks. Primary outcome was the number of clinic visits for an ear health check. Secondary outcomes included amount of ear discharge and TMP size.

Results
Two remote communities participated. 129 children were screened; 54 were randomized and 45 were seen at six weeks. MOP messaging was acceptable and motivating for the majority of families. Clinic attendance and ear assessments at 6 weeks will be reported.

Conclusion
This is the first randomised controlled trial of mobile phone messaging in an Indigenous language in a population at high risk of CSOM. This novel and culturally appropriate approach offers a new opportunity to enhance the case management of chronic diseasein these remote and disadvantaged communities. Greater NextG coverage should be supported.

Office Insertion of Tympanostomy Tubes Without Anesthesia in Young Children

Richard Rosenfeld, MD, Krishna Sury**,** Christopher Mascarinas, MD
Department of Otolaryngology, SUNY Downstate, Brooklyn, NY

Introduction
Tympanostomy tube insertion is one of the most common surgical procedures in children, with about 12,000 cases performed weekly in the United States. To improve child comfort, general anesthesia is typically used to conduct the procedure. General anesthesia for tube insertion, however, is associated with a 9% incidence of minor complications and 2% incidence of major adverse events. Moreover, early exposure of young children to general anesthesia may predispose to learning disabilities.
The purpose of this study is to report outcomes of office insertion of tympanostomy tubes, without anesthesia, in children age 18 months or younger and to compare those outcomes with a similar cohort of children who had tubes placed in the operating room under general anesthesia.

Methods
This study was conducted at a hospital-based pediatric otolaryngology practice in Brooklyn, New York. A historical cohort was identified by chart review of all children aged 3 to 18 months who had bilateral tympanostomy tubes inserted as a sole surgical procedure by the principal investigator (RMR) as part of routine clinical care for otitis mediabetween January 2006 and March 2009. The goal was to identify approximately 50 children who had tubes placed in the office setting and 50, for comparison, who had tubes placed in the operating room. Children with excluded if they had concurrent surgery other than tubes, tubes were inserted for an indication other than otitis media, the child had an syndrome or craniofacial anomaly, or the caregiver could not speak English. Institutional review board approval was obtained before starting the study.
All procedures were performed as part of routine clinical care using Armstrong beveled fluoroplastic tympanostomy tubes and a binocular microscope. Office insertion was accomplished using a papoose board for restraint and an assistant to steady the child's head. Parents were informed prior to the procedure that the child would likely cry immediately upon being restrained, but would only experience discomfort during the myringotomy and actual tube insertion, which would occupy about 1-2 minutes of the roughly 7-8 minute total procedure time. Tube insertion in the operating room was performed using midazolam premedication followed by mask anesthesia using nitrous oxide and sevoflurane under the supervision of a pediatric anesthesiologist.

The office charts were reviewed to obtain demographic data and information about the child's clinical history, which was recorded on a de-identified data form. The primary outcome was caregiver responses about changes observed after ear tubes using a 5-point Likert scale (agree strongly, agree, neither agree nor disagree, disagree, disagree strongly, don't know). The following questions were administered using a telephone interview by an impartial research assistant:
Getting the ear tubes inserted was pleasant for my child
Having my child get ear tubes was pleasant for me as a parent
My child recovered quickly after the tubes were placed
My child had nightmares or bad memories after the procedure
I am satisfied with the overall experience of getting the ear tubes
Secondary outcomes were obtained from the medical record and included (a) time to obstruction of first tube, (b) time to extrusion of first tube, (c) time to malfunction (obstruction or extrusion) of first tube, and (d) time to extrusion of both tubes. All statistical analyses were performed using SPSS software and comparisons were made with the Mann-Whitney U test, Fisher's exact test, Pearson chi-square, and Kaplan-Meier survival analysis curves. All tests were performed using a two-sided alpha level of 0.05 for statistical significance.

Results
The final sample included 46 children with tubes inserted in the office and 48 children with tubes inserted in the operating room. The two cohorts were comparable with the exception of a slightly younger age and greater prevalence of acute otitis media in the office-insertion group (Table 1). Tubes were inserted for recurrent acute otitis media (25%), otitis media with effusion (20%), or both (55%). Most children had bilateral effusions (82%) and a mild hearing loss (median pure tone average, 35 dB HL).

No differences were found (Tables 2 and 3) for tube insertion in the office vs. operating room for parent perceptions of child recovery (P=.386), overall satisfaction (P=.676), post-procedure nightmares or bad memories (P=.113), pleasantness of procedure for the parents (.848), or pleasantness of procedure for the child (P=.060). Similarly, no differences were found (Table 4) for median time to failure (extrusion or obstruction) of one tube (P=.252) or both (P=.445) No adverse events occurred during tube placement.

Discussion

Our study is the first to compare outcomes of tympanostomy tube insertion in young children without anesthesia in an office setting to traditional placement of tubes under general anesthesia in the operating room. Overall there were no differences in caregiver reports of satisfaction with the procedure, their child's recovery, or the duration of tube patency. In addition, those parents who had experienced the procedure in both settings (office and operating room) with different children universally preferred office placement because the procedure was rapid, their child did not have to fast, and there was immediate recovery with ability to console and feed the child.

The issue of where to perform tympanostomy tube placement in young children involves a process of shared decision-making between the clinician and caregiver, which also takes into account the clinician's experience and comfort level. In addition, caregiver values are an important consideration. For example, some caregivers are very concerned about potential adverse events of anesthesia, the need to keep their child without food prior to the procedure, and the potentially frightening emergence delirium that is typically lasts 5-15 minutes after surgery, but has been associated with agitation and regressive behavior lasting up to 2 days. Other caregivers may be less concerned with general anesthesia, but more worried about having their child be restrained in a papoose board and experience brief pain during the procedure. Most caregivers, however, are comfortable with child restraint if properly counselled.

Limitations of this study include non-randomized allocation, recall bias by caregivers, and allocation bias caused by self-assignment to procedure setting by the caregivers. We conclude, however, that the findings are sufficient to justify office insertion of tubes as a well-tolerated, reasonable alternative to general anesthesia for caregivers and clinicians who are comfortable with this choice.

Table 1.—Characteristics of 94 children under age 19 months receiving tympanostomy tubes

Characteristic	Inserted in office	Inserted in OR	Total	P value
Total subjects, n(%)	46(49)	48(51)	94(100)	—
Child age in months, mean (SD)	11.3(3.2)	13.4(3.6)	12.5(3.6)	.021†
Male gender, n(%)	19(40)	29(60)	48(51)	.098§
Developmental delay or in therapy, n(%)	9(20)	21(44)	30(32)	.012§
Otitis media duration in months, median(IQR)	6(5)	6(5)	6(5)	.811†
Prior AOM episodes, median(IQR)	5(6)	5(5)	5(5)	.730†
Indication for tubes, n(%):				
AOM or recurrent AOM only	19(41)	4(8)	23(25)	<.001§
OME only	10(22)	9(19)	19(20)	
Both AOM and OME	17(37)	35(73)	52(55)	
Middle-ear effusion, n(%):				
None	2(4)	4(8)	66)	.695§
Unilateral	5(11)	6(13)	11(12)	
Bilateral	39(85)	38(79)	77(82)	
Bilateral flat (B) tympanogram, n(%)	23(72)	24(63)	47(67)	.737§
Valid audiogram, n(%)	38(83)	44(92)	82(87)	.227‡
Hearing level in decibels, mean(SD):				
Pure tone average (0.5, 1, 2 kHz)	37(15)	36(10)	35(18)	.782†
Speech reception threshold	30(23)	33(14)	30(15)	.863†
Years until survey, median(IQR)	1.1(1.7)	1.6(1.7)	1.6(1.7)	.022†

AOM, acute otitis media; IQR, interquartile range; OME, otitis media with effusion; OR, operating room; SD, standard deviation

†Mann-Whitney U test
‡ Fisher's exact test
§ Pearson chi-square

Table 2.—Mean caregiver responses regarding the tube insertion experience

Survey item	Response, mean (SD)†		
	Inserted in office	Inserted in OR	P value‡
Getting the ear tubes inserted was pleasant for my child	3.2(1.5)	2.6(1.2)	.060
Having my child get ear tubes was pleasant for me as a parent	2.6(1.5)	2.6(1.4)	.848
My child recovered quickly after the tubes were placed	1.5(0.8)	1.6(0.7)	.386
My child had nightmares or bad memories after the procedure	4.2(1.0)	4.5(0.6)	.113
I am satisfied with the overall experience of getting the ear tubes	1.4(0.7)	1.3(0.6)	.676

OR, operating room; SD, standard deviation
† Range from 1 (agree strongly) to 5 (disagree strongly)
‡Mann-Whitney U test

Table 3.—Caregiver agreement with survey items regarding tube insertion experience

Survey item	Agreement, n (%)†			
	Inserted in office	Inserted in OR	Odds ratio (95% CI)	P value
Getting the ear tubes inserted was pleasant for my child	21/46(46)	28/45(62)	.51(.22, 1.18)	.113
Having my child get ear tubes was pleasant for me as a parent	25/44(57)	31/48(65)	.72(.31, 1.67)	.446
My child recovered quickly after the tubes were placed	43/46(94)	46/48(96)	.62(.10, 3.91)	.611
My child had nightmares or bad memories after the procedure	4/44(9)	0/42(0)	—	.117
I am satisfied with the overall experience of getting the ear tubes	44/46(96)	47/48(98)	.47(.04, 5.35)	.613

CI, confidence interval
†Includes "agree" and "strongly agree" responses

Table 4.—Outcomes of tympanostomy tube insertion

Outcome	Time to outcome (95% CI)†		
	Inserted in office	Inserted in OR	P value
Median months until one tube nonfunctional‡	12.9(8.3, 17.)	15.6(12.3, 18.8)	.252
Median months until both tubes nonfunctional‡	28.2(17.2, 39.2)	24.8(20.4, 29.1)	.445
Median months until repeat tube insertion (if occurred)	30.0(27.9, 33.6)	25.3(22.9, 27.7)	.138

OR, operating room; SD, standard deviation
†Kaplan-Meier survival analysis with log rank comparison
‡Nonfunctional defined as obstructed or extruded

Prevalence of Otitis Media in Cleft Palate Infants Is Affected by Diagnostic Technique

Allison Tobey, MD[1], **Cuneyt M. Alper, MD**[2], Todd Otteson, MD[2], Joseph E. Losee, MD[3], William J. Doyle, PhD[2]
[1]Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA, [2]Department of Otolaryngology, Division of Pediatric Otolaryngology, [3]Department of Plastic Surgery, Division of Pediatric Plastic Surgery, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction

Nonsyndromic clefts of the lip and/or palate (CL/P) are among the most common birth defects, affecting approximately 1 in 595 newborns in the United States[1]. Otitis media (OM) is a known complication of CL/P [2, 3] and early studies reported OM to be a nearly universal condition in young CL/P infants [2,4,5]. More recent studies demonstrated that OM in CL/P patients persists throughout childhood and into adolescence at a much higher frequency [6,7] and is associated with greater prevalences of hearing loss [6], language delays [8] and cholesteatoma when compared to an age-matched population with CL/P. However, recent studies reported a lesser OM prevalence in CL/P infants when compared to earlier studies and the reasons for this discrepancy has not been clarified.

Because of the increased risk of OM and its complications in CL/P patients, early, prophylactic myringotomy and tympanostomy tube insertion (M&T) has been recommended [9] and later studies documented lesser OM prevalences and improved hearing in CL/P patients treated with that procedure . However, controversy continues to exist regarding the long term efficacy and potential complications of this treatment strategy [3]. Other interventions have been evaluated in studies but documented no effects on OM and hearing of the timing of palatal surgery [10] and, with few exceptions, of the type of palatoplasty performed [7].

Because the data on the prevalence of OM in CL/P infants has not been updated in recent years and the treatment of OM in the CLP population remains controversial, the present study evaluated the prevalence of OM during the first year of life in CL/P

patients followed at the University of Pittsburgh Craniofacial/Cleft Palate Center (UPCCC) and explored the short-term effects of M&T on HL in those patients. It is anticipated that these results will be used to establish the potential population available for enrollment per year, and the sample size and power estimates for future clinical trials of the long-term efficacy of M&T and different palatoplasty procedures on OM and HL in CL/P patients

Methods and Materials
This is an IRB approved case series and medical record review in a tertiary-care academic children's hospital. All non-syndromic CL/P patients followed during first year of life between 1/2003 & 7/2008 are included.

Main outcome measures:
1) Demographics 2) Age at & findings from first otoscopic exams *(by pediatrician/nurse practitioner & by pediatric otolaryngologist)* 3) Age and findings @ time of M&T & palate surgery 4) Age of & finding obtained by tympanometry 5) Age of & findings obtained during hearing evaluations

Results
Of the 225 patients, 108 (48%) were female and the racial distribution was 207 (93%) Caucasian; 11 (5%) Black; 4 (2%) Asian and 3 (1%) other or mixed race. The patient's cleft types were classified as: 24 (11%) Veau I; 83 (37%) Veau II; 81 (36%) Veau III, and 37 (16%) Veau IV. Most patients (82%) were seen at the UPCCC within the first month of life. Information on newborn hearing testing (NBHS) was available for 170 patients. Seventy-five percent of patients passed their NBHS bilaterally, 2% failed in one ear and 23% failed in both ears.

Typically, otoscopic evaluations were done first by a physician or nurse practitioner (average age=1.9±2.9 months). Results were available for 72 patients and OM was documented in 28 (39%; 1 unilateral). In contrast, for an approximate age-matched (2.9$\pm$0.7 months) group of 100 patients examined by a pediatric otolarygologist, OM was documented by otoscopy in 71 patients (71%, 13 unilateral). The difference in ages between the two evaluations was not significant (Student's t=0.92, p=0.36) but the difference between OM prevalence diagnosed at the two assessments was significant ($\chi 2$=17.7, p<.001). Of interest, the results of the otoscopic exams done on 53 patients by a pediatric otolarygologist at a much earlier age of 1.0$\pm$0.4 months documented OM in 39 patients (73.6%, 9 unilateral) which is greater than the disease prevalence for diagnoses by a physician or nurse practitioner but similar to at that diagnosed by a pediatric otolaryngologist at the lager age range. Overall, pre-M&T otoscopy was done on 71 patients by non–ENT practions and 195 patients by a pediatric otolaryngologist. OM was diagnosed in 28 (39.4%, 1 unilateral) and 142 (73%, 17 unilateral) by non-ENT practioners and ENT physicians respectively.

Pre-M&T tympanograms were obtained on 130 patients with OM diagnosed in 113 (87%, 15 unilateral) patients. M&Ts were performed on 212 patients (94.2%); 210 patients were ≤ 16 months at M&T. The presence or absence of fluid was reported for M&Ts performed on 197 patients <16 months of age, with OM diagnosed in 187 (94.9%, 12 unilateral). The difference in the prevalence of OM diagnosed by diagnostic method was statistically significant ($\chi 2$=34.5, p<.001)

Patients with cleft lip typically underwent lip repair at 3-6 months of age (average=5.3$\pm$2.0 months). Primary palate repair was typically performed between 9-16 months of age (average=13$\pm$2.3 months). In most cases, an attempt was made to coordinate M&T with cleft surgeries to avoid additional anesthetic exposure. This is reflected in the age at which patients underwent their first M&T procedure. Specifically, the 119 patients with CL&P underwent M&T at 5.5$\pm$2.4 months as compared to 8.3 $\pm$6.2months for the108 patients with CP only (Students t=4.55; p<.001). Of the 100 patients with CP only who had undergone palatoplasty in the 1st year of life, 66 had M&T procedures done prior to their palatoplasty, 28 at the time of palatoplasty, 1 after palatoplasty and the remaining 5 did not have an M&T procedure.

Discussion
In this study, data were collected for the diagnosis of OM using different assessors and methods for disease assignment. The result showed a significantly lower prevalence of diagnosed OM when otoscopy was done by non-ENT physicians when compared to ENT physicians. This discrepancy is likely due to between-group differences in the use of pneumatic otoscopy and/or their level of training or experience. Also documented, were significant differences in the prevalence of OM by by otoscopy (for both non-ENT practitioners and ENT physicians), an intermediate value for diagnoses made by tympanometry and a high value for diagnoses made by M&T. It can be argued that our M&T population is biased, since the subset of patents in which M&T would be recommended are those likely to have fluid diagnosed in clinic. However, during the period of data collection, it was standard of care to perform M&T at time of cleft surgery (lip or palate) or sooner on all CLP patients regardless of middle ear status. This is reflected by the fact that 94.2% of the study population obtained M&T and therefore we feel that this biased is reduced.

The observation that different types of examiners and different diagnostic techniques produce different prevalence of OM raises an important issue regarding the comparability of the results for OM prevalence CL/P patients among studies as well as the criterion for how the presence of OM should be defined in the CL/P infant population.

The limitations of the present study are primarily related to the retrospective nature of the collected data and, specifically, the lack of a consistent, formal protocol used for the regular and systematic evaluations of OM in the CL/P patients followed at the UPCCC.

Conclusions
OM prevalence in CL/P infants is high and is affected by the otoscopic training of the assessor and by the type of diagnostic method used for assignment, but not by the age at the time of the evaluation or cleft type. OM prevalence was not associated with age or cleft type. Because this study was a chart review and the information recorded in each chart was not standardized or complete for all enrolled patients, these results should be considered preliminary. A more formal, prospective protocol for the study of OM prevalence in infant CL/P patients is recommended.

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Clinical Outcome of Pediatric Acute Otitis Media Caused by Beta-Lactamase Negative Ampicillin Resistant *Haemophilus influenzae*

Atsuko Masuno, MD, Muneki Hotomi, MD, PhD**,** Masamitsu Kono, MD, PhD**,** Shin Takei, MD**,** Yorihiko Ikeda, MD, PhD**,** Gen Sugita, MD, PhD**,** Akihisa Togawa, MD, PhD**,** Shinji Tamura, MD, PhD**,** Noboru Yamanaka, MD, PhD
Department of Otolaryngology, Wakayama Medical University, Wakayama City, Wakayama

Introduction
Haemophilus influenzae is one of the leading causative pathogens for acute otitis media. Recently there has been an alarming increase of antimicrobial resistant strains without production of beta-lactamase; beta-lactamase non-producing ampicillin resistant H. influenzae (BLNAR). In this study, we investigate the clinical outcomes of AOM with the nasopharyngeal colonization of BLANR.

Method
The clinical course of children with AOM colonized with H. influenzae in nasopharynx was evaluated in this study. The diagnostic criteria for AOM included the onset of one or more symptoms such as ear pain, crying or fever, and tympanic membrane changes of erythema, bulging or decreased landmarks. Exclusion criteria included another illness within 1 month, antibiotic use within 1 month, ≥4 episodes of AOM in the past, a previous history of tympanostomy tubes, craniofacial anomalies, immune deficiency, including immunosuppressive medications, a second focus of infection, and allergy to AMPC. All children were treated with AMPC (40 mg/kg) for the first 5 days followed by CDTR-PI (10 mg/kg) or CVA-AMPC (40 mg/kg).

Results
The failures of early improvement of AOM were associated with BLNAR. The clinical course of children colonized with BLNAR in nasopharynx was worse than children with susceptible strains of H. influenzae.

Conclusion
The current findings showed that the clinical features of AOM were closely associated with nasopharyngeal colonization with BLNAR. Nasopharyngeal colonization with BLNAR will be one of the risk factors for intractable AOM among children.

Myringotomy and Tympanostomy Tube Insertion in Adults: Initial Diagnosis, Risk Factors, and Follow-Up for Disease Recurrence

Richard Joseph M. Villardo, MD[1], William J. Doyle, PhD[1], Margaretha L. Casselbrant, MD, PhD[1], Barry Hirsch, MD[2]
[1]Department of Otolaryngology, Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA, [2]Department of Otolaryngology, Eye and Ear Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction
Otitis Media (OM) is a very common disease in children that also affects adults.[1,2] While most OM episodes are short-lived and self-limiting whether or not they are treated medically, some episodes persist from a few months to years, a presentation usually referred to as chronic otitis media with effusion (cOME).[3] COME is the most common cause of conductive hearing loss[4] and can cause permanent sensorineural hearing loss.[5,6] Established cOME is unresponsive to most medical treatments including antibiotics,[7] decongestants and antihistamines,[8] and steroids,[9] but myringotomy, and with/without tympanostomy tubes insertion (M w/wo TTI) effects symptom resolution in almost all treated ears.[10-11] Nonetheless, cOME often recurs after the TTs are extruded or become blocked with debris necessitating multiple TT reinsertion.[12] Spontaneous resolution do occur, as confirmed in a prospective study wherein the spontaneous resolution rate in cases of cOME with no obvious cause was 30% for children, and 37% for adults.[13]
Most evidence shows that the majority of new OM episodes are a complication of a viral upper respiratory tract infection.[14] A prospective study of cOME in adults showed a greater likelihood of spontaneous resolution of their effusion if there was a history of upper respiratory tract infection at the onset of hearing loss.[13] Less well understood are the patho-physiological processes that promote disease persistence as cOME, although in the adult population, nasopharyngeal cancers,[15] history of irradiation,[16] barotrauma,[17] and chronic sinusitis[1] have been implicated. In addition, in a 1989 study involving 35 adult patients who underwent M w/wo TTI for cOME, it was concluded that cOME was associated more with patients who had a higher incidence of previous middle ear surgery, ear disease in childhood, cigarette smoking, a family history of ear disease, and chronic nasal obstruction.[18]
Many investigators express the belief that that the etiology of cOME is multifactorial with eustachian tube dysfunction (ETD), allergic rhinitis or an atopic condition,[19] gastroesophageal reflux disease (GERD),[20] bacterial biofilms,[21] craniofacial anatomy,[22] and comorbidities associated with sinusitis and asthma being contributing factors. While cOME has been studied extensively in children, there was an initial paucity of information relating to the etiology and natural history of the disease in adults in the past. Recent studies have now come on board, increasing our understanding of the behavior of this disease on this population cohort, though these are assumed to be similar for the two age groups.[1-2] Like children with the disease, the usual treatment is M w/wo TTI. However, it is unknown how often repeated insertions are required and which type of TT is optimal for treatment. Tests are now available that can assign cOME episodes to specific etiologies thereby allowing specific treatments directed to resolving the underlying cause of the disease. For example, a surgical procedure that "debulks" the region of the anterior ET orifice was described for adults with cOME caused by ETD,[21, 23-24] newly developed monoclonal antibodies directed against the IgE molecule may prove useful in treating cOME caused by allergy,[25] and proton pump inhibitors may prove useful in treating cOME cause by GERD.[26] Because of their potential risks, these interventions should first be evaluated for efficacy and safety in adults prior to their application in children with cOME. This requires that the adult disease be better characterized, which is the primary goal of the present study.

Methods
A retrospective chart review from August 1, 2004 to July 31, 2008 of 101 patients from a tertiary university hospital treated with M w/wo TTI. The initial entry diagnosis, data on the incidence of cOME potentially attributable to ET dysfunction, the median duration of TTs in adult subjects, and the natural history of cOME with respect to disease recurrence (expected placebo effect) in adults treated with TTs was extracted.

Results
In 101 patients with a mean age of 56 years (17, 98) undergoing M w/wo TTI, chronic middle ear disease (CMED) was equally distributed in both genders at the initial diagnosis. Chronic otitis media with effusion (cOME) was the most common initial diagnosis (51%). When stratified by age, chronic rhinitis was the most the most commonly identified risk factor for CMED in the youngest age group (16%;17-44 y/o) and in the oldest age group (10%;66-99 y/o) groups, while chronic sinusitis was the most common risk factor in the middle age group (13%;45-65 y/o). Other identified risk factors for CMED included atopy, gastroesophageal reflux and head and neck cancers. The middle age group had the greatest number of CMED occurrences.

Compared to those with unilateral disease, patients with bilateral ear disease had a greater number of episodes but with a longer inter-episode interval. For treatment with tympanostomy tubes, the Armstrong grommet tube was most frequently used.

Figure 1: Distribution of Risk Factors vs. Age Group

Risk Factors (N=101)	17-44 y/o (n=31)	45-65 y/o (n=34)	66-98 y/o (n=36)
Asthma	8	4	6
Chronic Rhinitis	16	8	10
Chronic Sinusitis	12	13	10
GERD	12	8	8
Atopy/AR	12	10	10
OSA	2	4	2
Drug Allergy	6	2	5
Head & Neck Tumors	15	2	6
Immune Deficiency	0	0	0
Hyperbaric O2	0	0	0

Figure 2: Distribution of # of TTs vs. Mean ff-up period in between M and/or Ts (in weeks)

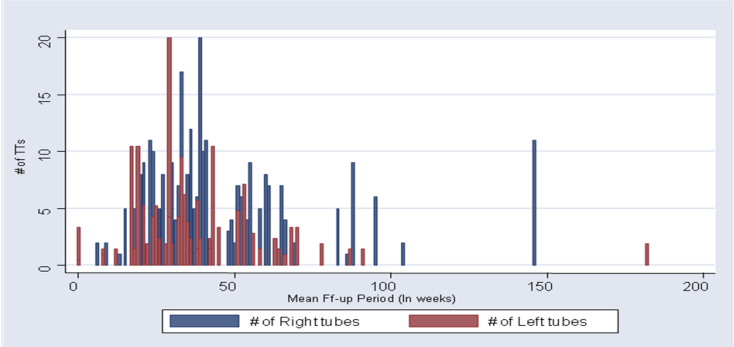
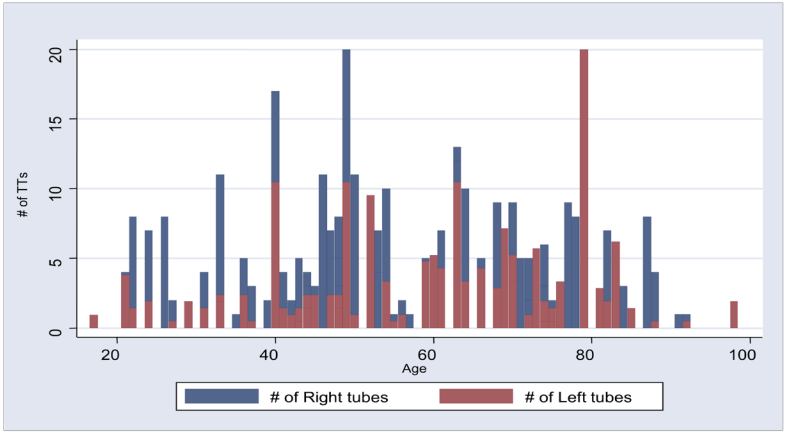


Figure 3: Distribution of # of TTIs vs. Age



Discussion

It is known that COME does not occur in isolation, and could be best described as an inflection point in the dynamic process of the formation of CMED. It is however, usually the focal and oftentimes, initial point of contact between an involved clinician, as its formation heralds symptomatic complaints from the patient. Therefore, it is often diagnosed as a disease whereas it is actually a symptom of a primary condition, foremost of which is ETD. The ET is the pivotal organ crucial to maintaining the equilibrium of the middle ear pressure (MEP) gradient in relation to the environment.[1] It fulfills this function by the cyclical active opening and closure of its nasopharyngeal orifice as mediated by the tensor veli palatini.[2] Any derangement in any of these components would lead to a pressure imbalance, and ultimately to fluid formation in the MES with its attendant sequalae. Although the most common entry diagnosis after COME was ETD in our study, the precise etiology of what caused the ETD, whether secondary to, anatomical/neuromuscular causes, infection, mucosal deficiencies, ciliary dyskinesia, and other acquired causes was more often than not, undefined and unexplored. Therefore, more efforts should e made in identifying underlying conditions that predispose to the occurrence of ETD.

As in past investigations, our results have validated the co-existence of allergy (allergic rhinitis, chronic rhinitis), sino-nasal disease states (chronic sinusitis), and GERD with COME. It is imperative that at a minimum: allergy/asthma testing, nasal/sinus culture, and GERD evaluation, in combination or in part, should be part of any comprehensive work-up for COME. However, as these conditions presents with similar prevalence patterns as in the general population,[27-30] it might be more cost-effective, to more extensively investigate other co-existent risk factors such as GERD, H&N Cancers, obstructive sleep apnea, and drug allergy, which were consistently present in our series. A diagnosis that should not be missed are cancers of the head and neck region.

The most common surgical treatment for COME is TT insertion which was demonstrated in large-scale, well controlled clinical studies to be effective in resolving the disease condition.[10] However, depending on the specific type of tube used, TTs remain

functional for a median of approximately 12 months and then are extruded. Recurrence of COME is a frequent consequence of tube extrusion and repeated intubations are common. These impacts greatly on the quality of life of affected patients.[31-32] In our series, the greatest number of patients needing 10 or more TTis were concentrated in the middle age cohort (11%), as well as on the late age/elderly group (6%). It may be good clinical foresight to account for this difference by making allowances in inserting longer-lasting ventilation tubes to these age groups, and instituting a strict schedule of follow-up for maintenance and/or replacement of tympanostomy tubes.

Conclusions

COME is a relatively frequent diagnosis in adults and is usually "treated" with M w/wo TTI. In this population, disease recurrence is high requiring multiple procedures. Varied associated "risk factors" were identified and may represent an alternative target for treatment.

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Eight Cases of Acute Otitis Media with Sensorineural Hearing Impairment Caused by Pneumococcus Mucosus

Kenzo Ohara, MD, Tatsuya Hayashi, MD**,** Yasuaki Harabuchi, MD
Otolaryngology-Head and Neck Surgery, Asahikawa Medical University, Asahikawa, Hokkaido

Introductions
Although acute otitis media caused by Streptococcus mucosus has received little attention in recent literatures in English, it is well known in Japan as an important disease entity with severe clinical symptoms including sensorineural hearing loss. The purpose of this study is to clarify the features of this disease entity from our eight adult cases and the reviews of literatures.

Patients and Methods
Eight adult patients with mucosus otitis were treated at Asahikawa Medical University Hospital and/or other related institutionsfrom 2003 to 2010. Clinical symptoms and the disease courses were collected from the patient records.

Results
Eight patients aged from 33 to 73 with median age 53.5 years visited our outpatient offices complaining severe otalgia and hearing loss. Audiograms showed sensorineural hearing loss in all cases and all the strains isolated from middle ear fluid or nasopharynx of the patients were mucoid-type *Streptococcus pneumoniae* (pneumococcus mucosus).
A gush of profuse otorrhea was found in all cases at the time of myringotomies. Tympanostomy tube insertion to 7 patients were carried out for disease control. Administration of predonisolone was required to 7 patients for the control of their moderate to severe sensorineural hearing loss.

In case 5, patient developed meningitis concomitant with the progress of mastoiditis. In all patients sensorineural hearing loss were improved after treatment.

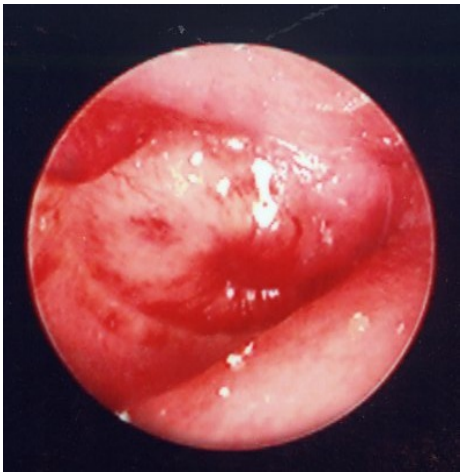
Case	Age	Sex	Side	Severe otalgia	A gush of otorrhea at the time of myringotomy	Pure tone threshold	Fever	Tympanostomy tube insertion	PSL (Initial dose)	Complications
1	56	F	R	+	+	50 d B	38.1℃	+	30mg/day	
2	33	M	B	+	+	R:37.5dB L:48.8dB	-	+ (bil.)	60mg/day	
3	51	F	R	+	+	51.3dB	-	+	40mg/day	
4	35	F	L	+	+	36.3dB	38.0℃	+	30mg/day	
5	37	F	R	+	+	66.3dB	-	-	-	Meningitis
6	73	M	R	+	+	60dB	-	+	40mg/day	
7	63	F	B	+	+	R:78.8dB L:65dB	-	+ (bil.)	100mg/day	
8	63	M	B	+	+	R:106.3dB L:106.3dB	-	+ (bil.)	80mg/day	

Case presentation

Case 1
56 year-old woman presented to our hospital with right otalgia and fullness of ear. Profuse otorrhea was found at the time of myringotomy. She was prescribed amoxicillin(AMPC) under the diagnosis of AOM. A week later, she was returned our hospital with right hearing loss. Augiogram showed sensorineural hearing impairment. Pneumococcus mucosus was isolated from otorrhea and nasopharynx.

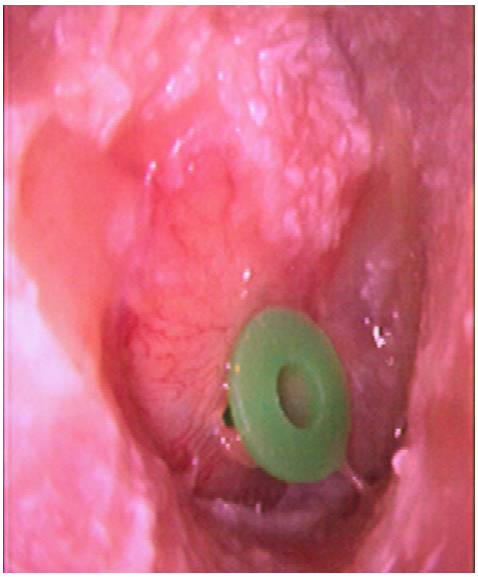
Otoendoscopic examination revealed a hyperemic tympanic membrane with marked bulging. A gush of serous otorrhea followed myringotomy.

Clinical course
Day 11, we performed tynpanostomy tube insertion. Day 14, her hearing loss was not improved, so we decided to systemic administration of panipenem/betamipron(PAPM/BP) and prednisolone. After admission, clinical symptons ware improved gradually.



Case 2

63 year-old woman visited a local ENT office with chief complains of bilateral otalgia and hearing loss. Pure tone audiogram showed bilateral sensorineural hearing loss. She was referredto our hospital under the diagnosis of bilateral labyrinthitis complicated by sever acute otitis media.



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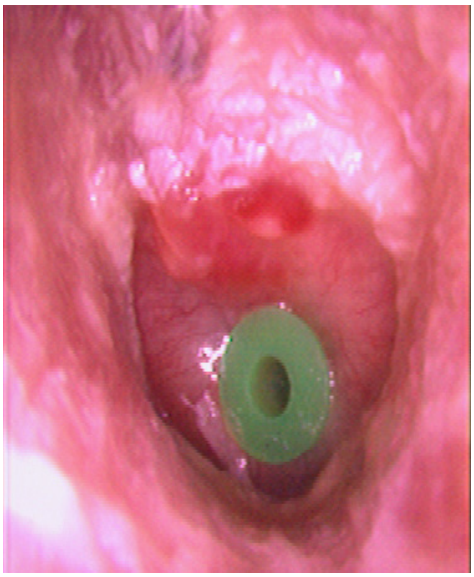

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Clinical course
At the time of her first visit(day 5), myringotomies were done and profuse otorrhea was found in both of ears. Day 7, laboratory stuff told us that Pneumococcus mucosus was isolated from otorrhea. We performed tympanostomic tube insertion and decided systemic administration of predonisolone. After admission, clinical symptoms ware improved gradually.

Discussion
We summarized the characteristics of Streptococcus mucosus otitis as following
1. Patients complain severe otalgia.
2. A gush of profuse otorrhea is found at the time of myringotomy.
3. SNHL develops.
4. SNHL improved by PSL iv (intravenous injection).

• More than 90% of Pneumococcus mucosus are serotype 3 and have *pbp2x* gene in Japan. Strains of serotype 3 of *Streptococcus pneumoniae* are isolated in more adults than children.
• Pneumococcus mucosus is easy to be recognized by the form of colony on agar in regular laboratory work.
• This type of otitis media is considered to be an important disease entity because SNHL often develops and requires systemic administration of PSL. SNHL improved in all of our cases by PSL.
• Prompt diagnosis with the clinical features and the result of bacterial cultures is considered as a key to success in treatment of the patients.

Conclusion
1.We reported 8 cases of AOM caused by Pneumococcus mucosus.
2. Characteristics of Streptococcus mucosus otitis were clarified.
3. We should pay much attention to streptococcus mucosus otitis for it's disease.

Genotype -> Phenotype: Cystic Fibrosis and Otitis Media? A Dyssynchronous Scientific Zeitgeist Where the Rubber hits the Road

Joseph Dohar, MD[1]**,** Kathryn Colman, MD[1]**,** Shean Aujla, MD[2]
[1] Division of Pediatric Otolaryngology, [2]Division of Pulmonary Medicine, Allergy and Immunology, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA

Introduction
In the not so distant past, an abnormal sweat test combined with a consistent clinical phenotype served as the gold standard for the diagnosis of Cystic Fibrosis (CF). Concomitant with the explosion of genetic testing and research has emerged a dyssynchrony between technology and biological truth. As of April 1, 2010, there were 1,850 mutations listed in the cystic fibrosis gene mutation database (CFGMD).[1] Such a morass and often oppositional array of diagnostic features has not only bewildered CF pulmonology experts but has also similarly challenged the traditional otolaryngic CF clinical phenotype including the long-accepted exclusion of otitis media as a comorbidity. This small retrospective case series from the Cystic Fibrosis Center at the Children's Hospital of Pittsburgh of UPMC presents such representative cases of divergence between phenotype and genotype in patients presumably diagnosed with CF by state-of-the-art means. This small series makes three points that are important for non-CF experts to understand. First, new CF genetic tests and diagnostic criteria notwithstanding, the diagnosis, even for the experts, can remain elusive and somewhat controversial in certain cases.[2] Second, patients now diagnosed with CF manifest markedly increased variability of disease expressivity.[3] Finally, the authors conclude that otitis media may now need to be included in the otolaryngic clinical phenotype of CF; a new merged zeitgeist between scientific insight and technology.

Report of Cases
LL is an 8 year old male with a history of Eustachian tube dysfunction, chronic otitis media with effusion, chronic sinusitis, and chronic cough. LL developed recurrent otitis media starting at less than 6 months of age, and he has had five operations for myringotomy and placement of PE tubes. A right-sided long term tube was placed at age 6. Thick, mucoid effusion was noted in the middle ear at the most recent procedure.

From a pulmonary standpoint, he had a normal newborn screen which included a newborn screen for cystic fibrosis. His cough started between the ages of 3 and 4 months and was described as wet and nonproductive. Chest xray was normal on two occasions, with one reported episode of pneumonia. He has had three episodes of croup which were treated with oral steroids. Treatments for cough have included Zyrtec, Zantac for presumptive gastroesophageal reflux, and occasional courses of prednisone. He was seen by Pediatric Allergy in 2006; skin tests and RAST testing were negative. At age 6, he underwent pulmonary function testing with spirometry, which was normal.

He has been seen for chronic rhinosinusitis with chronic rhinorrhea that usually is watery but sometimes is thick yellow or green. He is treated with antibiotics four to five times per year for acute on chronic sinusitis. He has had two sinus CT scans, the most recent of which was at age 8 and showed a right maxillary and right sphenoid sinus
retention cyst. A previous scan at age 3 was normal. At age 3 he underwent adenoidectomy and at age 4 he underwent tonsillectomy with revision adenoidectomy for recurrent infections.

Immunological workup was normal. At age 8 sweat chloride testing was performed. Results of this showed the chloride values on two sites of 63 and 45 mEq/L respectively. The value of 63 is clearly elevated. The value of 45 is in the intermediate range. Repeat sweat test showed chloride values of 68 and 64, which are clearly positive. However, genetic analysis found no mutations upon full sequencing of the CF gene. His family history is negative for cystic fibrosis. Chest physiotherapy was started using the ThAIRapy Vest twice a day, which is increased when he develops a respiratory infection. With this therapy, his chronic cough improved markedly. Of note, he has always been active in youth sports including wrestling and boxing. He also is pancreatic sufficient and does not require pancreatic enzymes, however his weight gain has been suboptimal.

In summary, he is an 8 year old boy with the clinical phenotype of mild lung disease, pancreatic sufficiency, chronic sinusitis, and chronic OME who has a positive sweat test but no mutations detected on full CFTR gene sequencing. He is being managed as if he has CF, and is responding to the therapy.

CD is a 3 year old who has a history of recurrent pneumonia, chronic cough, and pancreatic sufficiency. She had a normal newborn screen for cystic fibrosis. At 9 months of age she had a normal sweat test. However, at 20 months of age she had two elevated sweat tests; the first revealed chloride values of 63 and 63 and the second revealed chloride values of 68 and 68. Her full CF genetic mutation analysis did not identify any mutations. She was treated with vitamins, chest PT, albuterol, Pulmicort as well as antibiotics for respiratory infections with improvement in her symptoms. Flexible bronchoscopy was performed which did not reveal typical CF pathogens; Moraxella catarrhalis was identified in one sample. She has been treated for several pulmonary exacerbations and continues to be followed by the CF team for what appears to be atypical cystic fibrosis.

GH is a 16 year-old male with a history of pulmonary infections, pancreatic insufficiency, and recurrent acute otitis media as an infant. He had an equivocal sweat test but follow-up genetic testing revealed that he was homozygous for the delta F508 mutation. He has frequent lung infections and is treated with antibiotics for these. He also is pancreatic insufficienct. Interestingly, however, he had recurrent acute otitis media as a child. He outgrew his otologic disease and has not had recent episodes. Audiogram revealed normal hearing thresholds bilaterally. Nasal endoscopy revealed mucopurulent mucous bilaterally but no nasal polyposis. Current therapy includes high-frequency chest wall oscillation, vitamins, prophylactic antibiotics, albuterol, DNASE, and pancreatic supplements.

Discussion

Although a very small retrospective case series, these patients illustrate an evolving change in the current phenotype of patients diagnosed with cystic fibrosis. Although the precise histopathological mechanism underlying this ostensible change is unknown, this case series suggests the following points which deserve more systematic study. First, the time-honored abnormal sweat test combined with a consistent clinical phenotype that has traditionally served as the gold standard for the diagnosis of Cystic Fibrosis (CF) has now been supplanted by genetic testing which can play a significant role in diagnosis.[4,5] Concomitant with the ability to genotype patients has emerged a dyssynchrony between technology and biological truth. As of April 1, 2010, there were 1,850 mutations listed in the cystic fibrosis gene mutation database (CFGMD). This number is sure to rise with ongoing research in the field. The result of this diagnostic paradigm shift has not only bewildered CF pulmonology experts but has also similarly challenged the traditional otolaryngic CF clinical phenotype including the long-accepted exclusion of otitis media as a co-morbidity.[6--8] For non-CF experts, it is important to understand that in certain cases, despite an ability to genotype, the diagnosis may, nonetheless, remain elusive and somewhat subjective. Second, patients now diagnosed with CF manifest markedly increased variability of disease expressivity which now appears to include middle ear disease.[3] The authors conclude that otitis media may now need to be included in the otolaryngologic clinical phenotype of CF; a new merged zeitgeist between scientific insight and technology.

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S100A12 Is a Biomarker of Acute Otitis Media Caused by *Streptococcus pneumoniae* in Children

Keyi Liu, PhD, Michael Pichichero, MD
Research Institute, Rochester General Hospital, Rochester, New York

Objective
S100A12 is predominantly expressed in neutrophil granulocytes in response to infection or inflammation. The objective of this study was to quantitate S100A12 levels in children with acute otitis media (AOM) caused by *Streptococcus pneumoniae* (*S. pneumoniae*), *nontypeable Haemophilus influenzae* (*NTHi*) or *Moraxella catarrhalis*(*M. catarrhalis).*

Methods
ELISA was used to measure protein levels of S100A12 in serum, and real-time RT-PCR was used for quantitate mRNA levels in peripheral blood mononuclear cells (PBMCs).

Results
When children experienced AOM due to *Spn*, serum S100A12 levels became significantly elevated (32.38 ± 15.60 ng/mL, n=11) compared to controls (9.68 ± 2.12 ng/mL, n=19. *P* = 0. 001). Pair-wise study in 7 children showed the elevated serum concentrations of S100A12 returned to normal levels when the children recovered from infection. In contrast, serum levels of S100A12 were not significantly elevated with infection by either *NTHi* or *Mcat* AOM infection. The serum S100A12 concentrations in 8 AOM children infected by *NTHi*, and 8 children infected by *Mcat* were significantly lower than *Spn*-AOM (*P* = 0.04 and 0.004, respectively). The S100A12 protein changes in sera measured by ELISA were confirmed by evaluation of gene expression changes in PBMCs. Measure by RT-PCR. Serum levels of S100A12 in children with upper respiratory viral infections (n=3 infected by parainfluenzae) were not elevated compared to virus negative controls.

Conclusion
S100A12 may be a useful biomarker for distinguishing *Spn* as a causative pathogen in AOM (and other Spn infections), for monitoring successful antimicrobial therapy and evaluating *Spn* vaccine efficacy.

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A Simple Scoring System to Improve Clinical Assessment of Acute Otitis Media

Janet Casey, MD[1]**,** Stan Block, MD[2]**,** Pamela Puthoor[3]**,** Jim Hedrick, MD[2]**,** Anthony Almudevar, PhD[4]**,** Michael Pichichero, MD[5]
[1]Pediatrics, Legacy Pediatrics, Rochester, New York, [2]Pediatric Research, Kentucky Pediatric Research, Bardstown, Kentucky, [3]Research, [4]Biostatistics and Computational Biology, University of Rochester, Rochester, New York, [5]Research Institute, Rochester General Hospital, Rochester, New York

Objective
To evaluate an easy to use 10-point scoring system in clinical assessment of acute otitis media (AOM).

Study Design
Symptoms of AOM observed by validated otoscopists at 2 AOM research centers were tabulated and scored with a 10-point and a 30-point system at acute onset of illness and at the test-of-cure (TOC) 3 weeks later.

Results
330 children (x = 13.1 months) with AOM were studied. At AOM onset the mean 10-point score (5.3) and 30-point score (14.1); were highly correlated r=0.72; p<0.001**.** At TOC 256 children were cured, 69 failed and 5 were lost to follow-up. Mean 10-point scores were 0.5 and 4.4 for children with cure and failure; and 2.2 and 12.0 for the 30-point scoring, respectively; r = .94, p <0.001. The 10-point score was more had a sensitivity of 87%, specificity of 98%, positive predictive value of 91% and negative predictive value of 97% compared to the diagnosis by validated otoscopists.

Conclusion
A simple, easy to use 10-point AOM scoring system was shown to discriminate AOM cure and failure at TOC. The scoring system could prove useful to clinicians in differentiating cure and failure at follow-up after diagnosis of AOM.

Study supported by NIH NIDCD RO1 08671.

Prognosis for Children with Otitis Media Symptoms

Christina Ryborg[1], Jørgen Lous, MD[2], **Anders Munck, MD**[2], **Jens Soendergaard, PhD, MD**[2], **Jakob Kragstrup, PhD, MD**[2], **Janus Thomsen, PhD, MD**[2]
[1]Odense, [2]Institute of Public Health, University of Southern Denmark, Research Unit of General Practice, Odense

Introduction
At the age of 2,up to 90% of all children in Western countries have suffered from secretory otitis media (SOM) and by the age of 3,50-85% of all children in Western countries have had at least one episode of acute otitis media (AOM).[1,2] Most studies on otitis media have targeted either SOM or AOM, but in this study we focus on the progression of symptoms in children with SOM and/or AOM.

Aim
The aim was to analyze progression of ear symptoms among children with AOM and/or SOM during a 4-week period.

Method
We conducted a cohort study among general practitioners (GPs) in the Region of Southern Denmark and Region Zealand in the spring 2010. A total of 130 GPs included children between 0 and 6 years presenting with a new ear problem (no contacts associated with the ears for the last 4 weeks). The GPs were trained in using tympanometry and updated on the diagnostic criteria for AOM and SOM. The children were diagnosed based on the ear with the presumably worst diagnosis (e.g. in case of AOM in one ear and SOM in the other the chosen diagnosis was AOM). The GPs registered symptoms, results of otoscopy and tympanometry, diagnosis and treatment (defined as penicillin V, amoxicillin, other antibiotic, pain-relievers and nose drops). Subsequently, four weeks later the child was seen again by the GP in order to monitor the disease and register symptoms, diagnosis and treatment.

Results
A total of 730 children were included.The symptoms most of the children suffered from were sleeping problems (62%, 95% CI: 58-65), earache (50%, 95% CI: 47-54) and pulling the earsear pulling (48%, 95% CI: 44-51). Four weeks later 34% of the children were free of symptoms, while 22% (95% CI: 18-24) had sleeping problems, 9% (95% CI: 7-11) had earache, and 19% (95% CI: 16-22) were pulling ears (Table 1).

In the first consultation 54% (95% CI: 50-58) of the children were diagnosed as having AOM and 41% (95% CI: 37-45)were diagnosed with SOM. Four weeks later 11% (95% CI: 9-13)were diagnosed with AOM and 53% (95% CI: 49-57) were diagnosed with SOM (Table 2). Among children with SOM in the first consultation the distribution of diagnoses after 4 weeks was 60% (95% CI: 54-66) with SOM, 7% (95% CI:4-10) with AOM and 33% (95% CI: 28-38) with healthy ears (normal otoscopy and tympanometry). Among children with AOM in the first consultation the distribution of diagnoses after 4 weeks was 50% (95% CI: 45-55) with SOM, 14% (95% CI: 11-17) with AOM and 35% (95% CI: 30-40) with healthy ears (Figure 1). About 40% (95% CI: 36 to 44) of all children were prescribed antibiotics in the first consultation and 17% (95% CI: 14 to 20) in the second consultation.More than 60% (95% CI: 57-67) of all children diagnosed with AOM in the first consultationreceived an antibiotic in the first consultation, and about 7% (95% CI: 4-10) of the children diagnosed with SOM received an antibiotic (Table 3). A total of 35% (95% CI: 32 to 38) of all the children were diagnosed as having healthy ears after the 4-week period.

Discussion
After 4 weeks only 1/3 of the children werediagnosed as having healthy ears. This means that earproblemsmay be prolonged, and hence a burden for the families, and that these diseases pose a potentially large burden on society as well. It is important that GPs inform the parents sufficiently about the disease and the probability of a prolonged course when they diagnose the children with AOM or SOM.

The number of antibiotics prescribed in this study (to 40% of all children and 62% of the children with AOM) seems quite high compared to the recommendations for treatment of OM. The recommendations have changed over years, but today most experts recommend initial observation.[3] Most children with AOM recover spontaneously within a few days, and on average, 16 children have to be treated to prevent one child suffering from earache after 2 to 7 days (number-needed-to-treat (NNT)= 16).[4] Compared to other studies the number in this study isnot that high, as the rates of use of antibiotics for AOM varybetween 56% in the Netherlands and 95% in the USA and Canada.[5,6]

Only 28% of the children received painrelievers and 10% received nose drops. Taking into account that this should be the priority of drugs prescribed it seems quite low,compared to the number receiving antibiotics(40%).

Conclusion

Ear problems among small children may beprolonged, as 2/3 of the children are still suffering from ear symptoms after 4 weeks. The treatment prescribed to children with ear symptoms is not sufficient, as a huge number of the children receive antibiotics instead of the recommended pain-relieving treatment and nose drops.

Table 1

Symptoms	At first consultation (n=730)			At second consultation (n=730)		
	N	%	95 % CI	N	%	95 % CI
Sleeping problems	451	62	58-65	161	22	18-24
Earache	369	50	47-54	64	9	7-11
Ear pulling	348	48	44-51	137	19	16-22
Fever	292	40	36-44	42	6	4-7
Hearing loss	78	11	9-13	72	10	7-12
Earfluid discharge	57	8	6-10	21	3	2-4
Delayed language	42	6	4-7	40	5	4-7
Balance problems	19	3	1-4	18	3	1-4
Other things	131	19	16-22	149	20	17-23
Free of symptoms	7	0.1	0-0.03	25	34	31-38
Unknown	22	0.3	0-0.7	61	8	6-10

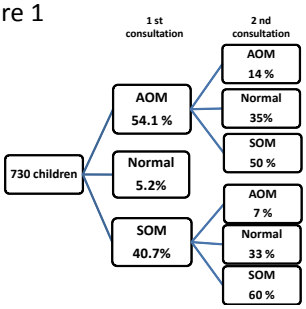
Table 2

Diagnosis	At first consultation			At second consultation		
	N	%	95 % CI	N	%	95 % CI
AOM	395	54	50-58	77	11	9-13
SOM	297	41	37-45	389	53	49-57
Normal	38	5	3-7	259	35	32-39
Missing	-	-	-	5	0.7	

Table 3

Drugs	At first consultation			At second consultation		
	N	%	95 % CI	N	%	95 % CI
Penicillin V	167	23	20-26	57	8	6-10
Amoxicillin	130	18	15-21	58	8	6-10
Other antibiotic	58	0.8	0-0.4	9	1.2	0-2
Painrelievers	197	28	24-31	70	10	7-12
Nosedrops	73	10	8-12	48	7	5-8
None of the above	231	32	28-35	488	67	63-70

Figure 1



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Clinical Efficacy of Middle Ear Ventilation Tube Insertion Against Intractable Acute Otitis Media

Akihida Togawa, MD, PhD, Muneki Hotomi, MD, PhD**,** Shin Takei, MD**,** Masaki Hayashi, MD, PhD**,** Masamitsu Kono, MD**,** Yorihiko Ikeda, MD, PhD**,** Gen Sugita, MD, PhD**,** Noboru Yamanaka, MD, PhD
Otolaryngotogy, Wakayama Medical University, Wakayama, Wakayama

Introduction
Acute otitis media (AOM) is one of the most common infectious diseases among children. Amoxicillin has been considered as the drug of first choice. Myringotomy is widely accepted for one of the effective treatment modalities against acute otitis media (AOM). Insertion of ventilation tube is an effective alternative treatment modality against intractable AOM. In this study, we studied the clinical efficacy of ventilation tubes against intractable AOM. The improvement of middle ear and mastoid cavity findings were evaluated by computed tomography (CT).

Methods
Pediatric patients with intractable case of AOM were enrolled into this study. Intractable case of AOM was defined as the case of AOM not improved even after the first treatment with amoxicillin and the second treatment with alternative antibiotics with/without myringotomy. We evaluated middle ear and mastoid findings before and after the insertion of ventilation tubes by CT.

Results
All pediatric patients with intractable AOM showed strong bulging of tympanic membrane and middle ear fluid occupying middle ear cavity and mastoid air cells evaluated by CT. The typical intractable AOM cases showed highly edematous and/or granulomatous changes of middle ear mucosa by trans-tympanic membrane microscopy. After insertion of ventilation tubes, the middle ear and mastoid findings of all the patients were improved within 3 months.

Discussion
There has been an alarming increase of intractable cases against antimicrobial treatments in recent years. The current CT and trans-tympanic membrane microscopic study suggested that extension of inflammation and exudative fluids in the middle ear and mastoid cavities likely would cause intractability of AOM. The insertion of ventilation tube rapidly improved both middle ear and mastoid pathology and prevented the prolongation of AOM. Insertion of ventilation tube would be one of the effective treatment modality against intractable AOM.

Cumulated Incidence of Ventilation Tube Insertion And It's Correlation to Subsequent Ear Surgery

Mikkel Attermann Bruhn, MD[1]**,** Janus Jespersen, MD[1]**,** Michael Gaihede, MD[1]**,** Mette Nørgaard, MD[2]**,** Rikke Bech Nielsen[2]
[1]Department of Otolaryngology, Head and Neck Surgery, [2]Department of Clinical Epidemiology, Aalborg University Hospital, Aalborg, Nordjylland

Objectives
Ventilation tube insertion (VTI) in secretory otitis media is a very common procedure based on the rationale of equilibrating the middle ear pressure (MEP) with ambient. Repeated VTI treatments are also common in recurrent cases. These cases represent more severe courses of disease as well as they are prone to the risk of permanent perforation of the tympanic membrane; thus reconstructive ear surgery may be relevant. The purpose of the present study was: 1) to describe the cumulative incidence of VTI in children born in 2000 in a Danish County and 2) the fraction of these subsequently admitted to a tertiary hospital setting for reconstructive ear surgery during an eleven-year period from 2000 to 2010.

Study Design
A population-based retrospective cohort study.

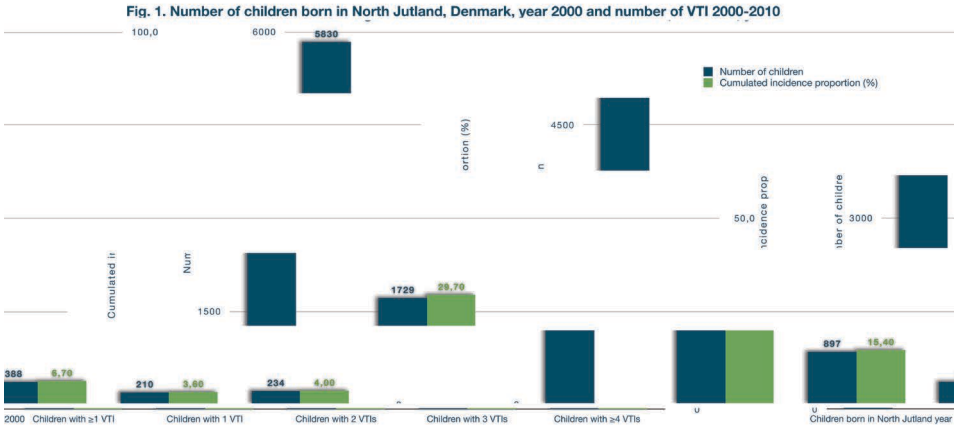
Materials and Methods
The study was conducted in North Jutland County, Denmark, from 2000-2010. Since 1968 a 10-digit civil registration number has been assigned to all Danish citizens at birth, which allows unambiguous identification of patients and linkage between datasets. From Statistics Denmark we obtained information on the number of children born in North Jutland County in the year 2000. The information about VTI's was obtained from the Health Authorities. This database covers all surgeries by private ENT-practioners in our county and includes information on civil registration number, date and type of surgery. The vast majority of VTI's in Denmark are performed by private ENT-practioners (>99%), but they are only in a negligible extent involved in proper ear surgery. In our department, all ear surgeries have been systematically and prospectively registered into an otosurgical database since 1993. By using the civil registration number we were able to determine the cumulated incidence of VTI in the study period

from 2000-2010 on every child born in North Jutland year 2000, and subsequently to correlate each of these individual cases to our otosurgical database to determine the subsequent risk of ear surgery after VTI.

Results

Among 5830 children born in 2000 a total of 1729 (29,7%) had at least one VTI during the study period 2000-2010 (Figure 1). In total, these 1729 children had 3548 VTI's. The incidence of VTI according to age was determined (Figure 2); peak-age was in the second year of life (1.5 years). In this group 51.8% had 1 VTI, 22.4% had 2 VTI's, 12.1% had 3 VTI's and 13.5% had 4 VTI's or more. For simplicity, both uni- and bilaterally VTI's have been included (overall ratio 1:5).

The 1729 children treated with VTI's in 2000 were followed during the period 2000-2010 and 34 of these were identified in our otosurgical database. Nine children had more than one surgery. In total 48 operations were performed. The distribution of oto-surgery was 46 tympanoplasties type I (including myringoplasties) and 2 tympanoplasty type II. Seven patients developed cholesteatoma during the follow-up period (20,6%). Overall 0.4% of the cohort treated with VTI developed cholesteatoma. There was significant increased risk for subsequent reconstructive ear surgery in children, who had ≥2 VTI's (RR 2.25; 95% CI, 1.1-4.6).Overall, among the children treated with VTI's, less than 2% were admitted to subsequent ear surgery (Table 1).



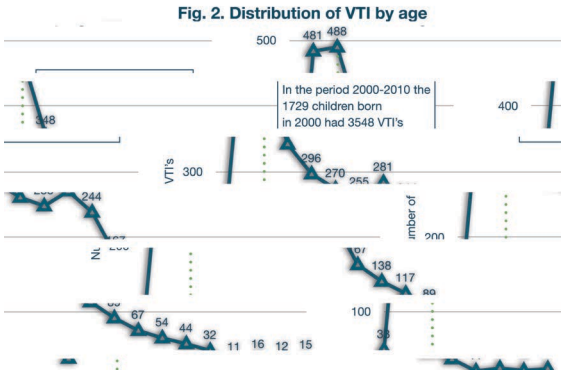


Table 1. Summary characteristics of the 1729 children who underwent at least one VTI in North Jutland, Denmark from 2000-2010 by surgery status

	Surgery (% of total)			Relative Risk (95% CI)	Fishers exact P-value
	Yes	No	Total		
Number of VTI procedures					
1	11 (1.23)	886 (98.77)	897	(ref)	(ref)
2	13 (3.35)	375 (96.65)	388	2.73 (1.23;6.04)	0.01
3	3 (1.43)	207 (98.57)	210	1.16 (0.33;4.14)	0.74
4+	7 (2.99)	227 (97.01)	234	2.44 (0.96;6.22)	0.07
≥2	23 (2.76)	809 (97.24)	832	2.25 (1.11;4.60)	0.02
Total	34 (1.97)	1695 (98.03)	1729		

Discussion

Almost one third of the children born in our County in 2000 were treated with at least one VTI from birth until the age of eleven. This proportion is considered to be representative for Denmark (data compared with population-based cross sectional sample from 2004 in Denmark). This is probably one of the highest cumulated incidences described in the literature. In Iceland 30% of preschool children have had at least one VTI compared with 9% of 3-year old in Norway and 8.4% in Australia. [1,2]

Several factors may explain the high incidence found in this study. In Denmark a relatively high proportion of children are attending day care, since the majority of mothers are working, and hence, the infection load of these children is high as well as parents tolerance may be low. Furthermore, access to ENT-practioners is relatively easy with one practioner per 30-40,000 inhabitants. Finally, our registration of insertions is almost 100 %, since our health system is predominantly public, and reimbursements of ENT-practioners are based on their registrations and reports of procedures to the County; hence, the enticement for reporting procedures is high.

We only received two percent for subsequent reconstructive ear surgery over the 11-year period, and the vast majority of these were simple closures of tympanic membrane perforations (96%). Previously, cholesteatoma has been reported in 1 % of cases after earlier VTI's; [3] we found only 0.4% in our cohort.

More studies have investigated the correlation between recent decade's increments in VTI's and the incidence of middle ear cholesteatomas, but have not been able to demonstrate any relationship.[4] Since VTI's may prevent pressure load of the tympanic membrane, which can be related to formation of atrophy,[5] as well as VTI's have been related to a higher degree of pneumatization of the mastoid in later life, [6] a higher incidence of VTI's could in theory result in a decreased incidence of cholesteatomas.

In fact, recent investigations in our department have demonstrated a significant decrease in primary middle ear cholesteatomas during 1993 to 2009 from 1.0 to 0.7 per 10,000 inhabitants (unpublished data). Whether these epidemiological data indicate a long-term benefit of VTI's remain to be documented by future studies. In conclusion, the VTI's are only related to subsequent reconstructive ear surgery in a smaller number of the cases (2%), and mostly to simple tympanic membrane closures, whereas only in 0.4 % cholesteatomas were encountered.

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Efficacy of Tosufloxacin, Oral Fluoroquinolone, for Pediatric Acute Otitis Media with Special Emphasis on Severe, Recurrent, or Prolonged Cases

Rinya Sugita, MD[1], Noboru Yamanaka, PhD[2], Muneki Hotomi, PhD[2], Yoshifumi Uno, MD[3], Shigeki Matsubara, MD[4], Yasuhiro Hayashi, MD[5], Shoichi Sawada, MD[6]

[1]Ear-Nose-Throat, Sugita-ENT-Clinic, ichikawa, chiba-ken, [2]Otolaryngology-Head and Neck Surgery, Wakayama Medical University, wakayama-shi, wakayama-ken, [3]Ear-Nose-Throat, Uno ENT Clinic, okayama-shi, okayama-ken, [4]Ear-Nose-Throat, Matsubara ENT Clinic, gifu-shi, gifu-ken, [5]Ear-Nose-Throat, Shirasagidai ENT Clinic, wakayama-shi, wakayama-ken, [6]Ear-Nose-Throat, Sawada ENT and Eye Clinic, kouchi-shi, kouchi-ken

Introduction

Clinical guideline for acute otitis media (AOM) has been widely used in Japan since it was developed in 2006 and has made a considerable contribution for the treatment strategy and suppression of drug-resistant microbes in AOM. The guideline has assigned high score on the diagnostic scoring system to age below 2 years of age due to AOM of this age shows intractable clinical course.

Objectives

In this study we examined clinical efficacy of tosufloxacin (TFLX) for intractable otitis media in children.

Study design: We enrolled 104 children who were diagnosed as severe based on the clinical scoring system and/or who showed no improvement after the 1st or 2nd line antibiotic therapy. All children were treated with TFLX, 6mg/kg/twice daily for 5 days. The patients were followed up on 3rd day, 5-7th day, and 12-14th day for efficacy, cure, and recurrence of AOM.

Results

44% of patients were below 2 years of age, 77 % of them attended day-care, and 56 % of them were treated with other antibiotics before enrollment. 66% of patients were classified into simple severe group and the other was classified into recurrent/prolonged group. *H. influenzae* was detected in 42 % as causative pathogen and *S. pneumoniae* was detected in 25 %. Drug-resistant strains in both pathogens were 87.5 % and 96 %, respectively. TFLX showed 100 % efficacy rate and no recurrence in 84 %.

Conclusions

TFLX showed superior efficacy for severe and/or recurrent/prolonged AOM and will be one of the drug of choice for them.

A Double-Blind Comparative Study of a Novel Oral Carbapenem Tebipenem Pivoxil (ME1211) Vs. Cefditoren Pivoxil in Pediatric Patients with Acute Bacterial Otitis Media

Aki Suzuki, MD[1], Kenji Suzuki, MD[1], Toshiyuki Fujisawa, MD[1], Seiichi Nakata, MD[1], Shunkichi Baba, MD[2], Kimiko Ubukata, PhD[3], Kyoichi Totsuka, MD[4], Seiji Hori, MD[5], Keisuke Sunakawa, MD[3]

[1]Department of Otolaryngology, Second Hospital, Fujita Health University School of Medicine, Nagoya, [2]Department of Otolaryngology, ME1211 Pediatric Study Group, Nagoya, [3]Department of Infection Control and Immunology, ME1211 Pediatric Study Group, Sagamihara, [4]Department of Infection Control, [5]Department of Pharmacology, ME1211 Pediatric Study Group, Tokyo

Objectives

Increase of penicillin-resistant S. pneumoniae (PRSP) and ƒÀ-lactamase-nonproducing ampicillin-resistant H.influenzae (BLNAR) has made the treatment of acute bacterial otitis media (ABOM) in pediatric patients more difficult. Tebipenem pivoxil (TBPM-PI) is a novel oral carbapenem possessing strong activity and broad spectrum against bacterial pathogens including PRSP and BLNAR. A randomized double-blind comparative study of TBPM-PI vs. CDN-PI was conducted to assess the efficacy and safety of TBPM-PI in pediatric ABOM.

Methods

To 216 pediatric patients with moderate to severe ABOM, TB (TBPM-PI 3.5-5 mg/kg b.i.d.) or CD (CDN-PI 4.2-6 mg/kg t.i.d.) were administered for 7 days. Clinical and bacteriological responses were evaluated at Day3 and the end of administration.

Results

The overall clinical efficacy rates in 204 patients in the per protocol set (mean age: 4.03 years) were 98.2% (108/110) for TB and 92.6% (87/94) for CD (95%C.I. of difference:-0.2, 11.5). Clinical efficacy rates in the infection cases with PRSP were 100%

(15/15) for TB and 77.8% (14/18) for CD. The bacteriological eradication rates at the end of administration including PRSP and BLNAR were 100% (69/69) for TB and 98.5% (64/65) for CD. Those at Day3 were 98.2% (55/56) for TB and 80.3% (53/66) for CD. There were no severe adverse drug reactions and major problems in safety in both groups.

Conclusion
It was confirmed that TBPM-PI had excellent clinical potential against PRSP and rapid bacteriological eradication. TBPM-PI is expected to be an innovative therapeutic agent for pediatric infections including PRSP and BLNAR.

Delayed Antimicrobial Treatment Does Not Change the Clinical Course of Non-Severe Acute Otitis Media

Akihisa Togawa, MD, Muneki Hotomi, MD, PhD**,** Masanobu Hiraoka, MD**,** Masamitsu Kono, MD**,** Yorihiko Ikeda, MD, PhD**,** Masaki Hayashi, MD, PhD**,** Gen Sugita, MD, PhD**,** Shin Takei, MD**,** Noboru Yamanaka, MD, PhD
Otolaryngology, Wakayama Medical University, Wakayama, Wakayama

Introduction
Acute otitis media (AOM) is the most common disease for which antibiotics are prescribed during childhood. However, due to their widespread use, we are witnesses to increased development of bacterial resistance to antibiotics. To judge the requirement of antibiotic treatment in patients with non-severe AOM, this case control study evaluated the clinical course of non-severe AOM depending on the treatment modalities.

Methods
The study included children with non-severe forms of AOM, aged between 6 months and 6 years. Children were divided into two groups: children who first received symptomatic therapy without antibiotics and then treated with antibiotics (delayed treatment group) and children received antibiotic treatment from the beginning (initial treatment group).

Results
The improvement of the tympanic membrane findings was observe in 60 -70 % of patients of delayed treatment group until day 14. None of the children developed complications that would require surgical treatment. In the initial treatment group of children with amoxicillin did not show any significant difference of the improvement from the delayed treatment group.

Conclusion
The wait-and-see approach is recommended in forms of AOM without serious signs and symptoms, because it significantly reduces the use of antibiotics and their potential adverse effects.

Association Between the Presence of a Tracheostomy in Children and the Need for Ventilation Tubes: Does One Exist?

Craig Derkay, MD[1]**,** J. Seth McAfee, MD[2]**,** Bryan Fine, MD[3]
[1]Otolaryngology and Pediatrics, [2]Otolaryngology, Eastern Virginia Medical School, Norfolk, Virginia, [3]Pediatrics, Children's Hospital of the King's Daughters, Norfolk, Virginia

Introduction
Eustachian tube dysfunction in tracheostomy-dependent children has not been well-studied.

Objectives
To determine if there is an association between the presence of a tracheostomy tube in children and otitis media to a degree that requires placement of ventilation tubes.

Methods
A retrospective 3 year chart review was undertaken at our regional tertiary care children's hospital to determine the frequency with which ventilation tubes were placed in children who have a tracheostomy. Simultaneously, the CHCA hospital database (PHIS) was queried regarding the association between ventilation tube placement in children with tracheostomies.

Results
At our children's hospital, 19 children out of a population of 69 with tracheostomies (27%) have undergone placement of ventilation tubes in the past 3 years. Thirteen of the 19 (69%) have significant developmental delay, cerebral palsy or severe mental retardation and 8/19 (42%) were born at <28 weeks gestation. Five of the 19 (26%) remain ventilator-dependent. The primary indication for placement of the ventilation tubes was for persistent middle ear effusion in 14/19 (72%).

Conclusions

There appears to be an association between the presence of a tracheostomy in children and the need for placement of ventilation tubes. These children have multiple co-morbidities contributing to their Eustachian tube dysfunction and influencing the decision to place the tubes. Clinicians need to be aware of the possible increased risk of otitis media compounding the communication difficulties in tracheostomy-dependent children.

Incidence of Ventilation Tube Treatments. The Largest Number of VT Insertions in the World?

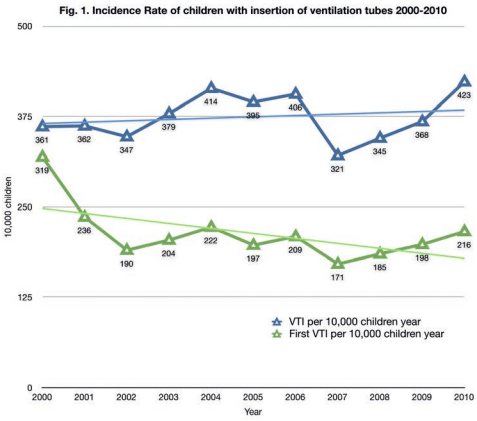
Janus Jespersen, MD[1], Mikkel Attermann Bruhn, MD[1], Michael Gaihede, MD[1], Mette Nørgaard, MD[2], Rikke Bech Nielsen[2]
[1]Department of Otolaryngology, Head and Neck Surgery, [2]Department of Clinical Epidemiology, Aalborg University Hospital, Aalborg, Nordjylland

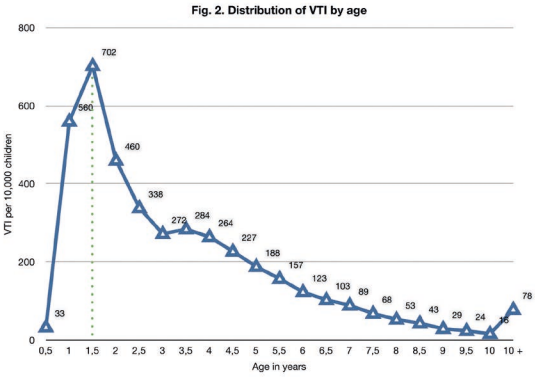
Objectives
Treatment with ventilation tube insertions (VTI) is a very common procedure in children with otitis media (OM), primarily secretory OM, where it is based on the rationale of equilibrating the middle ear pressure with ambient pressure. Repeated VTI's are also common in recurrent cases. We report here the current incidence of VT treatments in children from 2000 to 2010 in a Danish County, which demonstrates one of the largest incidences of VT insertions in the world.

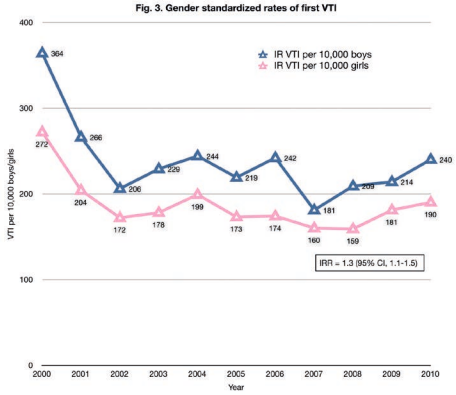
Methods
Database information was achieved from the County Health Authorities with information on all VTI's in children in primary otological practices for the study period; procedures included both uni- and bilateral cases. Information about population and age groups were obtained from Statistics Denmark. The incidences of VTI and reinsertions were determined and described.

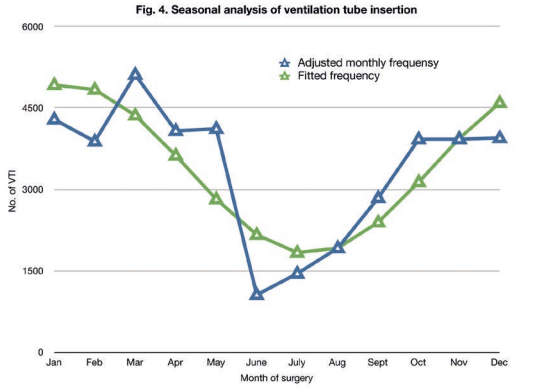
Results
A total of 22,996 children younger than <16 years of ageunderwent surgery with VTI's from 2000 to 2010. For simplicity, cases with uni- and bilateral insertions were pooled into one group, thus the number of VTI's represent the number of procedures; the ratio was 1:5 uni- versus bilateral.

During the study period the number of procedures was constant averaging 3664 per year (3544-4084); this corresponded to an averaged incidence rate of 375 procedures per 10,000 children <16 years (Figure 1). The age distribution of the patients is shown in Figure 2, which demonstrates a peak during the second year of life with 702 procedures per 10,000 children. Treatments in boys were significantly more frequent than in girls (IRR = 1.3; 95 % C.I. = 1.1-1.5) (Figure 3). Furthermore, a significant seasonal variation was observed. (Peak/low ratio = 2.75; 95 % CI = 2.6-2.8) (Figure 4).









Discussion

Only few data comparing national rates of VTI's have been reported. A Canadian study found an incidence rate varying from 33 to 213 per 10,000 person-years in children younger than 15 years within a single province.[1] In Norway, a peak incidence rate of 60 tubes per 10,000 children (<16 years) were reported compared to 51 tubes pr. 10,000 children (<16 years) in Finland.[2,3] Our study showed an incidence rate of 375 tubes per 10,000 children(<16 years). The ratio of 1:5 uni- versus bilateral cases is most probably reflecting indications for surgery, so that around 20 % of the cases represent acute OM, while 80 % of represent secretory OM. It was not possible to achieve information about diagnoses, but uni-lateral insertions are predominantly only accepted for AOM.

We have not found similarly high numbers of VTI in the literature. Several factors may explain the high incidence found in this study. In Denmark a relatively high proportion of children are attending day care (94 %), since the majority of mothers are working (80 %), and hence, the infection load of these children is high as well as parents tolerance may be low.[4] Furthermore, access to ENT-practioners is relatively easy with one practioner per 30,000 to 40,000 inhabitants. Finally, our registration of insertions is almost 100 %, since our health system is predominantly public, and reimbursements of ENT-practioners are based on their registrations and reports of procedures to the County; hence, the enticement for reporting procedures is high.

Factors associated with higher rates of VTI were younger age, male gender, and seasonal variation with peak during winter; these observations are consistent with previous studies.[5,6] Future studies employing longer observation time can describe the correlation between needs for subsequent otosurgery, and hence, provide wider perspectives to cost-effectiveness analysis and health economic decisions .[7]

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Acute Suppurative Labyrinthitis Caused by Actinomycosis

Mikkel Attermann Bruhn, MD[1], Janus Jespersen, MD, MD[1], Tove Ejlertsen, MD[2], Michael Gaihede, MD[1]
[1]Department of Otolaryngology, Head and Neck Surgery, [2]Department of Clinical Microbiology, Aalborg University Hospital, Aalborg, Nordjylland

Objectives
Actinomycosis is an uncommon infection of the middle ear. Actinomyces is a facultatively anaerobic or strictly anaerobic, nonacid-fast, Gram-positive bacilli, which can be found commensally in the oropharynx. Although actinomycosis has a propensity of involving the oral cavity, only 26 cases of actinomycosis of the middle ear have been reported in the literature.

Methods
We report a case of a 16-year old girl, who presented with clinical, audiometric and radiologic findings consistent with right-sided acute suppurative labyrinthitis.

Results
The microbiological examinationgave abundant growth of Actinomyces odontolyticus.

Conclusions
This is the first case of actinomycosis causing acute suppurative otogenic labyrinthitis. Treatment involves aggressive surgical debridement and long-term administration of antibiotics.Although actinomycosis of the temporal bone is rare, it should be considered in the differential diagnosis of acute suppurative labyrinthitis.

Changes in the Eustachian Tube Function with Growth and Development in Children with Repaired Cleft Palate

Aloka Singla, MD[1], James T. Seroky[1], J. Douglas Swarts, PhD[1], **Cuneyt M. Alper, MD**[1], Joseph E. Losee, MD[2], Ellen M. Mandel, MD[1], Julianne Banks[1], William J. Doyle, PhD[1]
[1]Department of Otolaryngology, Division of Pediatric Otolaryngology, [2]Department of Plastic Surgery, Division of Pediatric Plastic Surgery, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA
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Introduction
Otitis media with effusion (OME) is recognized as nearly universal in the population of infants and children with cleft palate (CP) and is often associated with long-standing conductive and, perhaps, sensorineural hearing losses. Most evidence suggests that OME in CP patients is a complication of inefficient Eustachian tube function (ETF).
The existing literature documents an important role for the Eustachian tube (ET) in the pathogenesis and/or persistence of otitis media (OM) and cross-sectional studies report a lower prevalence of OM in older children and better ET pressure-regulation in older children.
Previous studies have tried to relate the poor ETF in CP children to its anatomical substrate in the hope of identifying surgically modifiable conditions to reduce CP morbidity by decreasing OME prevalence. To date, no effect of any reconstructive procedure on OME prevalence has been demonstrated.
In one study, infant CP patients who had tympanostomy tubes inserted at 3 months of age as prophylaxis for OME were tested before and after repair of the soft palate at 18 months of age. In contrast to a documented 70% frequency of one measure of ETF inefficiency, tubal constriction, in older CP children and adolescents with tubes for persistent OME, the tests in the infants showed a 30% prevalence for that measure. However, no data exists documenting the longitudinal (over 5 years) ventilatory ET function following post-palotoplasty in CL/P children.

The specific aim of this study was to examine the growth related changes in Eustachian tube function (ETF) after palate repair, and then to follow the enrolled infants up to 5 years using yearly complete manometric Eustachian tube function (ETF) test protocols.
This longitudinal study will help in the development and application of methods for the early identification of children "at risk" for OM, for the prognosis of disease course and for alternative, patient-specific interventions to prevent/cure OM in post-palotoplasty CL/P children.

Methods and Materials
Infants from birth to 24 months with repaired cleft palate were tested and followed by yearly visits to the ENT Research Center clinic at the Children's Hospital of Pittsburgh through the age of 6 years. Subjects with known immune deficiency and cleft palate associated with syndrome were excluded.
At each visit, an interval history and a complete ENT examination with tympanometry were performed. Special emphasis was placed on ascertaining the patency of the TTs (if present) and the presence of MEE, AOM or otorrhea.
ET function testing in ears with patent tympanostomy tubes (or a non-intact tympanic membrane) was done using the Forced Response test (FRT) whereas ears with intact TMs were tested using a pressure chamber.
FRT: As shown in Fig. 1, the ME was first inflated using a constant flow rate until the ET opened, opening pressure (PO). Once opened, flow was continued until a steady-state pressure and flow rate were achieved (PS, QS). The steady-state resistance to airflow at this point is calculated as a ratio of pressure to airflow, i.e. RS=PS/QS. The subject was then instructed to swallow which perturbed the equilibrium state resulting in active pressure (PA) and flow (QA) from which active resistance is calculated, RA=PA/QA. The ratio of steady-state to active resistance during swallowing, RS/RA is a measure of the tubal dilatory efficiency (DE), i.e. the ventilatory function of the ET. Finally, after a swallowing event, the applied airflow is stopped and the pressure remaining at the time of ET closure (Q=0) is recorded as the ET closing pressure (PC), a measure of the periluminal pressure that keeps the ET lumen closed. ET dilatory efficiency (DE=RS/RA) values less than 1 are reflect ET constriction (EC) during swallowing.
Pressure Chamber (Pch) : Children with an intact TM and no OM underwent an inflation and deflation test (IDT) in a Pch using a tympanometry-sonotubometry protocol. Concurrent, ME pressure changes to ambient and the production of sonotubometric peaks (duration >200 msec, amplitude > 1dB), were categorized as successful ET dilations.

Results
A total of 29 CL/P repaired children (24 male, 5 female) with more than 2 ETF tests after palatoplasty were studied.
Table 1 shows the available data and test results grouped for each year following the first post-palatoplasty test. The second column (Days ± SD) summarizes the interval of time after palatoplasty included in each TEST YEAR. Of the 29 subjects, 25 had ETF evaluation within Year 1, and 20, 13, 5 and 1 subjects in Years 2, 3, 4, 5 respectively. The Table reports the number of ears for which specific FRT parameter data is available for each follow-up interval to Year 5. One subject in post–palatoplasty year 2, 4 subjects in Year 3, and 2 subjects in Year 4 were tested in the pressure chamber (PrCh). ET functional obstruction, that is tubal constriction, in post-palatoplasty CL/P children with patent tubes and intact TMs was found in 36%, 65% and 47% of ears in Years 1, 2 and 3. The analysis shows that Eustachian tube opening (PO) and closing (PC) pressures, passive resistance (RS), and, for ears with document ET dilation, the active resistance (RA) and dilatory efficiency (DE) did not change significantly over the 5 year interval.

Discussion
The incidence rate for Cleft Lip (CL) and CP in live births is usually cited in textbooks as between 1/1000 and 1/700 live births, and the results of recent studies support that estimate
Because the integrity of craniofacial skeleton controls and synchronizes the growth of the midface, the dysmorphologies associated with CP cause abnormal facial development that, in turn, affects the morphological fields for dental development and eruption and impose abnormal vector relationships on the ET-ME system.
Early studies of infants and children with CP documented a high prevalence of significant hearing losses over the speech frequencies that is not lessened by CP repair and which can persist into adulthood.
Like the prevalence of hearing loss, that of OME decreases with advancing age with a near universal prevalence of OM at ages less than 3 years, a decreasing prevalence between 3 and 5 years of age and a relatively stable prevalence, between 10 and 30%, from 6 years into adolescence. However, it is not known what factors influence the time to resolution of persistent OME in CL/P children.
The existing literature documents an important role for the Eustachian tube (ET) in the pathogenesis and/or persistence of otitis media (OM). Cross-sectional studies report a lower prevalence of OM in older children and better ET pressure-regulating function. Various observations suggest that OME in CP patients is consequent to poor ETF. The present study was designed to understand the effect of ET growth and development in repaired CL/P children on ETF, as tested by FRT or IDT in the pressure chamber at yearly intervals. The results showed that the average values of the variables representing the passive function, active resistance and dilatory efficiency of the ET where stable over the study period (Fig. 2,3,4,5,6), hence can not explain the cause of resolution

of persistent OME in CL/P children with time. ETF inefficiency, tubal constriction, in Post-palatoplasty CL/P children with patent tubes and intact TMs was found to be 36%, 65% & 47% in Year 1,2 and 3.

Conclusions

There was no significant change in ETF and pressure-regulating function of ET during growth and development CL/P children up to the third year of the study. Hence, the role of any change in ETF in decreasing the prevalence of OM after CP repair can not be established .

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POST PALATOPLASTY		NO OF SUBJECTS TESTED		EUSTACHIAN TUBE FUNCTION TEST RESULTS (Number of Ears)						
Test Year	Days ± SD	FRT	PR Ch	PO	PC	RS	RA	DE	Dilation	Constriction
1	140±80	24	0	41	31	33	21	22	14	0
2	483±88	18	1	31	21	28	25	26	9	17
3	891±67	9	4	13	10	13	12	17	9	8
4	1125±34	3	2	4	4	5	4	5	3	2
5	>1448	1	0	1	1	1	1	1	0	1

Changes in the Eustachian Tube Function with Cleft Palate Repair

Allison Tobey, MD[1], Joseph E. Losee, MD[2], **Cuneyt M. Alper, MD**[1], J. Douglas Swarts, PhD[1], Ellen M. Mandel, MD[1], James T. Seroky[1], William J. Doyle, PhD[1]
[1]Department of Otolaryngology, Division of Pediatric Otolaryngology, [2]Department of Plastic Surgery, Division of Pediatric Plastic Surgery, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA
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Introduction

Nonsyndromic clefts of the lip and/or palate (CLP/CP) are among the most common birth defects, affecting approximately 1 in 595 newborns in the United States[1]. Otitis media (OM) is a known complication of CLP/CP [2,3] and early studies reported OM to be a nearly universal condition in young CLP/CP infants[2,4,5]. The high prevalence of OM in the cleft palate population is thought to be secondary to Eustachian tube dysfunction. Multiple anatomic and tissue abnormalities contribute to Eustachian tube dysfunction within the cleft palate population such as midline soft palate diastasis with abnormal levator veli palatine insertion, abnormal tubal insertion of the tensor veli palatine and abnormal cartilage super structure [6-9]. The goal of palatoplasty is to recreate a functional soft and hard palate and thus restore speech and swallow function, recreate an oral/nasal barrier, and re-establish Eustachian tube function: mastoid aeration and drainage and middle/inner ear protection.
Previous studies have shown the prevalence of OM and otologic complications associated with OM in the CLP/CP population improve after palate repair, however other studies show OM and otologic complications associated with OM persists much longer in the CLP/CP population compared to their non cleft counterparts [2,10-12]. These studies indirectly suggest Eustachian tube function may improve after palate repair but may not be as functional as non cleft Eustachian tubes. Force response testing allows for direct measurement of Eustachian tube function. This study sought to prospectively investigate the direct relationship between palate repair and Eustachian tube function in the CLP/CP population.

Methods and Materials

The study was approved by the University of Pittsburgh Institutional Review Board (IRB). Prior to palatoplasty, the parent(s) of non-syndromic infants with CL/P were approached during a scheduled visit to the Cleft Palate Craniofacial Clinic (CPCC) at the Children's Hospital of Pittsburgh and an IRB approved Informed Consent was obtained.

The Forced Response Testing (FRT) requires the presence of a patent tympanostomy tube (or a non-intact tympanic membrane) with no evidence of otorrhea. Infants seen at CPCC have bilateral tympanostomy tubes placed prior to palatoplasty. On presentation for testing, otoscopy was done by a study physician to document the lack of otorrhea and the patency of the tympanostomy tube. For FRT testing, the child was seated in the parent's lap and gently restrained. A hermetically sealed plastic probe was introduced into the ear canal. The probe was coupled to a flow sensor, pressure transducer and, via a 3-way valve, to a variable-speed, constant flow pump. For testing, the constant flow pump was set to deliver ≈23 ml/min of air-flow to the middle ear.

Main outcome measures were demographics, type and date of palate repair, FRT before and after palate repair for measuring the passive resistance (PR), opening pressure (OP), steady state pressures (STP), steady state flow (STQ), closing pressures (CP), swallow pressures (SwP), and for calculating the swallowing flow (SwQ), active resistance (AR), dilatory efficiency (DEff).

Results

Table: Pre and Post Forced Response Testing Results for CLP/CP infants

	Pre Palatoplasty	Post Palatoplasty	t	p-value
Opening Pressure				
n	42	42		
Average ±st dev (mmH2O)	323.9±152.2	338.9±127.5	-0.48	0.63
Steady State Pressure				
n	36	36		
Average ±st dev (mmH2O)	226.0±138.9	200.5±128.9	0.80	0.43
Steady State Flow				
n	36	36		
Average ±st dev (ml/min)	21.8±3.7	21.0±6.6	0.61	0.54
Closing Pressure				
n	24	24		
Average ±st dev (mmH2O)	162.6±69.8	151.1±125.9	0.38	0.7
Swallow Pressure				
n	23	23		
Average ±st dev (ml/min	186.8±86.5	221.0±176.8	-0.81	0.42
Swallow Flow				
n	23	23		
Average ±st dev (ml/min)	11.6±5.5	40.9±61.4	-2.14	0.04
Passive Resistance				
n	28	28		
Average ±st dev **(mmH2O per ml/min)**	10.5±5.5	9.5±5.7	0.69	0.49
Active Resistance				
n	12	12		
Average ±st dev **(mmH2O per ml/min)**	49.4±11.3	26.8±32.2	1.37	0.19

Discussion

In a prospective study of 150 CP children enrolled between 2 and 18 months, Robinson and colleagues reported a prevalence of OME of 92% before palate repair, no short-term change after palate repair but a reduced prevalence to 70% at age 4 years [10]. Moller and colleagues described a universal prevalence of pOME at less than 3 years, a decreasing prevalence between 3 and 5 years and a stable prevalence from 6 years into adolescence [2].

In a longitudinal study on 24 children with CP (37 ears) utilizing the mIDT and FRT before and after palatoplasty showed that the passive properties of the tube were improved following palatoplasty. Active tubal function, which measures muscle-induced tubal dilation, was little affected by the procedure [12]. In that population, 84% of the ears tested increased the transET airflow of a pre-dilated ET (16% constriction) with swallowing. Together, and acknowledging that the frequencies of pOME and poor ET

function decreases with age, these results suggest that ET constriction in young CL/P children may be a prognostic marker of disease persistence into late childhood and early adolescence [12].
Current study demonstrated tubal constriction in 71% of the pre-repair CP population which decreased to 52% after the repair. Although not statistically significant, if the trend holds, increased number of subjects could demonstrate a significant change with the repair. While there was no apparent change in the opening pressure, steady state flow and passive resistance, swallow pressure and the active resistance increased considerably and there was a significant increase in the swallow flow after the palatoplasty. These results demonstrate higher proportion of tubal constriction at baseline and an apparent improvement with palatoplasty as opposed to the earlier report [12].

Conclusions
This study suggests that ETF improves with cleft palate repair. However, a larger patient population is needed to confirm this outcome. Further studies are also needed to demonstrate the impact of this change on the prevalence of OME after the cleft palate repair and the role of ongoing tubal constriction in the persistence of OME and continued need for ventilation tube insertion.

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A Novel Treatment for Middle Ear Fluid and Associated Hearing Impairment in Otitis Media

Steven Hefeneider, PhD[1], Sharon McCoy[1], Carol MacArthur, MD[2], Dennis Trune, PhD[2]
[1]Inflammation, 13therapeutics, Portland, OR, [2]Otolaryngology-Head and Neck Surgery, Oregon Health and Science University, Portland, OR

Objective
To reduce inflammation and subsequent hearing loss in otitis media

Introduction
AOM is characterized by inflammation and fluid in the middle ear, which can impact hearing. No current therapeutic addresses this fluid and accompanied hearing impairment. Pathogens such as viruses produce proteins during the infectious process which have been demonstrated to modulate the human immune response. We have identified a peptide, P13, derived from the vaccinia virus A52R protein. P13 demonstrates potent *in vitro* cytokine inhibition in response to toll-like receptor signaling.

Methods
Murine middle ear inflammation was induced by transtympanic injection of heat-killed *S. pneumonia.* Twenty-four hours post-bacterial injection, animals with documented middle ear inflammation were treated with either P13 or control administered systemically, or topically as ear drops. Inflammation was documented by middle ear histology, and ABRs used to monitor hearing.

Results
P13, administered after inflammation was established, decreased middle ear fluid area, number of cells in middle ear fluid, and tympanic membrane thickness. Most importantly, P13 decreased both the severity and duration of hearing impairment. Five days after bacterial injection, P13 reduced hearing loss approximately 50% as compared to controls. By day 13, hearing thresholds inP13 treated animals returned to normal, while control groups continued to demonstrate significant hearing impairment.

Conclusions
P13, administered topically as ear drops, reduced inflammation and reduced hearing impairment in a murine model of middle ear inflammation. Preclinical data suggests that P13 may be useful as a treatment for the fluid retention and hearing impairment seen in patients with otitis media.

Topical Application of Plasminogen, Mini-Plasminogen and Plasmin - All Heals Tympanic Membrane Perforations

[1], **Sten Hellström, MD, PhD**[1], Per-Olof Eriksson, PhD, MD[2], **Yong-zhi Guo, PhD**[3], Jinan Li, PhD[3], Tor Ny, PhD[3]
[1]Dept of Audiology and Neurotology, Karolinska University Hospital, Stockholm, Sweden, [2]Dept of Otorhinolaryngology, Inst of Clinical Sciences, Umeå, Sweden, [3]Dept of Medical Biochemistry, Umeå University, Umeå, Sweden

Introduction
Tympanic membrane (TM) perforations are commonly seen in clinical practice. The majority of these perforations heals spontaneously, but there are perforations that never heal and therefore will become chronic.The reason why some perforations heal, whereas others stay open has so far remained an enigma.

Wound healing is a complicated process involving three phases; inflammation, tissue formation and tissue remodelling. The plasminogen activator (PA) system has been suggested to play an important role in proteolysis of the extracellular matrix and inflammation. The key molecule of the PA system, plasmin, is generated from conversion of the precursor, plasminogen (plg).

Some years ago we showed that the healing of tympanic membrane perforations is completely arrested in plg-deficient mice. Inflammatory cells were recruited to the wounded area but there were no signs of tissue debridement. In addition, removal of fibrin, keratinocyte migration and in-growth of connective tissue were impaired. The study also showed that the healing of the TM perforations in the plg-deficient mouse could be initiated by intravenous substitution by plg.[1.]

Methods
In the present studies the role of plg and plasmin in the healing of tympanic membrane perforations was elucidated in mice as well as in rats. Furthermore plg treatment was tested in a chronic TM perforation rat model.

Results
It was shown that treatment with plg will accelerate the healing of TM perforations in wild-type mice as well as in rats. The healing can be initiated either by local injection close to the annulus fibrosus or by topical application and the effect of plg on the healing is dose dependent. We could also show that chronic TM perforations in rats healed following topical application of plg.

Interestingly, intra-membrane application of plg in plg-deficient mice also heals TM perforations. Furthermore, topical application of a plg derivative, mini-plasminogen, recombinant plasminogen, or active plasmin in plg-deficient mice results in a healing of TM perforations in these mice. When different doses of plg are topically applied onto the rice papers covering the TM perforations in plg-deficient mice, all of the doses showed positive effect with 2.5mg/ml being the most effective dose.

Conclusions
Our data suggest that local injection or in particular topical application of plg is an effective method for the healing of TM perforations. A clinical trial is in progress which will elucidate if local treatment by plg in future could replace surgical myringoplasty.

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Efficacy of Laser Assisted Myringotomy Against Acute Otitis Media

Muneki Hotomi, MD, PhD, Masamitsu Kono, MD**,** Atsuko Masuno, MD**,** Shin Takei, MD**,** Gen Sugita, MD, PhD**,** Akihisa Togawa, MD, PhD**,** Noboru Yamanaka, MD, PhD
Otolaryngology, Wakayama Medical University, Wakayama-shi, Wakayama

Introduction
Myringotomy is widely accepted for one of the important treatment modality against acute otitis media (AOM). However, there has been no clear evidence in long term that the surgical treatment is superior in improvement of AOM than conservative treatment using antibiotics. This study evaluates clinical efficacies on cases with intractable AOM among laser-assisted myringotomy (LAM), conventional myringotomy and medication with antibiotics without surgical procedures.

Study design.
We investigated the improvement of tympanic membrane (TM) using scoring system for AOM among laser-myringotomy (LAM) group (n=25), the conventional myringotomy group (n=21) and antibiotics only group (n=16). We compared the score of TM in each group at first visit and 2 weeks, 4 weeks, 12 weeks after treatment.

Result
TM scores at 2 weeks after treatment significantly improved in LAM and conventional myringotomy groups rather than antibiotics group. However, at 12 weeks after, the improvement of TM scores did not show significant difference among 3 groups. For severe AOM, both LAM and conventional myringotomy showed significant reduction of TM scores compared to antibiotics only.

Conclusion
Surgical drainage showed rapid improvement of intractable AOM. The relatively short period of fenestration by the conventional myringotomy, however, might result in limited ventilation effect on the middle ear cavity. LAM would keep the ventilation long enough 2-3 weeks and can be safety performed on very young children. The advantage of LAM comes from the slower healing and relatively long term maintenance of the fenestration. The LAM will be effective against sever, intractable AOM among children.

Surgical Techniques for BAHA Implantation in Children

Malou Hultcrantz, MD, PhD
Otorhinolaryngology, CLINTEC, Stockholm Sweden

Objectives
The method to anchor titanium and human bone tissue was introduced by Brånemark in the late 1970ties and was refined for hearing by Tjällström.[1] The surgical procedure has always been performed using a skin thinning step in order to reduce coetaneous tissue thickness, to minimize/avoid infections and to enhance hearing. A very common failure after skin penetration of the implant is the peri implant infections.[2,3] To avoid infections regular cleaning of the skin around the abutment is one of the most important factors. Better treatment options in order to avoid peri implant infections in osseointegration systems have been desired. Lately new information has spread in scientific meetings in the Baha field, indicating that no skin thinning is needed and Hultcrantz reported 2009 patient with good results after a 2 year follow up time.[4,5]
This surgical technique,in adults,has shown no increased inflammatory reactions after a non-skin thinning technique which makes the procedure easier, faster and more cost beneficial with no negative side effects.
Earlier, surgery was performed in children as a 2 step, non-skin thinning performance, then switching to using the dermatome and a 1-2 step procedure but from 2010 the novel surgical 1-2 step technique (depending on bone thickness) without the skin thinning procedure using a vertical incision, and a long abutment (6-9 mm) has been introduced. Objective was to evaluate the outcome of different surgical procedures over a 10 year period (2001-2011) at the Karolinska University Hospital. Also the extent of peri implant infections in contact with the device and clinical signs and symptoms after a novel surgical procedure using a vertical incision without skin thinning in children was noted.

Methods
The present study was a single-centre clinical investigation including 28 children operated during a 10 year period at the Karolinska University Hospital ENT clinic. Patient's medical records were collected and registered.
14 of the children was operated with a 2 step procedure, with 2 fixtures (one sleeping), where the site of implantation was posterior and superior to the external auditory canal and a based skin flap (0.6 mm thin) was raised either with the scalpel or with the dermatome. The subcutaneous tissue was removed down to the periosteum and the Baha set (Cochlear Nordic) was used to drill the hole for the screw in the skull and the bone anchored fixture was launched in place. After 3 month the second procedure was performed introducing the 5,5 mm long abutment to one of the fixtures. 14 children were operated without skin thinning, and

most of them with a 2 step operation. A single vertical incision was used, the fixture introduced in the bone under the skin edge, a hole punched in the skin where the abutment was fitted and the skin was closed with resorbable sutures. Longer abutments were used (6 and 8 mm). 6 children were operated as a single step procedure, without thinning of the skin.

Results

Many of the children suffered from syndromes and the reason for implantation is given belowe. Some children had more than one problem.

15 unilateral or bilateral microtia
5 Charge syndrome
5 severe hearing loss
3 OMC
2 Goldenhar syndrome
2 Cruzon
1 Treacher Collin
1 Cornelia de Lange
1 Down syndrome

During the 10 year follow up of implanted children the most common complications were: 2 screws lost probably due to infection, 5 recurrent infections, 3 revisions (overgrowth), 1 screw did not anchor after surgery (out of 2), 2 lost abutments after 5 and 8 years respectively, 2 removed (neuralgic pain, not used).

The vertical incision instead of the flap technique used (with or without dermatome) has facilitated the surgery (6). The BAHA procedure without skin thinning using the vertical incision, showed as good tissue response as earlier techniques, with no severe tissue related adverse events during the 2 year follow up, as compared to the earlier surgical results. It is faster and the surgical procedure easier, with reduced surgery time. The healing time of the operation area is faster and the risk offlap necrosis is reduced (7). There was no increase in abutment loss or in tissue related infections.

Discussion

In the Baha surgery, skin thinning is performed in order to avoid skin reactions in relation to the implant. (2,3). However, skin thinning seems to increases the surgical trauma, prolongs the surgical procedure as well as increase the time for healing and increase scar tissue. All factors negative for healing and inflammatory response in the skin. In the healing process around the abutment it is usually a reduction in vessels and an increase of fibroblasts giving the skin a pale and firm condition (8). It could be hypothesized that a normal skin, without the skin thinning, would react better in an infected situation.

Hultcrantz showed in an abstract report from 2009 (4) in a cohort of 20 patients from a randomized trial using the older common flap technique (no dermatome) as compared to a direct insertion without skin thinning with a 2 years follow up a better healing, less numbness and no increase in peri implant infections. These adult patients have now been followed for 3 years and show the same result. These results have also been supported by Soo, 2009 (5). In the present report, also children seem to benefit from not reducing the skin. This method can be selected both as a 1 and 2 step procedure, depending on the skull thickness at the time of implantation. No extra negative advents have been noted.

Conclusion

This human report support good findings using a newer surgical technique without the skin thinning step, even in children, and can report good tissue response with no surface related adverse events. There are benefits from not reducing the skin around the abutment: time for surgery is reduced, the healing time is reduced, there is no major numbness in the long time follow up, the peri implant infections do not increase, the site of the surgery looks cosmetically better and no problem have been noted by not removing the surrounding hair.

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Best Method of Repairing Anterior or Posterior Tympanic Membrane Perforation

Timothy Jung, MD, PhD[1,2], You Hyun Kim, MD[1,2], Seong Kook Park, MD[1,2]
[1]Division of Otolaryngology-Head & Neck Surgery, Loma Linda University School of Medicine, Loma Linda, CA, [2]Otolaryngology Research, Jerry L. Pettis Veterans Medical Center, Loma Linda, CA

Introduction

One of the common sequelae of chronic otitis media is tympanic membrane(TM) perforation, which can cause hearing loss and otorrhea. It is essential for every otolaryngologist to know how to repair TM perforation. The two traditional methods for reconstruction of TM perforation have been medial(underlay) or lateral (overlay) graft techniques. In the underlay technique, the graft is placed entirely medial to the remaining TM and annulus, which is perhaps the most common and easiest technique. It is typically used for posterior perforations.In the overlay technique, the graft is placed laterally to the annulus, and any remaining fibrous middle layer after the squamous layer has been carefully removed. The anterior canal wall is widened with a drill to minimize blunting, and the graft is placed just medially to the long process of malleus to prevent lateralization. Each of these techniques has its advantages and disadvantages. We have developed a new medio-lateral graft tympanoplasty technique which seems to be superior tothe traditional methods for the repair of anterior or subtotal TM perforation.

The anterior or subtotal TM perforation is difficult to repair because of less vasculature than posterior tympanic membraneand anterior bony overhang that blocks visualization. Because of the reduced vasculature in the anterior tympanic membrane, there is a greater risk of necrosis and re-absorption of the fascia graft.When the medial graft technique is used to repair anterior or subtotal TM perforation, the anterior portion of the fascia graft may fall away, resulting in re-perforation and obliteration of the anterior part of the middle ear cavity.Although the lateral graft technique has a higher success rate for the reconstruction of anterior or subtotal TM perforation, serious lateralization of graft may occur especially when the malleus is absent.

During the past sixteen years, we have developed and used the medio-lateral graft tympanoplasty for repair of anterior or subtotal tympanic membrane perforation. In the medio-lateral graft technique, the fascia graft is placed medially to the posterior half of the TM perforation and laterally to the anterior half of the perforation. This method is a hybrid of the medial and lateral graft techniques taking advantage of both methods. The purpose of this study is to describe and evaluate the medio-lateral graft tympanoplasty for anterior or subtotal TM perforation and medial graft tympanoplasty for reconstruction of posterior TM perforation.

Materials and Methods

Patients: The charts of 200 patients who underwent the medial tympanoplasty(100 cases) and medio-lateral tympanoplasty(100 cases) during the past 11 years(1995 to 2006) were retrospectively reviewed. The main outcome measure was intact TM without lateralization or anterior blunting.All patientsunderwent preoperative and postoperative audiograms. In addition, ossiculoplasty was also done as needed.

Surgical Technique

The procedure is usually performed under general anesthesia.Depending on the clinical situation,transcanal, endaural, or postauricular approaches can be used. A rim of tissue is removed from the perforation edge to de-epithelialize and encourage migration of the mucosal layer and epithelium. Vertical canal incisions are made at the 12- and 6-o'clock positions. The 6-o'clock incision can be extended right up to the annulus. The 12-o'clock incision is made down to a few millimeters above the annulus close to the short process of malleusto preserve blood supply when anterior canal skin is used as the superiorly based flap. A posterior tympanomeatal flap is elevated, and ossicles are evaluated.Mastoidectomy or ossiculoplasty are performed at the appropriate time if needed. In posterior TM perforation,temporalis fascia is grafted as a medial graft under the tympanic membrane perforation. In the medio-lateral tympanoplasty, first a horizontal incision is made in the anterior canal skin with a curved round knife. The distance of the anterior-horizontal canal incision from the anterior annulus should be about the same as the diameter of the perforation. After the incision, the anterior canal skin is elevated, then canalplasty is performed by drilling the anterior bony overhang with diamond burrs and suction irrigator until a full view of the anterior annulus is possible. The antero-medial canal skin flap is elevated up to the annulus. At the annulus, only the squamous epithelial layer of the TM is carefully elevated to the anterior half of the perforation edge, leaving the anterior annulus intact. The middle ear cavity is packed with Gelfoam soaked in non-ototoxic fluoroquinolone antibiotic otic drops. Unlike in the case of usual medial graft technique, the middle ear packing does not have to be tight. The temporalis fascia is grafted medially under the long process of malleus and

under the annulus in medial graft tympanoplasty.In medio-lateral tympanoplasty the temporalis fascia is grafted medially for the posterior half of the perforation and is grafted laterally over the annulus anterior half of the perforation. To avoid anterior blunting, the fascia graft is brought only up to the anterior sulcus on the annulus not passing beyond the annular sulcus. As a second layer of closure, antero-medial canal skin is rotated to cover the perforation and fascia as a superiorly based flap.Antero-lateral canal skin is replaced, and packings are placed. Traditional rosebud packing is inserted by using otosilk strips with a small to medium-sized cotton ball inside, soaked in the antibiotic otic drops. The rest of the ear canal is packed with a gauze strip soaked in antibiotic ointment or Xerofoam gauze. The incision site is closed in the usual manner.

Results

There were four failures (96% success rate) in the medial graft method for posterior TM perforation due to infection and re-perforation. By comparison, there were three failures (97% success rate) in the medio-lateral graft tympanoplasty. These three failures were caused by a postoperative infection, recurrent cholesteatoma, and anterior blunting, respectively. There was no lateralization or anterior fall-away of fascia graft. Two of the epithelial pearls on the tympanic membrane were easily removed in the office setting. It was noted that healing of the reconstructed TM took place much faster with the medio-lateral graft method compared with the case of the traditional medial or lateral graft technique.

There was no significant postoperative hearing loss compared with the preoperative hearing. More than 70% of the operated ears had hearing improvement of 0-40dB (0-10dB in 19% of ears,11-20dB in 44%,21-30dB in 7%, and 31-40dB in 4%), even without ossiculoplasty.With ossiculoplasty using either partial ossicular replacement prosthesis (PORP, 15%) or total ossicular replacement prosthesis(TORP,11%), there were various degrees of hearing improvement from 11 to 30 dB.

Discussion

The advantages of medial (underlay) graft include ease of learning the technique, avoidance of lateralization of the graft and blunting of the anterior sulcus, and high success rate especially for the posterior perforation. The disadvantages of medial graft are poorvisualization of the anterior tympanum, possible anterior graft fall-away, reduction of middle ear space with consequent increased risk of adhesions, and less suitability for reconstruction of anterior perforation. The lateral (overlay) graft provides superior exposure, suitable for all perforations, and minimizes reduction of the middle ear space. This technique has a high success rate and has been particularly effective for large, anterior perforations. The disadvantages of lateral graft technique include anterior blunting, possible lateralization of graft especially with absent malleus, tendency to create more epithelial pearls, need for malleus manipulation, longer healing time, increased operation time, and complexity for repair of small posterior perforations.

One of the most serious complications of the overlay graft technique is lateralization of graft and reconstructed TM. Lateralization of TM is a condition in which the visible surface of the TM is located lateral to the bony annular ring and loses contact with the conducting mechanism of the middle ear. Lateralization of TM may be associated with considerable morbidity, including hearing loss and cholesteatoma. Surgical repair is often necessary for significant underlying disease, but reestablishment of a normal TM can be challenging. Medio-lateral graft tympanoplasty avoids lateralization of the graft by placing fascia medially to the posterior half of the TM and perforationas well as the long process of malleus. In our study, there was no lateralization of graft or reconstructed TM.

The medio-lateral graft tympanoplasty is a hybrid between medial and lateral graft methodstaking advantages of both methods. It has many advantages over traditional medial or lateral grafttechniques, including: (1) prevention of anterior fall-away of fascia graft, (2)stability of the graft, like “a button in a button hole,” (3) no need for tight Gelfoampacking to support the graft anteriorly, (4) prevention of lateralization of graft, (5) better blood supply because anterior canal skin is rotated as a rotational flap rather than free graft, and (6) easier than overlay method because the epithelial layer of only the anterior half of the TM remnant is elevated rather than the entire TM.

Conclusion

The medial graft tympanoplasty is best suited for repair of the posterior TM perforation with high success rate. The medio-lateral graft method is superior to the traditional medial or lateral graft technique best for reconstruction of the large anterior or subtotal TM perforation. Success rate is very high since it takes advantages of both the medial and lateral grafting methods while avoiding their pitfalls. This new method should help otologic surgeons to improve outcomes of tympanoplasty for anterior or subtotal TM perforation.

2010 Update on the Recommendations for Clinical Care Guidelines in the Management of Otitis Media in Aboriginal and Torres Strait Islander Populations

Peter Morris, PhD[1]**,** Amanda Leach, PhD[1]**, Paul Torzillo, MD**[2]
[1]Child Health Division, Menzies School of Health Research, Darwin, Northern Territiroy., [2]Department of Respiratory Medicine, Royal Prince Alfred Hospital., Sydney, NSW

Background and Study Design
The Darwin Otitis Guideline Group (DOGG) in collaboration with the Office for Aboriginal and Torres Strait Islander Health (OATSIH) Otitis Technical Advisory Group (TAG) updated the 2001 version of the"OM Guideline" for the Office of Hearing Services. The aim of the update was to provide evidence based information for the delivery of comprehensive, effective and appropriate care for ATSIH people with otitis media. Each recommendation is explicitly linked to the source of the original relevant evidence (type and level) and any evidence-based guidelines that have made the same recommendation. Two main sources of information were: i) evidence-based clinical practice guidelines, evidence summaries, and systematic reviews, and ii) high quality primary research on otitis media and hearing loss. Each recommendation has been graded according to the most recent NHMRC grading system 2010.Sections include Prevention, Diagnosis, Prognosis, Medical Management, and Audiological Assessment and Management, Practical Health Care Delivery Considerations, and Prioritisation for regions with limited resources.

Results
Overall, we identified 51 evidence-based guidelines, reviews, and summaries: 12 evidence-based guidelines, 10 clinical evidence reports, 1 evidence-based text-book, 21 CochraneSystematic Reviews, and 7 other systematic reviews. Nearly all of these were new publications. Practical Treatment Plans for each diagnostic category include family education, Medical and Audiological recommendations are included. Eight algorithms and a set of Ten Key Messages are included.

Conclusion
Greater focus and resources for evidence based practice in otitis media management is underway in Australia. These programs should be evaluated.

Comparison of Amoxicillin/Clavulanate High Dose to Cefdinir in Treatment of Acute Otitis Media

Janet Casey, MD[1]**,** Stan Block, MD[2]**,** Jim Hedrick, MD[2]**,** Anthony Almudevar, PhD[3]**,** Michael Pichichero, MD[4]
[1]Pediatrics, Legacy Pediatrics, Rochester, New York, [2]Research, Kentucky Pediatric, Bardstown, Kentucky, [3]Biostatistics & Computational Biology, University of Rochester, Rochester, New York, [4]Research Institute, Rochester General Hospital, Rochester, New York

Introduction
To compare the clinical efficacy of amoxicillin/clavulanate high dose (Amox/Clav HD) as 10 days therapy to cefdinir as 5 days therapy for acute otitis media (AOM).

Study Design
Diagnosis of AOM was based on specific criteria by validated otoscopists at 2 AOM research centers. The outcome measure was resolution of all symptoms and signs of AOM except persistence of middle ear effusion at test-of-cure (TOC) 12-15 days after antibiotic treatment .

Results
330 children (x = 13.1 months) with AOM were studied. At TOC 256 children were cured, 69 failed and 5 were lost to follow-up. Amox/Clav HD-treated children had a better cure rate (86.5%) than cefdinir (71.0%), p=0.001. Clinical outcomes showed that amox/clav HD was correlated with more frequent cure outcomes and that cefdinir was correlated with less frequent cure outcomes as children increased in age between 6 and 24 months of age.

Conclusion
10 days amox/Clav HD is more effective than 5 days of cefdinir as therapy for AOM at TOC when fulfillment of entry criteria into a clinical study (bona fide AOM) and assessment of outcome are judged by validated otoscopists.

The Outcome of Simultaneous Adenoidectomy and Tympanostomy Tube Insertion in Patients with Otitis Media with Effusion and Factor That Influence the Recurrence

Kitirat Ungkanont, MD
Department of Otolaryngology, Siriraj Hospital, Mahidol University, Bangkok Thailand

Objective
To evaluate the outcome of the patients who had simultaneous adenoidectomy and tympanostomy tube insertion, in terms of recurrence of otitis media, adenoid-related diseases and the need for repeated surgery.

Method
A retrospective cohort study was performed in the patients who had adenoidectomy with tympanostomy tube insertion during 1994-2003. Medical records were reviewed from the time of surgery until the last contact of each patient. Data collection included indications for surgery, culture results, post-operative recurrence of otitis media , adenoid-related diseases and additional operations.

Results
There were sixty-six patients with 47 boys and 19 girls. The mean age was 6.27 + 3.05 years. Co-existing adenoidal diseases consisted of obstructive sleep disorder (42.4%), rhinosinusitis (54.5%) and adenotonsillitis (3%). Only 9 cases (13.6%) had no associated adenoidal disease and adenoidectomy was done with the insertion of their second set of tympanostomy tubes. The predominant bacteria from the adenoid culture were Streptococcus pneumoniae (21.7%), Pseudomonas aeruginosa (21.7%) and Streptococcus viridans (17.4%). Mean period of follow up was 23.8 months. . Forty-one patients (62.1%) had no recurrence of otitis media. Nine cases (13.6%) had repeated myringotomy and tube insertion. Significant correlation was found between recurrent rhinosinusitis and repeated tube insertion ($p = 0.008$). Relative risk of recurrent otitis media in patients with and without recurrent rhinosinusitis was 3.63 (95%CI 1.4 to 9.4).

Conclusion
Simultaneous adenoidectomy with myringotomy tube insertion produced satisfactory results in reducing recurrence of otitis media during the follow-up period. Recurrent rhinosinusitis was correlated with repeated myringotomy and tube insertion.

Auto-Inflation to Treat Chronic Otitis Media After Tympanoplasty

Takao Yabe, MD, PhD[1], Yuta Inoue, MD, MD[1], **Rie Yabe**[2], **Hideaki Sakata**[3]
[1]Otolaryngology, Tokyo Metropolitan Hiroo Hospital, Tokyo, Japan, [2]YABA ENT Clinic, Ginza, [3]Department of Speech, Language and Hearing Therapy, Mejiro University Clinic

Objectives
Auto-inflation treatment using the Otovent middle ear inflation kit forsecretory otitis media has become a commonly used therapeutic option, but reports on its clinical application for the postoperative treatment of chronic otitis mediaare still rare. Otoventconsists of a nasalbulb and special latexballoonsto improve middle ear ventilation. We investigated the efficacy of this postoperative treatment inchronic otitis media (COM) patients.

Methods
We studied 30 patients withCOM, 20 patients with cholesteatoma (CHO), 10 patients with adhensive otits media (ADO), and 10 patients with postoperative otitis media (POO).Ten patients with COMwho did not use Otovent served as the control (CON) group. They comprised 41 women and 39 men with a mean age of 46.0 (range 10 to 65) years. We evaluated the treatment effect based on the four-grade VAS system, microscopic ear drum observations and temporal bone CT scans.

Results
The average score in thefour-grade VAS system was 8.1 in COM, 5.8 in CHO, 4.5 in ADO, 6.1 in POO and 6.6 in CON patients.Well-pneumatized postoperative ear drums were seen in 28/30 COM, 15/20 CHO, 4/10 ADO, 7/10 POO and 9/10 CON patients. Postoperative CT scans showed good pneumatization of the mastoid in the COM, CHO,POO and CON groups.

Conclusions
The average score in thefour-grade VAS system for theCOM patients was better than that of CON, CHO, ADO and POO patients. We suggest that the former results indicated the efficacy of postoperative Otovent treatment, and the latter reflect the eustachian tube function in each type of lesions.

Clinical Efficacy of Tebipenem Pivoxil, Oral Carbapenem, for Pediatric Acute Otitis Media with Special Emphasis on Severe, Recurrent, or Prolonged Cases

Noboru Yamanaka, MD, PhD[1], Muneki Hotomi, MD, MD[1], Mitsuko Suetake, MD, PhD[2], Keiko Kanesada, MD, PhD[3], Rinya Sugita, MD, PhD[4], Yoshifumi Uno, MD, PhD[5], Shoichi Sawada, MD, PhD[6], Akihiro Uchizono, MD, PhD[7]
[1]Otolaryngology, Wakayama Medical University, Wakayama, [2]Otolaryngology, ENT Clinic Momo, Sendai, Miyagi, [3]Otolaryngology, Nonohana Clinic, Yamaguchi, [4]Otolaryngology, Sugita ENT Clinic, Chiba, [5]Otolaryngology, Uno ENT Clinic, Okayama, [6]Otolaryngology, Sawada ENT and Eye Clinic, Kochi, [7]Otolaryngology, Sendai ENT Clinic, Kagoshima

Introduction
High prevalence of drug-resistant S.pneumoniae and H.influenzae has been a worldwide problem. In Japan, those drug-resistant microbes have been frequently detected in children less than 2 years of age and caused intractable AOM. Tebipenemu-pivoxil is a newly developed oral carbapenem and is expected to be highly bactericidal to drug-resistant S.pneumoniae.

Objectivs
In this study we examined clinical efficacy of tebipenem-pivoxil (TBPM-PI) for intractable otitis media in children.
Study design: We enrolled 61 children with acute otitis media who were diagnosed as severe based on the clinical scoring system and/or who showed no improvement after the 1st or 2nd line antibiotic therapy. All children were treated with TBPM-PI, 4mg/kg/twice daily for 7 days. The patients were followed up on 3rd day, 7-10th day, and 14-21st day for clinical efficacy, cure, and recurrence of AOM.

Results
75% of patients were below 2 years of age, 84% of them attended day-care, and 67% of them were treated with other antibiotics before enrollment. 69% of patients were classified into recurrent/prolonged group and the other was classified into simple severe group. PCR study detected bacteria in 73% (19% with viruses), and viruses in 10% (rhinovirus, RS, bocavirus, etc). S.pneumoniae was detected in 52% (gPRSP, 82%) and H. influenzae in 46% (gBLNAR, 62%). Overall clinical efficacy rate of TBPM-PI was 82% and recurrence rate after the treatment was 12%.

Conclusions
Clinical efficacy of TBPM-PI against AOM was superior against severe and/or recurrent/prolonged AOM and will be one of the drug of choice for them.

Epidemiology

Otitis Media, Hearing Loss and Associated Disorders in Down Syndrome – The Forgotten Story?

Marit Erna Austeng, MD[1], Kari J. Kværner, PhD, MD[2]
[1]ENT-Department, Ostfold Hospital Trust, Fredrikstad, Norway, [2]Oslo University Hospital, Departmen for Research, Innovation and Education, University of Oslo, Oslo, Norway

Introduction
An increased risk of hearing loss in children with Down syndrome is well documented. In addition to cognitive retardation, the children are at risk of suffering additional learning- and developmental delay unless hearing rehabilitation take place. In Norway, no national consensus on screening or follow-up of these children exists.

Objective
To assess the prevalence of hearing loss, history of otitis media and associated diseases in a cohort of Down.

Study design
The cohort study includes all children born in 2002 in Norway with Down syndrome, a total of 65 children. Children with more than one parent of foreign ethnicity were excludied. In all children, clinical examination, otologic and audtiory examination was performed. Hearing loss was measured using pure-tone audiometry, according to recommended procedures, using a Madsen Auricle audiometer, calibrated according to ISO standards. Main outcome measures were hearing ability at age 8 measured with pure-tone audiometry, otoacoustic emissions, tympanometry and history and clinical presence of otitis media.

Results
Preliminary results showed that 67% had a uni- or bilateral hearing loss above 25 dB HL, estimated as the pure-tone air-conduction average (PTA) at 500, 1000, 2000 and 4000Hz. Mean PTA was of 30.5 dB HL. Otological examination revealed tympanogram type B in 40% of the children and 33% reported a history of otitis media. Symptoms of sleep apnea were present in almost all children. The data collection is ongoing and final results will be presented.

Conclusion
Preliminary results suggest that undiagnosed hearing loss and otitis media is frequent in Down children, and that associated sleep apnea symptomatology are surprisingly high.

Effects of Probiotics on the Presence and Persistence of Human Bocavirus in the Nasopharynx of Otitis-Prone Children

Liisa Lehtoranta[1], Hanna Smolander[3], Johanna Nokso-Koivisto, MD, PhD[2], Karin Blomgren, MD, PhD[2], Katja Hatakka, PhD[4], Tuija Poussa, PhD[5], Klaus Hedman, MD, PhD[3], Korpela Riitta, PhD[1], Maria Söderlund-Venermo, PhD[3], Anne Pitkäranta, MD, PhD[2]
[1]Faculty of Medicine/Department of Pharmacology, [2]Faculty of Medicine/Department of Otorhinolaryngology and Helsinki University Central Hospital, University of Helsinki, Helsinki, Uusimaa, [3]Department of Virology, Haartman Institute, Helsinki, Uusimaa, [4]R&D & Renewal, Valio Ltd, Helsinki, Uusimaa, [5]STAT Consulting, STAT Consulting, Tampere

Introduction
Viral respiratory infections play an important role in the pathogenesis of acute otitis media (AOM). Human bocavirus 1 (HBoV) has been frequently identified worldwide in children with respiratory tract infections, and its role in AOM has been suggested. The disease associations for the closely related bocaviruses, HBoV2-4, are yet unknown. Increasing evidence shows that probiotics might reduce the risk of AOM of viral origin.

Objectives
To examine i) the prevalence and persistence of bocaviruses in consecutive nasopharyngeal aspirates (NPA) of otitis-prone children, ii) whether probiotics might reduce the viral occurrence or persistence during the cold season, iii) how the findings correlate with the child's respiratory symptoms and the risk factors concerning AOM.

Study design
In a double-blind, placebo-controlled, randomised, six-months intervention study, 309 otitis-prone children (10 months to- 4 years) consumed daily either one capsule of probiotics (Lactobacillus rhamnosus GG, L. rhamnosus Lc705, Bifidobacterium breve 99 and Propionibacterium freudenreichii JS) (n=155) or placebo (n=154). The presence and persistence of HBoV1-4 DNA

in NPAs were determined by real-time quantitative PCR at the baseline and after three and six months and were adjusted by age, gender and day-care.

Results
Among the 152 children with a complete follow-up, 25 (16.4%) exhibited a high load (> 104 copies/ml) of HBoV1 in NPA, and 11.5% and 0.6% showed persistent shedding of HBoV1 for 3 and 6 months, respectively. However, none had DNA of HBoV2-4. The comparison between the intervention groups in 94 group-matched children showed a slight reduction in HBoV1 DNA prevalence in the probiotic group (probiotic vs. placebo: 8.5% vs. 17.1%, OR= 0.453, CI 95%= 0.127-1.625; p=0.23) and also in the persistence (OR 0.304, 95%: CI 0.089-1.038, p=0.057).

Conclusions
HBoV1, but not HBoV2-4, DNA occur often in the nasopharynx of otitis-prone children, and may persist for 6 months. Probiotic treatment might reduce the occurrence and persistence of HBoV1 DNA during the cold season.

Incidence Surveillance Study of Acute Otitis Media in Sado Island, Japan

Taketo Otsuka, MD, PhD[1], Osamu Kitami, MD[3], Koji Kondo, MD[4], Akio Tsuchiya, MD[2], Shinsuke Ohshima, MD, PhD[2], Atsushi Iwaya, MD[5], Minoru Okazaki, MD, PhD[1], Muneki Hotomi, MD, PhD[6], Noboru Yamanaka, MD, PhD[6]
[1]Pediatrics, [2]Otolaryngology, Sado General Hospital, Sado, Niigata, [3]Otolaryngology, Shiseido Otolaryngology Clinic, Sado, Niigata, [4]Internal Medicine, Kondo Clinic, Sado, Niigata, [5]Pediatrics, Ryotsu Hospital, Sado, Niigata, [6]Otolaryngology-Head & Neck Surgery, Wakayama Medical University, Wakayama, Wakayama

Objectives
Study of acute otitis media (AOM) in children in Sado Island. The study started January 2010 and is now in progress.

Methods
All outpatients aged 0-18 year-old were enrolled in this study. The study group consisted of all pediatricians (7 doctors) and otolaryngologists (3 doctors) in Sado Island. One week (surveillance-week) in each month was assigned for the study. AOM is diagnosed on the basis of clinical symptoms such as fever, irritability, otalgia, and tympanic membrane findings (redness, bulging, and obliteration of landmarks). AOM severity was diagnosed as mild, moderate or severe based on the scoring system of Japan AOM clinical guideline 2009. Specimens were collected from the nasopharynx and/or middle ear cavity and examined for bacterial and/or viral infection. *S.pneumoniae* and *H.influenzae* were analyzed for serotypes and antimicrobial susceptibility. Incidence rate of AOM in all outpatients was calculated.

Results
Among 3,353 outpatients, 154(4.6%) were diagnosed as having AOM in for 10 surveillance-weeks during January 2010 through October 2010. The incidence of AOM was higher in January and April compared to other months. The incidence and severity of AOM were highest in children of 1 year of age, which correlated to higher prevalence of *S.pneumoniae* and *H.influenzae* infection/colonization in the nasopharynx in this age group. The incidence and the severity of AOM tended to decrease in older children.

Conclusions
AOM incidence (episode/child/year) was the highest, 0.68, in children of 1 year of age in Sado Island.

Tympanic Membrane Retraction: Roles of Middle Ear Pressure and Intrinsic Pre-Disposition

Mahmood Bhutta, MD[1], Mark Haggard, PhD[2], MRC Multi-centre Otitis Media Group[2]
[1]Nuffield Department of Surgical Sciences, University of Oxford, Oxford, Oxfordshire, [2]Experimental Psychology, University of Cambridge, Cambridge

Objectives
Middle ear hypobaria is a necessary factor in the development of tympanic membrane retraction, but other factors, such as the intrinsic strength of the membrane, must play a role. The relative contributions of these determinants has not previously been estimated.

Methods
We have baseline data from nearly 3,000 children being assessed for recruitment to the TARGET trial in the UK. Potential participants underwent bilateral tympanometry and (blinded) otoscopic rating of TM retraction. Using these data we tested correlations between (Sadé/Tos) grade of retraction and (modified Jerger) tympanometric status to quantify this relationship for

subsequent analyses. We then tested for correlation between pars tensa / pars flaccida tympanic retractions of the ipsilateral or contralateral ear. We entered these variables into a multi-variate logistic regression model to determine effect sizes.

Results
As expected, there is a commensurate effect of middle ear hypobaria on the presence and degree of both pars tensa or pars flaccida retraction ($p<0.0005$). We found a high correlation between retraction of a site in one ear and retraction of a site elsewhere. Multi-variate modelling demonstrates that, after controlling for the effect of hypobaria, the odds ratio for the latter effect is large, and is greatest for the same anatomical sites in contralateral ears.

Conclusions
Our modelling demonstrates an important role for site-specific predisposition in aetiology, suggesting a role for intrinsic factors, possibly related to the ultrastructure of the tympanic membrane. Middle ear hypobaria is a necessary but insufficient factor in the aetiology of tympanic membrane retraction.

Incidence of Viral Upper Respiratory Tract Infection and Acute Otitis Media Complications in the First Six Months of Life

Pedro Alvarez-Fernandez, MD, Johanna Nokso-Koivisto, MD, PhD**,** Linda Ede, MD**,** James Grady, PhD**,** David McCormick, MD**,** Janak Patel, MD**,** Tasnee Chonmaitree, MD
Pediatrics, University of Texas Medical Branch, Galveston, Texas

Introduction
The common cold or upper respiratory infection (URI), a disease caused by a variety of viruses, is a universal illness. Particularly susceptible to URI are infants and young children. Onset of the symptoms usually begins 1–3 days after the exposure to the viral pathogen and the illness usually lasts 7–10 days. While URI in infants and young children is often complicated by acute otitis media (AOM),[1] respiratory tract viral infections in infants younger than 6 months of age often complicated by lower respiratory disease, which has received more attention than URI in this age group. The incidence of viral URI specifically for infants in the 0 to 6 months age group has not been well studied and the incidence of AOM following URI in young infants is unknown. Data suggest that infants in the first 6 months of life have lower incidence of AOM than in children 6-24 months of age.[2] However, children who experience AOM early in life are at greater risk for otitis media with effusion and recurrent otitis media.[3-5] The objective of this study was to determine the incidence of URI and its AOM complication in infants younger than 6 months of age and to identify risk factors associated with AOM occurrences.

Methods
In an ongoing prospective study, subjects were enrolled from near birth and followed to the 1st AOM episode or 6-12 months of age. Data were collected on family history, clinical risk factors, and TNF α^{-308} and IL-6 $^{-174}$ polymorphisms.Subjects were followed by visits or phone calls bi-weekly. They were evaluated by the study team during URI episodes and followed for AOM complication. During each sick visit, risk factors were updated, otoscopic examination and tympanometry were performed.At the first visits for each URI episode and at AOM visits, nasopharyngeal specimens were collected forviral studies.
Viral detection was performed by FilmArray® Respiratory Panel (Idaho Technology, Inc. Salt Lake City, UT), which targeted: adenovirus, human bocavirus (hBoV), coronavirus (OC43, 229E, NL63, HKU1), influenza A, Influenza A H1N1, influenza A H1N1 2009, influenza A H3N2, influenza B, human metapneumovirus (hMPV), parainfluenza 1, 2, 3, 4, respiratory syncytial virus (RSV) and human rhinovirus (HRV). For HRV, the test further specifies to HRV1, 2, 3 or 4 or Enterovirus 1, 2 groups. Test positive for both HRV and Enterovirus was reported as Rhinovirus/Enterovirus.

Results
A total of 129 children were followed to 6 months of age; 49% were males, 75% whites and 58% Hispanics. Six percent of the infants were breastfed to 6 mos., 3% attended daycare and 9% were exposed to smoke. Overall, 227 URI and 41 AOM episodes were documented (mean =1.76 URI and 0.32 AOM episodes/infant/6 mos.). Rate of AOM complicating URI is 18%. Table 1 shows demographic characteristics and risk factors of subjects with and without AOM. Male infants and those with 2 or more URI episodes were at risk for AOM.

Of the 227 URI episodes recorded, 137 (60%) episodes were seen by the study group and nasopharyngeal specimens were collected for viral studies. Of 41 recorded AOM episodes, 31(76%) were diagnosed by the study physicians. A total of 145 respiratory viruses were detected in nasopharyngeal secretions collected during URI episodes (Table 2). One or more viruses were detected from 118 (86%) episodes; no virus was detected in 19 (14%) episodes (Mycoplasma pneumoniae was detected in one virus-negative episode). Rhinovirus was the most common virus detected during URI (70% of all URI episodes).

The number of URI and AOM episodes occurred by month of age in 129 infants are shown in Figure 1. The incidence of URI (proportion of children with one or more episodes by age 6 mos.) was 81%; incidence of AOM was 26%. A total of

28(22%)infants had 3-7 URI episodes and 1 (1%) had 3 AOM episodes in 6 months. Infants at age of 6 months had the highest number of URI and AOM.

Summary and Conclusions

Infants in the first 6 months of life have an average of 1.8 URI episodes; rhinovirus is the most common virus detected. Incidence of URI in the first 6 months of life (proportion of children with at least one episode) is 81%; AOM incidence is 26%. Rate of AOM complicating URI is 18%. Male gender and having had 2 or more URI episodes were associated with AOM risk. The relatively low rate of AOM in this age group may due partly to maternal antibodies and low colonization rate with AOM pathogens.

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Table 1. Demographic characteristic and risk factor data of 121 infants

	Total No.	With AOM No. (%)	Without AOM No. (%)	Relative Risk	95% CI
Gender					
Female	66	11 (32)	55 (58)	Reference	
Male	63	23 (68)	40 (42)	2.19	1.17-4.11
URI Episodes					
0-1	58	6 (18)	52 (55)	Reference	
2 or more	71	28(82)	43 (45)	3.81	1.69-8.57
Birth weight					
Highest 3 quartile	95	21 (62)	74 (78)	Reference	
Lowest quartile	34	13 (38)	21 (22)	0.57	0.32-1.02
Season of Birth					
Spring (Mar-May)	27	7 (21)	20 (21)	Reference	
Summer (Jun-Aug)	35	7 (21)	28 (29)	0.77	0.30-1.93
Fall (Sep-Nov)	46	12 (35)	34 (36)	1.01	0.45-2.24
Winter (Dec-Feb)	21	8 (24)	13 (14)	1.46	0.63-3.4
Number of Siblings					
0	58	18 (53)	42 (44)	Reference	
1	35	5 (15)	30 (32)	0.47	0.19-1.16
>1	36	11 (32)	23 (24)	1.07	0.57-2.00
Day care to 6 m					
Yes	4	2 (6)	2 (2)	Reference	
No	125	32 (94)	93 (98)	1.95	0.7-5.44
Smoker exposure					
Yes	12	4 (12)	8 (8)	Reference	
No	117	30 (88)	87 (92)	1.3	0.55-3.06
Type of feeding (to 6 mos.)					
Breastfed or mixed	44	12 (35)	40 (42)	Reference	
Formula fed only	77	22 (65)	55 (58)	1.24	0.67-2.27
TNFα $^{-308}$ polymorphism					
No	90	22 (65)	68 (72)	Reference	
Yes	39	12 (35)	27 (28)	1.25	0.69-2.28
IL-6 $^{-174}$ polymorphism					
No	93	25 (74)	68 (72)	Reference	
Yes	36	9 (26)	27 (28)	0.93	0.48-1.79

Table 2: Respiratory viruses detected in 137 URI episodes seen by the study group

	No. (%) of URI episodes	
Virus	Episode with one or more viruses	Episodes with single virus
Rhinovirus	96(70)	76(55)
Parainfluenza 1-4	15(11)	6(4)
Human metapneumovirus	9(7)	4(3)
Coronavirus	9(7)	1(1)
Bocavirus	6(4)	1(1)
Rhinovirus/Enterovirus	4(3)	0
Adenovirus	4(3)	0
Respiratory syncytial virus	1(1)	0
Influenza A H1N1	1(1)	1(1)
Episodes with any virus	118(86)	89(65)
Episodes with no virus	19(14)	N/A
Total viruses	145	

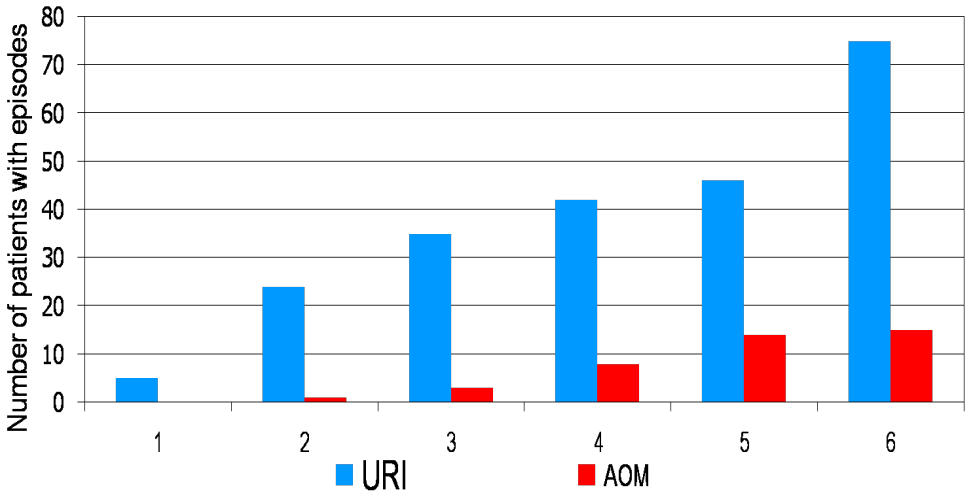

Figure 1. Number of URI and AOM episodes by month of age in 129 subjects (227 URI episodes)

Incidence of Otitis Media in Preterm Infants with Low Birthweight

Kelvin Kwong, MD[1], Livjot Sachdeva[1], Priyanka Shah[1], Bernard Gonik, MD[2], Roberto Romero, MD[3], James Coticchia, MD[1]
[1]Otolaryngology - Head & Neck Surgery, [2]Obstetrics and Gynecology, [3]Perinatology Research Branch, Wayne State University, Detroit, MI

Introduction

Acute otitis media (AOM) and Otitis Media with Effusion (OME) are the second most prevalent clinical problems in infants and children in US and the most common causes of hearing loss[1]. Prematurity, low birth weight (LBW) and in-utero infections have been reported as some of the intrinsic risk factors for otitis media (OM)[2]. There is a growing hypothesis of an association between subclinical intra-uterine infection/ inflammation and the development of OM in premature infants. However, no definite association between premature birth and OM has yet been established. Standard otoscopy provides extremely limited visualization, due to inadequate illumination, the small size of the ear canal and anatomy of horizontally oriented tympanic membrane (TM) in low birthweight infants weighted < 1500 grams[3].

Aims

To determine the incidence of ME disease in premature infants with or without intrauterine chorioamnionitis (HCA) using endoscopic otoscopy complemented with the use of tympanometry.

Methods

20 preterm infants (i.e. < 36 wks), 0 to 3 months of age, who were admitted to the Neonatal Intensive Care Unit (NICU) was enrolled in this study. Primarily, the presence of OM was detected using endoscopic otoscopy based on otoscopic morphology (Table 1) together with the use of tympanometry. Determination of the AOM and OME was based on the diagnostic criteria shown in Table 2. The presence of chorioamnionitis will be determined by the histological examination of placenta from the mother of preterm infants.

Table 1: Otoscopy Grading Scheme Based On Tympanic Membrane Morphologic Characteristics

Point Value	0	1	2
Color	Pearly Grey	White	Erythematous
Transparency	Transparent	Translucent	Opaque
TM Position	Neutral	Retracted	Bulging
TM Thickness	Normal	Mildly Thickened	Significantly Thickened

Table 2: Diagnostic Criteria Based On Otoscopy

Diagnosis	Points
Normal	0 points
Otitis Media with Effusion	1-5 points
Acute Otitis Media	≥ 6 points

Results

Demographics of the subject were summarized in Table 3. 12 (60.0%) of the 20 subjects were found to have AOM or OME. 6 (30%) of these patients with endoscopic findings suggesting AOM. 5 of 7 patients (71.4%) in HCA positive group were diagnosed with AOM or OME as opposed to the six (50.0%) out of 12 patients in the HCA negative subgroup

Table 3: Demographic and clinical profiles of preterm infants (N=20)	
Age at diagnosis (days)	
Average	17.5
Range	3-56
Male to female ratio	1:1
Gestational Age (weeks)	
Average	28.6
Range	23.9-34
Birth weight (grams)	
Average	1,420
Range	526-2806

Discussion

A wide range of incidences of congenital otitis media have been reported in various studies ranging from 3.3% of 300 normal unselected newborns to media in a population of 970 neonates in NICU to 30% in 125 critically-ill neonates[3-4] and 97.7% of neonatal ears in 44 NICU patients[3,5, 6]. All the above studies used traditional otoscopy technique. With the more horizontal orientation of the TM, it could be difficult and challenging to get a consistent and accurate assessment of TM and the middle ear status especially in preterm infants with very low or extremely low birth weight.

This is the first validated study documenting the incidence of AOM and otitis media with effusion in low birth weight preterm infants. This study, in addition to its exclusive subject enrollment of preterm infants, was also unique that most of the diagnostic endoscopy and tympanometry were performed within the first 2.5 weeks of age. The excellent agreement in findings between otoscopy and tympanometry in our study could potentially be explained by the optimal visualization and assessment of the TM in our patients using the endoscopic otoscopy techniques.

This study also provided preliminary evidence that the incidence of otitis media in LBW preterm infants with history of maternal HCA would potentially be higher than that of those with negative maternal HCA history. This finding raised a hypothetical question on the possible etiology of congenital otitis media which infected amniotic fluid could potentially be refluxed via the Eustachian tube.

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Factors Associated with Tympanostomy Tube Treatment Among Children with Chronic Otitis Media with Effusion And/or Recurrent Otitis Media

Kathleen Daly, PhD[1], Bruce Lindgren[2], Kelsey Walt[2], Frank Rimell, MD[3], James Sidman, MD[4], Timothy Lander, MD[5], Robert Tibesar, MD[6], Michele Sale, PhD[7]

[1]Otolaryngology, [2]Cancer Center, University of Minnesota, Minneapolis, MN, [3]Otolaryngology, Universtiy of MN, Minneapolis, MD, [4]Pediatric Otolaryngology, Minneapolis, MN, [5]Pediatric Otolaryngology, Minneapolis, MN, [6]Otolaryngology, Minneapolis, MD, [7]Dept Medicine (Cardiovascular Medicine), University of Virginia, Charlottesville, VA

Introduction

Otitis media (OM) is a common childhood disease, and often clusters in families.[1,2]About half of the children who participated in a survey of OM in nine countries had at least one OM episode by age five.[3] Children with OM often develop recurrent OM (ROM) episodes and/or chronic otitis media with effusion (COME). Genetic, demographic, and environmental risk factors were studied in participants treated with tympanostomy tubes to elucidate their role in the development of COME and ROM. Although pneumococcal conjugate vaccine has reduced overall incidence,[4,5]OM remains a common cause of childhood morbidity.[6,7]

Objective

To better understand the role of risk, demographic and genetic factors in tympanostomy tube treatment among participants with COME and ROM.

Methods

Participants were recruited from Fairview University Hospital, the University of Minnesota, and the Pediatric ENT Associates clinic in an ongoing genetic study at the University of Minnesota. Four sources of data for phenotyping were collected:parent or self-reported history, ear examination, tympanometry, and abstracted data from medical records. This information was used to classify participants as cases or controls. DNA was collected from saliva, buccal cells, and white blood cells to evaluate the role of single nucleotide polymorphisms (SNPs) in COME, ROM and tube treatment.

Study participants were classified as cases (those treated with tympanostomy tubes or middle ear surgery for COME/ROM) or controls (those without a history of COME/ROM or tube treatment; none of the available data sources were positive). Additional criteria for control status were:≥6 years old to verify the absence of COME/ROM during infancy and early childhood, theperiod of highest risk for OM, ≥ 1 additional negative source besides reported history, and no positive sources. Participants were not eligible if they had Down syndrome, another craniofacial anomaly (e.g. cleft palate); agenetic disorder with otologic complications, immune deficiency or disorder, or any other condition predisposing to COME/ROM. Factors potentially related to tube treatment were examined, including daycare, bottle or combination feeding, cigarette smoke exposure, race, ethnicity, gender, allergy, hayfever, and ear exam findings consistent with COME and/or ROM.

Results

574 cases and 312 controls have been enrolled beginning in 2004. In this group, 87 were found to be ineligible, leaving 799 for analyses (552 cases, 247 controls); 672 participants had sufficient data for multivariate analyses. 54% of participants were male, 16% were Asian, American Indian, or African American, and 5% were Hispanic. 73% had been in daycare, 35% were breastfed and 23% were exposed to secondhand smoke on a regular basis (Table 1).

Associations between demographic, genetic and environmental factors and case status were evaluated with univariate and multivariate analyses. Forty-eight SNPs were evaluated. Of these, six SNPs were related to tube treatment with p values <0.10, three of these were significantly (p <0.05) related to tube treatment in univariate analyses (Table 2).

SNP rs12271647_A and demographic and risk/protective factors significant in univariate analyses (p <0.05) were entered into logistic regression models. Female gender(OR 0.54, 95% CI 0.39, 0.75, p <0.001); bottle/combination feeding (OR 1.42, 95% CI 1.00, 2.00, p <0.047); daycare (OR 1.64, 95% CI 1.13, 2.37, p 0.009); white race (OR 1.64, 95% CI 1.01, 2.64, p <0.044) were all significantly related to case status. Of the six SNPs with p <0.10 in univariate analyses, only the rs12271647_A allele was significantly related to tympanostomy tube treatment in logistic regression analyses: OR 1.43, 95% CI 1.03, 1.98, p <0.034(Table 3).

Discussion

Maternalsmoking after tympanostomy tube treatment for OM among one to four year olds was associated withincreased risk of recurrent acute otitis media (OR 4.15, 95% CI 1.45, 11.9).[8]Although fathers were more likely to smoke than mothers, paternal smoking was not related to ROM, perhaps becausefathers spent more time out of the home. New York children (n = 682) who were treated with tubes often did not meet the Guidelines for tube treatment: 3 consecutive months with middle ear effusion (75%

of children had fewer than 42 consecutive days of OME) and/or <2 OM in the 6 months prior to the tympanostomy tube placement.[9,10]

Another study investigated risk factors for treatment with a second tympanostomy tube.[11]In this study, 20% of the original cohort of 2121 children treated with tubes had a second set of tubes. Factors associated with having a second set of tubes were: <18 months at the first tube surgery, which conferred a 2-fold risk, craniofacial anomalies, and family history of bilateral tubes, adenoidectomy or tonsillectomy. Adenoidectomy at first surgery was related to reduced likelihood of having a second tube. Data from this study suggest that 20% of children will need a second set of tubes.

Kogan et al studied 8285 three-year-olds from a national sample.[12] Nearly 7% of all children in this sample had tubes by age three. Factors associated with tube treatment in logistic regression were:continued health insurance, day care, white race, birthweight <1500g, and living in the Midwest or South. Black children with ROM were half as likely as their white counterparts to see a specialist and be treated with tubes.[13]Although both black and white children with ROM were equally likely to have insurance coverage, and to have a usual source of care, black children were more likely to have well child care or a physical exam in the past year, but less likely than white children to have access to prescriptions or see a specialist.[13]

The great majority of enrollees had COME and/or ROM treated with tubes, only 5% of those with these conditions did not have tubes. COME and ROM were so closely associated with tube treatment that they were not useful independent predictive variables. Environmental, demographic and genetic factors contributed to the increased risk of tube treatment for COME/ROM. Daycare, combination feeding (breast and bottle) and SNP rs12271647_A significantly increased the likelihood of tube treatment in multivariate analysis. Females were significantly less likely than males to be treated with tubes.

American Indian and Alaska Native children younger than age five have the highest annual rates of tube treatment (2.6/100/year) compared to all other Indian Health Service (IHS) regions (0.0, 0.2/100/year) and to all US children (1.8/100/yr).[6]Alaska Native children experienced a four-fold higher rate of outpatient visit rates for OM, and a 10-fold increased likelihood of tympanostomy tube insertions compared to any of the other IHS regions. Among 682 Children in New York City, 60.4% had tubes for MEE, 20.7% for AOM, and 10.6% for Eustachian tube dysfunction.[9]Tube treatment occurred after 3 ROM episodes in a year, but those with middle ear effusion had much earlier tube treatment than suggested by the Guidelines: 29 days of effusion compared to the 3 months proposed in the Guidelines.[9,10]

Conclusions

Environmental, demographic, and genetic factors all contributed to increased likelihood of tube treatment for COME/ROM. Although widespread immunization of infants with pneumococcal conjugate vaccine has reduced its incidence, OM remains a common cause of childhood morbidity. This study demonstrates a significant relationship between a SNP allele and tympanostomy tube treatment, providing further evidence of a role for genetic factors in COME/ROM. Evidence of the role of genetics in COME/ROM merits further study.

Table 1. History and demographic variables and case status

Variable	Odds ratio	95% Confidence interval	Logistic regression P-value
Hay fever	0.46	0.23, 0.90	0.023
Bottle & breastfed	1.25	0.93, 1.69	0.132
Daycare	1.53	1.12, 2.09	0.008
White race	1.77	1.20, 2.60	0.004
Female gender	0.58	0.44, 0.78	<0.001

Table 2. SNP evaluation with univariate analyses

SNPs with p values < 0.10 and relationship to tube treatment				
SNP	Allleles	Odds ratio	95% Confidence interval	P value
rs12271647_A:	AA vs AC	1.48	CI 1.09, 2.00	0.011
rs1629816_A:	AA vs AG	0.80	CI 0.64, 0.99	0.036
rs13025485_G:	GG vs AG	1.27	CI 1.00, 1.62	0.049
rs11259905_C:	CC vs AC	1.27	CI 0.97, 1.66	0.082
rs2621228_A:	AA vs AG	1.22	CI 0.98, 1.52	0.074
rs11856299_T:	TT vs CT	1.28	CI 0.96, 1.73	0.091

Table 3. History, demographic and genetic variables related to tube treatment
Logistic regression model

Variable	OR, 95% CI	P value
Hayfever	0.53 (0.26,1.09)	0.084
Bottle & breastfed	1.42 (1.00, 2.00)	0.047
Daycare	1.64 (1.13, 2.37)	0.009
White	1.64 (1.01, 2.64)	0.044
Female	0.54 (0.39, 0.75)	<0.001
rs12271647_A	1.43 (1.03, 1.98)	0.034
rs1629816_A	0.85 (0.67, 1.07)	0.170
rs13025485_G	1.25 (0.96, 1.63)	0.097
rs11259905_C	1.23 (0.89, 1.71)	0.215
rs2621228_A	1.11 (0.85, 1.44)	0.438

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Otitis Media and Pneumococcal Serotype and H. Influenzae Nasal Carriage Surveillance in 18 Months Prior to Introduction of PHiD-CV in October 2009

Amanda Leach, PhD[1]**,** Andre Wattiaux, MD[2]**,** Heidi Smith-Vaughan, PhD[1]**,** Ross Andrews, PhD[1]**,** Peter Morris, PhD[1]
[1]Child Health Division, Menzies School of Health Research, Darwin, Northern Territory, [2]CDC, Department of Health and Families, Darwin, Northern Territory

Background and Study Design

Synflorix (PHiD-CV) vaccine was introduced to the Northern Territory of Australia as a 2,4,6 and 18 month schedule in October 2009. From September 2008 to April 2010, children 0 to 6 years of age and living in a participating remote Aboriginal community were eligible for standardised ear assessments and nasal swab collection.

Results
304 children (mean age 16.6 months, 53% male) participated. Sixteen (5%) had received a single dose of PHiD-CV; 14 of these had also received at least one dose of PCV7. Almost all (89%) had OM; 60 (21%) had tympanic membrane perforation, TMP. Pneumococci were carried by 72% and *H. influenzae* by 75% children; 62% carried both pathogens and 15% had neither. Of 55 cultures of TMP, 15 (27%) were positive for pneumococcus and 22 (40%) were positive for *H. influenzae*. Dominant pneumococcal carriage serotypes were 16F (13%), 19F (10%) and 19A (9%). 40% of pneumococci had intermediate resistance to penicillin and 29% were azithromycin resistant. Most isolates of 19F, 19A, 23B and 16F had penicillin MIC > 0.12 microg/mL and most isolates of 23B, 6A, 7F and 11A had azithromycin MIC > 2 microg/mL.

Conclusion
In this high risk population, almost all children (89%) had OM, and TMP remains common (21%). This is associated with high rates of carriage of either pneumococcus or *H. influenzae* (85%) from infancy. Pneumococcal serotypes 16F, 19F and 19A predominate and antibiotic resistance is common. The best use of pneumococcal vaccines in this population is unclear.

Is Children's Use of ENT Physicians in Primary Care related to Social Marginalization?

Jorgen Lous, MD, PhD[1], Anker Lund Vinding, PhD[2], Karin Friis[3], Kirsten Fonager, PhD[4]
[1]Institue of Public Health, Research Unit for General Practice, University of Southern Denmark, Odense C, [2]Deptarment of Analysis, [3]Office of Analysis, Region North Denmark, Aalborg Ø, [4]Social Medicine, Aalborg Hospital, Aalborg
Extended abstract: format Windows (1997-2003)
Key words: Socially marginalized mothers, children, use of health care services, ENTphysicians

Introduction
An extensive body of literature has demonstrated an association between socioeconomic status and child mortality and morbidity. Studies have generally found an association between low socioeconomic status and more child health problems. This association has been observed for conditions as disparate as infections, accidents, asthma, and a range of chronic physical disabilities (including hearing and visual impairments) [1]. In countries with a mainly tax-funded healthcare system an association between socioeconomic disparities and more use of health care services has been found [2].
The aim of this study was to explore the association between social marginalization of the mothers and their children's use of the healthcare system in the year 2009.
Social marginalization is defined according to the official Danish Department of Occupational Affairs as "having received public social benefits (sick leaves, pension, unemployment benefits, or social benefits)for more than 80% of the year".

Method
In 2009 we conducted aregional registry-based cross-sectional study of use of different health care services amongchildren of marginalized mothers in the North Denmark Region, one of the five regions in Denmark.Region North has 580000 inhabitants.
On 1 July2009 a total of 114807 children aged 0-15 years wereidentified. A total of 111 942 (97.5%) children with 62 905 different mothers were included in the study.Data were linked to a national database containing information onpublic social benefits.Information on use of health care services was obtained on 111814 (99.9%) of these children.
In Denmark all types of public health care servicesare free for all Danes. The patients do not need a referral to be seen by an eye or an ENT-specialist in primary care. Referral is needed to visit other specialists or to attend the public hospitals.In Denmark both eye and ENTphysicians perform surgery with generalanesthesia in their surgeries some days each month, and more than 95% of all tympanostomy tubes are inserted in primary care.
The material was analysedusing the Statistical Analysis System, SAS, and Statistics with confidence [3].

Results
Overall, 10 232 (9.2%, 95% confidence interval (CI) 9.0 to 9.3) of the children lived with a socially marginalized mother. The variation between the 11 municipalities in the Region was from 3.3% to 13.3%.
Marginalization was strongly related to marital status. Compared to married mothers,widows (OR=4.9), divorced (OR=3.2), and unmarried mothers (OR=2.4) have a significantly higher risk of being marginalized (Table 1). Mothers from non-western countries were more frequently marginalized (OR=4.4) than mothers from Denmark.
Children with a marginalized mother had significantly more chronic medical diagnoses (OR= 1.22), they had more frequently been in contact with their general practitioner during the year, and they used the healthcare system more often than children of non-marginalized mothers, except in the case of ENTspecialists (14.3% versus 15.7%) (Table 2).
The marginalized children also had fewer contactsto an ENTspecialist. They had on average 5.3 versus 5.8 consultations in 2009 (Table 3). And surprisingly, children of marginalized mothers had significantlyless chance of having an operation where tympanostomy tubes (grommets) were inserted (17.6% versus 22.1%).

A total of 2.5% of all children (0-15 years) of marginalized mothers and 3.5% of all children of non-marginalized mothers had tympanostomy tubes in one or both ears in year 2009, including new as well as reoperations. The difference represents an OR of 0.75 (95% ci 0.66 to 0.87) for children of marginalized mothers to have a tube operation compared with children of non-marginalized mothers.

Discussion

In line with previous studies, this study also found that children of marginalized mothers in general used the health care system more than other children [1,2]. Surprisingly, we found that they visited the ENTphysician more seldom, and when they did attend they had a less intensive treatment, i.e. fewer consultations and less frequently tympanostomy tubes inserted.

No explanation for this finding has been found. Maybe the marginalized mothers are less aware of the children's ear condition and reduced hearing. Maybe they put less pressure on the physician to treat and operate than families where both parents have a job outside the home.

Another hypothesis could be that children of marginalized mothers more seldom have upper respiratory infections and less need of consultation at the ENTclinics, but this seems unlikely given the huge amount of research showing more illness in children from socially marginalized families. A recent study from the US showed an association between socioeconomic disparities and hearing impairment in children [4].

New research must explore thesesurprising findings further.

Conclusion

The marginalized mothers seem less aware of their children's ear problems compared to other health problems, and the ENTspecialists were less inclined to insert tympanostomy tubes when the child attended the surgery.

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Table 1

The frequency of marginalization in relation to the mother's civil status

Note: 95% ci = 95% confidence intervals

Mother's civil state	All children	Children of non-marginalized mothers	Children of marginalized mothers	
	Number	Number	Number	OR (95% ci)
Married	75 949	71 183	4 766	1.0 (reference)
Unmarried	25 362	21 795	3 567	2.4 (2.3 to 2.6)
Divorced	9 942	8 181	1 761	3.2 (3.0 to 3.4)
Widowed	561	423	138	4.9 (4.0 to 5.9)
Total	111 814	101 582 (90.9%)	10 232 (9.1%, 9.0 to 9.3)	

Table 2

The use of health care service in 2009
Note: PHC = Primary health Care, GP = General practitioner

Use of health care service	All children	Children of non- marginalized mothers	Children of marginalized mothers	
	Number (%)	Number (%)	Number (%)	OR (95% ci)
Contact to PHC	99 668 (89.1)	90 430 (89.0)	9 238 (90.3)	1.14 (1.07 to 1.23)
Contact to GP	98 027 (87.7)	88 922 (87.5)	9 105 (89.0)	1.15 (1.08 to 1.23)
Used hospital outpatient dept.	23 966 (21.4)	21 315 (21.0)	2 651 (25.9)	1.32 (1.26 to 1.38)
Used emergency dept.	9 796 (8.8)	8 588 (8.5)	1.208 (11.8)	1.45 (1.36 to 1.55)
Used hospital	12 241 (10.9)	11 108 (10.9)	1 133 (11.1)	1.01 (0.95 to 1.08)
Used eye physician in PHC	4 963 (4.4)	4 401(4.3)	562 (5.5)	1.28 (1.17 to 1.41)
Used ENTphysician in PHC	17 412 (15.6)	15 943 (15.7)	1 469 (14.4)	0.90 (0.85 to 0.95)
Used psychiatrist in PHC	619 (0.55)	518 (0.51)	101 (0.99)	1.95 (1.57 to 2.41)
Other specialist in PHC	16 607 (14.9)	15 045 (14.8)	1 562 (15.3)	1.04 (0.98 to 1.10)
Total	111 814 (100)	101 582 (100)	10 232 (100)	

Table 3
The use of ENTphysician in primary health care in 2009

Use of health care service	All children	Children of non- marginalized mothers		Children of marginalized mothers	
	Number (%)	Number (%)	95% ci	Number (%)	95% ci
Used ENTphysician in PHC	17 412 (15.6)	15 943 (15.7)	15.5 to 15.9	1 469 (14.4)	13.7 to 15.0
Mean number of ENTconsultations			5.7 to 5.9		5.1 to 5.5
Tympanostomy tube insertion	3 786 (3.4)	3 527 (3.5)*	3.4 to 3.6	259 (2.5)*	2.2 to 2.9
Frequency of grommet insertion in children seen by ENT	21.7%	22.1%*	21.5 to 22.8	17.6%*	15.8 to 19.7

* OR = 0.75 (95% ci: 0.66 to 0.87) for grommets in children of marginalized mothers compared with children of non-marginalized mothers.

Risk Factors for Bacterial Colonization and Acute Otitis Media; Birth Cohort Study in Sado Island, Japan

Taketo Otsuka, MD, PhD[1], Atsushi Iwaya, MD[2], Minoru Okazaki, MD, PhD[1], Muneki Hotomi, MD, PhD[3], Noboru Yamanaka, MD, PhD[3]
[1]Pediatrics, Sado General Hospital, Sado, Niigata, [2]Pediatrics, Ryotsu Hospital, Sado, Niigata, [3]Otolaryngology-Head & Neck Surgery, Wakayama Medical University, Wakayama, Wakayama

Introduction
The first step in a bacterial disease is establishment of nasopharyngeal carriage (colonization). Pneumococcal conjugate vaccine and *H. influenzae* type b vaccine are not implemented under the routine vaccination program in Japan.

Objectives
To evaluate the risk factors for bacterial colonization and its impact on acute otitis media (AOM).

Methods
We conducted a birth cohort study, the SADO-study, in Sado Island. Nasopharyngeal cultures were obtained at the 4-, 7-, 10-, and 18-month health checkups of subjects born in 2008. *S.pneumoniae* and *H.influenzae* were analyzed for serotypes and antimicrobial susceptibility. Information about gender, birth weight, demographics, day care attendance, breastfeeding, and their history of AOM were obtained.

Results
A total of 349 newborns were enrolled (98.3% of those eligible). The total of 1,339 samples provided 429 *S. pneumoniae* and 210 *H. influenzae* isolates. 48.1% of the *S. pneumoniae* isolates were penicillin-resistant and 45.5% of the *H. influenzae* isolates were ampicillin-resistant. Having older sibling(s) (OR=3.6-4.1), recent exposure to antibiotics (OR=2.8), and day-care attendance (OR=1.7-4.0) were found to be risk factors for bacterial carriage, though these factors did not correlate with drug resistance. Frequent serotypes of *S. pneumoniae* isolates were 6B (18.9%), 6A/C (15.3%), 23F (12.0%), and 19F (8.4%). AOM′s incidence correlated to the recent colonization with *S.pneumoniae* and *H.influenzae* in otherwise healthy children.

Conclusions
Our findings contribute to understand the relationship between bacterial colonization and AOM.

A Case-Control Study Examining Risk Factors for Otitis Media with Effusion: Methods and Rationale

Rebecca Walker, Jim Bartley, Ed Mitchell
Department of Paediatrics, University of Auckland, Auckland

Introduction
Otitis media with effusion (OME) is a common condition in children that results in hearing loss and may be associated with learning difficulties and developmental delays. The prevalence of OME in New Zealand may be higher in Mâori and Pacific Island children. The aetiology of OME is disputed, and controversy surrounds many reported risk factors. Recent research indicates that OME is associated with bacterial middle ear infection, possibly in conjunction with poor Eustachian tube function. Resistance to chronic OME may therefore depend on effective immune function, which could be impaired by allergy and micronutrient insufficiency.
Study Design: Using a case-control design we will examine risk factors for chronic OME by comparing 150 children aged 3-5 years undergoing tympanostomy tube insertion with 300 healthy aged- and sex-matched controls in Auckland, New Zealand.

Methods
Blood tests will be used to measure vitamin D, zinc, and iron levels and serum cytokine levels of interleukin 2,4,5,6,8,10,12, IFNg and TGFb. Skin prick tests for common inhalant allergens will be performed. Parents will complete questionnaires on their childrens' health and environment. Univariable and multivariable analyses of potential risk factors will be performed.

Rationale
This study will investigate whether ethnicity is a risk factor in New Zealand children in a multivariable analysis. By considering immune factors that regulate respiratory and middle ear immune function, atopy, micronutrients that are relevant to immune function, ethnicity and environmental influences together we aim to develop a fuller picture of the development of chronic OME, especially in relation to immune function.

Pathogenesis/Microbiology

Lactate Dehydrogenase Concentrations in Nasopharyngeal Secretions during URI and occurrence of Acute Otitis Media Complication

Linda Ede, MD[1], James O'Brien[1], Tasnee Chonmaitree, MD[1], Yimei Han[2], Janak Patel, MD[1]
[1]Pediatrics, [2]Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, Texas

Introduction

Acute otitis media (AOM) mostly occurs during or after viral upper respiratory tract infection (URI). The pathogenesis of AOM is multifactorial which includes complex interactions between the host, pathogen and environmental factors. Viral URI causes inflammation of the nasopharynx which is mediated by substances such as cytokines and inflammatory mediators. The inflammation leads to Eustachian tube dysfunction which causes a negative pressure in the middle ear allowing the bacterial and viral pathogens from the nasopharynx to enter the middle ear leading to the development of AOM[1,2].

Lactate dehydrogenase (LDH) is a membrane-associated enzyme found in body tissues which is released into the extracellular environment during tissue injury associated with inflammation. LDH is therefore often used as a marker of inflammation in many infectious conditions such as bacterial meningitis and empyema[3,4]. LDH is present in nasopharyngeal secretions of children with respiratory viral infections[5]. However, it is not known whether the concentrations of LDH in nasopharyngeal secretions (NPS) during viral upper respiratory infection (URI) reflect the degree of inflammation that could predict the risk for acute otitis media (AOM) complication.

Materials and Methods

In a prospective cohort study, healthy young children (6 to 36 months) were followed for 1 year. Children were evaluated at every episode of URI for the development of AOM within 28 days of URI onset. NPS samples were collected by suction catheter and diluted in 1ml of saline. Measurable volume of NPS samples (n=596) collected at the initial visit within 7 days of URI onset from 183 subjects were assayed for LDH concentrationsusing a commercial enzyme immunoassay. The LDH concentration range was between 0.9 to 1000mU/mL. Algorithms were used to extrapolate concentrations beyond this range. Final concentrations of LDH were adjusted to the dilution factor and reported in mU/ml of the undiluted NPS.

A subset of virus positive NPS samples (n= 271) were also analyzed for interleukin IL-1β, IL-6, and tumor necrosis factor (TNF) αconcentrations using a multiplex EIA[6]. Respiratory specimens were collected for virus studies at the initial URI visit and at subsequent visits only if AOM was diagnosed. Nasal swab specimens were analyzed for respiratory syncytial virus (RSV) antigen detection by EIA, tissue culture, and by molecular techniques[7]. In addition, the archived NPS samples were analyzed by quantitative real time PCR for RSV, human bocavirus (hBoV) and human metapneumovirus (hMPV*)* virus.

Results

Analysis using the GLMM suggested that LDH concentrations positively predictthe development of AOM(p= 0.01; Table 1). LDH concentrations were also significantly higher in samples positive for human bocaviruses, adenoviruses and rhinoviruses compared to virus negative samples (Table 2). For the first URI episodein each child, there was a positive correlation between LDH concentrations and IL-1β, IL-6 and TNF α concentrations (p = 0.008, 0.003, 0.01 respectively).The effect of LDH on AOM was highest on day 2 of URI and lasted up to 4 days. There was a significant stepwise increase in AOM occurrence with increasing concentrations of LDH (p value of 0.012; Figure 1).Children in the upper third of LDH concentrations had 43% rate of AOM as compared to 31% rate in the lowest third.

AOM risk was analyzed in relation to LDH concentrations in different age groups(< 12, 12 to 24 and > 24 months). Children without AOM had an increasing amount of LDH concentration during URI episodes as they got older. Butchildren with AOM had a persistently high LDH concentration that was similar in all three age groups (median range 2435 – 2576mU/mL).

Discussion

In the present study, we show that LDH concentration in NPS is a significant marker of risk for development of AOM complicating URI. There was a stepwise increase in AOM rates with increasing concentrations of LDH concentrations. The relationship between LDH concentrations and AOM is primarily during the first four days of URI, and was highest on the second day.

The direct correlation between concentrations of LDH and acute phase cytokines indicates that LDH is a marker of acute inflammation associated with URI. We have previously shown that concentrations of IL-1β in NPS are positively associated with the risk for AOM.[6] It is likely that more severe inflammation or tissue injury during viral URI leads to increased Eustachian tube dysfunction, thereby leading to the development of AOM.

Additionally, our data show that the LDH concentrations increase with the age of the children, suggesting that inflammatory injury induced by either direct cytopathic effect of the virus or by host immune response increases with age. Interestingly,

however, the median LDH concentrations associated with AOM were high in all age groups. We also show that LDH concentrations are higher in URI associated with adenoviruses, bocaviruses, rhinoviruses and mixed viruses when compared to other viruses and when the viruses could not be identified. Furthermore, exposure to cigarette smoke exposure and duration of breastfeeding did not influence the concentrations of LDH while these factors increased or reduced the AOM risk, respectively.

Clinically, it may be useful to have a predictive biochemical marker of risk of AOM during early stage of viral URI. Such a marker would allow the clinician to develop a follow-up evaluation strategy for children with URI. In summary, concentrations of LDH during URI episodes correlate with the risk for AOM suggesting that severity of cellular injury in the nasopharynx plays a role in the pathogenesis of AOM.

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Table 1: Risk factors that predict LDH concentrations in 596 episodes of URI in 183 children.

Characteristics	N (%)	Median LDH concentration (mU/ml)	Estimate (95% CI)	p-value
Age at visit			0.0 (-0.00 to 0.03)	0.04
Gender				
Female	94 (51)	4421	-0.04 (-0.16 to 0.08)	0.52
Male	89 (49)	5240	Reference	
Race				
Black	51 (28)	5449	-0.00 (-0.34 to 0.33)	0.98
White	37 (20)	6498	0.16 (-0.16 to 0.48)	0.32
Other	19 (10)	6348	0.13 (-0.21 to 0.47)	0.46
Hispanic	76 (42)	3077	Reference	
AOM†				
Yes	223 (37)	2439	0.29 (0.06 to 0.51)	0.01
No	373 (63)	1550	Reference	
Cig Smoke				
Yes	58 (52)	6312	0.00 (-0.26 to 0.28)	0.95
No	125 (68)	4122	Reference	
Fever‡				
Yes	288 (48)	1471	-0.19 (-0.42 to 0.03)	0.09
No	308 (52)	2111	Reference	
Virus type §				0.02
Day of URI			0.05 (-0.02 to 0.11)	0.17
Breast feeding				
0 – 6 months	28(15)	4077	0.18 (-0.19 to 0.56)	0.33
> 6 months	68(37)	5553	-0.04 (-0.32 to 0.25)	0.80
None	87(48)	3991	Reference	

† AOM development within 28 days of onset of URI symptoms;
‡ Presence of fever on the day of sample collection.
§ Additional virus type results are shown in Table 2.

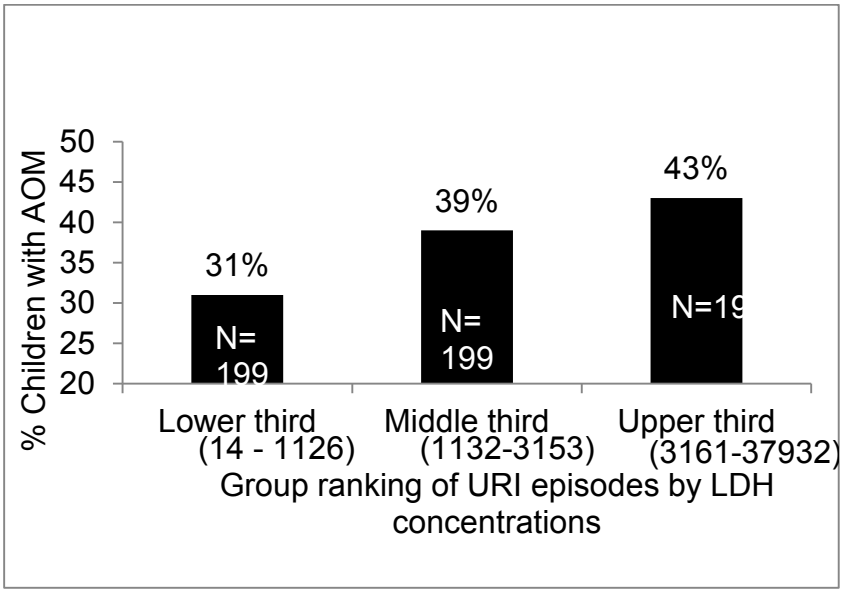
Median values shown above were derived by back-transformation of natural log data as described in Methods. GENMODE procedure was applied for above data analysis using log-transformed data; this model took into account multiple episodes of URI in the same child.
The presence of AOM, fever and viruses were calculated based on the 596 episodes of URI. Race, gender and cigarette smoke were calculated based on 183 subjects. Children who had fever at the time of sick visit were compared to children who did not have fever at visit. Children who had CMV were removed from the analysis as it is not considered a causative virus of URI.

Table 2: Virus type as a risk factor that predicts LDH concentrations in NPS in 596 episodes of URI in 183 children.

Virus type	Episodes N (%)	Median LDH concentration (mU/ml)	Estimate (95% CI)	P value
Coronavirus	8 (1)	4778	0.64 (-0.03 to 1.31)	0.06
HBoV	39 (7)	2869	0.63 (0.17 to 1.08)	0.01
Adenovirus	42 (7)	2816	0.75 (0.31 to 1.19)	<0.001
Rhinovirus	54 (9)	2229	0.49 (0.06 to 0.92)	0.03
Influenza A	16 (3)	2111	0.48 (-0.17 to 1.13)	0.14
Mixed Virus	187 (31)	2076	0.44 (0.12 to 0.77)	0.01
Parainfluenza	22 (4)	1790	0.37 (-0.26 to 0.99)	0.25
hMPV	24 (4)	1396	-0.42 (-1.12 to 0.27)	0.23
Enterovirus	24 (4)	1325	-0.09 (-0.64 to 0.45)	0.73
RSV	34 (6)	1307	0.08 (-0.46 to 0.63)	0.76
Virus negative	146 (24)	1305	Reference	

Above virus types were arranged in descending order of median LDH concentrations in NPS; the median values shown above were derived by back-transformation of natural log data as described in Methods. GENMODE procedure was used for analysis of log transformed data; this model took into account multiple episodes of URI in the same child.

Figure 1: AOM rates in relation to increasing concentrations of LDH during 596 episodes of upper respiratory tract infection (URI) in 183 children.



The values in parenthesis represent the range of LDH concentrations in each of the three equal groups arranged in increasing order of LDH concentrations. Log-transformed data were used for Cochran-Armitage trend test to calculate the relationship between increasing concentrations of LDH and rates of AOM; $p = 0.012$.

Bacterial Eavesdropping in Polymicrobial Otitis Media

W. Edward Swords, PhD
Microbiology and Immunology, Wake Forest School of Medicine, Winston-Salem, NC

Objectives
This study aims to define determinants of persistence and disease in polymicrobial otitis media caused by Haemophilus influenzae and Moraxella catarrhalis.

Methods
Polymicrobial biofilms were established in vitro using relevant static and continuous-flow model systems, and analyzed by confocal laser scanning and electron microscopy. Antibiotic resistance was determined by plate-count of planktonic and surface-adherent bacteria. Persistence and virulence were assessed in the chinchilla infection model for otitis media.

Results
Haemophilus influenzae and Moraxella catarrhalis established polymicrobial biofilms that had elevated resistance to antibiotic and host killing. For M. catarrhalis, this resistance phenotype was conferred by diffusible quorum signals secreted by H. influenzae, which altered production of M. catarrhalis factors that promote biofilm formation. Similar experiments with other otitis media pathogens will also be discussed.

Conclusions
Interspecies quorum signaling is an important virulence determinant for M. catarrhalis, and may represent an attractive pharmaceutical target.

Streptococcus pneumoniae Induced Inflammation-Associated Molecular Patterns Under Conditions Simulating Eustachian Tube Obstruction

Ha-Sheng Li-Korotky, PhD, MD, Chia-Yee Lo**,** Juliane Banks**,** J. Douglas Swarts, PhD
Pediatric Otolaryngology, Children's Hospital of Pittsburgh, Pittsburgh, PA

Objective
Streptococcus pneumoniae, a leading cause of otitis media (OM), adapts to the host environment and undergoes spontaneous intra-strain variations in colony morphology. Transparent (T) variants are more efficient in colonizing the nasopharynx while the opaque (O) variants exhibit greater virulence during systemic infections. We recently demonstrated that T variants exhibited a higher growth rate and greater epithelial adherence and destruction than did O variants during interactions with human middle ear (ME) epithelial cells (MEECs). Eustachian tube obstruction (ETO) leads to increased negative pressure in the ME cavity that could modify MEEC responses to S. pneumoniae infection. The underlying molecular mechanisms remain poorly understood.

Methods
Human MEECs were preconditioned for 24 hr under the simulated ME gas/pressure condition [physiological ME gas composition and subambient pressure (-250 mm H2O)] that reflect ME condition with ETO, subsequently exposed to a dose (~107 CFU/mL) of S. pneumoniae (6A) T variants, and then incubated for 1 and 3 hr. Gene expressions coding for inflammation-associated molecular patterns (IAMPs) were assayed using real-time PCR.

Results
At the end of experiment after ETO conditioning (without bacterial infection), matrix metallopeptidase 1 (interstitial collagenase) (Mmp1) was significantly induced, followed by prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (Ptgs2), interleukin 10 (Il10), tumor necrosis factor (Tnf), interleukin 1b (Il1b), heme oxygenase (decycling) 1 (Hmox1), interleukin 6 (Il6), and matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase) (Mmp9). Interestingly, synergistic effects of ETO and pneumococcal T variants on IAMP expressions in MEECs were observed for Ptgs2, Il6, IL1b, Hmox1, and Mmp9.

Conclusions
Conditions characteristic of the ETO intensify the pneumococcus-induced inflammatory responses in MEECs.

Synergistic Effects of Pneumococcal Phase Variation and Tympanostomy Tube Placement on Inflammation-Associated Molecular Patterns in Simulated Otitis Media

Ha-Sheng Li-Korotky, PhD, MD, Chia-Yee Lo**,** Juliane Banks
Pediatric Otolaryngology, Children's Hospital of Pittsburgh, Pittsburgh, PA

Objective
Streptococcus pneumoniae, a leading cause of otitis media (OM), adapts to the host environment and undergoes spontaneous intra-strain variations in colony morphology. Transparent (T) variants are more efficient in colonizing the nasopharynx while the opaque (O) variants exhibit greater virulence during systemic infections. We recently demonstrated that T variants exhibited a higher growth rate and greater epithelial adherence and destruction than did O variants during interactions with human middle ear (ME) epithelial cells (MEECs). More than half a million tympanostomy tubes (TTs) a year are inserted to alleviate OM. Nevertheless, as many as 50% of the children who receive TTs can have an episode of otorrhea and about 39% patients with otorrhea and TTs were found culture positive for S. pneumoniae. Predominant selection of the pneumococcal variants and associated virulence in TT related otorrhea were undefined previously.

Design
Human MEECs were preconditioned for 24 hr under the simulated ME gas/pressure conditions [approximately ambient gas composition (21% O2, 5% CO2, and 74% N2) and pressure] that reflect ME conditions with TT placement. Cells were exposed to S. pneumoniae (6A) T or O variants (~107 CFU/mL) and incubated for 1 hr and 3 hrs under TT condition. Gene expressions coding for inflammation-associated molecular patterns (IAMPs) were assayed using real-time PCR.

Results
At the end of experiment after TT conditioning (without bacterial infection), matrix metallopeptidase 3 (stromelysin 1, progelatinase) (Mmp3) was significantly induced in MEECs, followed by matrix metallopeptidase 1 (interstitial collagenase) (Mmp1), interleukin 10 (Il10) and tumor necrosis factor (Tnf). Synergistic effects of TT and T variants on IAMPs in MEECs were observed for Tnf, Mmp3, and Il10, whereas synergistic effects of TT and O variants on IAMPs in MEECs were observed for Mmp1, Il10, and Tnf.

Conclusions
Middle ear condition characteristic of the TT insertion intensifies the pneumococcal-induced inflammatory responses in MEECs.

Middle Ear Cytokine Gene Production in Response to Bacterial and Steroid Challenges

Carol MacArthur, MD, Fran Hausman**,** Beth Kempton**,** Dennis Trune, PhD
Otolaryngology, OHSU, Portland, OR

Objective
The middle and inner ear have been shown to be able to respond to inflammation with local production of cytokines. Steroids are often used trans-tympanically for their anti-inflammatory impact on the inner ear, but the local effect of steroids on inflammatory gene production has not been studied.

Methods
The middle ears of Balb/c mice weretranstympanically injected with 0.5 µl of either heat killed *H. influenza*,prednisolone or dexamethasone. Untreated and saline (PBS) injected mice were used as controls. Mice were euthanized at 6, 24 and 72 hours, the bullae harvested and total RNA isolated from the middle ear tissues. Cytokine genes (Mip-2, IL-6, IL-1β, IL-10, TNF, IL-1α, MIP-1α and KC) were analyzed with real-time qRT-PCR.

Results
Heat killed bacteria cause a robust upregulation of all studied cytokine genes that peaks at 6 hours and then progressively declines. PBS causes a much weaker response, with gene production up only at 24 hours. Prednisolone (a mixed glucocorticoid and mineralocorticoid) causes an intermediate upregulation in several cytokine genes (MIP-2, IL-6 and IL-10), principally at 6 hours, while most genes were downregulated and not measurable at 24 and 72 hours. Dexamethasone (a pure glucocorticoid) shows a similar effect.

Conclusions
The middle ear is capable of local production of cytokines by gene upregulation in response to inflammation. In this study, steroids are shown to cause downregulation of cytokines in the middle ear in comparison to control, PBS- and bacteria-injected ears. These findings supportknown anti-inflammatory and fluid regulatory properties of glucocorticoids and mineralocorticoids.

Microbial Flora in the Middle ear of Children with Acute Tympanostomy Tube Otorrhea

Thijs van Dongen, MD[1], Maroeska Rovers, PhD[1], Alice van Zon[2], Debby Bogaert, PhD[3], Anne Schilder, MD, PhD[2]
[1]Julius Center for Health Sciences and Primary Care, [2]ENT, [3]Microbiology, University Medical Center Utrecht, Utrecht, Utrecht
Extended abstract oral presentation OM2011

Introduction
Over half a million children have tympanostomy tubes placed each year in the United States, making it the second-most common operative procedure in childhood.[1] The main complication of TT's is developing acute tympanostomy tube otorrhea (ATTO), occurring in about 1 in 4 children.[2] ATTO can occur as part of an upper respiratory tract infection in which pathogens ascend to the middle ear from the nasopharynx, but ATTO may also occur when pathogens from the external ear canal enter the middle ear through the tympanostomy tube, such as after swimming.[1]Known pathogens involved in ATTO are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxhella catarrhalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[3]
In recent years, changes in health care practice, such asthe introduction of routine pneumococcal vaccination in children, have led to changes in the pathogens causing acute otitis media in various countries.[4-5]From the perspective of treatment as well as prevention, it is important to know whether this has alsoled to changes in the pathogens causing ATTO.

Objectives
The objective of the present study was to establish the current prevalence of the pathogens in the middle ear fluid of children with ATTO.

Materials and Methods
The study population consisted of all children participating in an ongoing pragmatic randomized controlled trial comparing eardrops, oral antibiotics and watchful waiting for treatment of ATTO.Inclusion criteria were: age 1 to 9 years, having had tympanostomy tubes for at least 2 weeks before onset of otorrhea and having had symptoms of otorrhea for less than 7 days. Exclusion criteria were: having used ototopical or systemic antibiotics in the last 14 days and having had an a previous episode of otorrhea in the last 28 days.
Otorrheal swabs were taken from all children at baseline by swabbing the discharge in the external ear canal while avoiding skin contact. The samples were inoculated onto sheep blood (5%), *Haemophilus*, and MacConkey agar plates within 24 hours after the sample was taken. The culture plates were incubated aerobically at 37°C (MacConkey agar) and less than 5% carbon dioxide (blood and *Haemophilus* agars) and were examined at 24 and 48 hours.
If bilateral otorrhea was present, only the culture results from the ear that drained first were used. Results were stratifiedaccording to age (1-2 years, 3-4, 4-9) and pneumococcal vaccination status(yes vs no).

Results
Table 1 shows the characteristics of the 141 children included in the study between June2009 and April2011.
Table 2 shows the culture results of the otorrheal samples taken at baseline in this group of children (note: all untreated).*H. influenzae* was the most frequently cultured pathogen (42%), followed by *S. aureus* which was present in about one third of the patients. *S. pneumoniae* was remarkably low: 9%.
S. pneumoniae, *M. catharralis* and *H. influenzae*were more prevalent in the younger children, hence also more prevalent in the vaccinated children than in the non-vaccinated children (Table 3). *P. aeruginosa* and *S. aureus*were more prevalent in the higher age group.

Discussion
In our study, *H. influenzae*is the most prevalent pathogen, followed by *S. aureus*. The prevalence of *S. pneumoniae*is very low.

To relate our results to those of previous studies on ATTO, we reviewed the literature of the last decade and found 5 recent studies comprehending at least 1124 patients for each pathogen (the various studies did not culture all pathogens).[6-10] The prevalence of*S. pneumoniae*isconsiderably lower in our study compared to the previous studies (9% vs. 22%), whereas*H. influenzae*ismore prevalent in our study (42 vs. 22).*S. aureus*is more often cultured in our population than in the previous publications (36 vs. 14).
Reasons for these differences could be twofold: First, the mean age of the children in our trial is higher (4.3 years vs. 2.2 years). And secondly, the sampling period of our trial was a few years after introduction of a routine pneumococcal vaccination in The Netherlands, while in all the other studies it was not yet introduced. Stratification of the results however, shows that the high mean age of our population does not explain the big differences we found. The effect of the vaccination status is difficult to interpret since all vaccinated children are younger, but it can very well explainpart of the differences. Only half of the children in our trial were vaccinated, but herd immunity can play a role since the vaccination rate in The Netherlands is more than 94%.[11]This supports Block et al. who also described a possible herd effect when they found a decrease in pneumococcal

infections and an increase in the prevalence of *H. Influenzae* in children with acute otitis media after introduction of routine pneumococcal vaccination.[4]

A potential limitation of our study is the sampling technique. We sampled the otorrhea from the external ear canal and not directly from the middle ear. Evidence about the correlation of these sampling methods is contradictory: some suggest a good correlation while others report that samples from the ear canal can be misleading.[12,13]

Our results reflect the current prevalences of pathogens in a unique and big group of children with untreated ATTO for 1-7 days. In addition, our population represents the situation after introduction of a routine pneumococcal vaccination.

Conclusion

The results of our study indicate a change in pathology that occurred after introduction of the pneumococcal vaccination in The Netherlands. *H. Influenzae* was the main bacterium present in the otorrhea of children with untreated ATTO. Compared to earlier studies, we found a decrease of *S. pneumoniae* in the otorrhea and an increase of *H. Influenzae* and *S. Aureus*.

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Tables

Table 1 Patient characteristics

Number		141 children
Mean age (range)		4.3. years (1-9)
Gender	male	57%
	female	43%
Season	Oct-Apr	68%
	May-Sept	32%
PCV7-vaccinated*		49%
Bilateral otorrhea		20 children

*PCV7 = 7-valent pneumococcal vaccine

Table 2 Number and percentage of positive otorrheal cultures

Bacteria	n	%
S. pneumoniae	12	9
H. influenzae	59	42
M. catarrhalis	7	5
S. aureus	50	36
P. aeruginosa	22	16
No bacteria	14	10

Table 3 Percentages of positive otorrheal cultures stratified by age and vaccination status

PCV7-vaccination	Yes		No
Age (years)	1-2	3-4	4-9
Number of patients	37	32	71
Bacteria	%	%	%
S. pneumoniae	11	13	4
H. influenzae	51	50	32
M. catarrhalis	11	3	3
S. aureus	32	31	39
P. aeruginosa	5	16	21

Nasopharyngeal Carriage in Young Otitis-Prone Children

Marie Gisselsson-Solén, MD[1], Ann Hermansson, MD, PhD[1], Åsa Melhus, MD, PhD[2]
[1]Dpt of Otorhinolaryngology, Head & Neck Surgery, Clinical Sciences, Lund University, Lund, [2]Section of Clinical Bacteriology, Department of Medical Sciences, Uppsala

Objective
Acute otitis media (AOM) is the most common bacterial infection in children, and 10-15% of all children develop recurrent disease (rAOM). The purpose of this study was to investigate the patterns of nasopharyngeal carriage in children with a high risk for developing rAOM.

Methods
96 children taking part in a vaccination study at Lund University Hospital constituted the study group. All had an onset of AOM before six months of age, implicating a high risk for developing rAOM. The children visited the study clinic every other month during the first year, and, in addition, whenever their parents suspected a new AOM episode. After three years, a final visit was made. Otomicroscopy and nasopharygeal cultures were performed at all visits.

Results
No difference was found in nasopharyngeal carriage between vaccinated and non-vaccinated children. *Streptococcus pneumoniae* and *Moraxella catarrhalis* were more frequently found during illness in very young children, whereas *Haemophilus influenzae*

was more common in older children. Children with siblings in public day care carried AOM pathogens more often. Between October and June, when AOM is more common, *H. influenzae*, *M. catarrhalis* and *Streptococcus pyogenes* were more often present in nasopharyngeal cultures than during the summer. Comparing the children who developed rAOM with those who did not, the former group carried *H. influenzae* more often.

Conclusions
This cohort of young otitis-prone children has been studied closely during their first years of life with frequent ear examinations and nasopharyngeal cultures during healthy periods as well as during AOM.

Nasopharyngeal Cultures Taken During Antibiotic Treatment

Ann Hermansson, PhD, MD[1], Marie Gisselsson-Solén, MD[1], Åsa Melhus, PhD, MD[2]
[1]ENT, University of Lund, Lund, Skåne, [2]Medical microbiology, Uppsala Universitet, Uppsala

Objective
To investigate to what extent the simultaneous administration of antibiotics affect the results of nasopharyngeal cultures in small children.

Method
In a population of small otitis-prone children in a study of pneumococcal vaccination 101 nasopharyngeal cultures obtained during treatment were compared to 1049 samples obtained without simultaneous antibiotic treatment. Middle ear status was recorded at all visits as well as medication type and duration. The group consists of children vaccinated with a 7-valent pneumococcal vaccine and a control group of unvaccinated children. Standard laboratory procedures for culture were used.

Results
In brief, the presence of pneumococci was significantly lower ($p<0,001$) in the antibiotic treatment group both when comparing vaccinated and unvaccinated children. In the case of other major AOM pathogens, no significant differences were seen.

Conclusion
Antibiotic treatment affects the results of samples from nasopharynx in small children. The effects are discussed concerning both actual treatment effect and the possibility of an effect on cultures.

Middle Ear Cd14 Gene Expression During Eustachian Tube Obstruction and Acute Otitis Media Induced by *Streptococcus pneumoniae*

Ha-Sheng Li-Korotky, PhD, MD[1], Chia-Yee Lo[1], Allison Cullen-Doyle[1], Juliane Banks[1], Sancak Yuksel, MD[2]
[1]Pediatric Otolaryngology, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]Otorhinolaryngology - Head & Neck Surgery, University of Texas Medical School at Houston, Houston, Texas

Objectives
Streptococcus pneumoniae, a leading cause of otitis media (OM), adapts to the host environment and undergoes spontaneous intra-strain variations in colony morphology. Transparent (T) variants are more efficient in colonizing the nasopharynx while the opaque (O) variants exhibit greater virulence during systemic infections. Eustachian tube (ET) obstruction (ETO) leads to increased negative pressure in the ME cavity that could modify ME response to S. pneumoniae infection. The underlying molecular mechanisms remain poorly understood.

Design
Male Sprague-Dawley rats were randomly assigned to 7 groups, including a control group without treatment, a ETO sham group, a ETO group, 2 pneumococcal challenged groups infected by either T variants or O variants, and 2 ETO plus pneumococcal infected groups. For ETO rats, the left, bony ET was obstructed via cauterization. For pneumococcal infected rats, ME bulla was inoculated via a surgical approach with either 25 µL phosphate-buffered saline (PBS, pH 7.2) or PBS containing 1x 107 CFU/mL of S. pneumoniae 6A (T or O variants). Rats were sacrificed at 1, 2 or 4 days post-manipulation and ME tissues were harvested for total RNA extraction. Relative gene expression levels encoding Cd14 receptor were assessed using real-time PCR.

Results
For ETO sham groups, Cd14 expression levels were significantly higher in pneumococcal-infected middle ears than that in control groups. Cd14 gene up-regulation was higher in O variant challenged group than that in T variant challenged group, suggesting that pneumococcal O variants are more virulent than T variants in vivo. For ETO groups, Cd14 molecular activity was significantly induced for all groups, including both control and bacterial infected groups from day 1 to day 4. All O variant exposed rats died at day 3 or 4 post infection.

Conclusion
ETO up-regulates Cd14 gene expression in the ME and intensifies pneumococcal virulence of both phase variants during ME infection.

E. Coli Surface Expression of Pneumococcal Adhesins

M. Nadeem Khan, PhD, Michael Pichichero, MD
Research Institute, Rochester General Hospital, Rochester, New York

Objective
To express pneumococcal antigens on the surface of *E coli* in order to study their role in adhesion to epithelial cells.
Streptococcus pneumoniae (Spn) is a major otopathogen. Outer membrane proteins of pneumococci are key antigens for induction of protective immunity . The impaired ability of children to make functional antibodies against *Spn* adhesins perhaps makes them vulnerable to acute otitis media (AOM). Most studiescharacterizing adhesins are done with knockouts of the gene of interest. However, the role of individual adhesins is a challenge to assess as there are multiple adhesins on the surface of *Spn*. We have developed an *E. coli* surface expression system whereby key surface adhesins of *Spn* have been expressed in a strain of *E. coli* that is non-adherent to human nasopharyngeal and middle ear epithelial cells.

Method
The *E coli* expression was controlled by providing a set of different physical conditions in order to prevent expressed proteins to form inclusion bodies. The surface expression of expressed proteins in *E coli* was confirmed by flow cytometry using FITC labelled secondary antibody.

Result
Using this system we have demonstrated that *E. coli* with surface expressed choline-binding and histidine triad proteins of *Spn* bind to nasopharyngeal epithelial cells (D562).

Conclusion
The choline binding and histidine triad proteins of *Spn* are adhesins and studies are underway to use this *E. coli* surface expression system to evaluate antibody responses against *Spn* adhesins. The study was supported by NIH RO1 08671 and sanofi pasteur.

Differential Expression of Non-Typeable *Haemophilus influenzae* Key Virulence Genes from Nasopharyngeal and Middle Ear Fluid Samples of Children with Acute Otitis Media

Ravinder Kaur, PhD[1]**,** Janet Casey, MD[2]**,** Michael Pichichero, MD[1]
[1]Research Institute, Rochester General Hospital, Rochester, New York, [2]Pediatrics, Legacy Pediatrics, Rochester, New York

Introduction
Nontypeable *Haemophilus influenzae* (*NTHi*) is one of the most common otopathogens that causes acute otitis media (AOM) in children in the United States and worldwide. In this study, we have determined the expression of key *NTHi* virulence genes Protein D, P6, OMP26, P5, PilA, and LuxS in the nasopharynx (NP) and middle ear fluid (MEF) of children with AOM caused by *NTHi*. Expression of these genes among different clinical isolates was also studied. Bacterial RNA was extracted from in-vitro grown *NTHi* strains, NP and MEF samples and assayed by real-time RT-PCR. All genes expression was normalized by 16S rRNA gene. Compared to their respective in-vitro grown strains, from the 20 NP samples of children at the time of AOM, the mRNA of protein P6 was over-expressed in 60% of the NP samples for P6, in 80% of the samples for OMP26, 55% samples for protein D, in 57% samples of PilA (6 of the NP samples didn't show any expression for PilA), in 75% samples for P5 and in 60% of the samples for LuxS. Interestingly the expression pattern of these proteins in MEF was different from NP samples where all showed less over-expression compared to in-vitro grown strains. PilA expression was not observed in 4 of the MEF samples and in 3 of both NP and MEF samples from children. The expression of these vaccine candidates was also compared among different clinical isolates; compared to *NTHi* 86-028 strains, different isolates showed differential expression of six proteins studied. When the results were compared between NP and MEF samples from the same child, mRNA was present at significantly higher levels in the NP samples than in the MEF. These findings illustrate the dynamic nature of *NTHi* virulence gene expression *in vivo*. Such analysis will help unravel the complex interactions between *NTHi* and its host environmental niches during disease development. According to our knowledge this is the first study showing gene expression profiles of virulence factors of *NTHi* directly in host samples during AOM infection.

Table 1: Target Virulence Proteins studied:

Protein	Characteristics
Protein D[1]	Protein D (ProD) is a highly conserved 42 kDa surface lipoprotein, shown to impair ciliary function in a human nasopharyngeal tissue culture model. Mechanism of ProD virulence is its glycerophosphodiesterase activity, which leads to the release of phosphorylcholine from host epithelial cells.
P6[2,3]	P6 is a highly conserved 16-kDa lipoprotein of *NTHi*, elicits antibodies that are bactericidal and protective in experimental models of infection. Intranasal immunization with P6 was shown to confer antigen-specific mucosal immunity and enhance mucosal clearance of *NTHi* in a mouse model.
OMP26[4,5]	A 26 KDa protein, is relatively conserved among isolates of *NTHi*. It is highly efficacious as a mucosal immunogen, inducing significantly enhanced clearance in immunized rats upon direct challenge of middle ear and lung.
PilA[6]	*NTHi* PilA is a type IV pilus adhesion protein. It plays a role in adherence to human respiratory epithelial cells, in colonization of the mammalian upper respiratory tract, and in biofilm formation.
P5[7,8]	P5 is a outer membrane protein of *NTHi*, also called fimbrin P5. It has both immunodominant and host-adhesive domains and has been shown to bind to human mucin, as well as to surface-expressed carcinoembryonic antigen-related cell adhesion molecule 1.
LuxS	Auto inducer-2/ luxS plays a role in Quorum sensing (QS), or cell-to-cell communication in bacteria.

Methods

Bacterial RNA was extracted from 20 in-vitro grown *NTHi* strains, NP and MEF samples and assayed by real-time RT-PCR. All genes expression was normalized by 16S rRNA gene. Comparative Ct method was used to determine relative target gene expression. The data was represented as fold change ($2^{-\Delta(\Delta Ct)}$) which is
The amount of target, normalized to an endogenous reference and relative to a calibrator (control) (Δ(ΔCt method).
Figure 1: Showing the primer sequences used in RT-PCR for the target genes

Protein	Primer	Sequence 5'-3'	Position in gene	Amplicon size
16S rRNA	forward	GTATTCACCGCGACATTCTG	165	69
	reverse	GAGTCTGCAACTCGACTCCA	214	
	probe	TCGCGATTACTAGCGATTCCGACTTC	187	
Protein D	Forward	CCCAAAGGGTTATTGGGTAA	259	105
	Reverse	ATATACCAACCTGGGCCAAC	374	
	probe	CCACTTCTGCCATTGCACCAGG	292	
P6	forward	CCAGCATCAACACCTTTACC	101	133
	reverse	CAACGCCAGCTGCTAAAGTA	214	
	probe	TGCATCTGCACGACGTTGGC	139	
OMP26	forward	TTTATTAATTTCCTGTTGGCGTT	277	110
	reverse	AAATTGCTGCTGCTCGTAAA	367	
	probe	TGCACCTCGCTTACGTCAAGCTG	309	
PilA	forward	TCCACCCGTACAGTTTGTTG	226	140
	reverse	CCACTATCGCAATTCCCTCT	346	
	probe	TTACTGCAAGCGTCAGCGCCTT	288	
P5	forward	TGCAACTGGCTACGGTAAAG	951	119
	reverse	CGTTTACCGCGATTTCTACA	1050	
	probe	TCGCTTGTCTTGCTCCAGACCG	1025	
LuxS	forward	GATTGGCACACCAAATGAAC	273	128
	reverse	GTTCCGTATAGCTTCCGCAT	381	
	probe	TGCATTGAAGCTAACCAAGCCTCAGA	301	

Results

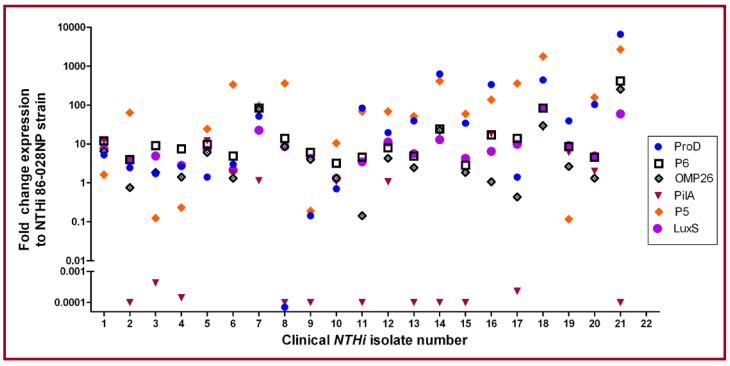
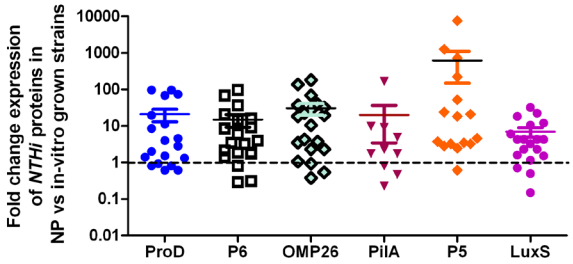


Figure 2:Expression of virulence genes in different clinical isolates of *NTHi* from children with AOM NTHi were grown in-vitro to their stationary phase (OD=0.8)
* Data is represented as fold change gene expression relative to the standard NTHi strain 86-028NP.



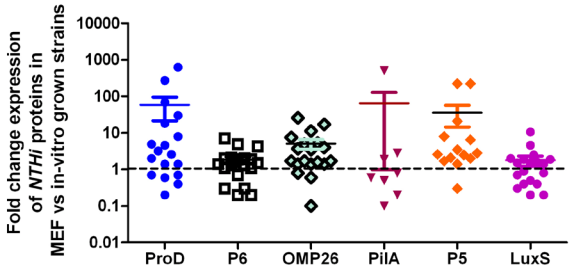


Figure 3: Expression of virulence genes in the NW and MEF samples of children during AOM infection due to NTHi compared to the in-vitro grown NTHi.

Data represent fold change in normalized transcript levels for each gene in samples collected from NP and MEF, relative to the corresponding *NTHi* strains grown aerobically to stationary growth phase in BHI broth.

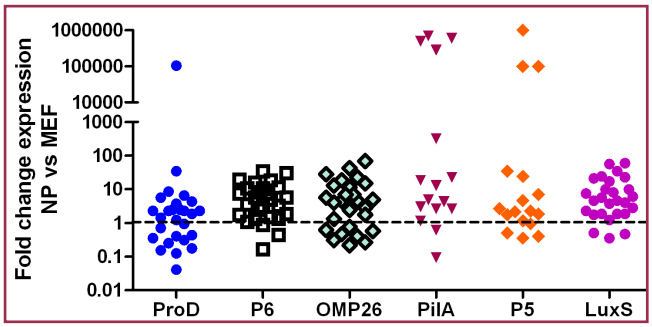


Figure 4: Virulence gene expression of *NTHi* in NP samples of children with AOM relative to normalized MEF samples. Most of the genes were expressed more in NP samples compared to MEF.

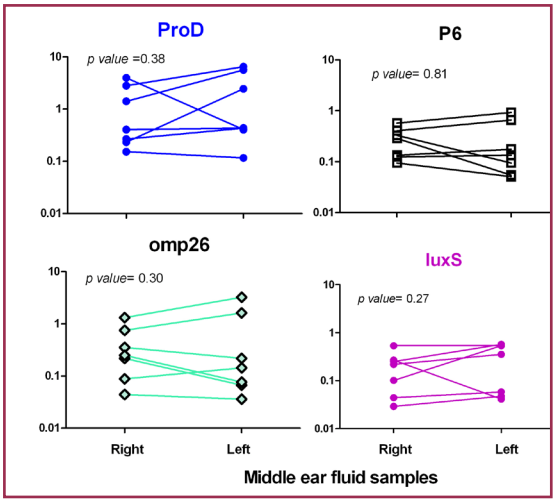


Figure 5: *NTHi* Virulence gene expression in the right and left MEF of 7 children with AOM indicating similar patterns of gene expression from both ears.
* Data is represented as fold change in MEF gene expression relative to NP.

Conclusions

Different clinical isolates of *NTHi* showed differential virulence genes expression.
More expression of *NTHi* virulence genes was observed directly in the host at the site of infection (both NP & MEF) compared to in-vitro grown strains.
Similar patterns of gene expression profiles were observed in both ears of one child, validating the expression data. PilA and P5 were more expressed in NP samples compared to MEF samples, consistent with their role as adhesins.

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Innate Sensing of Pathogen/Damage-Associated Molecular Patterns in Pseudomonas Lipopolysaccharide Challenged Middle Ear Epithelial Cells

Allison Cullen-Doyle**, Ha-Sheng Li-Korotky, PhD, MD**
Pediatric Otolaryngology, Children's Hospital of Pittsburgh, Pittsburgh, PA

Introduction

Pseudomonas aeruginosa, a human opportunistic bacterial pathogen, is frequently isolated from patients with chronic middle ear (ME) diseases [1], including chronic suppurative otitis media (CSOM) [2, 3], cholesteatomatous otitis media (OM)[4], and mastoiditis [5]. *P.aeruginosa* is one of the most common bacterial isolates in infectious otitis externa and the associated purulent otorrhea [6, 7]. Furthermore, *P.aeruginosa* is a major biofilm-forming pathogen, leading to a high rate of persistent otorrhea after insertion of

tympanostomy tubes or other ME prostheses [1, 8]. *P.aeruginosa* is also responsible for complications of acute OM in children [9]. With increasing antimicrobial resistant *P.aeruginosa* strains, the treatment of these diseases caused by *P. aeruginosa* is challenging and costly [5, 10].

Inflammation of the middle ear mucosa (MEM) during bacterial infection is caused both by direct effects of the metabolic processes of live bacteria as well as by indirect effects mediated by the host responses to conserved molecules presented by live bacteria and/or toxic molecules released from killed bacteria, such as lipopolysaccharide (LPS). Related bacteria contain similar structural motifs that can be readily detected by host innate immunity as molecular patterns of infectious non-self, termed pathogen-associated molecular patterns (PAMPs). Host defense against bacteria consists of an early, innate immune response and, if infection persists, a later, adaptive immune response. It is known that primary effectors of ME innate immunity include: (1) the physical barrier of the MEM; (2) non-specific, antimicrobial substances produced by MEM; (3) phagocytic cells that migrate into the MEM, and (4) local proinflammatory cytokines and other chemical mediators/signals released by those inflammatory cells in responding to pathogens [11]. Only recently has it become apparent that host germ-line-encoded pattern recognition receptors (PRRs) recognize PAMPs and activate both innate and adaptive immunity while also executing a variety of direct functions, including phagocytosis, opsonization, chemotaxis and endocytosis[12].

LPS released by *P.aeruginosa* is a key virulence factor triggering both innate and acquired host responses to infection [13]. LPS of *P.aeruginosa* was used to effectively induce otitis media with effusion (OME) and CSOM in animal models [14, 15]. *P. aeruginosa* LPS-induced cell/organ specific expression patterns of PRRs and downstream molecular/cellular signaling pathways and associated pathological outcomes have been explored in lethal sepsis [16], pulmonary cystic fibrosis [17], and corneal infection [18]. Nevertheless, the innate PRRs of the MEM responding to *P. aeruginosa* infection are unknown.

We hypothesized that the ME epithelial cells (MEECs) possess a unique set of PRRs that recognizes and responds to *P. aeruginosa* LPS, a PAMP, leading to MEM inflammation. To test this hypothesis, gene expression patterns coding for innate PRR molecules were investigated in MEECs challenged with *P. aeruginosa* LPS. Specifically, we studied the time-dependent effect of the LPS on PRR gene expression in mouse MEECs. We expect that the information gained from this study will help to develop the strategies for the control and modulation of the innate PRR molecular activities and associated downstream inflammatory processes in CSOM.

Materials and Methods

Mouse middle ear epithelial cell culture. The mouse middle ear epithelial cell line (MEEC) was previously established [19] and generously provided by Dr. Ji-Zhen Lin (Otitis Media Research Center, University of Minnesota School of Medicine). The MEEC cells were seeded onto 12-well plates at a density of 3.5×10^5 cells/well in a humidified 10% CO_2 incubator at 33°C for proliferation and then transferred to a humidified 5% CO_2 incubator at 39°C for differentiation, in a mediumcomposed of Ham's F-12K media, sodium bicarbonate (7.5%), L-glutamine (200 mM), epidermal growth factor (10 ng/ml), insulin-transferrin-sodium selenite (10 µg/ml), glucose (2.7g/L), hydrocortisone (500 ng/ml), non-essential amino acids (100 mM), and 4% fetal bovine serum (FBS).

P. aeruginosa LPS challenge. The MEEC monolayers at 80–90% confluence were challenged with either *P. aeruginosa* LPS (100 µg/ml, Sigma L-9143) or a control medium (equal volume of PBS) for6, 12, 24, and 48 hrs. At the end of each time point, monolayers were washed 3 times and detached from the surface by adding 100 µl of 0.25% trypsin/0.02% EDTA. Cells were collected by centrifugation and stored at –80°C for gene expression assays. Quadruplet wells of MEECs were tested for each condition per each time point and experiments were repeated for 2 times.

Gene expression assay. Cell pellets were individually homogenized using a TissueLyzer (Qiagen). Total RNA was extracted with RNeasy Mini Kit (Qiagen) following manufacturer's instructions.We focused on 12 genes coding for PRRs, including Cd14, LPS-binding protein (Lbp), toll-like receptors (Tlrs) 1-9, and toll interacting protein (Tollip). Gene-specific primers were designed using OLIGO *Primer Analysis Software (*v6.3, Molecular Biology Insights, Inc., Cascade, CO). Expression levels of the gene transcripts were quantified and analyzed using real-time polymerase chain reactions (PCR) and the Sequence Detection Software (Applied Biosystems) as previously described [20].

Statistical analyses. A 2 x 4 two-way between-condition analysis of variance (ANOVA) was performed on gene expression levels of the mouse MEECs (dependent variable) as functions of independent variables (treatment and time) using PASW software package, Statistics 18 (SPSS Inc., Chicago, IL, USA). Post hoc analyses with Bonferroni adjustments were performed. The α level was set at 0.05.

Results

The basal expression pattern encoding innate PRRs in control MEECs at 6 hrs revealed an order of the molecular intensities from the highest to the lowest levels of: Tollip > Tlr4 > Tlr5 > Cd14 > Lbp > Tlr9 > Tlr1 > Tlr7 > Tlr2 > Tlr6 > Tlr3 > Tlr8 (Fig. 1). Note that a specimen that contains more copies of a gene transcript has a lower Ct value because the cDNA copy number and Ct are inversely related. Interestingly, our results showed later that the basal expression levels of the innate PRRs in normal MEECs cannot predict the LPS-induced PRR molecular responses in the MEECs, suggesting that the activation of the PRRs is PAMP-

specific. Of 12 PRR genes assessed, only 5 PRR molecules in the MEECs were significantly up-regulated by *P.aeruginosa* LPS challenge (Fig. 2).

Cd14 expression levels in LPS-exposed MEECs were significantly higher than that of the control groups at all time points ($p < .001$). Lbp expression levels in LPS-challenged MEECs were significantly higher than that of the control groups at all time points ($p \leq .013$). Results showed consistent and moderately high levels (2 – 3 fold up-regulations) of Lbp gene expression in the LPS-treated MEECs. The consistent upregulation of the Lbp expression pattern is in agreement with that of the Cd14 expression, indicating a coordinate response of the MEEC-Cd14/Lbp to *P.aeruginosa* LPS exposure. Tlr2 expression levels in LPS-inflamed MEECs were significantly higher than that of control groups at 6 hrs, 12 hrs, and 24 hrs ($p \leq .003$), but not at 48 hrs ($p = .16$). Results demonstrated a significantly initial induction of the Tlr2 molecular activity at 6 hrs that declined over time. Our results suggest that there is a co-regulation of Cd14/Tlr2 during the early event of *P.aeruginosa* LPS challenge in the MEECs. The Tlr4 molecular activity in LPS-challenged MEECs was significantly higher than that of control groups only at 48 hrs ($p = .032$), suggesting a late response of the Tlr4 to LPS-induced MEEC inflammation and damage. Tlr9 gene activity in LPS-inflamed MEECs was significantly higher than that of the control groups at 6 hrs ($p = .021$) and 48 hrs ($p = .001$). These two peaks of the Tlr9 up-regulation in the LPS-exposed MEECs indicate that the Tlr9 in the MEECs recognizes and responds to both LPS and LPS-induced cell damage.

A total gene dose for each ME mucosal innate PRR that was significantly activated as a function of time after LPS exposureshowed that Cd14 is the most active PRR in the MEECs, followed by Tlr2, Lbp, Tlr9, and Tlr4 during the course of the *P. aeruginosa* LPS challenge, further suggesting that the MEM innate PRRs including Cd14, LBP, Tlr2, Tlr4 and Tlr9 recognize and respond to *P. aeruginosa* LPS and associated MEM damage (Fig. 3).

Discussion

Time-dependent MEEC-specific expression patterns of the PRRs were investigated in the mouse ME epithelial cells challenged with *P.aeruginosa* LPS. Among 12 PRR molecules assessed, seven PRR genes that were relatively, abundantly expressed in the LPS-free ME epithelial cells were Tollip, Tlr4, Tlr5, Cd14, Lbp, Tlr9, and Tlr1 ($\Delta Ct < 25$ cycles). Interestingly, only 5 PRR molecules were significantly induced post LPS challenge; Cd14, Lbp, Tlr2, Tlr4, and Tlr9. Our results provide strong evidence that the ME epithelial cells express a cluster of PRR molecules that are selectively activated by *P.aeruginosa* LPS. The time course and intensity of these PRR activations in the LPS-challenged MEECs are independent of PRRs' basal expression levels.

Cd14, ranking at the 4th position based on its abundance in the MEECs, was the earliest and the most responsive molecule that was up-regulated in the LPS-stimulated MEECs. The protein encoded by the Cd14 gene is a surface antigen expressed on both myeloid (monocytes/macrophages) and non-myeloid cells (endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, spermatozoa and pancreatic islet β cells) [21]. CD14 (terms with capital letters indicating human genes or proteins in the text) is a single-copy gene encoding 2 protein isoforms: a 50- to 55-kD glycosylphosphatidylinositol-anchored membrane protein (mCD14) and a monocyte or liver-derived soluble serum protein (sCD14) that lacks the anchor [22]. Both cell bound and soluble forms of the CD14 protein bind LPS and participate in the LPS signaling [23]. Changes in CD14 expression and serum sCD14 levels are being associated with an increasing number of human diseases, including LPS associated shock, allergy and OM through gene-gene and/or gene-environment interactions [24-27]. Recent studies also showed that CD14 is a central PRR molecule in maintaining normal innate immunity and host defense by the recognition and clearance of 'non-self' PAMPs as well as unwanted 'self' components that are cell-damage released endogenous molecules named damage-associated molecular patterns (DAMPs) [28].

In this study, time-dependent Cd14 expression in LPS-challenged MEECs showed an early induction at 6 hrs and a peak at 24 hrs, suggesting that Cd14 may not only directly respond to the bacterial endotoxin (early recognition at 6 hrs) but also to the unwanted 'self' components, DAMPs (delayed recognition at 24 – 48 hrs). The involvement of the Cd14 in the recognition of apoptotic cells was demonstrated using an anti-CD14 mAb that inhibited the binding of apoptotic cells to macrophages [29]. The clearance of apoptotic cells is delayed in Cd14 null mice as compared with wild-type mice [30]. In addition, CD14 appears to recognize necrotic cells in addition to LPS and apoptotic cells[31], suggesting that CD14 might be a universal responder to PAMPs and DAMPs. Indeed, our study showed the consistent high levels of the Cd14 molecular activity in the MEECs during the course of the LPS-induced inflammation, suggesting that the Cd14 receptor is involved in the recognition of both PAMPs (LPS) and DAMPs (inflamed MEEC released endogenous molecules).

Our study demonstrated a co-coordinated and consistent up-regulation of both Cd14 and Lbp (in a ratio 4:1) in LPS-challenged MEECs, suggesting that the Cd14 and Lbp are synergistically involved in the recognition of the *P. aeruginosa* LPS. Both CD14 and LBP were involved in host recognition of LPS [32] and synergistically induced TNF production in monocytes exposed to polysaccharides derived from *P. aeruginosa*[33]. Previous studies also showed that LBP mediates the binding of minute amounts of LPS to mCD14 that, in turn, activates macrophages for limiting infection [34, 35]. Interestingly, it was reported that low concentrations of LBP enhance responses to LPS, whereas high LBP concentrations can inhibit LPS bioactivity *in vitro* and *in vivo*[23, 36]. The relatively consistent ratio between Cd14 and Lbp (4:1) in LPS-challenged MEECs suggests that the intensities of Lbp and Cd14 molecular activities are modulated by undefined mechanisms including LPS concentration, LPS-host interactions, host cell released DAMPs.

CD14 does not contain a cytoplasmic domain and, therefore may not be capable of initiating a trans-membrane signal in the absence of other PRRs, such as TLRs [24]. This requirement greatly expands the specificity of CD14 recognition of pathogen PAMPs by combinational engagements of multiple PRRs, thereby allowing for selective activation of different downstream defense pathways.

While most previous studies showed coordinated Cd14/Tlr4 activations during *P. aeruginosa* infections in pulmonary cells/tissues [37], Kupffer cells of liver [38], macrophages [39], and human corneal cells [40], our study revealed unique time-dependent and tissue-specific activation patterns of the Cd14, Tlr2 and Tlr4 in LPS-inflamed ME epithelial cells.

In this study, Tlr2 is expressed in the control MEECs with much lower abundance by comparison with that of the Tlr4 and Tlr9. However, Tlr2 was significantly induced, along with the Cd14, at 6 hrs in the MEECs post LPS stimulation. Our results suggest that Cd14/Tlr2 complex, not Cd14/Tlr4 complex, is involved in MEEC recognition of*P. aeruginosa* LPS. Interestingly, nontypeable *Haemophilus influenzae*-stimulated human ME epithelial cells activated Tlr2 and the associated signaling pathways [41]. Our findings and the previous study in ME epithelial cells suggest that Tlr2 is a ME PRR responding to Gram-negative pathogens and PAMPs.

Interestingly, the early induced Tlr2 expression decreased with time post LPS exposure, suggesting that Tlr2 may not be sensitive to LPS-induced DAMPs. However, the delayed up-regulations (at 24 – 48 hrs) of the Tlr4 was significantly different between control and LPS-treated ME epithelial cells, indicating that the Tlr4 may be a Cd14's partner participating in the recognition of the DAMPs released by LPS-damaged MEECs. Indeed, Tlr4 was found to partially mediate NF-kappaB activation in response to necrotic cell lysates [42]. Furthermore, Tlr4 antagonists were proposed as immunosuppressive strategies in organ transplantation [43]. Therefore, we postulate that Cd14/Tlr2 complex participates in the early recognition of *P. aeruginosa* released LPS (PAMP), whereas Cd14/Tlr4 complex engages in the late recognition of the cell-damage released endogenous molecules (DAMPs).

TLR9 detects bacterial DNA, mostly unmethylated CpG dinucleotides (CpG DNA), and elicits both innate and adaptive immunity [44, 45]. Recent evidence indicates that TLR9 is expressed in more diverse cell types than initially thought. In this study, the Tlr9 was expressed in the control MEECs and was induced during both early and late stages after *P. aeruginosa* LPS stimulation. Our findings suggest that the *P. aeruginosa* LPS used in this study may be contaminated by the *P. aeruginosa* DNA that is a key component of biofilms of this pathogen [46]. Indeed, using *P. aeruginosa* unmethylated CpG DNA as a proinflammatory stimulus, TLR9 was functionally activated in a human tracheal epithelial line [47].

As discussed above, DNA from bacteria has stimulatory effects on mammalian immune cells, which depends on the presence of the unmethylated CpG dinucleotides in the bacterial DNA. In contrast, mammalian DNA has a low frequency of CpG dinucleotides, and these are mostly methylated; therefore, it was suggested that Tlr9 has a capability to discriminate microbial DNA from mammalian DNA [44]. Recently, the concept of the CpG motif specificity of the TLR9-mediated detection has been challenged. It was showed that a cationic antimicrobial peptide (CAMP) binds extracellular self-DNA fragments into aggregated particles that enter plasmacytoid dendritic cells and trigger robust type I interferon responses by activating endosomal TLR9 [48]. This process was linked to autoimmune diseases [49, 50]. Whether or not the later induction of the Tlr9 is linked to autoimmune diseases or immunodeficiency disorders associated recurrent OM[51, 52] is worth of investigation.

Conclusions. The gene expression levels of the innate PRRs in normal MEECs cannot predict the time course and intensity of the PRR molecular responses in LPS-challenged MEECs. The Cd14 is an early and the most responsive molecule in the LPS-inflamed MEECs, followed by Tlr2, Lbp, Tlr9 and Tlr4. The Cd14 receptor may be involved in the recognition of both *P.aeruginosa* LPS (PAMP) and LPS-induced MEEC damage released endogenous molecules (DAMPs). The consistent upregulation of the Lbp is in agreement with that of the Cd14, indicating a coordinate response of Cd14/Lbp for LPS binding. Furthermore, the Cd14/Tlr2 complex associated signaling pathways may be involved in the early stage of the MEEC responses to LPS while as the Cd14/Tlr4 pathway involved in the later stage of the ME epithelial cell damage. The Tlr9 in the MEECs may recognize and respond to both *P. aeruginosa* PAMPs, possibly unmethylated CpG DNA, and LPS-induced DAMPs, possibly CAMP-bound extracellular self-DNA fragments.

Future directions. Our study is the first step toward defining the properties of the innate immunity in the ME epithelial cells, deciphering the course of the bacterial OM, characterizing the mechanisms of PRR actions during pathogen-host interactions, and the discovery of novel pharmacologic approaches to modulation of appropriate immune responses during the initiation of the OM.

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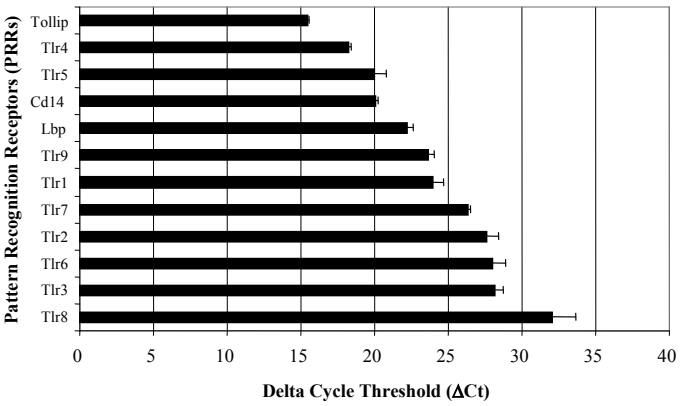

Fig. 1. Basal expression levels encoding PRRs in the control MEECs at 6 hrs. The ΔCt (r SD) represents the cycle threshold (Ct) of the target gene that was normalized to that of tl mouse 18S rRNA of the same specimen. A sample that contains more copies of a gene transcript has lower Ct because the cDNA copy number and Ct are inversely related.

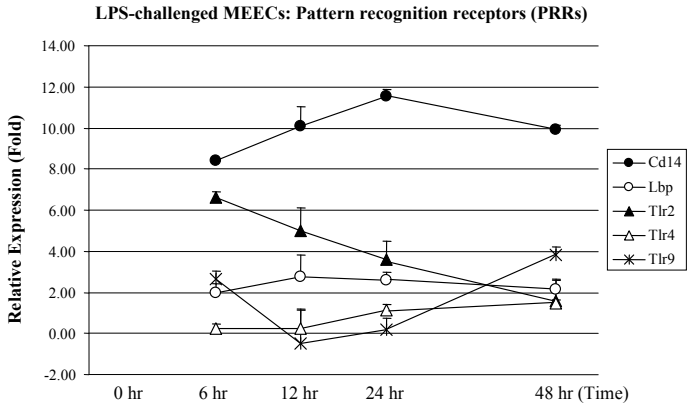

Fig. 2. Relative gene expression levels (in fold, mean ± SD) of the innate PRRs in mouse MEECs as a function of time. Data were normalized to the gene expression levels of the control group at the same time point (depicted as a line of 0 fold).

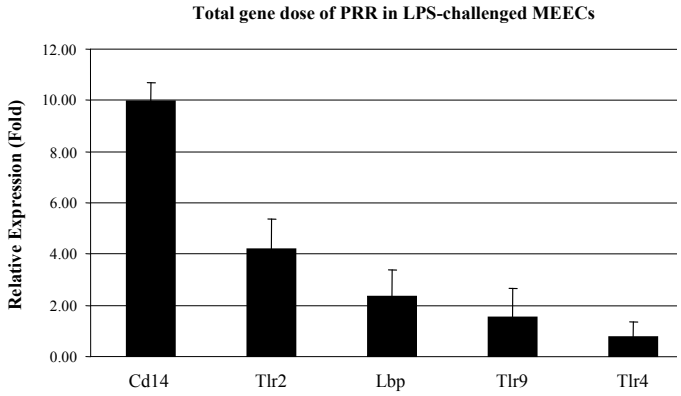

Fig. 3. A total gene expression levels (in fold, mean ± SD) across time points in *P. aeruginosa* LPS challenged MEECs. Data were normalized to gene expression levels of the control groups across time points (depicted as a line of 0 fold).

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Inflammation-Associated Molecular Patterns in Mouse Middle Ear Epithelial Cells Exposed to Pseudomonas Aeruginosa Lipopolysaccharide

Allison Cullen-Doyle[1], **Ha-Sheng Li-Korotky, PhD, MD**[2]
[1]Pediatric Otolaryngology, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]Pediatric Otolaryngology, University of Pittsburgh, Pittsburgh, PA

Introduction

Pseudomonas aeruginosa, a human opportunistic bacterial pathogen, is frequently isolated from patients with chronic middle ear (ME) diseases [1], including chronic suppurative otitis media (CSOM) [2-4], cholesteatomatous otitis media (OM) [5], and mastoiditis [6]. *P.aeruginosa* is one of the most common bacterial isolates in infectious otitis externa and the associated purulent otorrhea [7, 8]. Furthermore, *P.aeruginosa* is a major biofilm-forming pathogen, leading to a high rate of persistent otorrhea after insertion of tympanostomy tubes or other ME prostheses [1, 9, 10]. *P.aeruginosa* is also responsible for complications of acute OM in children [11, 12]. With increasing antimicrobial resistant *P.aeruginosa* strains, the treatment of these diseases caused by *P. aeruginosa* is challenging and costly [6, 13, 14].

Inflammation of the middle ear mucosa (MEM) during bacterial infection is caused both by direct effects of the metabolic processes of live bacteria as well as by indirect effects mediated by the host responses to conserved molecules presented by live bacteria and/or toxic molecules released from killed bacteria, such as lipopolysaccharide (LPS). LPS released by *P.aeruginosa* is a key virulence factor triggering both innate and acquired host responses to infection [15]. LPS of *P.aeruginosa* was successfully used to induce otitis media with effusion (OME) and CSOM in animal models [16-18]. *P. aeruginosa* LPS-induced cell/organ specific expression patterns of PRRs and downstream molecular/cellular signaling pathways and associated pathological outcomes have been explored in lethal sepsis [19], pulmonary cystic fibrosis [20, 21], and corneal infection [22]. We recently identified a subset of innate PRRs that were selectively activated in mouse middle ear epithelial cells (MEECs) exposed to *P. aeruginosa* LPS. This study further characterized LPS-induced inflammation-associated molecular patterns (IAMPs) in MEECs.

The time-dependent expressions of IAMPs in mMEECs exposed to *P. aeruginosa* LPS revealed molecular cascades underlying both proinflammatory and anti-inflammatory responses in MEECs. We expect that the information gained from this study will help to develop disease-specific modulations of cytokine/chemokine activities and for immunotherapy.

Materials and Methods

Mouse middle ear epithelial cell culture. The mouse middle ear epithelial cell line (mMEEC) was previously established [23] and generously provided by Dr. Ji-Zhen Lin (Otitis Media Research Center, University of Minnesota School of Medicine).The mMEEC cells were seeded onto 12-well plates at a density of 3.5×10^5 cells/well in a humidified 10% CO_2 incubator at 33°C for proliferation and then transferred to a humidified 5% CO_2 incubator at 39°C for differentiation, in a mediumcomposed of Ham's F-12K media, sodium bicarbonate (7.5%), L-glutamine (200 mM), epidermal growth factor (10 ng/ml), insulin-transferrin-sodium selenite (10 µg/ml), glucose (2.7g/L), hydrocortisone (500 ng/ml), non-essential amino acids (100 mM), and 4% fetal bovine serum (FBS).

P. aeruginosa LPS challenge. The MEEC monolayers at 80–90% confluence were challenged with either *P. aeruginosa* LPS (100 µg/ml, Sigma L-9143) or a control medium (equal volume of PBS) for6, 12, 24, and 48 hrs.At the end of each time point, monolayers were washed 3 times and detached from the surface by adding 100 µl of 0.25% trypsin/0.02% EDTA. Cells were collected by centrifugation and stored at –80°C for gene expression assays. Quadruplet wells of MEECs were tested for each condition per each time point and experiments were repeated for 2 times.

Gene expression assay. Cell pellets were individually homogenized using a TissueLyzer (Qiagen). Total RNA was extracted with RNeasy Mini Kit (Qiagen) following manufacturer's instructions. After initial screening we focused on genes coding for inflammatory cytokines and signaling molecules. Gene-specific primers were designed using OLIGO *Primer Analysis Software (*v6.3, Molecular Biology Insights, Inc., Cascade, CO). Expression levels of the gene transcripts were quantified and analyzed using real-time polymerase chain reactions (PCR) and the Sequence Detection Software (Applied Biosystems) as previously described [24].

Statistical analyses. A 2 x 4 two-way between-condition analysis of variance (ANOVA) was performed on gene expression levels of the MEECs (dependent variable) as functions of independent variables (treatment and time) using PASW software package, Statistics 18 (SPSS Inc., Chicago, IL, USA). Post hoc analyses with Bonferroni adjustments were performed. The α level was set at 0.05.

Results

P. aeruginosa LPS induced MEEC-specific IAMPs. Among those, nitric oxide synthase 2 (Nos2) was significantly augmented in MEECs averaged across the time, followed by chemokine (C-X-C motif) ligand 2 (Cxcl2), tumor necrosis factor (Tnf), interleukin 6 (Il6), heme oxygenase 1 (Hmox1), interleukin 1 beta (Il1b), interferon alpha 1 (Ifna1) and interferon beta 1 (Ifnb1) (Fig. 1).

There was a significant difference on MEEC gene expression levels between control and LPS groups averaged across time points for Nos2, Cxcl2, IL6, Hmox1, and Il1b ($p \leq .032$). Gene expression levels in LPS-challenged MEECs were significantly higher than that of the control groups at all time points for Nos2 and Cxcl2 ($p < .001$). Time-course for these expressions was different among the IAMPs, early inductions at 6 hrs for Nos2, Cxcl2, Tnf, Il6, Hmox1, and Il1b ($p < .007$), and late up-regulations at 48 hrs for Ifna1 and Ifnb1 ($p < .05$). The early-induced genes Nos2, Cxcl2, Tnf, Hmox1 and Il1b remained elevated at 48 hrs (Figs. 2 and 3).

Discussion

Our results showed that mouse middle ear epithelial cells have capacities to produce Nos2, Hmox1, cytokines (Tnf, Il6, Il1b, Ifna1 and Ifnb1) and chemokine Cxcl2 post *P. aeruginosa* LPS challenge. Most IAMPs are acute-phase responding molecules against LPS, a pathogen-associated molecular pattern (PAMP). Sustained responses may indicate LPS-induced MEEC damage, damage-associated molecular patterns (DAMPs). Nos2 was significantly up-regulated across the time, with a peak at 6 hrs, suggesting that Nos2 has a strong response to PAMPs and remained that response to subsequent DAMPs released from LPS-challenged epithelial cells. Tnf also responds to both PAMPs and DAMPs. For sensing endogenous danger signals (DAMPs) at the later time points, the same remained true for Hmox1, Ifna1 and Ifnb1. Interestingly enough, a recent study suggested that

epithelial NOS2 is not responsible for epithelial barrier dysfunction during inflammation, but may contribute to restoration of epithelial integrity [25].
Although a multifunctional proinflammatory cytokine Tnf can induce apoptosis, it is a key regulator of mucin 1 (Muc1), an anti-inflammatory molecule, during airway *P. aeruginosa* infection [26]. In this study, Tnfr 1(-/-) mice failed to increase Muc1 levels after *P. aeruginosa* infection. Thus, the later induction of Tnf may mediate epithelial cell apoptosis as well as Muc1 production to protect ME epithelial cells. Clearly, Il6 is an early response cytokine and induced by LPS challenge. Cxcl2 is a small chemoattractant peptide for the recruitment of leukocytes to the site of inflammation. Indeed, Cxcl2 was upregulated in response to *P. aeruginosa* induced lung injury [27].
Hmox1 has functions regulating oxidative stress, apoptotic cell death and cytokine expression profiles by positive regulation of I-kappaB kinase/NF-kappaB cascade [28]. This enzyme has shown cytoprotective, antioxidant and anti-inflammatory properties, provides immunosuppression through its expression by regulatory T cells or antigen-presenting cells. Upregulation of the Hmox1 pathway has a significant protective effect against spontaneous or induced autoimmune diseases, and allergy [29].
Interestingly, Il1b, Ifna1 and Ifnb1 were not immediately responsive to *P. aeruginosa* LPS infection, whereas they showed later inductions post challenge. IL1B gene polymorphisms demonstrated a consistent association with the severity of cystic fibrosis lung disease [30], indicating that Il1b may have a protective role in normal lung function. Ifna1 and Ifnb1 are type I interferon that plays a critical role in the innate immunity against viral infection. In addition, the production of type I interferon may be associated with TLR9 signaling pathway by sensing the unmethylated CpG dinucleotides in the bacterial DNA [31]. Recently, the concept of the CpG motif specificity of the TLR9-mediated detection has been challenged. It was showed that a cationic antimicrobial peptide (CAMP) binds extracellular self-DNA fragments into aggregated particles that enter plasmacytoid dendritic cells and trigger robust type I interferon responses by activating endosomal TLR9 [32]. Therefore, the late induction of the type I interferon may be associated with DAMPs in LPS-challenged MEECs.

Conclusions The time-dependent expressions of IAMPs in MEECs exposed to *P. aeruginosa* LPS revealed molecular cascades underlying both proinflammatory and anti-inflammatory responses in MEECs. This information lays the foundation for the development of disease-specific modulations of cytokine/chemokine activities and for immunotherapy.
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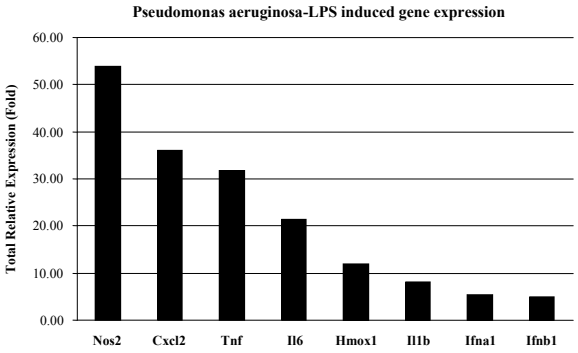

Fig. 1. A total gene expression levels (in fold, mean ± SD) across time points in *P. aeruginosa* LPS challenged MEECs. Data were normalized to gene expression levels of the control groups across time points (depicted as a line of 0 fold).

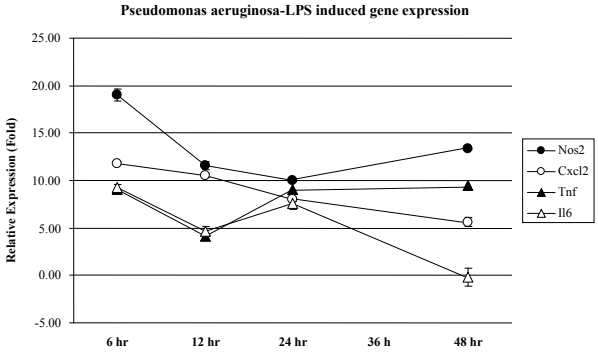

Fig. 2 Relative gene expression levels (in fold, mean ± SD) of the inflammation-associated molecular patterns in mouse MEECs as a function of time. Data were normalized to the gene expression levels of the control group at the same time point (depicted as a line of 0 fold).

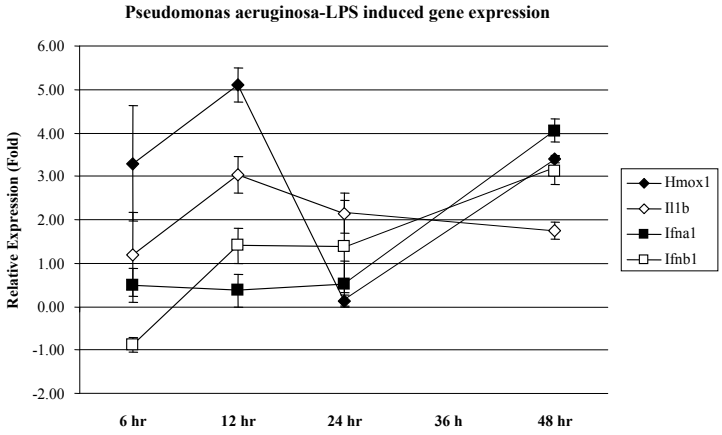

Fig. 3 Relative gene expression levels (in fold, mean ± SD) of the inflammation-associated molecular patterns in mouse MEECs as a function of time. Data were normalized to the gene expression levels of the control group at the same time point (depicted as a line of 0 fold).

Inhaled Nitrous Oxide Gas Exchange Method to Determine the Role of the Mastoid in Middle Ear Pressure Regulation

Cuneyt M. Alper, MD[1], Dennis J. Kitsko, DO[1], J. Douglas Swarts, PhD[1], Brian Martin[2], Sancak Yuksel, MD[3], Richard J.M. Villardo, MD[1], William J. Doyle, PhD[1]

[1]Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA, [2]Division of Pediatric Dentistry, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Dental Medicine, Pittsburgh, PA, [3]Division of Pediatric Otolaryngology, Department of Otorhinolaryngology-Head &Neck Surgery, University of Texas Medical School at Houston, TX ,

Introduction

The maintenance of the equilibrium of the middle ear pressure (MEP) gradient in relation to the environment entails a homeostatic balance between various anatomic and physiologic factors. The ME can be anatomically and functionally subdivided into two communicating airspaces, the anterior tympanum and the posterior mastoid air-cell system (MACS). The tympanum is primarily involved in hearing; while the MACS is a multiple-partitioned, cellular, air space that increases ME volume and surface area, but does not participate directly in sound transduction, and whose ultimate function is still unknown. It is currently hypothesized that it functions as a ME gas reserve such that MEs with larger MACS require less frequent ET openings to maintain near-ambient total pressure.[1] This mechanism requires that the rate of transMEM N2 exchange per ME volume is greater for the tympanum than for the MACS; and that the blood perfusion rate per surface area is less for the MACS when compared to the tympanum.

The maintenance of balance between the physiologic gases present in the ME space, specifically - O2, CO2, N2 and H2O, is paramount for a healthy ME. The ME exchanges gases with four compartments, the inner ear via the round window membrane, the ambient environment via the tympanic membrane,[2] the local blood via the ME mucosa (MEM),[3] and the nasopharynx via the

eustachian tube (ET).[4] The first three pathways are passive processes[5] ; whereas, the fourth pathway is an active, total pressure gradient-driven, bolus exchange of mixed gases in the air-phase.[4] Because the only substantial physiological gradient to drive transMEM gas exchange is an excessME N2 pressure relative to blood,[6] the rate of transMEM N2 exchange is the main determinant of the rate of total MEP decrease between ET openings.[3] Thus, an accurate estimate of the transMEM N2 exchange rate is fundamental to understanding the demand for gas placed upon the ET and to determining the efficiency of MEP-regulation under different extant conditions. Previously, a protocol for measuring the rate of blood to ME N2O exchange in anesthetized monkeys under well-controlled laboratory conditions was described,[7] and a formal mathematical analysis of the underlying physiology was developed that prescribes the requisite conditions for deriving time constants for transMEM N2O and N2 exchanges from the resulting data.[8] The transMEM time constant for a gas is defined as the rate of change in ME gas partial pressure divided by its transMEM partial-pressure gradient and is a normalized measure of the partial-pressure change at any specified gradient. This report describes the adaptation of those methods to study transMEM inert gas exchange in non-sedated human subjects, presents estimates for the N2O and N2 transMEM time constants, and tests the hypothesis that the MACS acts a gas reserve in healthy, adult human volunteers.

Methods and Materials

Study Population : The protocol was approved by the institutional review board at the University of Pittsburgh.

Healthy adult subjects were recruited, and informed consent was acquired for study participation. Volunteer subjects provided a short medical history; were given a standard ear, nose, and throat examination; had bilateral tympanometry; and women had a urine pregnancy test. *Exclusion criteria were:* pregnancy, chronic illnesses, who were currently taking prescription medications for any condition other than birth control, had extant unilateral or bilateral otitis media or low tympanic membrane compliance, or with a previous adverse reaction to breathing gas mixtures containing N2O were excluded.

Twenty-one subjects (age range, 20– 40 years; average age, 26.2.0 6 6.2 years; seven males) were enrolled. Twenty subjects had computed tomography (CT) done, but one of these did not complete the breathing experiment. Eighteen subjects completed the breathing experiments, but two did not have CT.

CT –Scan Study Protocols:

Twenty subjects with an expectedly wide range of MACS volumes based on the variability in their MACS surface are measured from a Schuller projection x-ray were selected for the study. Each had a CT scan of the ME in the transverse plane at a resolution of 0.31 mm/pixel and a slice thickness of 0.63 mm using a GE LightSpeed VCT system (GE Healthcare,Waukesha,WI). No ME pathologies were noted on the CT scans for any subject.

From each CT scan, a set of transverse images through the bilateral MACS regions at 0.25-cm intervals was selected for study. For the tympanum, every image was included in the reconstruction (i.e., interval ¼ 0.63 mm). Using Image J software (www.rsbweb.nih.gov/ij/), these sections were imported, and the left and right MACS and tympanums were identified, segmented out, and analyzed.

For each MACS and tympanum section, the perimeter and area of all air cells were highlighted, measured, summed across images, and multiplied by the section interval to yield MACS and tympanum surface areas (cm2) and volumes (mL).10 The average volume of the MACS for these 20 subjects was 5.24 6 4.76 mL (range, 0.65–21.4 mL) and of the tympanum was 0.67 6 0.12 mL (range, 0.48–0.96 mL).

Nitrous Oxide Inhalation Study Protocols:

Eighteen subjects had N2O breathing experiments done in the dental clinic at the Children's Hospital of Pittsburgh.

Subjects were seated comfortably in an exam chair that was reclined to approximately 30 to the horizontal and fitted with the finger probe of a pulse oximeter (Massimo RDS-1, Masimo Corporation, Irvine, CA) and with the upper arm cuff of an automated blood-pressure monitor (Critikon Dynamap 1846SX, GE Healthcare). A plastic anesthesia mask with a non-rebreathing valve (Breath Tech BT9005- Size 5 Adult, Cardinal Health,Dublin, OH) was placed over the nose and mouth.

The delivery `system supplying the mask was configured such that three gas sources could be placed online to the gas reservoir (breathing bag); room air, 100% O2, or a N20 gas mixture (20% O2, 25% N20, 75% N2). O2 and the N2O gas mixture were supplied as tank gas at certified composition (www.airgas.com) and were pressure regulated to near ambient at the gas reservoir.

The experiment consisted of three sequential phases:

1.) 20-minute acclimation period (period 1, room air breathing)
2.) 30-minute experimental period (period 2, gas mixture breathing),
3.) 30-minute recovery period (period 3, room air breathing).

At the completion of the experiment, all subjects breathed 100% O2 for a minimum of 5 minutes. Throughout the 80 minutes of the experiment represented by periods 1 to 3, bilateral MEP was recorded by tympanometry at 2-minute intervals. For safety, O2 saturation, heart rate, and blood pressure were checked at 5-minute intervals. At the end of the O2 breathing, the subjects were given a brief physical examination, and when recovered from the effects of breathing the N2O gas mixture were released from the study.

Statistical Methods

We tested the hypothesis that the MACS serves as a gas reserve by first calculating the slope of the function relating the time constant to ME volume using least-squares linear
regression.

Then, we evaluated the significance of this relationship under the hypothesis that the slope was negative and significantly different from a value of 0 (i.e., no relationship) using a 1-tailed Student t test.

The Pearson correlation coefficient was used to evaluate the relatedness of paired variables and the Student t test was used to evaluate the significance of differences for betweengroup comparisons and comparisons of the value of a variable with an expected value (e.g., 0). The standard descriptive statistics of average 6 standard deviation and range were used to summarize the data throughout.

Results

The results shows the MEP time function for the left and right ears of subjects 18 and 11 during the gas breathing experiment. For *the acclimation period* (0 to 20 minutes), MEP showed no directional change and varied about a mean value. In contrast, for *the experimental period* (20–50 minutes) MEP showed an increase interrupted by sharp decreases attributable to ET openings. Although the data for subject 18 did not evidence tubal openings until late in that period, those for subject 11 were characterized by a repeated series of such events. *The recovery period* was characterized by a variable response with a trend for all ears to decrease with and without ET openings. There was a sufficiently long period of linear increase to calculate the bilateral slopes of the MEP-time function for subject 18, but this was not true for subject 11. Of the 20 experiments, a linear period of increase characterized the data for 16 right and 11 left ears (10 bilateral) and the presentation below includes only those ears.

For those 27 ears, the average, standard deviations and range for the slopes of the MEP-time function, the fraction of the total variance in MEP explained by the regression on time (r2), and the time constant for N2O were 4.256±2.2 daPa/minute (range, 1.2–9.8 daPa/minute), 0.858±0.097 (range, 0.56–.096), and 0.002± 0.001 daPa/minute/daPa (range 0.001 to 0.005 daPa/minute/daPa), respectively (Table).

For the 10 subjects with bilateral data, the time constants for the left and right ears were significantly correlated (r2 ¼ 0.87, P < .001). Paired data for the time constant and ME volume were available for 23 ears.

Discussion

Controlling variability is the foundation of the validity of a scientific inquiry. This applies more so in this study as the various cause-and-effect relationship of the various ME gases with each other, and its surrounding anatomic structures such as the blood (intravascular compartment) and the ME space (MES), could not possibly be duplicated in toto in a controlled laboratory setting. Therefore we have to make an assumption that among the physiologic gases in the ME, N2, as represented by and its more tissue-soluble, perfusion-limited inert, isomeric gas - NO2,[5-7-9] could be used as a surrogate marker of the effect of the dominant transMEM gas exchange pathway in affecting pressure-gradient-driven, bolus exchange of mixed gases between the nasopharyngeal environment and the MES. This is based on the observations that: 1) the transMEM gas exchange pathway dominates the other passive pathways in effecting MEP change, [10] and 2) N2 is the only physiological ME gas not in equilibrium with the local blood.[6]

The results of the present study show that the N2O (and N2) time constant decreases curvilinearly with increasing ME (and MACS) volume to approach an asymptote at a ME volume of approximately 10 mL. Because the tympanum volume is relatively fixed and MACS volume was highly variable in this population, this effect can be attributed to the differences in MACS volume. As noted, this pattern is consistent with the hypothesis that the MACS functions as a ME gas reserve. Under those conditions, the frequency of effective ET openings required to prevent pathological underpressures is decreased, and ears with constitutionally moderate or even poor ET function are protected from the development of pathology

A limitation of the study is that only adults were included, although otitis media is a disease that primarily affects children in whom the ME geometry and MEP response to breathing gas mixtures has not been studied. This is a goal for future research. Nonetheless, the general protocol and model can be adapted to specific experimental conditions for purposes of testing predictions and/or hypotheses related to MEP-regulation. However, the model requires significant modifications when used to

characterize transMEM reactive gas exchange and is not useful for studying transMEM inert or reactive gas exchanges when new exchange compartments (e.g., a ME effusion) are introduced into the simple, two-compartment (blood, ME) model system.

Conclusions
The results show that inert gas time constants decrease with increasing ME volume, which supports the hypothesis that the MACS acts as a gas reserve for the ME. Thus, larger MACS volumes will partially protect the ME from the development of pathological underpressures by allowing for a decreased frequency of ET openings and/or less efficient ET openings when compared to smaller MAC volumes.

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TABLE:Results of all subjects

	AVG	STD	Min	Max
Slope	4.256	2.203	1.200	9.800
r2	0.858	0.097	0.560	0.960
N2O	0.002	0.001	0.001	0.005

Nitrous Oxide Gas Inhalation Anesthesia May Lead to Significant Middle Ear Pressure Changes and Risk for Post-Operative Otalgia and Otitis Media

Cuneyt M. Alper, MD[1], J. Douglas Swarts, PhD[1], Brian Martin[2], Julianne Banks[1], William J. Doyle, PhD[1]
[1]Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA, [2]Division of Pediatric Dentistry, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Dental Medicine, Pittsburgh, PA

Introduction
Nitrous oxide inhalation anesthesia is associated with increase in middle ear pressure (MEP).[1-5] There has been a number of reports on complications or sequalae related to the nitrous oxide anesthesia, including temporary or permanent hearing loss, hemotympanum, ossicular discontinuity, stapes disarticulation, tympanic membrane perforation, middle ear effusion, and displacement of tympanic membrane graft.[2,4,6,7]
During the nitrous oxide inhalation phase high MEPs have been reported.[8] On the other hand, after the termination of anesthesia during the elimination of nitrous oxide significant negative MEPs have been reported.[4,9-11]
Breathing N2O causes changes in MEP that can be accurately predicted.[12-15] These changes are due to diffusion of nitrous oxide gas in and out of the middle ear through middle ear and mastoid mucosa and is affected by the mucosal thickness, the surface area and the partial pressure gradient.
A study was conducted to determine the rate of MEP change with N2O gas inhalation in adult volunteers, and to make simulations on MEP increase during prolonged anesthesia and based on the predicted gas composition at the end of anesthesia, simulations on MEP decrease in children with poor Eustachian tube function.

Methods and Materials

The protocol was approved by the Institutional Review Board at the University of Pittsburgh. Healthy adult subjects were recruited by advertisement and all interested subjects signed an institutionally approved informed consent for study participation. Presenting subjects provided a short medical history; were given a standard Ear Nose and Throat examination; had bilateral tympanometry, and women had a urine pregnancy test. Pregnant women and persons presenting with chronic illnesses; or who were currently taking prescription medications for any condition other than birth control; or with extant unilateral or bilateral otitis media or low tympanic membrane compliance, or with a previous adverse reaction to breathing gas mixtures containing N2O were excluded. Twenty-one subjects (age range=20 to 40, average age=26.2.0$\pm$6.2 years; 7 Males) were enrolled.

Eighteen subjects had N2O breathing experiments done in the Dental Clinic at the Children's Hospital of Pittsburgh. Subjects were seated comfortably in an exam chair that was reclined to approximately 30 degrees to the horizontal and fitted with the finger probe of a pulse oximeter (Massimo RDS1) and with the upper arm cuff of an automated blood-pressure monitor (Critikon Dynamap 1846SX). A plastic anesthesia mask with a non-rebreathing valve (Breath Tech BT9005- Size 5 Adult) was placed over the nose and mouth. The delivery system supplying the mask was configured such that three gas sources could be placed "on-line" to the gas reservoir (breathing bag); room air, 100% O2 or a N20 gas mixture (20%O2, 25% N20, 75% N2). O2 and the N2O gas mixture were supplied as tank gas at certified composition (airgas.com) and were pressure regulated to near ambient at the gas reservoir.

The experiment consisted of 3 sequential phases: a 20-minute acclimation period (Period 1, room air breathing); a 30-minute experimental period (Period 2, gas mixture breathing), and a 30-minute recovery period (Period 3, room air breathing). At the completion of the experiment, all subjects breathed 100% O2 for a minimum of 5 minutes. Throughout the 80 minutes of the experiment represented by Periods 1-3, bilateral MEP was recorded by tympanometry at 2-minute intervals. For safety, O2 saturation, heart-rate and blood-pressure were checked at 5-minute intervals. At the end of the O2 breathing, the subjects were given a brief physical examination and, if recovered from the effects of breathing the N2O gas mixture, were released from the study.

The slopes of the MEP-time functions during N20 breathing were calculated to the first observation of Eustachian tube opening and divided by the estimated blood-ME N2O gradient to yield a N2O time-constant. Sufficient data were available for 16 right and 11 left MEs to calculate the time-constant.

Results

MEP did not change during the baseline period but, within 10 minutes of breathing the N2O mixture, showed a progressive increase. The right-left correlation for the time-constant was 0.87 (n=10 ears, p=0.001).

For the baseline period (0 to 20 min), MEP showed no directional change and varied about a mean value. In contrast, for the experimental period (20-50 minutes) MEP showed an increase interrupted by sharp decreases attributable to ET openings.

Period 3 was characterized by a variable response with a trend for all ears to decrease with and without ET openings. There was a sufficiently long period of linear increase to calculate the bilateral slopes of the MEP-time function for some subjects but this was not true for other subjects. Of the 20 experiments, a linear period of increase characterized the data for 16 right and 11 left ears (10 bilateral) and the presentation below includes only those ears.

For those 27 ears, the average, standard deviations and range for the slopes of the MEP-time function, the fraction of the total variance in MEP explained by the regression on time (r2), and the time-constant were 4.3$\pm$2.2 (range= 1.2 to 9.8) daPa/minute, 0.9$\pm$0.1 (range=0.6 to 1.0) and 0.0022$\pm$0.0011 (range 0.0006 to 0.0050) daPa/min/daPa, respectively. For the 10 subjects with bilateral data, the time-constants for the left and right ears were significantly correlated (r=0.87, p<0.001).

Based on these calculations, computer simulations were made for MEP changes with 25% (Fig1) and 50% (Fig 2 and 3) N2O, and with 21% (Fig1 and 2) and 50% (Fig 3) O2 inhalation for 1 hour followed for total of 12 hours during which there was no active opening of the Eustachian tube.

Discussion

Increasing MEP during surgical procedures where anesthesia is induced by inhalation of gas mixtures containing high concentrations of N2O (usually ≈ 50%) is a well recognized phenomenon.[12,16] Although less recognized, significant MEP decrease and resulting complications and sequalae have also been reported.[2,6,7,10,12] ME physiology and physical laws and limitations regarding trans-mucosal gas exchange help understanding these phenomenon.[17-18]

The efficiency of the ME transmission of tympanic membrane vibrations to the oval window depends on absence of pressure gradient between either side of the TM. The ME exchanges gases with four compartments, the inner ear via the round window membrane, the ambient environment via the tympanic membrane, the local blood via the ME mucosa and the nasopharynx via the Eustachian tube (ET). Gas exchange across the first three pathways is a passive, partial-pressure gradient-driven diffusive exchange across an inert barrier, while the fourth pathway is an active, total pressure gradient-driven, bolus exchange of mixed gases in the air-phase. Because of the physiological ME-local blood partial-pressure gradients, the net effect of the passive gas transfers is to deplete ME gas volume and progressively lower its pressure (ref. ambient), while that of transET gas transfer is to add sufficient gas volumes to rebalance ME and ambient pressures.

In order to demonstrate the effect of N2O inhalation anesthesia on MEP, computer simulations were conducted with a number of assumptions: 1) N2O time constant as measured in this dataset, other gasses calibrated using monkey data in ratios; 2) ET passively opens if/when MEP reaches +300 mmH2O 3) After opening ET passively closes when MEP drops to +100mmH2O 4) There is no gas mixing with ET opening (back flow) 5) Loss of ME gasses are proportional to each gases representation in the ME. 6) There is no active ET opening not only during the anesthesia but at the post-operative period simulating children with poor ET function.

Simulations demonstrated in Figures represent actual potential clinical scenarios of N2O inhalation at 25-50% concentration with 21-50% O2 and balance N2 (other anesthetic gasses are ignored for the sake of simplicity) for 1-4 hours. While the 25% N2O inhalation for 1 hour leads to only relatively minor decrease in MEP (Fig 1), prolonged inhalation of even 25% lead to significant negative MEP when ET does not open. This MEP drop is much more prominent with higher N2O concentrations (Fig 2) that is enhanced further with adding the 50% O2 (Fig 3).

While in adults, under normal conditions and normal ET function, this rate of MEP decrease can easily be compensated with more frequent and more effective ET openings, unless they have pre-existing ET dysfunction or a temporary condition like cold or allergies that have affected their ability to open ET. On the other hand, infants and children are known to have poor ET function, which get even worse with the frequent colds, making them much more prone to post-anesthesia MEP drop, and resulting otalgia, ME effusion, hemotympanum, hearing loss and even tympanic membrane perforation.

Conclusions

Adults with ET dysfunction especially when they have a cold and children with or without cold are at risk for post-N2O anesthesia complications and sequalae from significant negative MEP, especially after a prolonged anesthesia. Physicians should be aware of this risk, should inquire about the past history of OM, inform such patients with the risk, be aware of the post-operative symptoms including otalgia and hearing loss, and in patients with risk factors, consider avoiding anesthesia or use of N2O inhalation during anesthesia

Supported in part by NIH Grant DC007667

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Figure 1. Computer simulation for MEP changes with 25% N2O, and with 21% O2 inhalation for 1 hour followed for total of 12 hours during which there was no active opening of the Eustachian tube.

Figure 2. Computer simulation for MEP changes with 50% N2O, and with 21% O2 inhalation for 1 hour followed for total of 12 hours during which there was no active opening of the Eustachian tube.

Figure 3. Computer simulation for MEP changes with 50% N2O, and with 50% O2 inhalation for 1 hour followed for total of 12 hours during which there was no active opening of the Eustachian tube.

Figure 1.

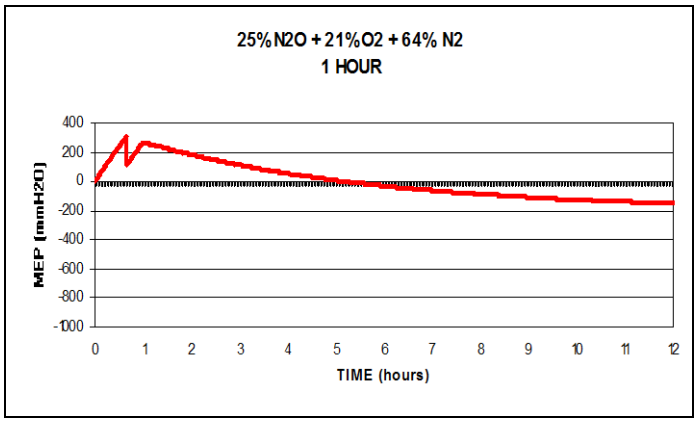


Figure 2

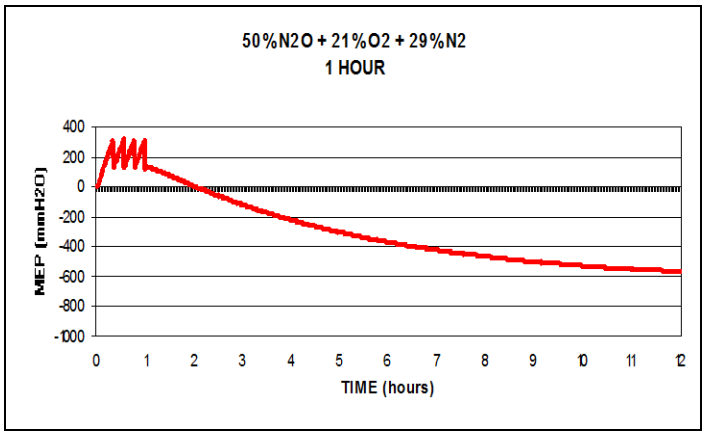
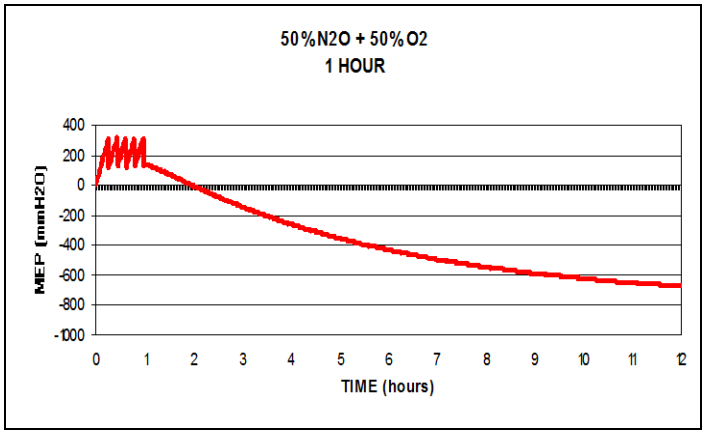


Figure 3



Expression of Inflammatory Mediators in the Middle Ear of Immunodeficient Mice with OME Induced by Eustachian Tube Obstruction

Patricia Hebda, PhD, Ha-Sheng Li-Korotky, MD, PhD**,** Mark Barsic**,** Chia-Yee Lo**,** Selma Cetin-Ferra, MD**,** Sancak Yuksel, MD**,** Joseph Dohar, MD
Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, Department of Otolaryngology, University of Pittsburgh School of Medicine

Introduction

Eustachian tube obstruction (ETO) induces middle ear (ME) pressure dysregulation reflected as under-pressures, causing increased vasculature permeability and ME effusion. This sterile ME effusion contains inflammatory mediators, which over time

may contribute to pathological changes in ME tissues. Our laboratory has shown that even in the absence of infection, inflammatory cytokines and chemokines are present in ME mucosa and effusions, although they are expressed over an extended period of time and at lower levels than have been observed in acute OM.[1] We postulate that this subtle, persistent inflammatory process contributes to the sequelae of OME, and should be targeted for therapeutic interventions to reduce the long-term adverse effects of OME on ME structure and function.

AIM

To use immunodeficient mice in our mouse model of OME, induced by ETO alone, to determine the contribution of host immune response to changes in structure and in expression of key inflammatory mediators in a mouse model of OME.

Methods

All work was reviewed and approved by the Institutional Animal Care and Use Committee. Unilateral ETO was induced by electrocautery of the Eustachian tube in wild type (WT) C57BL/6 and severe-combined immunodeficient (SCID) mice, as previously described.[2] Sham animals underwent the surgery to access the ME bulla and Eustachian tube with cautery to the adjacent tissues but without ETO. Weekly otoscopy assessed clinical indicators of OME.

Real-time PCR

Total RNA extraction. Middle ear tissue was individually homogenized using a TissueLyzer (Qiagen, Valencia, CA) and total RNA was extracted with RNeasy Mini Kit (Qiagen) following manufacturer's instructions. RNA quality and quantity were evaluated by gel electrophoresis on a 1.5% agarose/Tris-Borate-EDTA (TBE) gel via ethidium bromide staining and a 260/280 absorbance (ABS) ratio in a Beckman DU 600 Spectrophotometer (Beckman, Fullerton, CA, USA).

Reverse transcription and real-time PCR. Gene-specific primers (Table) were designed using OLIGO Primer Analysis Software *(*v6.3, Molecular Biology Insights, Inc., Cascade, CO) (Table). Expression levels of the gene transcripts were quantified using real-time PCR as previously described.[3] Briefly, the reverse transcription reaction was set up with 250ng of DNA-free total RNA, random primers, and SuperScript III (Invitrogen, Carlsbad, CA). The reaction was incubated at 25ºC for 10min, 50ºC for 50min, and 85ºC for 5min in a 2700 thermocycler (Applied Biosystems Inc., Foster City, CA). The real-time PCR reaction was set-up with SYBR Green PCR reagents (Applied Biosystems), including 5µl of 10x SYBR PCR Buffer, 6µl of 25mM MgCl2, 4µl of each dNTPs (blended with 2.5mM dATP, dGTP and dCTP, and 5mM dUTP), 2.5µl of each gene-specific primer (5µM), 0.5µl of AmpErase UNG (0.5U), 0.25µl of AmpliTaq Gold (1.25U) and 5µl of cDNA in a final volume of 50µl. The conditions for the real-time PCR were as follows: 50ºC for 2min, 95ºC for 12min, and 40 cycles at 95ºC for 15sec, and 60ºC for 1min in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems).

Results

Clinical and Histological Assessment

Following surgical creation of ETO in both wild type (WT) and SCID mice, otoscopy indicated retraction of the tympanic membrane and development of middle ear effusion, indicative of OME. (Figure 1) These changes were followed by weekly otoscopic examination and were found to be stable over the 4 week duration of the experiment. At the end of 4 weeks, the animals were euthanized and middle ear tissues were collected for analysis. Representative animals from each group were selected for histological evaluation. (Figure 2) In both the wild type and SCID mice, ETO ears presented with a middle ear effusion in the obstructed ear, whereas sham-operated control ears showed no effusion. In the WT-ETO ears, the ME effusion was serous, as expected, but the SCID-ETO ears there was a scant amount of cellular infiltrate, indicative of a more pronounced inflammatory response.

Data collection and normalization. The 7900 Sequence Detection Software (Applied Biosystems) was used for instrument control, automated data collection and data analysis. Relative quantification (fold difference) of the expression levels of each transcript was calculated using the 2-ΔΔCt method[4] with modifications[5].

Statistical analyses

A 2 x 3 two-way between-groups analysis of variance (ANOVA) was performed on gene expression levels of middle ear tissues (dependent variable) as a function of independent variables of the treatment (control, sham, and ETO) using PASW software package, Statistics 18, (SPSS Inc., Chicago, IL, USA). Post hoc analyses with Bonferroni adjustments were performed. The α level was set at 0.05.

A: Normal Otoscopy: **B: ETO Otoscopy:**

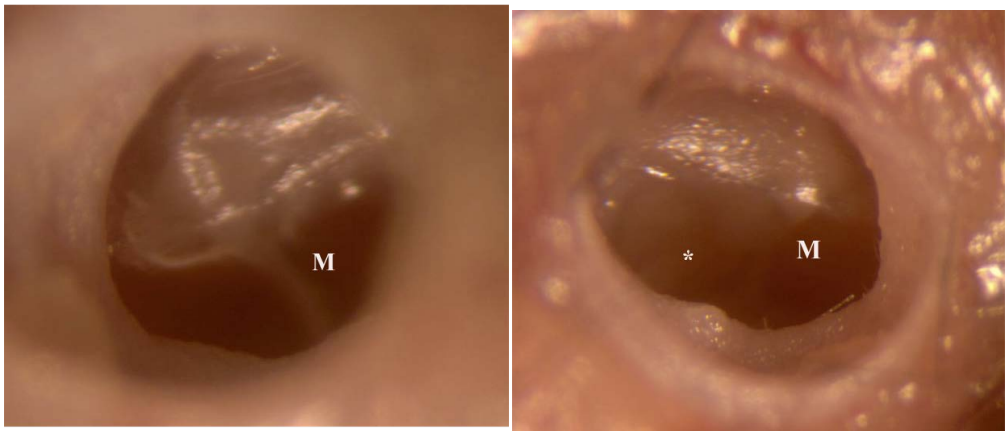


Figure 1. Otoscopy of the mouse ear before (Normal) and after ETO-induced OME.

Otoscopic examination of the mouse ears following Eustachian tube obstruction reveals hallmark changes associated with OME: retraction of the tympanic membrane and malleus (M) and development of middle ear effusion (*= ME effusion in the ETO ear). The effusion was serous with no indication of purulence or infection.

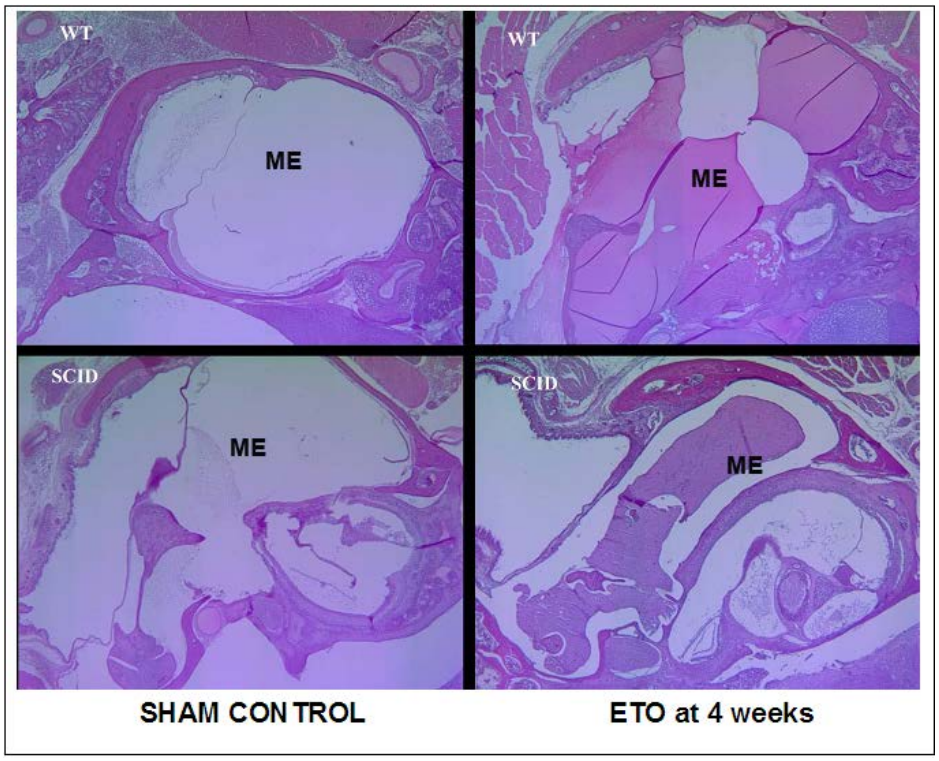


Figure 2

Table 1. Gene-specific primers for inflammatory markers

Gene	5'-Sequences	3'-Sequences
18S rRNA	AAG CCA TGC ATG TCT AAG TAC GCA	AAG TAG GAG AGG AGC GAG CGA CCA
Tnf	CGG TGC CTA TGT CTC AGC CTC TT	CAC TTG GTG GTT TGC TAC GAC GTG
Ifng	AGT CTC TTC TTG GAT ATC TGG AGG	GTG TGA TTC AAT GAC GCT TAT GTT
Il1b	GAA ATG CCA CCT TTT GAC AGT GAT	GCT CTT GTT GAT GTG CTG CTG TGA
Il6	CCT GGA GTA CAT GAA GAA CAA CTT	AGA GCA TTG GAA ATT GGG GTA GGA
Il10	CGG GAA GAC AAT AAC TGC ACC	AAC CCA AGT AAC CCT TAA AGT CCT
Tgfb1	GAA ACG GAA GCG CAT CGA AGC CAT	AGC ACG CGG GTG ACC TCT TTA GCA
Ccl2	TTA AGG CAT CAC AGT CCG AG	TGA ATG TGA AGT TGA CCC GT
Cxcl1	ATT TAA CGA TGT GGA TGC GTT TC	ACA CGA TCC CAG ACT CTC ATC TC
Ptgs2	GGG CAG GAA GTC TTT GGT CTG GTG	TTG AAG TGG TAA CCG CTC AGG TGT
Cxcl2	CAG TGA ACT GCG CTG TCA AT	TCT TTG GTT CTT CCG TTG AGG

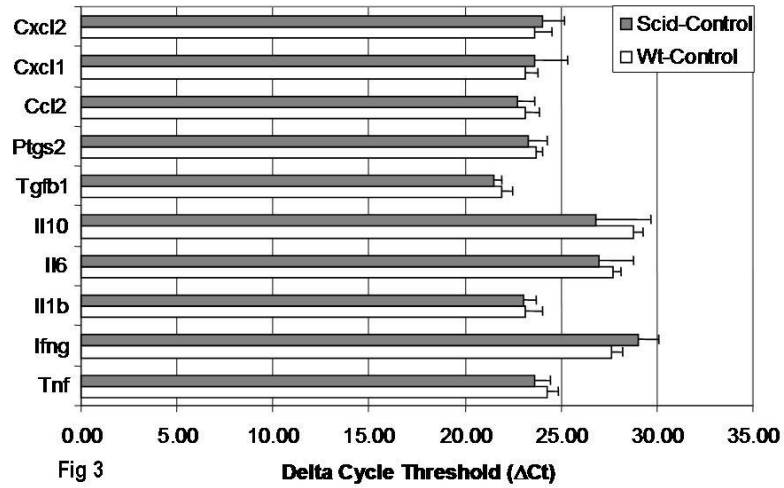


Figure 3. Baseline gene expression in control (normal) middle ears

Basal expression levels encoding inflammatory cytokines and modulators in control middle ear tissues. Significant genotype-specific difference in ME gene expression between Wt and Scid mice was observed for Ifng (Wt > Scid, p = .003, partial $\eta 2$ = .26),

The ΔCt (mean ± SD) represents the cycle threshold (Ct) of the target gene that was normalized to that of the mouse 18S rRNA of the same specimen. A sample that contains more copies of a gene transcript has lower Ct because the cDNA copy number and Ct are inversely related (fewer cycles of amplification are needed to reach threshold detection).

Real-time qRT-PCR

A panel of inflammatory markers were used to determine changes in gene expression in the middle ear mucosal tissues, listed in Table 1. All of these mediators showed similar baseline expression in both WT and SCID mice (Figure 3), with the exception of interferon-γ, which was showed slightly less baseline expression in SCID mice. Thus changes in gene expression for this panel should reflect changes attributable to the development of OME.

Interestingly, these changes (shown in Figure 4a-j) indicate that the SCID animals had a hyperinflammatory response with the development of OME. For each of the pro-inflammatory cytokines, TNFα, IFNγ, IL-1β, IL-6, and pro-inflammatotry mediator COX-2 (Ptgs2), there was a markedly greater increase in expression in the SCID animals. The chemokine CCL-2 (aka MCP-1) showed similar up-regulation in both mouse phenotypes; upregulation of chemokines CXCL-1 and CXCL-2 was much greater in SCIDs. The anti-inflammatory cytokine IL-10 showed less up-regulation in the SCID animals; TGFβ, also an anti-inflammatory mediator, showed a slight (< 2-fold) increase in both phenotypes.

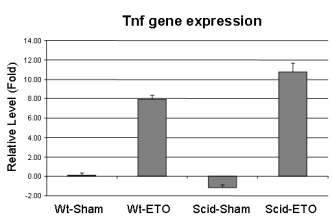


Fig 4a

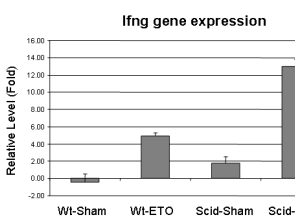


Fig 4b

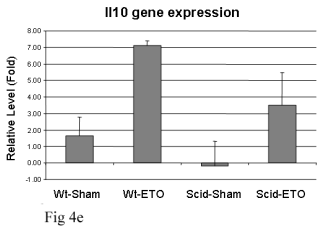


Fig 4e

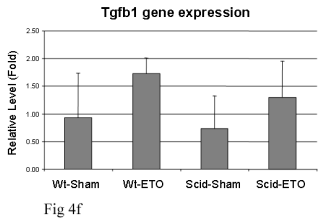


Fig 4f

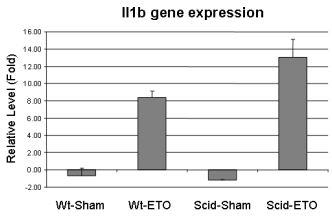


Fig 4c

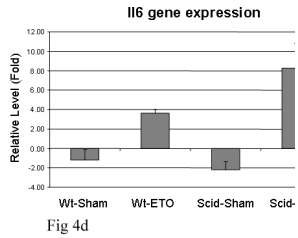


Fig 4d

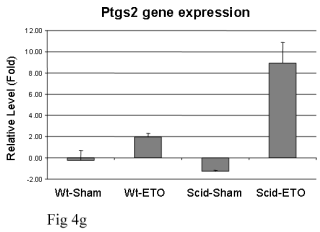


Fig 4g

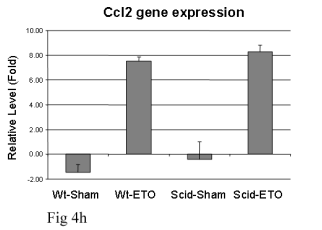


Fig 4h

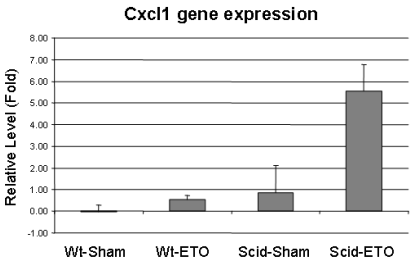


Fig 4i

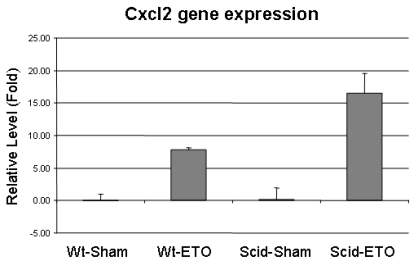


Fig 4j

Figures 4 a-j. Relative gene expression levels (in fold, mean ± SD) of the middle ear tissues as a function of genotype (Wt, Scid) and treatment (sham, ETO). Data were normalized to the expression levels of the control group (depicted as a line of 0 fold). In this form, higher values indicate greater gene expression.

Statistical Analysis

Fig 4a: The interaction effect between genotype and ETO on Tnf gene expression was significant ($p < .001$, partial $\eta 2 = .39$) and main effects of genotype and ETO on Tnf gene expression were significant as well ($p < .001$, partial $\eta 2 \geq .41$). Tnf expression levels in ME of the ETO groups were significantly higher than that of control and sham groups in both strains of mice 4 weeks after the operation ($p < .001$, partial $\eta 2 \geq .86$). Furthermore, ETO-induced Tnf expression in the ME was significantly higher in Scid mice than that in Wt mice ($p < .001$, partial $\eta 2 = .54$).

Fig 4b: The interaction effect between genotype and ETO on Ifng gene expression was significant ($p < .001$, partial $\eta 2 = .58$) and the main effect of ETO on Ifng gene expression was significant ($p < .001$, partial $\eta 2 = .89$). Ifng expression levels in ME of the ETO groups were significantly higher than that of control and sham groups in both strains of mice 4 weeks after the operation ($p < .001$, partial $\eta 2 \geq .59$). Furthermore, ETO-induced Ifng expression in the ME was significantly higher in Scid mice than that in Wt mice ($p < .001$, partial $\eta 2 = .51$).

Fig 4c: The interaction effect between genotype and ETO on Il1b gene expression was significant ($p = .012$, partial $\eta 2 = .26$) and main effects of genotype and ETO on Ill1b gene expression were significant as well ($p \leq .025$, partial $\eta 2 \geq .16$). Il1b expression levels in ME of the ETO groups were significantly higher than that of control and sham groups in both strains of mice 4 weeks after the operation ($p < .001$, partial $\eta 2 \geq .69$). Furthermore, ETO-induced Il1b expression in ME was significantly higher in Scid mice than that in Wt mice ($p < .001$, partial $\eta 2 = .35$).

Fig 4d: Main effects of genotype and ETO on Il6 gene expression were significant ($p \leq .012$, partial $\eta 2 \geq .19$). Il6 expression levels in ME of the ETO groups were significantly higher than that of control and sham groups in Scid mice ($p < .001$, partial $\eta 2 = .59$), and higher than that of sham group in Wt mice ($p = .018$, partial $\eta 2 = .24$). Furthermore, ETO-induced Il6 expression in ME was significantly higher in Scid mice than that in Wt mice ($p = .001$, partial $\eta 2 = .3$).

Fig 4e: For Il10 gene expression, only main effect of ETO was significant ($p = .001$, partial $\eta 2 = .38$). Il10 expression level in ME of Wt ETO group was significantly higher than that of control and sham groups ($p = .002$, partial $\eta 2 = .34$). Furthermore, ETO-induced Il10 expression in ME was significantly higher in Wt mice than that in Scid mice ($p = .041$, partial $\eta 2 = .13$).

Fig 4f: Only main effect of ETO on Tgfb1 gene expression was significant (p = .012, partial $\eta 2$ = .26). Tgfb1 expression level in ME of Wt ETO group was significantly higher than that of control group (p = .047, partial $\eta 2$ = .18).
Fig 4g: The interaction effect between genotype and ETO on Ptgs2 gene expression was significant (p< .001, partial $\eta 2$ = .49) and main effects of genotype and ETO on Ptgs2 gene expression were significant as well (p< .001, partial $\eta 2 \geq$ .38). Ptgs2 expression levels in ME of the ETO groups were significantly higher than that of control and sham groups in Scid mice 4 weeks after the operation (p< .001, partial $\eta 2$ = .76). Furthermore, ETO-induced Ptgs2 expression in ME was significantly higher in Scid mice than that in Wt mice (p< .001, partial $\eta 2$ = .61).
Fig 4h: Main effects of genotype and ETO on Ccl2 gene expression were significant ($p \leq$ .019, partial $\eta 2 \geq$ .17). Ccl2 expression levels in ME of the ETO groups were significantly higher than that of control and sham groups in both Wt and Scid mice (p< .001, partial $\eta 2$ = .77).
Fig 4i: The interaction effect between genotype and ETO on Cxcl1 gene expression was significant (p = .014, partial $\eta 2$ = .25) and the main effect of ETO on Cxcl1 gene expression was significant (p = .002, partial $\eta 2$ = .33). Cxcl1 expression levels in ME of the ETO groups were significantly higher than that of control and sham groups in Scid mice (p< .001, partial $\eta 2$ = .45). Furthermore, ETO-induced Cxcl1 expression in the ME was significantly higher in Scid mice than that in Wt mice (p = .002, partial $\eta 2$ = .27).
Fig 4j: The interaction effect between genotype and ETO on Cxcl2 gene expression was significant (p = .003, partial $\eta 2$ = .32) and main effects of genotype and ETO on Cxcl2 gene expression were significant as well ($p \leq$.046, partial $\eta 2 \geq$ .13). Cxcl2 expression levels in ME of the ETO groups were significantly higher than that of control and sham groups in both Wt and Scid mice 4 weeks after the operation (p< .001, partial $\eta 2 \geq$ .45). Furthermore, ETO-induced Cxcl2 expression in ME was significantly higher in Scid mice than that in Wt mice (p< .001, partial $\eta 2$ = .38).

Discussion

The ability to induce a similar OME following ETO in SCID vs WT mice indicates that B and T cells are not critical to the pathogenesis OME as reflected by our animal model, at least in the acute and early chronic phases up to 4 weeks. Therefore, other sentinel cells are playing a central role in this process.
Experimental OME in the SCID mice showed a distinct pattern of inflammatory gene expression compared with OME in WT mice. These results suggest that in the absence of intact immuno-regulation, the inflammatory response is compensatorily exaggerated, as indicated by the cellular infiltrate found in the ME effusion of the SCID-ETO mice and by the overall pattern of expression of pro- and anti-inflammatory mediators.
The fine balance between pro-inflammatory cytokines and immuno-suppressive cytokines promotes an efficient immune response without excessive damage. However, when stimuli continue, as in OME and the cells and ME mucosa are chronically activated, various processes of response to injury, healing and remodeling occur. There is evidence that the upregulation of pro-inflammatory cytokines and other inflammatory mediators is a key contributory factor in the development and persistence of OME. Therefore, a comprehensive analysis of this process may contribute to a better insight into the pathogenesis of OME.
Our group has previously reported on the histopathologic changes in this model of ETO-induced OME.[6] Over several weeks and months, there is thickening of the lamellar bone and mucosal lining of the ME bulla which may be exacerbated by the elevated inflammatory signaling cascade. The clinical relevance of this finding needs to be considered, and attempts made to control inflammation so as to minimize the adverse changes that may be occurring with persistent or recurrent OME.

Summary and Conclusion

Interestingly, there were notable differences in ETO-induced mediator expression in the SCID phenotype vs WT with greater induction of pro-inflammatory signals in the SCID ETO mice. The SCID mouse may be a valuable model to study ETO-associated immune modulation in OME; by looking at the host response in the absence of classic immuno-modulating cells, one can focus on the contribution of resident tissue cells to the inflammatory process and the morphological changes in the middle ear brought about by ETO.

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Nontypeable *Haemophilus influenzae* Promotes Pneumococcal Survival and Biofilm Formation Inhibiting Competence Development and Autolysis

Wenzhou Hong, PhD[1], Steve R. Taylor, MD[1], Christy Erbe[1], Pawjai Khampang[1], Joseph E Kerschner, MD[2]
[1]Otolaryngology, Medical College of Wisconsin, Milwaukee, WI, [2]Otolaryngology, Division of Pediatric Otolaryngology, Medical College of Wisconsin, Children's Hospital of Wisconsin, Milwaukee, Wisconsin

Otitis media (OM) is a most common childhood illness and caused by multiple microorganisms. Polymicrobial infection including *Streptococcus pneumoniae* (SP), nontypeable *Haemophilus influenzae* (NTHi) is involved in more than 70% of OM cases. Polymicrobial infection can promote pathogen survival and persistence *in vivo* and enhance bacterial resistance to host immune clearance and antibiotic treatment. There is much unknown about microbial interfere in polymicrobial infection and the impacts on disease outcomes. In present study, the impacts of NTHi on SP survival and biofilm formation in co-culture condition were investigated. The amounts of viable SP cells weren't significant different between SP growing alone and SP co-growing with NTHi at 16 hours after initiation of culture. But the amounts of viable SP cells as planktonic and biofilm were significant higher in co-culture with NTHi than SP alone at 24 hours after initiation of culture. Compared to SP culture alone, co-culture bacteria formed more complicated biofiilm structures visualized by confocal laser scanning microscope. Expressions of pneumococcal genes *cbpD* and*lytA*, which facilitate pneumococcal autolysis and fratricide, were detected by a quantitative PCR. The expression level of *lytA* was not significant difference at 16 hour time point but significant lower in SP co-cultured with NTHi than SP culture alone at 24 hour time point after initiation of culture. Expressions of pneumococcal *cbpD* gene in SP co-cultured with NTHi were significant lower than Sp culture alone at 16 and 24 hour time points after the initiation of culture. NTHi also inhibited expression and release of pneumococcal pneumolysin in co-culture condition. The results in this study suggested that NTHi may enhance SP survival and biofilm formation by inhibiting pneumococcal autolysis and fratricide by down-regulating pneumococcal cell wall hydrolase expressions.

Allergic Mastoiditis An Unrecoginized Entity

David Hurst, MD, PhD
Otolaryngology, Tufts University, Scarborough, ME

Objective
Assessment of 1) the atopic status of patients with intractable chronic effusion or drainage from their mastoid cavity and 2) the efficacy of allergy immunotherapy in maintaining patients free of mastoid disease.

Introduction
This previously unreported subgroup from a study of 101 patients with chronic OME. Allergy has not been recognized as an etiology for chronic drainage from mastoid cavities.

Study Design
Prospective, cohort study in a private community practice.

Methods
History, examination, audiogram, tympanometry and recurrence of effusion/infection among 7 adult patients, 6 male and 1 female, referred with previous mastoidectomy who experienced (1) effusion found to warrant myringotomy and ventilation tubes (M&T), or (2) a chronicly wet mastoid cavity in which the moisture, continued to drain through a perforation of the tympanic membrane or from the mastoid cavity itself despite various therapies. All were evaluated for allergy by intradermal skin testing according to AAOA criteria. A control cohort of 2 patients who refused therapy was included. Testing and immunotherapy targeted dust, pollen, and molds. Recurrence or persistence of drainage following 3 to 8 years of therapy was compared to patient's pretreatment status.

Results
All 7 patients were found to be atopic. All five treatment patients (six mastoids) responded to immunotherapy. Two controls failed.

Conclusion
This study, though too small to evaluate efficacy of immunotherapy, suggests that allergy is possibly an important, previously unrecognized etiology for the failure of mastoid cavities to remain free of effusion or drainage and may explain the chronicity of some patient's mastoid disease.

Activation of Epidermal Growth Factor Receptor Is Required for NTHi-Induced NF-κB-Dependent Inflammation in Otitis Media

Xiangbin Xu, PhD[1], Rachel R Steere[1], Christine A Fedorchuk[1], Jinjiang Pang[2], **Ji-Yun Lee**[1,3], Jae Hyang Lim[1,3], **Haidong Xu**[1,3], Jian-Dong Li[1,3]
[1]Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, [2]University of Rochester Medical Center, Aab Cardiovascular Research Institute, Rochester, NY, [3]Center for Inflammation, Immunity and Infection, Georgia State University, Atlanta, GA

Background
Inflammation is a hallmark of many important human diseases. Nontypeable Haemophilus influenzae (NTHi) is an important human pathogen causing respiratory tract infections in both adults and children. NTHi infections are characterized by inflammation, which is mainly mediated by nuclear transcription factor kappaB (NF-κB)-dependent production of proinflammatory mediators. Epidermal growth factor receptor (EGFR) has been shown to play important roles in regulating diverse biological processes, including cell growth, differentiation, apoptosis, adhesion, and migration. Its role in regulating NF-κB activation and inflammation, however, remains largely unknown.

Methodology/Principal Findings
In the present study, we demonstrate that EGFR plays a vital role in NTHi-induced NF-κB activation and the subsequent induction of proinflammatory mediators in human middle ear epithelial cells and other cell types. Importantly, we found that AG1478, a specific tyrosine kinase inhibitor of EGFR potently inhibited NTHi-induced inflammatory responses in the middle ears and lungs of mice. Moreover, we found that MKK3/6-p38 and PI3K/Akt signaling pathways are required for mediating EGFR-dependent NF-κB activation and inflammatory responses by NTHi.

Conclusions
Significance: Here, we provide important evidence that EGFR plays a critical role in mediating NTHi-induced NF-κB activation and inflammation in vitro and in vivo. Given that EGFR inhibitors have been approved in clinical use for the treatment of cancers, current studies will not only provide novel insights into the molecular mechanisms underlying the regulation of inflammation, but may also lead to the development of novel therapeutic strategies for the treatment of respiratory inflammatory diseases and other inflammatory diseases as well.

Use of Bacterial Load Measures to Investigate the Role of Alloiococcus Otitidis in Acute Otitis Media Affecting Indigenous Australian Children

Robyn Marsh, Jemima Beissbarth**, Michael Binks, Peter Christensen,** Peter Morris, MD, PhD**,** Amanda Leach, PhD**,** Heidi Smith-Vaughan, PhD
Child Health Division, Menzies School of Health Research, Institute of Advanced Studies, Charles Darwin University, Darwin, NT

Introduction
Otitis media is endemic in many Indigenous communities of the Northern Territory of Australia. *Alloiococcus otitidis* is an outer ear commensal and putative middle ear pathogen that has not previously been tested for in this population.

Objective
To determine if *A.otitidis* is present in Indigenous children with acute otitis media with perforation (AOMwiP).

Methods
Matched nasopharyngeal (NP) and ear discharge (ED) swabs from 31 children with AOMwiP were tested by qPCR for *A.otitidis* and total bacterial load. All *A.otitidis* PCR-positive samples were cultured using standard methods with incubation for 14 days.

Results
A.otitidis was detected in ED from 12/31 children (39%) but was not detected in NP swabs. It was cultured from 4/12 positive children. All *A.otitidis* PCR-positive samples were culture-positive for other bacteria.

A.otitidis bacterial load ranged from 10^4-10^8 cells/swab. In 4/12 children the *A.otitidis* load was 9-45% (median 24%) of total bacterial load, suggesting *A.otitidis* may be contributing to the pathology. In the remainder of children (8/12) the *A.otitidis* proportional load was <3% (range 0.02%-2.86%; median 0.83%).

Conclusion
A.otitidis can be a dominant species in ear discharge of Indigenous children with AOMwiP. Its absence in NP swabs suggests the ear canal as the likely primary reservoir. The significance of *A.otitidis* at low proportional loads is unclear. However, at high proportional loads *A.otitidis* is likely to be contributing to the pathology, possibly as a secondary pathogen.

High Detection Rates of Rhinovirus in the Middle Ear of Children with Recurrent Acute Otitis Media

Selma Wiertsema, PhD[1], Glenys Chidlow[3], Lea-Ann Kirkham, PhD[1], Eva Mowe[1], Karli Corscadden[1], Shyan Vijayasekaran, MD[2], Harvey Coates, MD[2], Gerald Harnett, PhD[3], Peter Richmond, MD[1]
[1]School of Paediatrics and Child Health, [2]Department of Otolaryngology, Head and Neck Surgery, University of Western Australia, Perth, Western Australia, [3]Molecular microbiology, PathWest Medicine WA, Perth, Western Australia

Objective
The importance of specific respiratory viruses in recurrent acute otitis media (rAOM) pathogenesis remains unclear. The aim of this study was to compare detection rates of common respiratory viruses in asymptomatic children with or without a history of rAOM and to investigate the presence of these viruses in the middle ear of children with rAOM.

Methods
Using multiplex real-time PCR we investigated asymptomatic viral infection of the nasopharynx with human rhinovirus, respiratory syncytial viruses, bocavirus, adenovirus, enterovirus, coronaviruses, influenza viruses, parainfluenza viruses, human metapneumovirus and polyomaviruses in children between 6 and 36 months of age either with (n=180) or without (n=66) a history of recurrent AOM. The presence of these viruses in 238 middle ear effusions collected from 143 children with rAOM was also investigated.

Results
Compared with healthy children rhinovirus (61.1% vs 42.4 %), bocavirus (52.2% vs 19.7%), parainfluenza virus (29.4% vs 9.1%), adenovirus (25.0% vs 6.1%), respiratory syncytial virus (27.8% vs 9.1%) and polyomavirus (36.1% vs 15.2%) were significantly more often detected in the nasopharynx of children with a history of rAOM. Rhinovirus was present in the middle ear of 46.2% of children with rAOM, whereas all other viruses were found in ¡Ü10%.

Conclusions
In comparison with healthy children asymptomatic carriage of respiratory viruses is significantly higher in children with rAOM. Rhinovirus is the predominant virus detected in the middle ear, suggesting that this virus may play a role in rAOM pathogenesis.

Acute Otitis Media Development after Upper Respiratory Tract Infection associated with Human Metapneumovirus: The role of Viral Load

Johanna Nokso-Koivisto, MD, PhD[1], Richard B. Pyles, PhD[2], Aaron L. Miller[1], Janak A. Patel, MD[1], Mike Loeffenholz, PhD[3], Tasnee Chonmaitree, MD[1,3]
[1]Pediatrics, [2]Microbiology and Immunology, [3]Pathology, University of Texas Medical Branch, Galveston, TX

Introduction
Human metapneumovirus (hMPV) is a respiratory virus which belongs to Paramyxoviridae family and to Pneumovirinae subfamily along with RSV. HMPV was detected the first time in 2001[1], and since then many studies have shown that hMPV is a common virus that infects almost all children by the age of 5 years, and can cause respiratory infections ranging from mild cold to severe lower respiratory tract infections in all age groups all over the world[2-9].
As hMPV is genetically related to RSV with similar clinical manifestations[1,3], it can be suspected that hMPV has the same tendency to induceacute otitis media (AOM) as RSV, which has been suggested to be an ototropic virus[10,11].However, the role of hMPV ininducing AOM in children requires further studies.During AOM, hMPV has been detected from 8% to 13% of NPS samples[12,13] and in 2.3% of middle ear fluids (MEF)[13].In a large cohort of children with respiratory symptoms hMPV was detected in 3.5%; overall 41%,and in children less that 3 years of age 61% of hMPV infections were complicated by AOM[5]. Accordingly,

in a retrospective study of 1532 children with URI, 5% of nasal wash specimens tested were positive for hMPV and almost half of these children were diagnosed with AOM[14].
The aim of this study was to compare the rate of AOM complicatingURI associated with hMPV to that associated with other respiratory viruses, and to determine if hMPV viral load is associated with URI outcome and AOM complication.

Methods
This was a prospective, longitudinal study of children to determine the incidence and characteristics of URI complicated by AOM. The study was performed from January 2003 through March 2007 at the University of Texas Medical Branch in Galveston, Texas, USA, and approved by the Institutional Review Board. The study protocol has been previously described [10]. In brief, healthy children were enrolled at the age of 6 months to 3 years into the study; they were followed for one year each for occurrences of URI and AOM. The parents informed the study personnel when the child developed URI symptoms (nasal congestion, rhinorrhea, cough and/or sore throat, with or without fever). Children were seen by a study physician as soon as possible, and followed for the occurrence of AOM. At each visit, otoscopic and physical examinations were performed, and tympanometric data were recorded. AOM complicating URI was considered when AOM occurred within 28 days of the onset of URI. AOM was defined as 1) acute onset of symptoms, 2) signs of tympanic membrane inflammation and 3) the presence of fluid in the middle ear as documented by pneumatic otoscopy and/or tympanometry.

Respiratory specimens for viral studies were collected at the initial URI visit and when AOM was diagnosed. Nasal swabs were collected for viral culture and nasopharyngeal secretions (NPS) were collected for other viral studies. NPSs collected during RSV season were also analyzed for RSV antigen detection by enzyme immunoassay (EIA). Culture and RSV-EIA –negative samples were analyzed by real time polymerase chain reaction (PCR) for adeno-, entero-, rhino- and corona-viruses (OC43, 229E and NL63) and by microarray PCR for RSV A and B, parainfluenzavirus types 1-3 and influenzavirus A and B performed at the Medical College of Wisconsin, Milwaukee, USA. Frozen archived NPS specimens were pulled for quantitative real-time polymerase chain reaction (qPCR) for hMPV, human bocavirus (hBoV), and RSV.

Results
A total of 700 archived NPS samples from 200 children were available for hMPV study. This accounts for 81% of 864 URI episodes which were followed by the study group and specimens were collected for viral studies. Demographic characteristics and risk factor data are presented in Table 1.

Respiratory viruses were detected in 534 (76%) NPS samples, and of these 303 (43%) episodescontained single virus only. HMPV was detected from 48 (7%) samples; in 25 (3.6%) hMPV was a single virus. In this study hBoV was the most common virus detected (24% of the samples), followed by adenovirus (23%), rhinovirus (20%), RSV (15%), and enterovirus (14%). HMPV was detected year-round except July, but highest incidence was from January to March (Figure 1).A total of 45 (23%) children had hMPV infections during the study period; 3 children had hMPV detected twice.
Overall, 37% of URI episodes were complicated by AOM. HMPV was detected alone in 25 episodes, and 6 of those episodes were complicated by AOM. Therefore, of URI associated with a single virus, the rate of AOM complicating URI was in hMPV – positive episodes was 24%, which was the lowest compared to other viruses. The rate of URIs complicated by AOM for other viruses was highest with hBoV, followed by adeno-, corona-, RS-, entero-, influenza-, parainfluenza and rhino-virus (55, 48, 46, 44, 32, 31, 27 and 27%, respectively).

Of 642 URI episodes available for viral load analysis, 47 URI NPSs were positive for hMPV. The median hMPV viral load was $3.2x10^7$ copies/ml of original volume; the mean $9.2x10^8$ copies/ml (range $2.7x10^4$ – $1.0x10^{10}$ copies/ml). HMPV viral load was significantly higher (median 4.4×10^8 copies/ml of original sample) in children with fever compared to those without (4.4×10^5) ($p=<0.001$). In children with AOM complicating URI, hMPV viral load was lower (median 3.8×10^6 copies/ml) than those without AOM (2.3×10^8), but the difference was not significant ($p=0.11$).

Conclusions
HMPV was detected in 48 (7%) URI episodes; 3.6% as the onlyvirus. The rate of AOM complicating hMPV-induced URI is 24%; thisis the lowest rate compared to that of other respiratory viruses. Viral load was associated with presence of fever, but not withAOM development.

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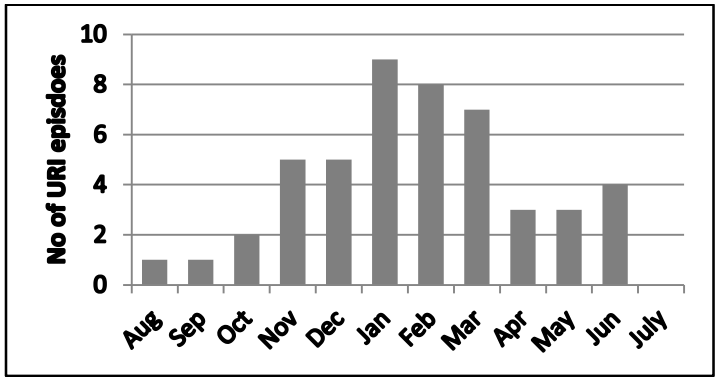
Table 1. Demographic and individual characteristics of 200 study children.

	Children (n=200)	%
Female	98	49
Median age at the visit (mos.)	18	
Race		
Asian	6	3
Black	56	28
Biracial	19	10
White	119	60
Ethnicity: Hispanic/Latino	89	45
Child care arrangement		
Home	131	66
Home day care	16	8
Day care center	52	26
Breast feeding (any)	105	53
Cigarette smoke exposure (none)	138	69
Otitis prone children	13	7
Family history of otitis media	89	45
History of prior otitis media episodes	112	56

Figure 1. Seasonal distribution of 47 upper respiratory tract infection (URI) episodes positive for human metapneumovirus (hMPV).



Minimal Biofilm Eradication Concentration of Antimicrobial Agents Against *Haemophilus influenzae* Isolated from Otitis Media

Shin Takei, MD, Muneki Hotomi, PhD, MD**,** Satomi Moriyama, PhD, MD**,** Masaki Hayashi, PhD, MD**,** Yorihiko Ikeda, PhD, MD**,** Shunji Tamagawa, MD**,** Noboru Yamanaka, PhD, MD
Department of Otolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama city, Wakayama

Introduction
Nontypable Haemophilus Influenza (NTHi), one of the leading causative pathogen for acute otitis media (AOM), has been reported to form biofilm. The antimicrobial susceptibility would be different between planktonic and biofilm-forming conditions. In this study, we evaluated the minimal inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) against NTHi.

Methods
The MIC and MBEC of cefems, penicillins, macrolides and quinolones were evaluated for NTHi isolates obtained from pediatric patients with AOM. The 96-pin replicator assay produces 96 equivalent biofilms for the antimicrobial susceptibilities. Susceptibilities were determined by microbroth dilution methods. We further evaluated the inhibition of biofilm formation by these antimicrobial reagents by scanning electron microscope (SEM).

Results
84 % clinical isolates of NTHi were biofilm-forming strains. The level of biofilm formation was significantly higher in NTHi isolated from AOM cases who did not improve with amoxicillin (AMPC) than NTHi isolated from AOM cases who improved with AMPC. The antibiotic sensitivities of planktonic organisms tested by the MIC assays were significantly higher than those of the same organisms in their biofilm state, as tested by the MBEC assays. The MBEC assay demonstrated that higher concentration of a certain antimicrobial agent to be effective for biofilms.

Conclusion
In the biofilm state, NTHi is much less susceptible to antimicrobial agents compared to the planktonic state. The current results suggest that production of biofilm by NTHi would be associated with clinical outcome of AOM. The MBEC is useful for the rational selection of antimicrobial agents against microbial biofilms and for the screening of new effective antimicrobial compounds.

Detection of Respiratory Virus in Pediatric Acute Otitis Media

Shunji Tamagawa, MD[1], Muneki Hotomi, MD, PhD[2], Levent B Beder, PhD, MD[2]**,** Masashi Ogami, PhD, MD[2]**,** Yuki Tatsumi[2]**,** Noboru Yamanaka, PhD, MD[2]
[1]Department of Otolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama, Wakayama ken, [2]Department of Otolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama

Introduction
Acute otitis media (AOM) is a leading infectious disease in childhood. Although AOM has been generally considered a bacterial infection and treated with antibacterial agents, recent studies have documented the close association between AOM and viral upper respiratory tract infection. In the pathogenesis of acute otitis media (AOM), contribution of viruses and their co-infection with bacterial agents has been emphasized recently based on several mechanisms. In this study, we investigated the prevalence of respiratory virus in children with AOM and further evaluated the virus prevalence together with clinical manifestations and bacterial findings.

Methods
We have conducted a prospective study organized by nationwide study group; Advanced Treatments for Otitis Media Study (ATOMS) consisted of 20 otolaryngology clinic since 2006. We employed RT-PCR to identify RS virus, influenza virus (type A and B), adenovirus, human boca virus (hBoV) and human metapneumovirus (hMPV) in nasopharyngeal swabs (NPS) and middle ear fluids (MEF) of AOM children.

Results
The respiratory viruses were identified in 47 cases (25.4 %) out of 185 patients. They were 20 (12.6 %) in MEF and 48 (27.2 %) in NPS. The pathogenic viruses were 2 (10.5 %) influenza virus, 5 (26.3 %) RS virus, and 12 (63.2 %) hMPV. hBoV was found in the nasopharyngeal aspirates of 14 children (6.3 %) and in the MEE of 6 children (2.7 %). The resolution time of AOM was significantly longer and rate of fever symptom was also higher in hBoV-positive group. Furthermore, we found positive correlation between detection of hBoV and Streptococcus pneumoniae in the MEF.

Conclusion
AOM, which is generally considered a bacterial disease, is more likely co-infection of virus and bacteria or the secondary bacterial infections after virus upper respiratory infections. However, a virus agent alone will cause signs and symptoms of AOM. A better understanding of the mechanism of viral and bacterial interaction in AOM will lead new strategies for more effective treatment.

Using Proteomics to Identify a Nontypeable *Haemophilus influenzae* Specific Signature in Infectious Diseases of the Upper Airway

Lucia Rosas[1], Subinoy Das, MD[2], Lauren Bakaletz, PhD[3]
[1]Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital & The Ohio State University College of Med, Columbus, Ohio, [2]Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital & The Ohio State University College of Med., Columbus, OH, [3]Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital & The Ohio State University College of Med, Columbus, OH

Otitis media, sinusitis, bronchitis, pharyngitis, and nonspecific upper respiratory tract infections (URTI) account for approximately 75% of outpatient antibiotic prescriptions in the USA. Antibiotic use remains high despite the fact that greater than 85% of these infections are due to viruses and resolve without complication. Nonetheless, those remaining infections that are indeed due to bacterial pathogens require more effective management than is currently available. Bacterial cultures provide limited diagnostic value because the most common bacteria responsible for URTI are also often commensal organisms in the nasopharynx. A diagnostic test that could discriminate between commensal and pathogenic bacteria would promote judicious use of antibiotic therapy.

We hypothesized that due to unique growth characteristics, bacterial biofilms secrete a distinct set of proteins into nasopharyngeal secretions that could be used to distinguish between commensal and pathogenic states. To investigate this, we cultured biofilms produced by nontypeable *Haemophilus influenzae* (NTHI) over 72 hours and performed two-dimensional nano-liquid chromatography mass spectrometry and MudPIT protein identification on supernatants. Several hundreds of proteins and their relative concentrations were determined. We selectively investigated the temporal characteristics of protein secretion in supernatants of NTHI biofilms and identified several attractive biomarker candidates. We are currently analyzing fluid obtained from the nasopharynx, mesotympanum, and sinus cavities of chinchillas experimentally infected with NTHI, as well as samples of nasopharyngeal lavage fluid of healthy individuals and patients with URTI complications. Collectively, our studies will support the development of a clinical diagnostic for early and rapid identification of NTHI-associated URTIs, leading to a more effective choice of treatment and improved outcomes. Supported by NIDCD/NIH R01DC05847-06S1 SD/LOB

The SapF ATPase Enhances Haemophilus Innate Immune Resistance, Nutrition Acquisition, and Biofilm Formation

Andrew Vogel, PhD[1], **Kevin Mason**[2]
[1]Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital, Ohio, replace, [2]School of Medicine, The Ohio State University, Columbus, Ohio

Commensal microorganisms, such as nontypeable Haemophilus influenzae (NTHI), have evolved several strategies to evade the innate immune response and colonize host tissues. As an opportunistic pathogen, NTHI causes upper airway infections and will exacerbate cystic fibrosis and chronic obstructive pulmonary disease. Previously, we demonstrated that the inner membrane Sap transporter (sapABCDFZ) confers resistance to antimicrobial peptides (APs) and uptake of essential nutrients. In addition, the SapD and SapF ATPases provide distinct functions that suggest unique biological roles for each. Herein, we demonstrate that SapF is essential for bacterial survival to high concentrations of human beta defensin-3. Further, the crystal violet retention assay indicated that biomass formation is enhanced on plastic surfaces in the presence of SapF. Additionally, SapF contributes to the density, height, and architecture of biofilm growth. We next determined the contribution of SapF during otitis media using a chinchilla model. SapF appears to be important in the initial colonization of the middle ear and nasopharynx, but may be dispensable in later stages of the disease. Collectively, these data highlight the important roles of SapF for proper biological function of NTHI related to growth, nutrient acquisition, and resistance to host immune components during disease. Further studies aim to understand SapF dependent substrate transport and protein-protein interactions which lead to NTHI pathogenicity and therefore can be targeted for effective treatment of Haemophilus diseases.

Upper Respiratory Tract Infections and Acute Otitis Media associated with Human Bocavirus

Johanna Nokso-Koivisto, MD, PhD[1], Richar Pyles, PhD[2], Aaron Miller[2], Janak Patel, MD[1], Michael Loeffelholz, PhD[3], Tasnee Chonmaitree, MD[1,3]
[1]Pediatrics, [2]Microbiology and Immunology, [3]Pathology, University of Texas Medical Branch, Galveston, TX

Introduction

Human bocavirus (hBoV) is a parvovirus that was first discovered in 2005[1]. Despite intensive research,the clinical significance of hBoV is still unclear. Based on the first studies of patients with upper or lower respiratory tract illnesses, hBoV was assumed to be a respiratory virus[1-4]. Acute otitis media (AOM) is one of the most common infections in children. AOM usually occursconcurrently withor after viral upper respiratory tract infection (URI).To date, the significance of hBoV in AOM pathogenesis is not well defined; children with hBoV detected at the time of respiratory infection have been diagnosed with AOM[2,5], and hBoV have been detected in 2.7% of middle ear fluids (MEF) in children with AOM[6].

We investigated the presence of hBoV in nasopharyngeal secretions (NPS) in healthy children younger than four years of age with URI. The purpose was to determine the relationship between hBoV in URI and AOM in young children, andto associate hBoV viral load with presence or absence ofAOM complication.

Methods

Study design and subjects

This was a prospective, longitudinal study of children to determine the incidence and characteristics of URI and its AOM complication. The study was performed from January 2003 through March 2007 at the University of Texas Medical Branch in Galveston, Texas, USA, and approved by the Institutional Review Board. The study protocol has been previously described[7]. In brief, healthy children were enrolled at the age of 6 months to 3 years into the study; they were followed for one year each for occurrences of URI and AOM. The parents informed the study personnel when the child developed URI symptoms. Children were seen by a study physician as soon as possible, and followedfor the occurrence of AOM. At each visit, otoscopic and physical examinations were performed, and tympanometric data were recorded.AOM complicating URI was considered when AOM occurred within 28 days of the onset of URI.AOM was defined as 1) acute onset of symptoms, 2) signs of tympanic membrane inflammation and 3) the presence of fluid in the middle ear as documented by pneumatic otoscopy and/or tympanometry.

Virologic studies

Respiratory specimens for viral studies were collected at the initial URI visit and when AOM was diagnosed. Nasal swabs were collected for viral culture and nasopharyngeal secretions(NPS) were collectedfor otherviral studies.NPSs collected during RSV season were also analyzed for RSV antigen detection by enzyme immunoassay (EIA). Culture and RSV-EIA –negative samples were analyzed by real time polymerase chain reaction (PCR) for adeno-, entero-, rhino- and corona-viruses (OC43, 229E and NL63) and bymicroarray PCR for RSV A and B, parainfluenzavirus 1-3 and influenzavirus A and Bperformed at the Medical College of Wisconsin, Milwaukee, USA.Frozen archived NPS specimens were pulled for quantitative real time polymerase chain reaction (qPCR) for hBoV, human metapneumovirus (hMPV) and RSV.

Results

Archived NPS specimens for hBoV studies were available from 707 URI episodes from 201 children; this accounts for 82% of 864 URI episodes which were followed by the study group and specimens were collected for viral studies in the original report[7].Demographic characteristics and risk factor data are presented in Table 1.

Using all methods described, viruses were detected in 542 (77%) NPS samples; of these 303 (43%)contained single virus only. HBoV was the most common virus detected in 172 (24%) episodes, followed by adenovirus, rhinovirus and RSV. In 44 (6%) URI episodes hBoV was the only respiratory virus detected. HBoV was detected year-round; 63% were detected between October and March.

Overall, 37% of URI episodes and 39% of virus-positive URI episodes were complicated by AOM. Of URI associated with the presence of a single virus, the rate of AOM complicating URI was highest in hBoV-positive episodes (52%), followed by adenovirus, coronavirus, RSV, enterovirus, influenzavirus, parainfluenzavirus, rhinovirus and hMPV (48, 45, 44, 32, 31, 27, 25 and 24%, respectively) (Figure 1). Two children had two AOM episodes associated with hBoV alone without any URI between.

In children with AOM complicating URI, hBoV viral load was lower (median 6.2 x 10^5 copies/ml of the original sample) than those without AOM (4.5 x 10^6), but the difference was not significant (p=0.62). In addition, the viral load did not differ whether hBoV was detected alone (9.5x10^5 copies) or concurrently with other viruses (3.8x10^6, p=0.16).

Of 94 (47%) children with hBoV infection,hBoV was detected more than once in 42 (45%) children. In 23 children, hBoV was detected twice; 10 children, three times; five children, four times; two children, five times; one child, six times; and one child had eight hBoV positive URI episodes during the study period.

Conclusion

In conclusion, hBoV was the most common virus detected (24%) alone or in combination with other respiratory viruses in children with URI. hBoV may have prolonged presence in the respiratory tract. Among cases of URI associated with presence of single virus, hBoV- associated URI had the highest rate of AOM complication. HBoV viral load in NPS was not associated with AOM risk. The results suggest an important role of hBoV in the pathogenesis of virus-induced AOM.

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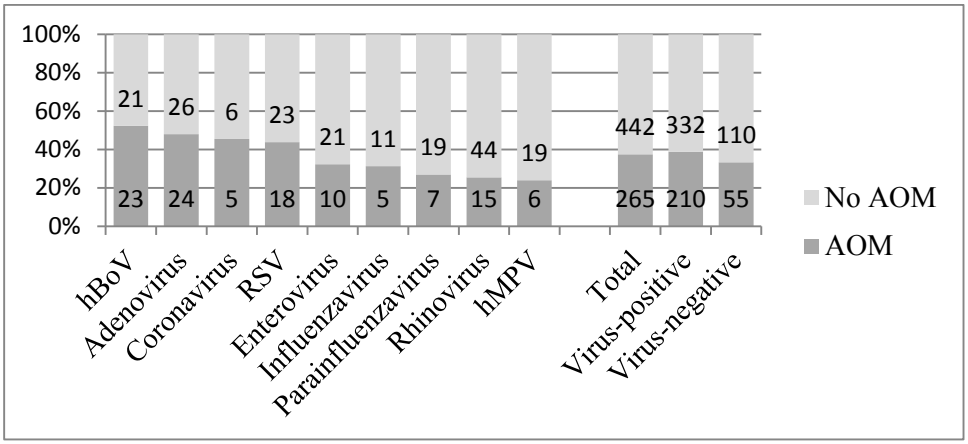
Table 1. Demographic and individual characteristics of 201 study children.

	Children with hBoVinfection[a] (n=94)	%	Children negative for hBov[a](n=107)	%
Female	45	48	54	51
Median age at the enrollment (mos.)	12		12	
No. of URI episodes /child year[b]	4.8		2.4	
No. of URI episodes with ≥2 viruses /child year[b]	2.1		0.4	
Race				
Asian	3	3	3	3
Black	28	30	29	27
Biracial	8	9	11	10
White	55	59	64	60
Ethnicity: Hispanic/Latino	37	39	52	49
Child care arrangement				
Home	60	64	72	67
Home day care	7	7	9	8
Day care center	26	28	26	24
Breast feeding[c]	50	53	55	51
Cigarette smoke exposure, none	60	64	78	73
History of prior otitis media episodes[b]	65	69	48	45

[a]One or more positive result(s) during one year study period.
[b]Significant difference between the groups, p<0.001.
[c]Any breast feeding irrespective of the duration

Figure 2. The proportion of acute otitis media (AOM) complicating upper respiratory infection (URI) associated with a single specific virus.



The numbers in columns represent the numbers of URI episodes in each group. Cases of simultaneous multiple viruses were excluded.

Treating Glue Ear Biofilms: An In-Vitro Model

Mat Daniel[1], Cheryl Rahman[2], Waheed Ashraf[3], Saif Al-Zahid[1], Jane McLaren[3], Helen Cox[2], Heather Fortnum[4], Neil Fergie[5], Kevin Shakesheff[2], John Birchall[1], Roger Bayston[3]

[1]Otorhinolaryngology Head & Neck Surgery, [2]Pharmacy, [3]Orthopaedic and Accident Surgery, [4]National Biomedical Research Unit in Hearing, The University of Nottingham, Nottingham, Notts, [5]Otorhinolaryngology Head & Neck Surgery, Nottingham University Hospital, Nottingham, Notts

Objectives

Glue ear (otitis media with effusion, OME) is caused by biofilms with reduced susceptibility to conventional antibiotic therapy. Treatment with grommets removes the effusion, but does not address underlying biofilm infection. As a quarter of children require further surgery, better treatments based on understanding of OME aetiopathogenesis are required. This project aimed to develop an *in-vitro* model of biofilm infection, and test effectiveness of antibiotics against biofilms.

Methods

Biofilms were established by incubating silicone discs in nutrient broth suspension of *Staphylococcus aureus* isolated from OME, and confirmed with Scanning Electron Microscopy and Confocal Laser Scanning Microscopy. Biofilms were exposed to rifampicin and clindamycin for different time periods and at various concentrations above minimum inhibitory concentration (MIC; minimum concentration that inhibits growth of the inoculum bacteria in their free planktonic state). Eradication was confirmed by removing the antibiotics and re-incubating the discs.

Results

Biofilms could be distinguished from planktonic bacteria by their reduced susceptibility to antibiotics. Eradication of biofilms required treatment with antibiotics lasting between 2-3 weeks, at antibiotic concentrations between 1,000xMIC and 10,000xMIC. Importantly, biofilms did not re-grow once antibiotics were discontinued.

Conclusion

Eradication of biofilms was possible, but required prolonged treatment with high antibiotic doses. Delivering high-dose antibiotics by controlled-release biodegradable polymers directly into the middle ear is now being explored as a potential novel strategy to combat OME.

Pneumococcal Surface Protein a (PspA) Family Distribution, Antimicrobial Resistance, and Serotype Composition of *Streptococcus pneumoniae* Isolated from Upper Respiratory Tract Infections in Japan

Masanobu Hiraoka, MD[1], **Yorihiko Ikeda, MD**[2], Muneki Hotomi, MD[2], Gen Sugita, MD[2], Atsuko Masuno, MD[2], Shin Takei, MD[2], **Masamitsu Kono, MD**[2], Levent Beder, MD[2], Akihisa Togawa, MD[2], Noboru Yamanaka, PhD[2]
[1]otolaryngology, Wakayama medical university, Wakayama, Wakayama, [2]otolaryngology, Wakayama medical university, wakayama city, wakayama

Introduction
The protection against pneumococcal infections provided by currently available pneumococcal polysaccharide conjugate vaccines are restricted to the limited number of the serotypes included in the vaccine. In the present study, we evaluated the distribution of the pneumococcal capsular type and surface protein A (PspA) family of pneumococcal isolates from upper respiratory tract infections in Japan.

Methods
A total of 251 S. pneumoniae isolates were examined in this study. Pneumococci were classified into three PspA families by polymerase chain reaction.

Results
In 251 pneumococci studied, the majority (51.0 %) was identified as belonging to PspA family 2, while most of the remaining isolates (43.8 %) belonged to family 1. There was no significant difference between the distributions of PspA1 versus PspA2 isolates based on the age or gender of the patient, source of the isolates or the isolates f susceptibilities to penicillin G. In contrast, the frequency of the mefA gene presence and of serotypes 19F, 23F and 15 were statistical more common among PspA2 strains.

Conclusion
The vast majority of pneumococci isolated from the middle ear fluid or nasopharynx represented PspA families 1 and 2. Capsular serotypes were generally not exclusively associated with certain PspA, although families of some capsular types showed a much higher proportion of PspA2 than the population of strains as a whole. A PspA-containing vaccine would potentially provide high coverage against pneumococcal infectious diseases because it would be cross-protective and immunogenic against the majority of pneumococci infecting both children and adults.

Phase Variation of *Streptococcus pneumoniae* simultaneously Isolated from Middle Ear Fluid and Nasopharynx of Children with Acute Otitis Media

Jun Arai, MD[1], Muneki Hotomi, MD, PhD[1], Masamitu Kono, MD[1], Masashi Ogami, MD, PhD[1], Yumi Ueno, MD, PhD[1], Susan Hollingshead, MD, PhD[2], David Briles, PhD[2], Noboru Yamanaka, MD, PhD[1]
[1]Otolaryngology-Head and Neck Surgery, Wakayama Medical University, wakayama-shi, wakayama, [2]2.Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama

Introduction
Streptococcus pneumoniae is a leading cause of acute otitis media (AOM). The pathogen undergoes spontaneous intra-strain phase variations in colony morphology depending on the capsular synthesis. Transparent variant is more efficient in colonizing the nasopharynx while the opaque variant exhibits greater virulence on systemic infections. However, there is little information about the pneumococcal phase variation in AOM. In this study, we evaluate the capsular phase variations of pneumococci simultaneously identified from middle ear and nasopharyngeal.

Methods
Both middle ear fluids and nasopharyngeal swabs were collected from pediatric patients with AOM before the antimicrobial treatment. The serially diluted specimens were plated on the Trypticase soy agar (TSA) plates with 6300 U/plate catalase. After the 16 h incubation at 37 degrees with 5% CO2 atmosphere, the pneumococcal phase variations were determined under the phase contrast microscopy.

Results
Eighteen pairs of middle ear isolates and nasopharyngeal isolates of *S. pneumoniae* were evaluated in this study. All of the middle ear isolates were classified into the opaque variant, while the predominant pneumococcal phase variation of nasopharyngeal isolates were classified into the transparent variant. The nasopharyngeal isolates showed mixed phase variation with transparent and opaque variant. We further quantitative evaluated the capsular polysaccharide phase variation of pneumococci.

Conclusion
The current results suggest that the opaque variant is more highly adapted to middle ear rather than the transparent variant. In contrast, vast majority of pneumococci in the nasopharynx showed transparent phase variant. The changes of pneumococcal phase in the nasopharynx or the middle ear fluids play a role in selecting the pneumococcal variant that can efficiently infect the middle ear mucosa.

Biodegradable Controlled-Release Antibiotic Middle Ear Implant for the Treatment of Otitis Media with Effusion

Rob Chessman[1], Mat Daniel[2], Cheryl Rahman[5], Waheed Ashraf[9], Saif Al-Zahid[3], Jane McLaren[4], Brian Richards[2], Helen Cox[5], Heather Fortnum[6], Neil Fergie[10], Kevin Shakesheff[7], John Birchall[10], Roger Bayston[8]
[1]Biomaterials-Related Infection Group, Orthopaedics and Accident Surgery, Queens Medical Centre, The Univeristy of Nottingham, Nottingham, [2]Biomaterials-Related Infection Group, Orthopaedics and Accident Surgery, [3]Biomaterials-Related Infection Group, Othopaedics and Accident Surgery, [4]Biomaterials-Related Infection Group,Orthopaedics and Accident Surgery, Queens Medical Centre, The University of Nottingham, Nottingham, [5]Centre for Biomolecular Sciences, [6]Otorhinolaryngology, [7]School of Pharmacy, [8]Orthopaedics and Accident Surgery, The University of Nottingham, Nottingham, [9]Biomaterials-Related Infection Group, Orthopaedics and Accident Surgery, [10]Otorhinolaryngology, Queens Medical Centre, Nottingham

Objectives
Bacterial biofilms, the causative agents of Otitis Media with Effusion (OME), cannot be eradicated unless exposed to high doses of antibiotics for prolonged time periods. This project aimed to develop a biodegradable implant suitable for use in the middle ear, locally releasing antibiotics (rifampicin and clindamycin chosen for their activity against OME pathogens, anti-biofilm properties and minimisation of resistance) for up to 21 days.

Method
The implant was based on the biodegradable polymer poly(lactic-co-glycolic acid) (PLGA, 50:50 ratio, 56kDa) produced using the emulsion method with mean particle size of 12µm. The carrier solution was varied (carboxymethyl cellulose (CMC) or Pluronic F127), as were the concentrations of antibiotics. Antibiotic release was assessed using Serial Plate Transfer Testing, with implants being transferred daily to plates that were freshly inoculated with *Staphylococcus aureus*, and the zones of inhibition of bacterial growth measured.

Results
All the implant formulations showed large zones of inhibition early on, followed by a large reduction in zone size over the first week. The zones then stabilised around day 10 and were still present by 21 days. Increasing the concentration of antibiotics gave larger zones of inhibition initially, but this was not prolonged throughout. Using Pluronic as a carrier appeared to result in larger zones than CMC throughout.

Conclusions
All the PLGA-based formulations showed a release of antibiotics over the desired 21 days. This modified-release antibiotic formulation for use in the middle ear as a treatment of biofilms appears to be a promising new strategy.

Biofilms Formed *in Vitro* by Bacterial Strains That Cause Otitis Media Are Eradicated by Treatment with Antibodies Directed Against Integration Host Factor Protein

Kyle Obergfell[1], Joseph Jurcisek[2], Steven Goodman, PhD[1], Lauren Bakaletz, PhD[2]
[1]Herman Ostrow School of Dentistry, University of Southern California, Los Angeles, CA, [2]Center for microbial pathogenesis, The Research institute at Nationwide children's Hospital & The Ohio State University College of Med, Columbus, Oh

Recently, the ability to form a biofilm in the middle ear has been identified as an important step in the disease progression of chronic otitis media (OM). The biofilm extracellular polymeric matrix (EPS) of many bacterial strains including the causative agents of OM nontypeable *Haemophilus influenzae* (NTHI), *Moraxella catarrhalis*, and *Streptococcus pneumoniae*, provides resistance to effective treatment. Extracellular DNA (eDNA) is an important component of biofilm matrices. We have shown that integration host factor protein (IHF), a nucleoid associated protein, bound to eDNA present within the biofilm matrix. We hypothesized that the binding of IHF to eDNA provides structural stability to the biofilm matrix and that removal of these proteins would destabilize the EPS. Herein, we have employed an *in vitro* biofilm assay to show that exposure to antibodies directed against IHF (anti-IHF) eradicated existing and robust biofilms formed by a wide-array of both Gram-positive and Gram-negative bacterial strains, including the causative agents of OM. Furthermore, NTHI biofilms previously resistant to antibiotic treatment showed an increased susceptibility to antibiotics when treated concurrently with anti-IHF.

A better understanding of the role of IHF in bacterial biofilm matrices will be important for developing novel therapeutics for OM and a multitude of other biofilm-dependent diseases.
This work was funded by discretionary funds to LOB

Pretreatment of Epithelial Cells With Poly (I:C) Enhances The Adherence of Streptococcus Pneumoniae

Masaki Kawabata, MD[1], Yuichi Kurono, MD, PhD[2]
[1]Otolaryngology, Head and Neck Surgery, Kagoshima University, Kagoshima, Kagoshima, [2]Otolaryngology, Head and Neck Surgery, Kagoshima University, Kgoshima, Kgoshima
Masaki Kawabata

Introduction
Viral upper respiratory tract infectins are often followed by secondary bacterial infections. *Streptococcus pneumoniae* (Spn)are commensal microoraganism and are alsomajor causative bacteria of acute otitis media (AOM) and paranasal sinusitis. However, the mechanisms how commensal bacteria can be a pathogen of those infectious diseases are not fully understood.Recently,several studies have revealed the potential for respiratory viral infections to enhance Spninfections in lower respiratory epithelial cells. Spn adheres to the airway epithelial cells and vascular endothelial cells via binding to receptor for platelet-activating factor (PAF-R) [1,].

Objectives
In the present study, we examined the impact of pretreatment with viral agent, Poly(I:C), in the adherence of Spn to human pharyngeal epithelial cells.

Material and Methods
Bacteria: Spn strain isolated from the nasopharynx of a patient with OME was used.

Cell Culture: Detroit 562 cells (ATCC CCL-138), a human pharyngeal carcinoma cell line, were used. To determine the effects of Poly (I:C) on Detroit 562 cells, Poly (I:C) were diluted in culture medium.

Adherence assays: Epithelial monolayers in 12-well plates were incubated for 48 hours with Poly(I:C). The cell monolayers were then washed with PBS. FITC-labeled Spn were added to the monolayers. After allowing for adherence at 37℃ for 60 min, the wells were washed with PBS. A PAF-R binding antagonist (ABT-491) was used at 1μM to treat confluent Detroit 562 cells for 60 min before and during adherence assays. The number of adherent FITC-labeled Spn was counted by using an immunofluorescence microscope.

Results
Pretreatment of Detroit 562 cells with Poly(I:C) enhanced the adherence of Spn. The PAF-R antagonist reduced the increased adherence of Spn that was induced by Poly (I:C) treatment.

Conclusions
Pretreatment with Poly (I:C) increased the expression of PAF receptor in Detroit 562 cells and enhanced the adherence of Spn.
These results suggest that increased adherence of Spn is mainly associated with the increase of PAF-R. The results suggest that viral infection affects the adherence of Spn to the epithelial cells. This might be one of the mechanismsunderlying the susceptibility to commensal bacterial infections.

Biofilm Formation on Coated Silicone Tympanostomy Tubes

Carol Ojano-Dirain, PhD[1], **Patrick Antonelli, MD**
Otolaryngology, University of Florida, Gainesville, FL

Introduction
Biofilm formation on tympanostomy tubes (TTs) may lead to refractory otorrhea and TT occlusion.[1-3]TT occlusion and recurrent post-TT otorrhea may require TT removal or replacement and cause patient suffering, inconvenience, and expense. TT composition and surface coatings have been shown to affect TT occlusion, and microbial adherence.[4-7] Polyvinylpyrrolidone (PVP) hydrogel coatings have been shown to reduce bacterial adherence, the first step in biofilm formation, by more than 90% over standard silicone TTs.[8] Silicone TTs coated with PVP are commercially available (Microgel®, Medtronic ENT, Jacksonville, FL). In a recent study, our laboratory showed that *Pseudomonas aeruginosa* biofilm formation on Microgel®TTs was significantly less than silicone TTs[9]. Other surface coatings such as silver oxide are also available. The objective of this study was to determine if PVP-coated or/and silver oxide-coated silicone TTs are less prone to biofilm formation by pathogens commonly found in post-TT otorrhea, *P. aeruginosa* and *S. aureus*, compared to standard silicone.

Materials and Methods

Silicone TTs with and without PVP (Microgel®) or/and silver oxide (Activent®) were dipped in 200 µL human plasma. Plasma exposure was done to mimic blood contamination that occurs in vivo which has been shown to promote biofilm formation[10]. TTs were aseptically transferred in each well of a 96-well microtiter plates filled with an early log phase PA (strain PAO1) or SA (ATCC strain 29213) in 200 µL of tryptic soy broth (TSB, MP Biomedicals, Solon, Ohio). After a 4-day culture period, gentamicin (Sigma, St. Louis, MO) or oxacillin (Fluka, Steinheim, Germany) over 100x the minimum inhibitory concentration were added to the media for 24 hours to eradicate planktonic (ie, non-biofilm) PA and SA, respectively.There were a total of 4 treatment groups per bacterial pathogen, with 22 TTs per group (20 TTs for quantitative bacterial counts and 2 TTs for SEM). Bacterial biofilmswere dispersed from the TTs by ultrasonication and quantitative bacterial counts were performed using standard microbiology techniques. Scanning electron microscopy (SEM) was performed on two TTs from each test condition to evaluate biofilm architecture. Bacterial counts were compared using one-way ANOVA followed by Student's t-test (JMP™ 7.0, SAS Institute Inc., Cary, NC). A statistical value of $p \leq 0.05$ was considered significant.

Results

PVP, silver oxide and PVP with silver oxide coatings reduced *P. aeruginosa* biofilm formation relative to silicone by over 1 log ($p<0.0001$) (Figure1). When only surface-coated TTs were compared, PVP was superior to silver ($p=0.04$) and PVP with silver ($p<0.0001$). Electron microscopy revealed *P. aeruginosa* bacteria in an extracellular matrix, typical of biofilms. Plain silicone TTs (Figure 2A) demonstrated the most robust *P. aeruginosa* biofilm. Consistent with the bacterial counts, fewer bacteria were seen with PVP (Figure 2B).

In contrast, PVP and PVP with silver coatings increased *S. aureus* biofilm formation nominally ($p=0.01$ & 0.003) (Figure 3). Very robust *S. aureus* biofilm was observed by electron microscopy after *S. aureus* culture such that there were no qualitative differences in *S. aureus* biofilm between the treatment groups (Figure 4, A to D).

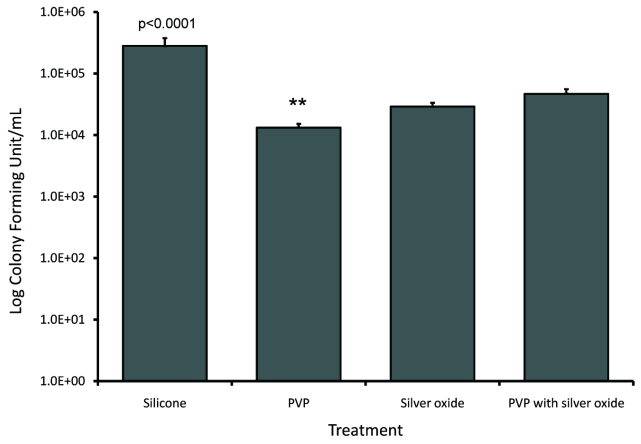


Figure 1. *P. aeruginosa*colony counts on silicone, PVP-, silver oxide-, and PVP with silver oxide-coated silicone TTs.*P. aeruginosa*counts on silicone TTs were over 1 log higher compared to all TTs with surface coatings ($p=0.0001$). **PVP was superior to silver ($p=0.04$) and PVP with silver ($p=0.001$.). Error bars represent standard error.

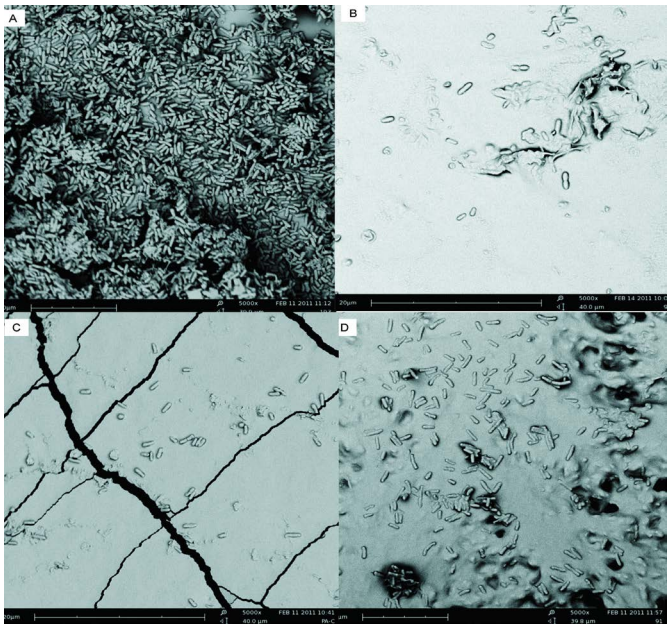


Figure 2. Scanning electron micrographs of silicone TTs (A) and silicone TTs coated with PVP (B), silver oxide (C), and PVP with silver oxide (D) after culture with *P. aeruginosa*.

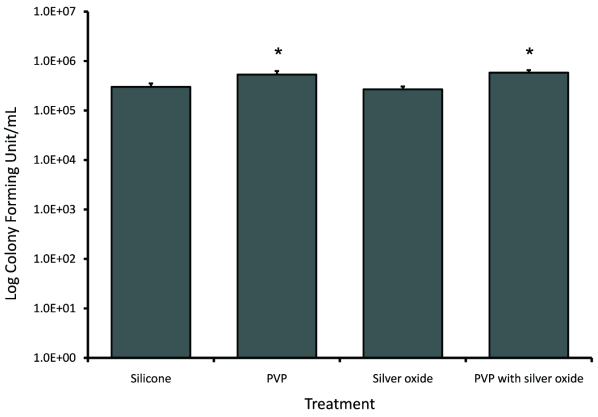


Figure 3. *S. aureus*colony counts on silicone, PVP-, silver oxide-, and PVP with silver oxide-coated silicone TTs. *PVP and PVP with silver coatings increased*S. aureus*biofilm formation nominally (p=0.01 & 0.003).Error bars represent standard error.

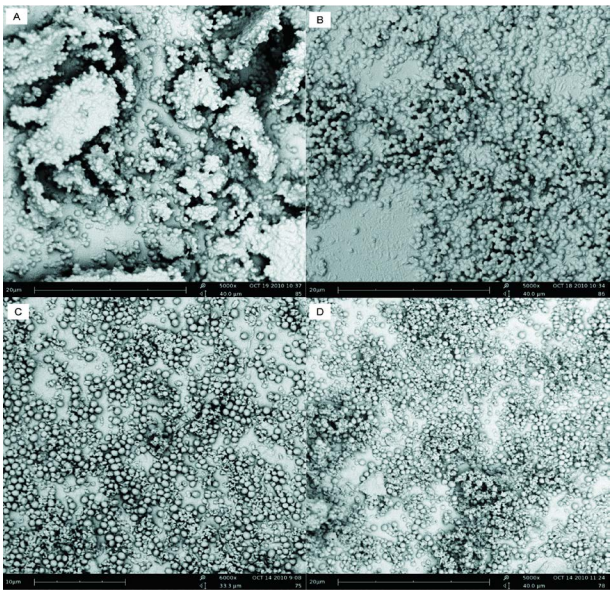


Figure 4. Scanning electron micrographs of silicone TTs (A) and silicone TTs coated with PVP (B), silver oxide (C), and PVP with silver oxide (D) after culture with S. *aureus*.

Conclusions

PVP and silver coatings reduce *P. aeruginosa* biofilm formation on silicone TTs. Combined PVP and silver coating does not improve biofilm resistance. TT surface coatings warrant further study through clinical trials.

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Bacterial and Viral Carriage in Australian Urban Children Undergoing Tympanostomy Tube Insertion

Rebecca Rocket[1], Theo Sloots, PhD[1], **Helen Massa, PhD[2],** Michael Nissen, MD[1], Chris Perry, MD[3], Allan Cripps, PhD[2]
[1]Queensland Paediatric Infectious Disease Laboratory, Sir Albert Sakzewski Virus Research Centre, Queensland Children's Medical Research Institute, Brisbane, Queensland, [2]Griffith Health Institute, Griffith University, Gold Coast, Queensland, [3]Children's Hospital Health Service, Royal Children's Hospital, Brisbane, Queensland

Objectives
This study aimed to examine bacterial and viral carriage in nasopharyngeal swabs (NPS) and middle ear fluid (MEF) and adenoid tissue (AD) from children undergoing tympanostomy tube insertion.

Methods
Samples were collected from urban children aged 2-7 years who underwent tympanostomy tube insertion. Samples were examined using PCR to identify *Streptococcus pneumoniae* (Pnc), non-typeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis* (Mc), Parainfluenza 2 & 3 (HPIV-2, HPIV-3), respiratory syncitial virus (RSV), human metapneumovirus (hMPV), rhinovirus (HRV), adenovirus (HAdV), influenza virus A & B and WU virus.

Results
NTHi was the predominant bacterium observed in both NPS (54%) and MEF (23%) from 13 patients. All three bacteria were present in NPS samples from 31% of patients and Pnc and Mc were shown in 38% and 31% patients.Most viruses were present in adenoids (79% of samples) compared to samples from NPS (54%) or MEF (35%). For all samples examined, HRV was the predominant virus observed (77%) compared to WU and hMPV (both 54%) and HAdV (38%). NPS samples showed WU and HRVequally frequent (23% patients) but WU always coincident with another virus in nasopharynx.

Conclusions
NTHi, HRV and WU virus frequently coinfect the upper respiratory tract and middle ears of urban children undergoing tympanostomy tube insertion for OM.

Monocyte Chemotactic Protein-1 Contributes to Middle Ear Inflammation in a Mouse Model of OME Induced by Eustachian Tube Obstruction

Patricia Hebda, PhD[1], Jennifer McLevy, MD[1], Ha-Sheng Li-Korotky, MD, PhD[1], Selma Cetin-Ferra, MD[2], Mark Barsic[2], Allison Cullen Doyle[1], Chia-Yee Lo[1], Sancak Yuksel, MD[1], Joseph Dohar, MD[1]
[1]Otolaryngology / Pediatric Division, [2]Otolaryngology/Pediatric Division, University of Pittsburgh, Pittsburgh, PA

Introduction
Eustachian tube obstruction (ETO) causes middle ear (ME) pressure dysregulation, which in turn leads to increased permeability of the mucosal vasculature and ME effusion, resulting in otitis media with effusion (OME). Our group previously reported ETO-associated histo-morphologic changes to ME structure.[1] However, the mechanism for transducing signals associated with underpressure and initiating ME mucosal inflammation is unknown. We postulate that this subtle, persistent inflammatory process contributes to the sequelae of OME, and should be targeted for therapeutic interventions to reduce long-term adverse effects of OME on ME structure and function.
Previous research into the role of the chemokine, monocyte chemotactic protein 1 (MCP-1), has lent support to the critical role of MCP-1 in monocyte recruitment during inflammatory responses.[2] MCP-1 is structurally related to the CXC subfamily of chemokines. MCP-1 induces chemotactic activity in monocytes and basophils but not in neutrophils or eosinophils. In addition it attracts memory T-cells and is involved in helper T-cell development.[2] It has been implicated in the pathogenesis of diseases characterized by monocytic infiltrates and global adaptive immunity. MCP-1 knockout mice, therefore, were selected for our study to further elucidate the inflammatory mediators and signaling pathways involved in ETO-induced OME.

Aims
The first aim was to assess the establishment of ME inflammation via our ETO model in MCP-1 deficient knock-out (KO) mice. The second aim was to assess the contribution of macrophage/ monocyte recruitment and activity, by comparing wild type and the MCP-1 KO mice, in our mouse model of ETO-induced OME.

Methods
Animal model of ETO (Fig 1)
All work was reviewed and approved by the Institutional Animal Care and Use Committee. Unilateral ETO was induced by electrocautery of the Eustachian tube in wild type (WT) C57BL/6 and MCP-1 KO mice, as previously described.[1] Briefly, the surgery began with a horizontal incision in the left cervical region, just posterior to the angle of the mandible. The incision was

carried through the skin, superficial fascia, platysma, and deep fascia. Using primarily blunt dissection, the triangle defined by the mandibular angle, the posterior belly of the digastric muscle, and the cervical great vessels were identified. Care was taken to identify and preserve all neurovascular structures visualized. Any bleeding was controlled with direct pressure and very focal electrocautery. Once the triangle was defined, blunt dissection was employed to identify the bony bulla. The blunt dissection was carried more anteriorly and inferiorly along the bulla until the bony Eustachian tube (ET) was identified and isolated. In the sham procedure, after ensuring hemostasis throughout, the incision was suture-closed with 4-0 nylon, and the mouse was allowed to recover from the anesthesia. In the ETO group, after left bony ET isolation, the bony ET was grasped with forceps. Electrocautery was applied to the forceps, producing the cauterized obstruction of the Eustachian tube. Weekly otoscopy assessed clinical indicators of OME. Starting on day 7, each mouse underwent bilateral otomicroscopy on a weekly basis to evaluate development of OME. Additionally, each mouse was weighed and monitored for signs of infection. After 4 weeks of ETO, animals were euthanized and samples taken for histology and molecular analysis.

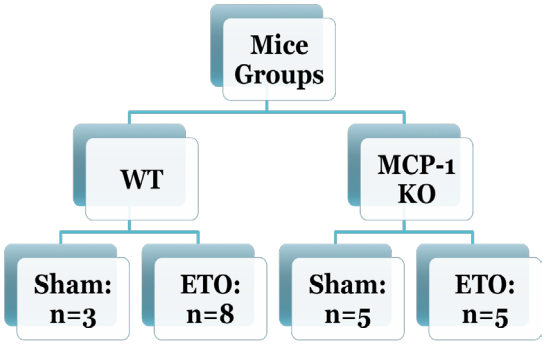

Figure 1. Experimental Groups

Histology

One representative specimen from each group was prepared for histological analysis. Specimen decalcification was achieved using 10% formic acid. The heads were bisected into anterior and posterior halves through the plane of the ME bulla and external auditory canal. They were then embedded in paraffin blocks, sliced coronally and stained with hematoxylin and eosin (H&E).[2] Sections were microscopically measured for thickness of the mucosa and bone at the hypotympanum using Metamorph computerized imaging software (Molecular Devices, Sunnydale, CA). Adjacent sections underwent immunohistochemical staining for macrophages using a monoclonal anti-macrophage antibody (RM0029-11H3, Abcam, Cambridge MA).

Real-time qRT-PCR

The mice were euthanized and the ME mucosa with bony bulla was collected and snap-frozen and total RNA was extracted in liquid nitrogen using TRIZol method as described previously.[3,4] Relative gene expression levels were analyzed using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) with gene-specific primers encoding inflammatory factors: tumor necrosis factor-α, (TNF-α), interleukin (IL)1-β, IL6, IL10, cyclooxygenase-2 (COX-2, gene name Ptgs2), and chemokine ligand-2 (Cxcl2). (Table 1) Relative quantification (fold difference) of the expression levels of each transcript was calculated using the 2-ΔΔCt method with modifications.[5,6]

Table 1. Primers for inflammatory markers used for RT-PCR analysis

Gene	5'-Sequences	3'-Sequences
18S rRNA	AAG CCA TGC ATG TCT AAG TAC GCA	AAG TAG GAG AGG AGC GAG CGA CCA
Tnf	CGG TGC CTA TGT CTC AGC CTC TT	CAC TTG GTG GTT TGC TAC GAC GTG
Ifng	AGT CTC TTC TTG GAT ATC TGG AGG	GTG TGA TTC AAT GAC GCT TAT GTT
Il1b	GAA ATG CCA CCT TTT GAC AGT GAT	GCT CTT GTT GAT GTG CTG CTG TGA
Il6	CCT GGA GTA CAT GAA GAA CAA CTT	AGA GCA TTG GAA ATT GGG GTA GGA
Il10	CGG GAA GAC AAT AAC TGC ACC	AAC CCA AGT AAC CCT TAA AGT CCT
Tgfb1	GAA ACG GAA GCG CAT CGA AGC CAT	AGC ACG CGG GTG ACC TCT TTA GCA
Ccl2	TTA AGG CAT CAC AGT CCG AG	TGA ATG TGA AGT TGA CCC GT
Cxcl1	ATT TAA CGA TGT GGA TGC GTT TC	ACA CGA TCC CAG ACT CTC ATC TC
Ptgs2	GGG CAG GAA GTC TTT GGT CTG GTG	TTG AAG TGG TAA CCG CTC AGG TGT
Cxcl2	CAG TGA ACT GCG CTG TCA AT	TCT TTG GTT CTT CCG TTG AGG

Results and Discussion

The first objective of the study was to assess whether the cautery ETO model could be recreated in the MCP1 KO mice. As noted in the weekly otomicroscopy (see Figure 2), 4 out of the 5 MCP1 KO-ETO mice developed MEE ipsilateral to the side of the

ETO; only 1 animal in each of the Sham cohorts presented with transient effusion (Figure 3). Both the otomicroscopy and the histological evaluation support the use of the electrocautery technique to create ETO and subsequent MEE in MCP1 deficient mice.

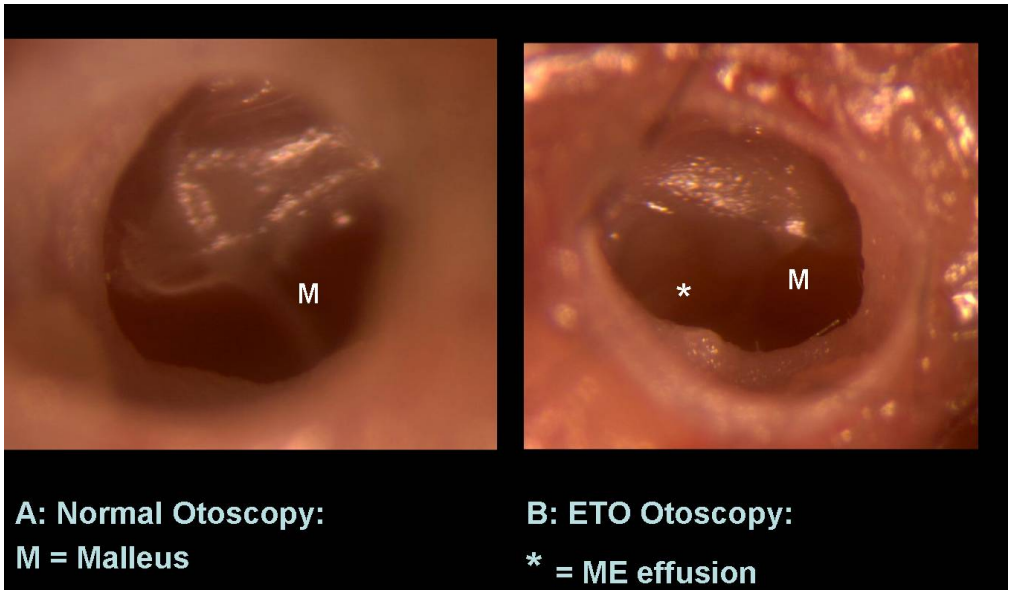

Figure 2. Otoscopy of the mouse ear before and after ETO-induced OME.

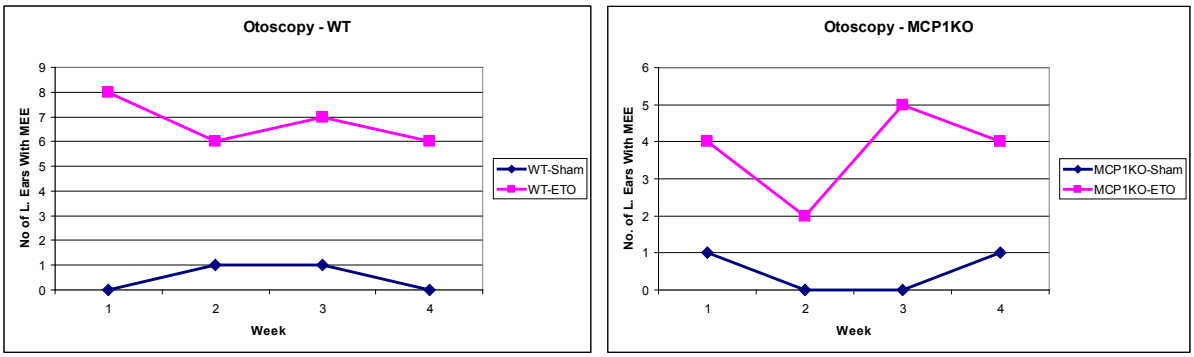

Figure 3. Number of animals in each group presenting with OME by weekly otoscopic examination

The second objective was to evaluate the role of MCP1 in the overall development of ME inflammation. We investigated the contribution of macrophage/monocyte recruitment and activity to ETO induced OME and subsequent sequelae by comparing the relative cytokine and chemokine production in the WT-ETO and MCP1 KO-ETO mice.

Figure 4 shows the normal histology of the transected middle ear (4a) and that same region following ETO with ME present in the lumen (4b). Immunohistochemical staining for macrophages (Figure 4b)revealed that while both WT and MCP-1 KO mice developed ME effusion, there was minimal presence of macrophages in the MCP-1 KO group, thus implicating MCP-1 as being responsible for macrophage recruitment in the pathogenesis of OME.

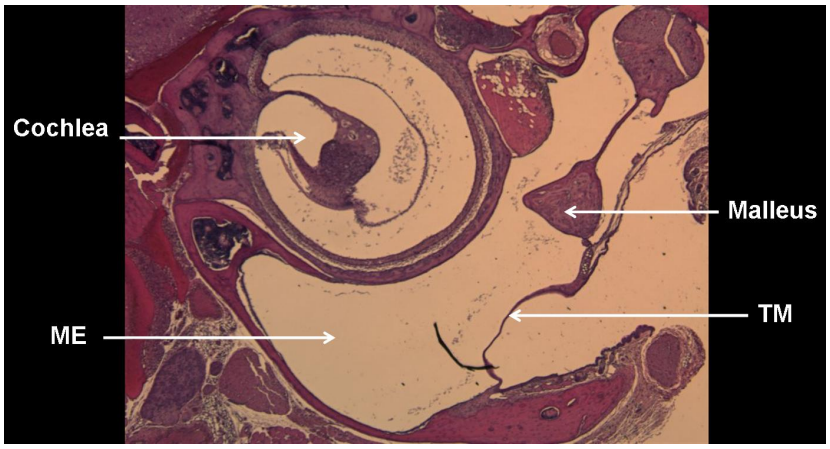

Figure 4a. Normal ME histology stained with hematoxylin & eosin (H&E).

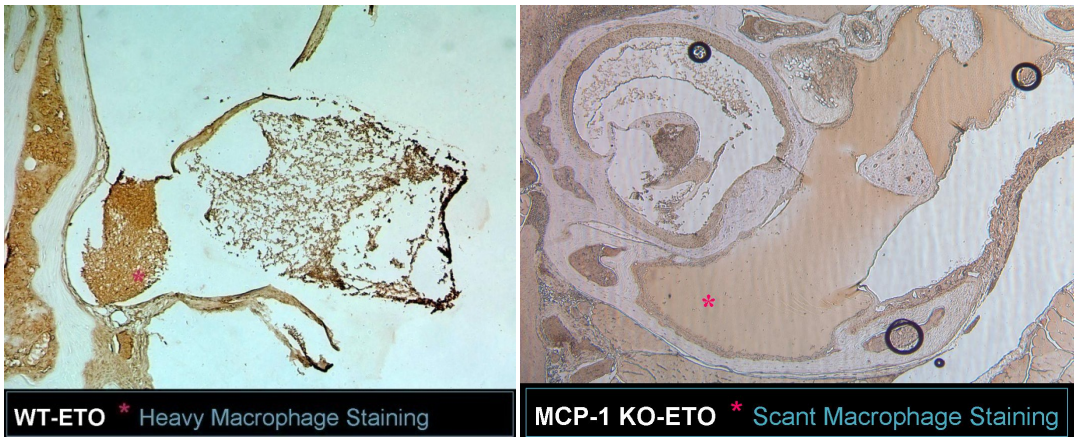

Figure 4b. Immunohistochemical staining for macrophages showed that while both WT and MCP-1 KO mice developed ME effusion, there was minimal presence of macrophages in the MCP-1 KO group, thus implicating MCP-1 in the pathogenesis of OME. See Figure 4a for normal histology of the middle ear.

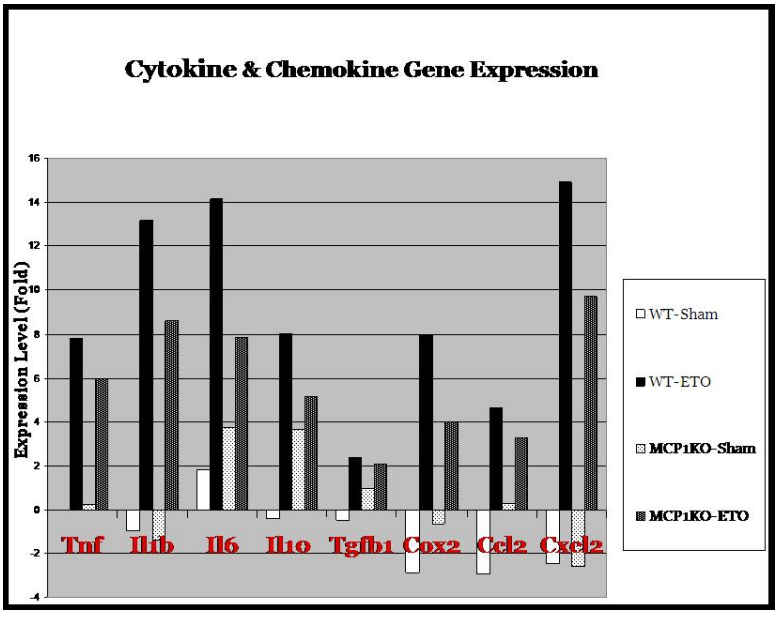

Figure 5. Cytokine and chemokine expression, as fold change compared to normal control ears, in the WT-Sham, WT-ETO, MCP1 KO-Sham, and MCP1 KO-ETO animals. Note that these inflammatory markers, while elevated after ETO, were consistently lower in the MCP-1 KO-ETO mice compared with the WT-ETO, with the exception of TGF-β1, which showed a 2-fold increase in both WT and KO mice following ETO.

Figure 5 presents the summarized results of mRNA expression of key inflammatory mediators following ETO. The cytokines TNFa, IL1-b, IL6, and IL10, as well as COX-2, and chemokine CXCL-2 were strongly up-regulated in the ME mucosa of WT mice compared with MCP-1 knockouts. Therefore, the WT-ETO mice appear likely to be more susceptible to ETO-associated inflammatory changes, as reflected by gene expression profiles coding for cytokine/chemokine clusters. MCP-1 appears to modulate the inflammatory response such that in its absence (in MCP-1 KO mice) the inflammatory response was less robust. While the inflammatory cascade is complex and no single cytokine can be ascribed predominance, this work supports the premise that MCP-1 and macrophages (recruited by this chemokine) are significant participants in the inflammatory response to ME pressure dysregulation initiated by ETO.

Summary and Conclusion
In this study the absence of MCP-1 resulted in a reduced recruitment of macrophages to the ME following ETO. This coincided with a less robust inflammatory response.

These results suggest that monocyte / macrophage participation contributes to the establishment of ETO-associated ME inflammation and OME. These findings may have clinical applicability for future management of OME.

This work was supported in part by NIH grant #DC007437 (PAH), and the Children's Hospital of Pittsburgh Foundation awards: the Lester A. Hamburg Endowed Fellowship and the Eberly Family Endowed Chair in pediatric otolaryngology.

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Biofilm in Chronic Otitis Media Among Greenlandic Patients

Marcus Wessman, MD[1], Thomas Bjarnsholt, PhD[2,3], Helle Krogh Johansen, MD[2], Preben Homøe, MD, PhD[1]
[1]Department of Otolaryngology, Head and Neck Surgery, [2]Department of Clinical Microbiology, Rigshospitalet, Copenhagen, [3]Department of International Health, Immunology, and Microbiology, Faculty of Health Sciences, University of Copenhagen, Copenhagen

Introduction
Chronic infections and bacteria in biofilms have been increasingly associated with each other. We have previously found morphological evidence of biofilms in chronic suppurative otitis media[1]. A biofilm infection in the middle ear could explain the recurrent and recalcitrant episodes of otorrhea in chronic otitis media (COM), a disease otherwise characterized by a dry tympanic membrane perforation.

Objectives
To examine middle ear biopsies from Greenlandic COM-patients for the existence of biofilms and bacteria.

Material and Methods
Middle ear biopsies from 32 Greenlandic COM-patients admitted to ear surgery were investigated. If possible, biopsies were examined by means of bacterial culturing, polymerase chain reaction (PCR) and peptide nucleic acid – fluorescent in situ hybridization (PNA-FISH). 23 skin biopsies served as controls.

Results
Staphylococci were the most common cultured bacteria.
PCR analyses detected bacteria in 7 out of 20 (35%) middle ear biopsies, and in 2 out of 10 (20%) control samples.
PNA-FISH showed morphological signs of biofilms in 13 out of 31 (42%) analyzed middle ear biopsies. In the control biopsies there were signs of biofilms in 7 out of 23 biopsies (30%).

Discussion
Staphylococci were the most frequently cultured bacteria in both the middle ear biopsies and the control samples, but *S. aureus* were most frequently seen in the middle ear biopsies. No correlation was found between findings of biofilms and bacterial culturing. The findings could be related to contamination. We consider the validity of the culturing in this study as low.
PCR analysis detected bacteria in 35% of the middle ear biopsies even though the biopsies were very small.
The PNA-FISH analysis showed evidence of bacterial biofilms in 42% of the middle ear biopsies, although biofilms were also found in 30% of the control biopsies. *S. aureus* is a common pathogen in COM, but can also be found as a part of the normal flora on the skin. Differentiation between pathogenic and non-pathogenic biofilms with PNA-FISH technique is difficult, but the dimensions of the biofilm and distribution of bacteria may serve as an indicator.

Conclusion
These findings can neither support nor dismiss the theory of biofilms being part of the pathogenesis of COM, but they rather suggest that further investigations are necessary.

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Physiology

Middle Ear Pressure Measurements by Tympanometry in a Pressure Chamber for Subjects with Balloon Occluded Eustachian Tubes

Cuneyt M. Alper, MD[1], Richard J. M. Villardo, MD[1], Julianne Banks[1], J. Douglas Swarts, PhD[1], William J. Doyle, PhD[1]
[1]Department of Otolaryngology, Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA

Introduction

The Eustachian tube (ET) is the pivotal organ crucial to maintaining the equilibrium of the middle ear pressure (MEP) gradient in relation to the environment.[1]It fulfills this function by the cyclical active opening and closure of its nasopharyngeal orifice as mediated by the tensor veli palatini.[2]The middle ear space (MES) space act as an air-filled space with an constant pressure determined by a finite number of moles at any given time of the physiologic gases – O2, CO2, N2 and H2O in its homeostatic, ambient state.[3] The passive mechanisms involved in maintaining this balance are: 1. the local blood via diffusion across the ME mucosa; 2. the atmosphere via diffusion across the tympanic membrane; and 3. the inner ear via diffusion across the round window membrane.[4] The ET is considered to be the only active regulator of MEP. Any derangement in any of these components would lead to a pressure imbalance, and ultimately to fluid formation in the MES with its attendant sequalae. The main diagnostic method of measuring the MEP is by tympanometry. The advantages of tympanometry are mainly its non-invasiveness, its wide availability, cost-effectiveness, ease of operation, and accuracy.[5-7]However, recent research on mathematical models for the ME had put into question its validity to accurately determine the MEP.[8,9] It is the purpose of this study to refute or to agree to this prediction, and in order to do so, isolation of the MES from the outside environment is essential for any data gathering. Animal experiments in rhesus and cynomolgus monkeys demonstrated that deep anesthesia, BOTOX™ injection to and excision of the TVP causes ET obstruction (ETO);[10-12] however, there is still no ETO model for humans that exists at this time. These invasive methods are ethically unacceptable for human volunteer subjects and therefore, we have adapted the method of balloon occlusion used successfully in epistaxis control to induce ETO in our study subjects.[13] The specific aims of this study therefore are: 1.) to determine the effectiveness of an inflated nasopharyngeal balloon catheter for causing ETO in 10 adult subjects, and if successful, 2.) to determine the accuracy of tympanometry for measuring MEP over a wide range of ambient-MEP differences.

Methods and Materials

Adult volunteers with normal middle ear were enrolled. Of the 8 subjects the average age was 27.8±6.7, 5 males and 3 females, 5 whites and 3 blacks. After applying topical anesthesia and decongestant to the nasal passages, a posterior epistaxis balloon was inserted unilaterally (test ear) into the nasopharynx and positioned to block the Eustachian tube (ET) orifice when filled with 3 cc of saline. Repeated, bilateral MEP measurements were done by tympanometry inside a pressure chamber and applied chamber pressures were varied between +400 and -600 daPa in 50-100 daPa steps.

Results

Eight subjects completed the study. Despite attempted unilateral balloon occlusion of the ET orifice, there was no evidence of ET obstruction for either ear in 2 subjects. For 3 subjects both ears and for 3 subjects the test ear appeared to have ET obstruction. Data from these 6 subjects were included in the analysis. During the negative pressure cycle of the pressure chamber, the average MEP measured by tympanometry for the test and the control ears were 37±14 and 28±35, 63±32 and 55±48, 98±41 and 71±59 daPa for chamber pressures of -100, -200 and -300 daPa, respectively (Table). During the positive pressure cycle, those measurements were -56±47 and -31±49, -75±83 and -31±57, -101±79 and -59±59, -108±73 and -51±62, -168±126 and -107±95, -168±137 and -74±77 daPa for chamber pressures of +100, +200, +300, +400, +500, and +600 daPa respectively (Figure).

Discussion

The MEPs ware relatively more congruent between control and study subjects during the initial negative pressure cycle of -100, -200 and -300 daPa respectively, of the pressure chamber tests. The measurements began to diverge upon initiation of the positive pressure cycle starting at the +100 daPa point, and this difference was maintained throughout, the cycle from the +200, +300, +400, +500, and +600 daPa atmospheric levels. The study group showed consistently more negative MEPs. Various causes that could have skewed the results are: inhibition of TVP muscle contraction and therefore, ET opening, secondary to the compressive force exerted by the dilated balloon; increased surface tension within the ET lumen; possible inhibitory effect of increased nasal secretions; and general decrease in the effort of swallowing due to the discomfort brought about by the dilated balloon. This represents higher magnitude of error compared to the earlier study we conducted on monkeys.[7]We encountered numerous limitations in the use of balloon obstruction in inducing ETO. The first subject developed an allergic reaction to the balloon latex, and the second one could not tolerate any prolonged inflation of the balloon in the nasopharynx. ETO could not be achieved in two subjects; while, surprisingly, three subjects presented with bilateral ETO. It is uncertain if this was due to slippage of the

balloon, or secondary to a specific anatomical constriction with regards to a narrow skull base. It was also apparent that the balloon promotes increased production of nasal secretions, adding to the discomfort of the subjects with observed increased movements which might have promoted inadvertent dislodgement of the balloon from its initial occluded position.

Conclusions

Balloon obstruction has various limitations that limit its use in experimentally-inducing ETO in human volunteer subjects. Measurement of MEP by tympanometry in balloon obstructed ETs does not accurately track the predicted MEPs created in a pressure chamber. More studies should be conducted to determine the underlying reason and the actual magnitude of the error in tympanometric measurements.

Supported in Part by: National Institute of Health P-50 Grant DC007667

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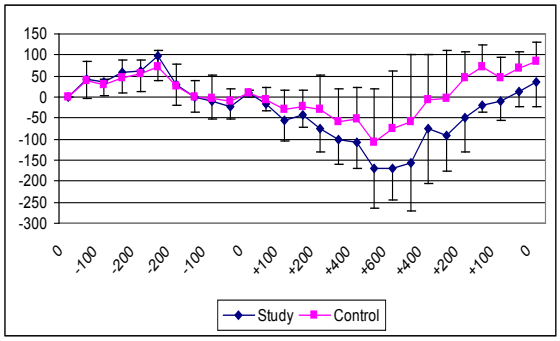
TABLE: Average and standard deviation of selected MEP measurements In Study (occluded) and Control (non-occluded) ears.

	Study	Control
Negative pressure cycle		
- 100 daPa	37 +/- 14	28 +/- 35
- 200 daPa	63 +/- 32	55 +/- 48
- 300 daPa	98 +/- 41	71 +/- 59
Positive pressure cycle		
+ 100 daPa	- 56 +/- 47	- 31 +/- 49
+ 200 daPa	- 75 +/- 83	-31 +/- 57
+ 300 daPa	- 101 +/- 79	- 59 +/- 59
+ 400 daPa	- 108 +/- 73	- 51 +/- 62
+ 500 daPa	- 168 +/- 126	- 107 +/- 95
+ 600 daPa	- 168 +/-137	- 74 +/- 77

FIGURE:Complete set of MEP measurements in the pressure chamber (average and standard deviations) of the Study and Control ears



The Mastoid/Eustachian Tube Morphometry of Crania with Chinese and Inuit Affinities

Jacob Sedgh, MD[1], J. Douglas Swarts, PhD[2], Brendan Cullen-Doyle[2], Jeffrey Laitman, PhD[3], Rolf Quam, PhD[4,5], Charles Bluestone, MD[2]

[1]Otolaryngology-Head and Neck Surgery, Penn State S. Hershey medical Center, Hershey, PA, [2]Pediatric Otolaryngology, Children' s Hospital of Pittsburgh of UPMC, Pittsburgh, PA, [3]Department of Anatomy, Mount Sinai School of Medicine, New York, NY, [4]Department of Anthropology, Binghamton University (SUNY), Binghamton, NY, [5]Comportamiento Humanos, Centro UCM-ISCIII de Investigacion sobre la Evolucion, Madrid

Introduction

The middle ear (ME) mastoid region of the temporal bone is of interest to a range of disciplines from systematics, to bioengineering, to otolaryngology. Features of this region, such as the presence or absence of a mastoid process, have been used in the phylogenetic analysis of primates and to discriminate among species of the genus *Homo*. From a bioengineering perspective, the development of a mastoid process and its internal structure, a consequence of insertion of the sternocleidomastoid muscle (SCM), are significant for an understanding of head posture and trauma due to whiplash.[1-2] The presence, size and internal structure of the mastoid process reflects the forces exerted on the temporal bone by the SCM. These forces balance the weight of the face and calvarium acting across a fulcrum, the occipital condyles. Lastly, from the perspective of otolaryngology, the cellularity (pneumatization) of the ME-mastoid impacts middle-ear (ME) pressure due to its continuity with the middle-ear space.[3-5] This is significant because persistent subatmospheric ME pressures elicit effusions which degrade the sound transmission qualities of the tympanic membrane and ossicular chain. Because features of the mastoid region of the temporal bone contribute to so many aspects of human biology a thorough understanding of its ontogeny and evolution would be useful.

For clinicians and biomedical scientists interested in the development and distribution of otitis media, mastoid pneumatization has always been of concern. Poor mastoid pneumatization characterizes humans with chronic middle-ear disease. Numerous radiographic studies, initially employing x-rays,[6-8] but more recently utilizing CT scanners,[9-11] have documented the correlation between the amount of the middle-ear disease and the degree of mastoid pneumatization. This correlation has been extended to include archaeological populations.[12-14] Unfortunately, the directionality of the causal relationship between these two features has not been established.

It is well known that certain racial groups around the world have a higher incidence of otitis media than others.[15] The etiology of these differences has been variously attributed to heredity, social, environment, microbiology and/or anatomy.[16] Whether these differences are reflected in the variability of mastoid pneumatization and ET morphometry has not been clearly delineated (but see Doyle,1977). Hypothetically, populations with a high prevalence of OM should have a higher mastoid volume variance and correlative ET morphometric featues.

The goal of this study was to establish a protocol in which anthropological collections of cranial material would be used to evaluate the variability of mastoid pneumatization and ET morphometry using nondestructive imaging and analysis techniques.

Materials and Methods:

Sample

This pilot study evaluates the feasibility of a broader and more in depth analysis of the mastoid features, by assessing crania from populations at high and low risk of CSOM.[17] To represent populations with high and low rates of CSOM we used Inuit and Chinese crania of known provenance from American Museum of Natural History in New York. Seven adult male skulls from each population were scanned.

Methods

Scanning Protocol

The specimens were scanned in the same orientation as a living subject would be, that is, supine with bregma entering the scanner first. A sagittal localizing scan, two data acquisitions (axial and coronal) encompassing the cranial base, and two data scans (axial and coronal) of each temporal bone were performed.

For the ME-mastoid region, the matrix size was 756 X 682 with a 20 X 18 cm (Field of view) FOV (Resolution = 0.26 mm). The axial scan covers the entire temporal bone with 1 mm slices incremented at 0.2 mm. The resulting cubic voxel had 0.254 mm sides. For the ET morphometry scans, the 1012 X 718 pixel matrix, had a 26.7 cm X 19 cm FOV, encompassing the CB from greater palatine foramina of the hard palate to the sigmoid sinus for the axial scan (Resolution = 0.26 mm). For the coronal scan, the FOV spanned the distance between the anterior clinoid processes superiorly to the tip of the mastoid process inferiorly. Slice thickness was 1.00 mm, with a 0.5 mm increment.

ME-mastoid temporal bone image analysis
For analysis every 5th image was imported into ImageJ then the entire stack was thresholded, separating the air spaces from bone, segmented and binarized (Figure 1). From each image of the stack, the perimeter and area of each air cell was measured. These perimeters and areas were summed and multiplied by the inter-slice interval (1 mm) to calculate the total surface area and volume of the mastoid air cell system.

Cranial base scans.
Points digitized on the cranial base and from the coronal images (Figure 1) include: the osseous orifice of the ET (diamonds, image 133) represented by the sphenoid eminence, the root of the medial pterygoid plate (triangle, image 117), medial pterygoid lip (arrowheads, image 86) and the hamalus of the medial pterygoid plate (square, image 86). The 3 dimensional coordinates (x, y in the image plane, z image number) of these points were used to calculate the distances between the structures. Those lengths are used as the input data for the calculations of the ET morphometric[18]model illustrated in Figure 1. Because there is ahigh correlation between right and left sides for most of the variables, only the values for the left side were used for the comparison of the two populations. Except where noted the mean ± the standard deviation are used throughout.

Results
The results for the ME-mastoid volume and surface area where highly correlated for the interobservercomparison, supporting the robustness of the method (Figure 2). There was also a significant correlation between the right and left sides for volume (Figure 3, $r = 0.93$, $p<0.01$) and surface area ($r = 0.72$, $p<0.01$), but not for the SA / Vol ratio ($r = 0.40$, $p > 0.05$). The Chinese and Inuit ME-mastoid geometric parameterswere not different: volume (25 ± 17 ml, 23 ± 9 ml; $t=0.30$, $p=0.77$), surface area (160 ± 80 cm^2, 142 ± 36 cm^2 ; $t= 0.54$, $p = 0.60$) and SA/Vol (7.1 ± 1.7 /cm, 6.8 ± 1.9 /cm; $t=0.38$, $p = 0.71$).
With respect to the ET morphometry, as with the ME-mastoid parameters, there were strong and significant correlations between the right and left sides for both the measured and calculated variables. For the 14 bilaterally expressed variables, the median correlation coefficient was 0.79 with an interquartile range of 0.67 to 0.85. However, except for the bihamular (Chinese 4.39 ± 0.55, Inuit 4.84 ± 0.38; $t = 1.81$, $p= 0.05$), bipterygoid (Chinese 3.64 ± 0.26, Inuit 3.9 ± 0.24; $t = 1.92$, $p= 0.04$) and staphylion-basion (Chinese 3.9 ± 0.81, Inuit 4.62 ± 0.17; $t = 2.32$, $p= 0.03$)distances none of the variables were significantly different between the two populations.

Discussion
In this study we established a CT scanning-image analysis protocol that allows us to evaluate ME-mastoid pneumatization and ET morphometrics of archaeological skeletal populations. The inter-observer correlations for mastoid volume and surface area were strong, supporting the robust nature of the image analysis protocol.

With respect to the ME-mastoid data itself, there were high correlations between right and left sides consistent with previous studies. However, both the ME-mastoid volume and surface areas are larger than those reported for living subjects. This can partially be attributed to the absence of the mucosal lining of the ME-mastoid system, but may also reflect an inability to discriminate cancellous bone spaces from the ME-mastoid air-cells. The lower surface area/volume ratio reflects this relatively larger ME-mastoid volume. Lastly, none of the three calculated variables were different between the populations assessed.

Quantitative description of the ET cranial base course was accomplished using the Doyle model.[18] As with the ME-mastoid measures, the parameters from this part of the study also showed relatively strong correlations between the sides and all the correlations were significant. Except for three variables there were no significant differences between these populations. The three significant variables, bihamular, bipterygoid and the staphylion-basion distance, are bilateral measures of nasopharyngeal width or nasopharyngeal depth. Sources of error for this section of the study include ambiguity of point location, resolution (minimum length that can be discriminated) and not meeting the assumptions of the trigonometric functions.

Upon further reflection, the choice of osteological populations to study was probably unfortunate. From a historical perspective these two populations are probably more closely related than most others you could pair either of them against. This obtains despite the differences reported for CSOM prevalence. In order to address this issue we are expanding the samples to include CT scans from new crania of Caucasians from Hungarian archaeological sites.

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Figure 1

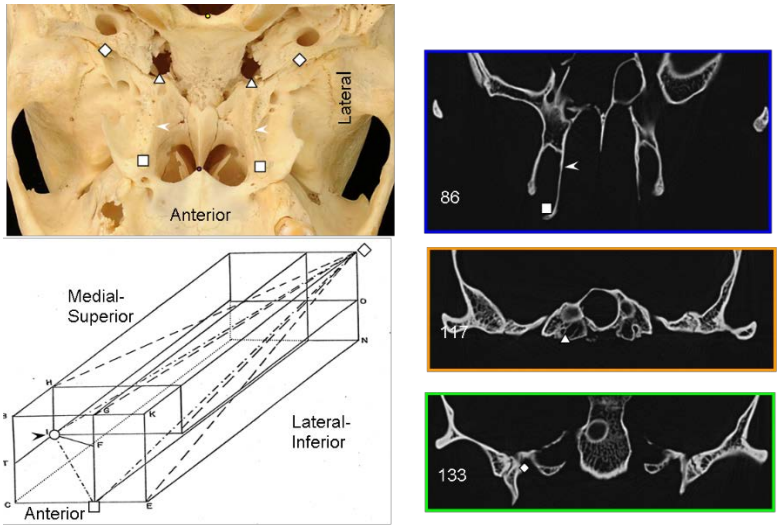

Figure 2

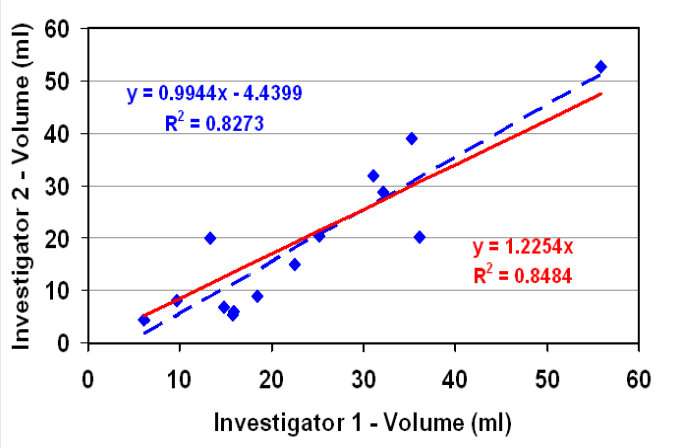

Figure 3

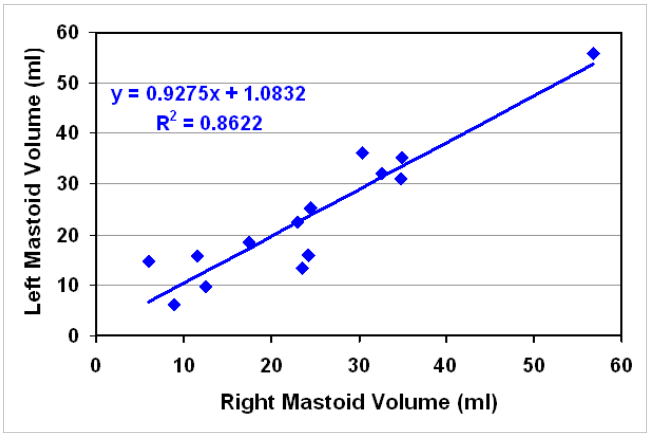

Figure 1: The location of the cranial base structures (identified on skull) digitized from the CT scans (images 86, 117, 133) and their location in the ET model.

Figure 2: Inter-observer correlation between two investigators measuring the ME-mastoid volume.

Figure 3: Correlation between right and left ME-mastoid volumes of all the specimens.

Hominoid Temporal Bone Pneumatization the Context for the Human Condition

J. Douglas Swarts, PhD[1], **Brendan Cullen-Doyle**[1], Charles Fitz, MD[2], Charles Bluestone, MD[1]
[1]Pediatric Otolaryngology, [2]Department of Pediatric Radiology, Children' s Hospital of Pittsburgh of UPMC, Pittsburgh, PA

Introduction

The mastoid region of the temporal bone (TB) is of interest to a range of disciplines from systematics, to bioengineering, to otolaryngology. Features of this region, such as the presence or absence of a mastoid process, have been used in the phylogenetic analysis of hominoids. From a bioengineering perspective, the development of a mastoid process and its internal structure, a consequence of insertion of the sternocleidomastoid muscle (SCM), are significant for an understanding of head posture and trauma due to whiplash.[1-3] The presence, size and internal structure of the mastoid process reflect the forces exerted on the temporal bone by the SCM. These forces balance the weight of the face and calvarium acting across a fulcrum, the occipital condyles. Lastly, from the perspective of otolaryngology, the cellularity (pneumatization) of the TB may impact middle-ear (ME) pressure due to its continuity with the middle-ear space.[4-9] This is significant because persistent sub atmospheric ME pressures elicit effusions which degrade the sound transmission qualities of the tympanic membrane and ossicular chain. A large body of literature discusses the relationship between TB pneumatization and the development of otitis media, an inflammatory condition of the ME space. Because features of the mastoid region of the TB contribute to so many aspects of human biology a thorough understanding of its ontogeny and evolution would be useful.

Hominoids, that is primates colloquially known as the great apes, are the comparative group which, from an evolutionary perspective, form the outgroup (context) for our explanations of human anatomy and physiology. The cranial airspaces, especially the sinuses, have been studied in an effort to understand the phylogenetic relationships among the hominoids, humans and our fossil ancestors (eg. Lucy, *Australopithicusafarensis*). Sherwood in a series of abstracts and publications[10-11] qualitatively described the development and extent of TB pneumatization in *Pan* (chimpanzee) and *Gorilla* based on lateral radiography and CT scans. He discussed the potential functional consequences of the extensive TB pneumatization and detailed Wittmer's epithelial hypothesis to explain the development of these spaces. More recently, Hills employing high resolution CT scanning and a method developed for trabecular bone structural analysis to assess the TB pneumatization of the hominoids.[12]
In our effort to understand human TB pneumatization, we thought it would be useful to evaluate the status of our closest phylogenetic relatives, the hominoids (apes) and an Old World monkey, using methods we apply when studying the human condition. To that end we CT scanned either all of the complete cranial specimens (*Pongo*, *Gorilla*) or a representative sample (*Pan*, *Colobus*) available from the Carnegie Museum of Natural History (CMNH).

Materials and Methods

Sample

The sample included the skulls of 4 gorillas, 2 orangutans (*Pongo*), 4 chimpanzees (*Pan*) and 3 monkeys (*Colobus*).

Image Analysis

The CT scans, including the both middle ears and TB pneumatic spaces were acquired in the transverse plane using a GE LightSpeed VCT system (General Electric Health Care). The images were obtained using a field of view which included both temporal bones (284 to 168 mm) using a 512 X 512 matrix. The resolution averaged over the entire sample was 0.048 cm per pixel with a slice thickness of 0.63 cm. From each CT scan, the complete set of transverse images through the TBs (superior to inferior) was used for the reconstructions. Using ImageJ software, these sections were imported, and the left and right TB were identified, and the pneumaticized areas segmented out and analyzed (Figure 1, (http://rsbweb.nih.gov). For each TB, the perimeter and area of all air-cells were calculated, corrected with the appropriate calibration factor, and summed across images. These sums were multiplied by the section interval to yield TB air-cell surface area (cm^2) and volume (ml). This procedure is essentially identical to that used previously to measure mastoid air-cell system (MACS) volume, surface area and the surface area/volume ratio in adult human subjects over a wide range of MACS volumes.

Results

Qualitative Observations

In contrast to the human condition, the TB of hominoids is highly pneumaticized including the petrous, squamous and zygomatic portions (Figure 1). The pneumaticized areas are air-filled as demonstrated in Figure 2, a post mortem CTscan of a gorilla. Soft tissue, primarily muscle extracranially and fluid in the nasal cavity, and cancellous bone (arrows) are easily distinguished from the air filled cavities. Lastly, the hominoid TB condition is a primitive retention from the common ancestor with Old World Monkeys, as represented by *Colobus.*

Quantitative Results

Except for the fully adult male *Gorilla* and *Pongo*, the volume and surface areas are of the same order of magnitude as seen humans (Table 1). *Gorilla* and *Pongo* show the expected sexual dimorphism; fully mature adult males can have body size twice that of females. The surface areas male TBs are approximately twice that of the female. The SA/V ratios are similar in

magnitude to those derived for Chinese (Descriptive statistics in last row of the Table) and Inuit skeletal populations. The SA/V ratio seems to track body size as defined by sex and age grade.

Discussion

This preliminary study quantifies the TB air-cell volume and surface area in a small sample of hominoid crania. Visually, the crania of the primates surveyed with CT scanning show extensive pneumatization throughout the TB, including the petrosal, squamous and zygomatic portions. Human TB pnuematization is not as extensive as that seen in hominoids. Furthermore, the cortical bone surrounding the spaces appears thinner in the hominoids. According to Sherwood[11] this pneumatization does not extend across suture boundaries. It is clear from the CT scan of a postmortem gorilla head that these spaces are indeed air filled cavities. Quantitatively, the hominoids possess TB pneumatic spaces, both volume and surface area, that are at least as large as the largest human we've measured in skeletal material. The SA/V ratio is largest in the adult male suggesting that surface areas are smaller which is consistent with Sherwood's observation that older *Pan* and *Gorilla* seem to have larger air-cells.

The shortcomings of this study as it stands are obvious. There were only a limited number of specimens available in the collection. Even the largest sample (*Pan*) includes only a single specimen from a subadult. To develop a more complete understanding of the changes in hominoid pneumatizaion during development would require many more specimens. However, the ubiquity of CT scanners and the sophistication of the image analysis software bode well for a multi-institution research project.

We would like to acknowledge the support of John Wible, Curator of Mammals and Susan McLaren, Collections Manager of Mammals CMNH in the execution of this study. Supported in Part by: NIH P- 50 Grant DC007667.

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. CMNH No.	Species	Age	Sex	Lt Vol (ml)	Lt SA (cm^2)	RtVol (ml)	Rt SA (cm^2)	Lt SA/Vol (/cm)	Rt SA/Vol (/cm)
42761	G. gorilla	Juvenile	Uk	18	151	18	141	8.40	7.74
1786	G. gorilla	Adult	F	26	160	21	121	6.06	5.74
42754	G. gorilla	Adult	M	25	374	25	386	14.84	15.60
42756	G. gorilla	Adult	M	35	407	36	423	11.76	11.80
61335	P. pygmaeus	Juvenile	Uk	2	28	3	29	11.57	10.28
22292	P. pygmaeus	Adult	M	70	611	72	601	8.77	8.40
42758	P. troglodytes	Juvenile	F	9	86	9	83	9.64	9.15
20918	P. troglodytes	Adult	F	11	93	11	91	8.61	7.98
42757	P. troglodytes	Adult	F	15	179	14	183	11.71	12.91
57888	P. troglodytes	Adult	M	9	152	5	89	17.50	18.92
	H. sapiens	Adult	M	Vol = 25 ± 17		SA = 160 ± 80		SA/Vol = 7.1 ± 1.7	

Vol is volume, SA is Surface Area. Uk means unknown.

Figure 1

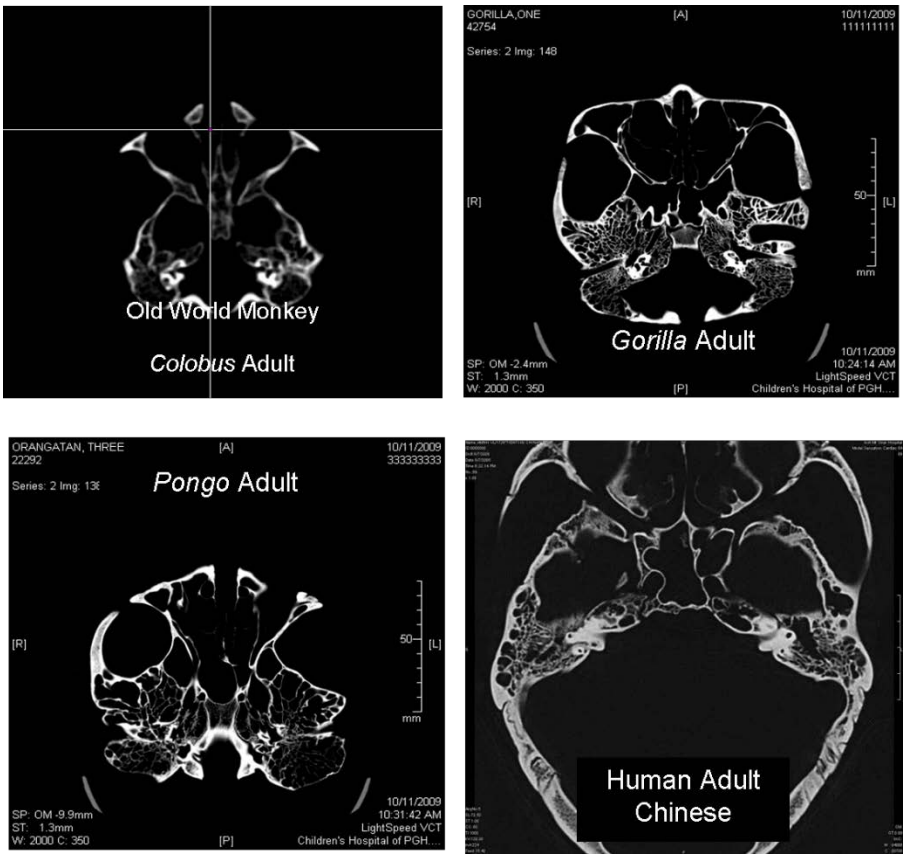

Figure 1. Axial CT images of an Old World Monkey *Colobus, Gorilla, Pongo*and *Homo* illustrating the extensive TB pneumatization in nonhuman primates.

Figure 2

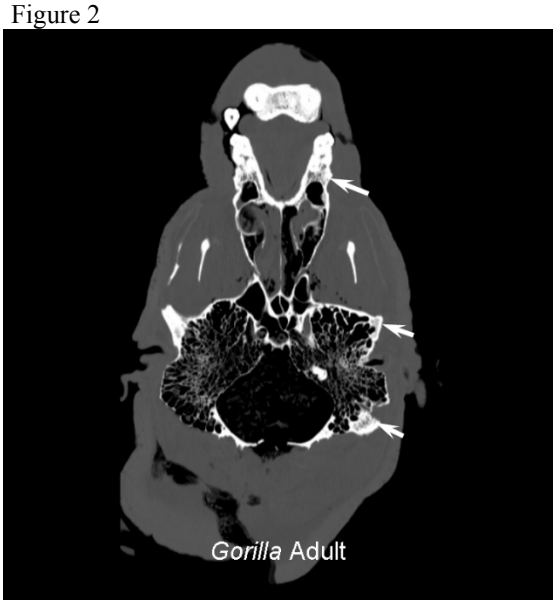

Figure 2. Post mortem CT scan of a gorilla demonstrating the patency of the pnuematized TB relative to the soft tissues (muscle) and fluid (nasal cavity). The brain was removed at autopsy.

The Growth and Development of Middle Ear Pneumatization in Subjects with and Without Otitis Media

Sean Foley[1], J. Douglas Swarts, PhD[1], Cuneyt Alper, MD[1], William Doyle, PhD[2]
[1]Pediatric Otolaryngology, Children' s Hospital of Pittsburgh of UPMC, Pittsburgh, PA, [2]Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA

Objectives
The middle-ear (ME) is composed of two compartments that communicate in the air-phase, the tympanum (TYM) and the mastoid air cell system (MACS). While the TYM is essentially a single cavity, the MACS is a multiply partitioned, cellular, air-space. These spaces are lined with mucosa that embeds a network of blood vessels. ME geometry dictates its physiology with respect to gas exchange and, therefore, the ME pressure. While ME geometry was studied in adults, little is known of that geometry in infants, children and adolescents. In this study, we assessed ME geometry of subjects at different ages with and without a significant history of otitis media (OM).

Methods
CT scans of seventy-two subjects with and without OM were identified from the clinical population of Children's Hospital of Pittsburgh of UPMC. Two with and two without OM were chosen for yearly intervals from 1 to 18. Using ImageJ (NIH), the axial CT images were thresholded, binarized, and the TYM-MACS complex segmented out. For each image, the measured variables included the numbers of air-cells, their perimeters and areas. These variables were used to calculate the ME surface area (SA), volume (Vol) and SA/Vol ratio.

Results
For Vol and SA, left-right measures were highly correlated, but subjects with OM were more variable (for non-OM group, Vol r2 =0.85, SA r2 =0.94, for OM group, Vol r2= 0.58, SA r2 =0.69). For the SA/Vol ratio, the sides were correlated for the non-OM but not for the OM group (r2 =0.66 and r2 =0.04 respectively). Regression of the variables on age yielded coefficients in the range of 0.2 to 0.3, with normal subjects having greater slopes (Vol 0.61 vs 0.45, SA 10 vs 7.3). The SA/Vol ratio was constant (15.2 ± 2.4) after 3 years of age and independent of OM status.

Conclusions
The ME (TYM+MACS) Vol and SA grow more slowly and show more variability in individuals with a history of OM. However, the SA/Vol ratio is independent of age and OM status after 3 years of age. Supported in part by grant DC007667 from the NIH.

Digital Cephalometrics: Accuracy and Reliability with Respect to the Impact of Craniofacial Growth on the Persistence of Otitis Media

Yusuf Kemaloglu, MD[1], J. Douglas Swarts, PhD[2], Cuneyt M. Alper, MD[2], Margaretha L. Casselbrant, MD[2], William J. Doyle, PhD[2]

[1]Department of Otolaryngology, Gazi University Faculty of Medicine, Ankara, Turkey, [2]Department of Otolaryngology, Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, PA

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Introduction

The impact of craniofacial (CF) growth on the development and risk of otitis media in childhood has been a long standing issue.[1-6] As reported by Mew and Meredith (1992), Hippocrates, first, noted the relationship between head type, malocclusion and ottorhea in his sixth book of epidemics.[2] Recently, evolutionary aspects of this relationship have also been suggested.[7,8]

One of the main tools to analyze CF structures in living humans is cephalometric analysis of lateral cephalogams (LCGs).[1-6,9] However, it has been reported that it is difficult to measure variations in the nose and pharynx on cephalograms with accuracy.[2]

The digitizing and measurement of LCGs is time consuming and subject to errors[10-12]. Hence, recently, digital tracing and measurement on either scanned digital copies of the hard copy x-ray or direct digital cephalometric images (DDCIs) have been implemented and are becoming more widely used in research studies.[11,13,14] Furthermore, digital images provide an option for multicenter research studies, since they can be electronically transferred in various picture formats easily[11]. Particularly, when an online cephalometrics service is used, not only the problem of sharing the x-rays is facilitated, but also the digital tracings and measurements can be done independently at distant centers. Nevertheless, for researchers who are used to pencil tracing hard copy images, the transition to digital images, either scanned or direct ones, by using a mouse on a computer screen may be problematic. Therefore, the purpose of this study was to determine whether a web based cephalometric service (CephX) is a good research tool regarding the accuracy and reliability of obtaining LCG data.

Methods

DDCIs of 13 cases (Male: 8, Female: 5; mean age: 6,15+/-2.27, minimum and maximum ages are 3 and 11 years, respectively) and their hard copies (HCs) were traced using both the CephX web based system and hand tracing. Eleven anatomical landmarks were defined by a single investigator (Table 1, figure 1) and 20 linear and 7 angular variables related to the cranial base, nasopharynx, jaws and Eustachian tube (ET) were calculated (Table 2).

The DDCIs in jpeg format and their HCs were taken from the archive of the department in Ankara. The subjects were free of any craniofacial abnormality and had no recorded surgical procedure. These x-rays were requested for evaluation of adenoidal hypertrophy by the first author.[15] The selection of these images was only based on their quality, and no differentiation was made for age, gender, occlusal type, or skeletal pattern. The images were acquired in a standardized manner using the same direct digital cephalometer (Planmeca2002cc) set at ×1.20 magnification, as recommended by the manufacturer. DDCIs were stored as jpeg format in a CD and delivered to the physicians together with HCs. HCs of the images were also printed in the same dental x-ray center by using e-program software program, resized to 1:1 scale and printed using a Konica Drypro793 printer.

For this study, first, DDCIs in CDs were imported to the CephX-Online Cephalometrics and Storage Service via internet (CephX Inc, Las Vegas, Nevada, USA). Then, by using specific sub-menus, a custom analysis was created for the measurements, and landmarks were set on each image by using a mouse on the screen after all images were calibrated by digitizing two points on the ruler of each LCG image (the observer was able to adjust the image using enhancement functions for magnification, brightness, and contrast) (figure 2a). At the end of tracing, this service automatically generates measurements of created custom analysis for each case as pdf files, separately. Manual tracing was performed on clear acetate placed over the printed image using a 0.35 mm lead pencil (figure 2b).

The first author of the study performed the processing including uploading to CephX, calibration, digital and manual tracings and manual measurements. Tracings and measurements in 10 cases were repeated twice on both DDCIs and HCs for detection of the intra-researcher variability.

All hard and soft tissue landmarks were traced with bilateral structures averaged to make a single landmark. For statistical analyses, first, intra-researcher variability was tested for each parameter on both materials by intra-class correlation coefficients (ICCs) separately. Then differences between the measurements obtained on DDCIs and HCs were evaluated using a Wilcoxon Signed Ranks Test, with a significance level of $p < 0.05$. Finally, Spearman correlation analysis was run for each paired parameter for DDCI and the HC images.

Results

Intra-researcher variability analysis showed that the ICCs were above 0.90 for all parameters, with the exception of AIFH on the HCs (0.85). As seen in Table III, the data obtained from DDCIs were not different from those of HCs with the exception of PL, which was found to be shorter on DDCIs.

Further, Spearman analysis showed that PL measurements on DDCIs and HCs were not correlated with each other while most of the other parameters had very high correlations (Table 3). PL was visually compared on the DDCIs and HCs and it was found that both pns and ans were traced more anteriorly on DDCIs, and ans also presented small vertical differences.

Discussion

Our data suggest that DDCIs and computerized tracings are convenient, time saving and user friendly instruments for researchers. Although, in this study we used some non-standard landmarks such as mep, sep and sos which are not commonly used in daily orthodontics, cephalometric analysis. The non-standard points related to the SB and ET were found to be highly reproducible for both methods in this study. We therefore believe that most, if not all, of the standard landmarks can be measured using DDCIs conveniently. Surprisingly, the greatest differences in landmark localization between methods were the hard palate points, pns and ans, which are both important in routine cephalometrics. Also, PL was significantly longer on HCs than DDCIs which suggests that the identification of either ans or pns, or both were made closer to each other on DDCIs, or farther on HCs. The data for the vertical parameters related with either ans or pns disclosed no mean differences while some had lower correlations (vTVPL, PSFH and AIFH). On the other hand, ASFH using ans disclosed high ICC and correlation values. Hence, it is likely that differences in tracing of ans and pns were mostly related with antero-posterior direction, rather than supero-inferior direction. Visual comparison supported this assumption. Both of these landmarks were located much more anteriorly on DDCIs than on HCs, most probably because the boundary between the bone and soft tissue shadows were identified better in DDCIs via the enhancement functions for magnification, brightness, and contrast offered by the in CephX system. In previous papers, ans and me (landmarks of AIFH) were described as related anatomical points with low levels of reproducibility.[12-14] Lagrevere and colleagues reported that pns and ans could be less reliable landmarks because they are located in structures with lower densities.[16]

In summary, DDCIs via an on-line service provides the ability to change magnification, brightness and contrast, allows for the ability to quickly add new parameters and measurements, eliminates chemical and environmental risks due to film processing, and provides easy storage and sharing of the images. Also, our results show that this system provides accurate and reliable evaluation for the specific parameters of SB and TB related with the ET.

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Table 1. Landmarks identified on Lateral Cephalograms.

ans	Anterior nasal spine; the most anterior point of the hard palate.
ba	Basion; the most anterior inferior point on the margin of the foramen magnum, located on the most inferior of the basillar part of the occipital bone (on the intersection point of the nasopharyngeal and posterior surfaces of the basillar part of the occipital bone)
go	Gonion; the most postero-inferior point on the outline of the mandibular angle. Midpoint of the right and left sides' images was used.
me	Menton; The most inferior point of the the mandibular symphysis;
mep	Middle ear point; The most antero-superior point of the middle ear image; Midpoint of the right and left sides' images was used.
n	Nasion; the intersection of the internasal and frontonasal sutures
pns	Posterior nasal spine; the most posterior point of the hard palate; the junction between the hard and soft palate was used.
ptm	Pterygo-maxillare; the most inferior point of the pterygo-maxillary fissure, located where the anterior and the posterior outline of the inverted teardrop merge with each other. Midpoint of the right and left sides' images was used.
s	Sella; geometric center of the sella turcica.
sep	Spheno-ethmoidal point; the most superior point of intersection of the sphenoid and ethmoid bones.
sos	Spheno-occipital synchondrosis; the most cranial end of the spheno-occipital synchondrosis, located on the sphenoidal line of the synchondrosis.

Table 2. Calculated Linear and Angular Parameters for this Study.

Linear parameters		
s-n	Anterior Cranial Base Length	ACBL
n-me	Anterior Facial Height	AFH
ans-me	Antero-inferior Facial Height	AIFH
n-ans	Antero-superior Facial Height	ASFH
s-sep	Anterior SB Length	ASBL
mep.ptm	This distance fits to the length of the region in which ET is located	ETL
ba-ptm	Nasopharyngel depth-1	ND1
ba-pns	Nasopharyngel depth-2	ND2
sos-ba	Occipital Cranial Base Length	OCBL
s-ba	Posterior Cranial Base Length	PCBL
s-sos	Posterior Sphenoid Bone Length	PSBL
s-pns	Postero-Superior Facial Height	PSFH
pns-ans	Palatal Length	PL
s-go	Posterior Facial Height	PFH
sos-ptm	Postero-inferior Sphenoid Bone Length	PISBL
sos-sep	Sphenoid Bone body Length	SBbL
sep-ptm	Vertical Sphenoid Bone Length -1	VSBL1
s-ptm	Vertical Sphenoid Bone Length -2	VSBL2
n-ba	Total Cranial Base Length	TCBL
ptm-pns	This distance fits to vertical length of the Tensor Veli Palatini muscle	vTVPL
Angular parameters		
^n.s.ba	Cranial base angle	^CBA
^ba.s.ptm	Nasopharyngeal angle-1	^NA1
^ba.s.pns	Nasopharyngeal angle-2	^NA2
^s.ba/pns.ans	Angle of the s.ba (posterior CB) line to pns.ans (palatal) line	^PCB/PL
^sos.ptm.sep	Sphenoidal angle	^SA
^mep.ptm.pns	ET angle-1	^ETA-I
^mep.ptm.s	ET angle-2	^ETA-II

Table 3. Average and Standard Deviation (SD) of the DDCI and HC Parameters and the Spearman Correlation (r) and Associated Probability Level (P) for the Paired Values

	HCs		DDCIs		Correlation Analysis	
	Mean	SD	Mean	SD		
Linear parameters					R	P
ACBL	57.99	3.50	58.04	3.38	0.97	< 0.001
PCBL	34.25	3.32	34.61	3.54	0.91	< 0.001
TCBL	85.36	4.90	85.44	4.79	0.97	< 0.001
OCBL	21.53	1.94	21.98	2.19	0.86	< 0.001
ND-1	37.71	2.16	37.68	1.81	0.71	< 0.01
ND-2	34.66	2.26	34.31	2.24	0.80	< 0.001
SBL-1	30.72	4.24	30.00	2.34	0.81	< 0.001
SBbL	35.28	4.25	34.53	2.60	0.96	< 0.001
ASBL	22.15	1.96	22.03	1.95	0.95	< 0.001
PISBL	30.72	4.24	30.00	2.34	0.78	< 0.002
PSBL	13.58	1.86	13.51	1.91	0.95	< 0.001
VSBL-1	30.85	4.18	30.56	3.62	0.88	< 0.001
VSBL-2	29.26	3.59	29.11	3.42	0.93	< 0.001
ETL	34.20	3.19	34.49	2.76	0.99	< 0.001
vTVPL	8.91	1.57	8.97	1.62	0.73	< 0.01
PL*	40.42	4.39	34.04	3.67		-
PFH	57.32	6.74	57.84	6.40	0.97	< 0.001
PSFH	36.96	3..06	37.92	3.51	0.78	< 0.002
AFH	95.12	9.75	95.99	9.40	0.97	< 0.001
ASFH	40.61	3..92	41.10	4.13	0.90	< 0.001
AIFH	55.32	5.77	57.03	5.71	0.61	< 0.03
Angular parameters						
^CBA	132.66	4.50	133.19	4.82	0.78	< 0.002
^NA-1	63.48	5.29	62.76	579	0.63	< 0.02
^NA-2	65.63	5.45	64.88	5.05	0.87	< 0.001
^PCB/PL	53.24	4.23	53.78	4.81	0.83	< 0.001
^SA	152.17	6.01	152.50	672	0.72	< 0.01
^ETA-1	58.00	10.12	55.98	8.46	086	< 0.001
^ETA-2	47.34	4.45	46.87	4.36	0.91	< 0.001

*Wilcoxon Signed Ranks Test; Z: - 0.283, p= 0.005.

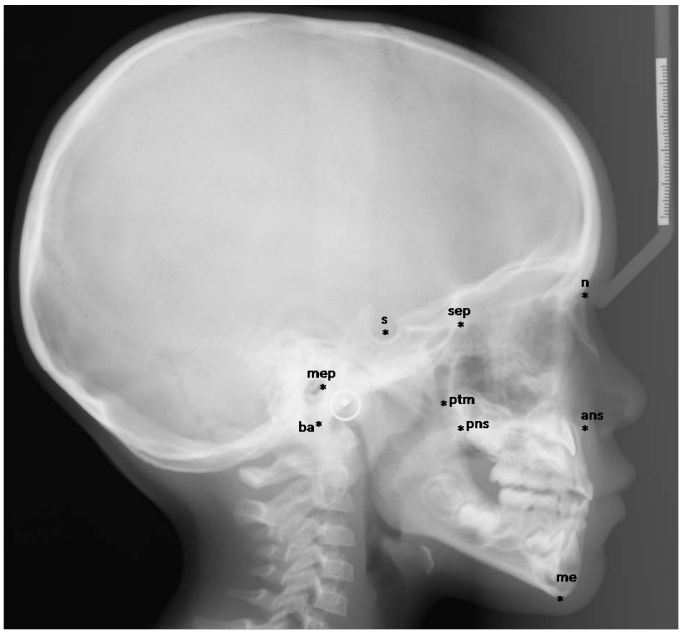

Figure 1. Landmarks digitized on the lateral cephalograms in this study.

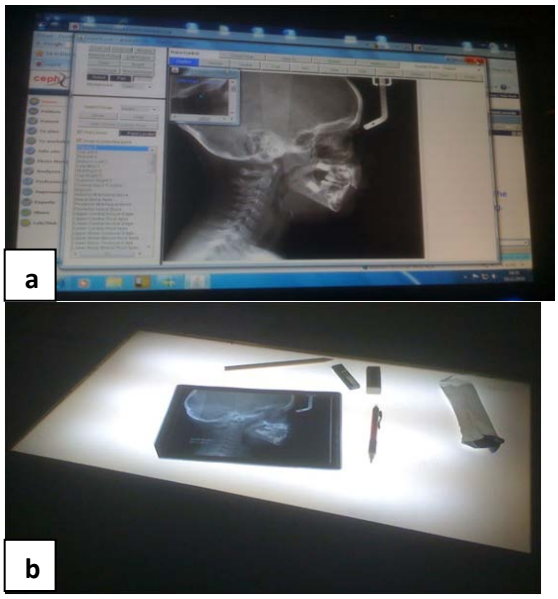

Figure 2. Digitizing process of a lateral cephalogram (a) on direct digital image by using a mouse, and (b) on hard copy by using a pencil.

Round Window Membrane Mesothelial Cells Protect the Inner Ear Against Middle Ear Infection

Patricia Schachern[1], Vladimir Tsuprun, PhD[1], Cureoglu Sebahatin, MD[1], Patricia Ferrieri, MD[2], David Briles, PhD[3], Michael Paparella, MD[4], Steven Juhn, MD[1]
[1]Otolaryngology, [2]Pediatrics and Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN, [3]Microbiology, University of Alabama, Birmingham, AL, [4]Otolaryngology, Paparella Ear Head and Neck Institute, Minneapolis, MN

Introduction

The round window membrane (RWM) has been shown to be the main conduit for the passage of bacteria and bacterial products from the middle to the inner ear in otitis media.[1-3] The structure of the RWM is similar in various species of mammals.[4,5] and in humans.[5,6] It is a three- layered structure, composed of an outer epithelial layer that faces the middle ear cavity, a middle connective tissue layer, and a mesothelial layer that faces the scala tympani and is in contact with the perilymphatic fluids. The mesothelial layer is of particular interest, as this layer appears to provide the last line of defense against bacterial penetration into the scala tympani. Mesothelial cells of different organs in mammals are considered similar regardless of anatomical site or species.[7]Although the primary function of the mesothelium is protection of a non-adhesive surface, it is also involved in transport of solutes and cells across serosal cavities, antigen presentation, inflammation and tissue repair, coagulation and fibrinolysis, and tumor adhesion.[8] Our goal in this study was to investigate the role of the mesothelial cells of the RWM in inflammation and protection of the inner ear from bacterial infection in the middle ear.

Methods and Materials

Streptococcus pneumoniae D39 type 2 were grown in Todd-Hewitt broth (Bacto Todd-Hewitt Broth, BD Diagnostics, Sparks, Maryland) containing 0.5% yeast extract (Bacto Yeast Extract, BD Diagnostics). Bacteria were plated on sheep blood agar plates and stored in 10% glycerin at -80^0 C. The colonies were grown overnight, transferred into broth and incubated at 37^0 C until early log phase. They were centrifuged at 2,000 RPM for 15 minutes and suspended in 0.15M phosphate buffered saline. Optical densities were measured at 660 nm on a spectrophotometer and diluted to the desired concentration in phosphate buffered saline. Bacterial concentrations were confirmed by plating multiple 10-fold dilutions on blood agar plates. They were incubated overnight at 37^0 C in 5% - 10% carbon dioxide and viable cells counted.

Chinchillas weighing 250-350 gm were housed and fed under standard conditions at our Institutional Animal Care Facility. The Institutional Animal Care and Use Committee of the University of Minnesota, Minneapolis approved the care and use of the animals. The animals were anesthetized with a combination of 40 mg/kg ketamine and 0.4 mg/kg acepromazine prior to bilateral intrabullar inoculation of 0.5ml of *S. pneumoniae* serotype 2 strain D39 (NCTC 7466) with concentrations ranging from 9.6 x 10^3 to 3.4 x 10^7 CFU/ml.Animals were killed 2 days after inoculation using an overdose of pentobarbital. Cochleae were removed and perfused via the apex and oval window with 2% glutaraldehyde in 0.2 M phosphate buffer with a pH of 7.4, and their fixation was continued by immersion for 2 hours. The samples were decalcified for 2 weeks in EDTA, washed in buffer, and post-fixed in 1% phosphate buffered osmium tetroxide for 1 hour. Samples were again washed in buffer and dehydrated in a graded series of ethanol, followed by propylene oxide. Tissue was embedded in epoxy resin, cut at a thickness of 1 μm, and stained with toluidine blue for light microscopic assessment.

Results

Compared to the normal, non-infected animals (Fig. 1a), mesothelial cells in animals infected in the middle ear with *Streptococcus pneumoniae*formed balloon-like structures that contained bacteria and leukocytes, confining them within the round window membrane (RWM) and preventing their escape into the scala tympani (Fig. 1b). They were also observed to extend long cellular processes that seemed to provide a surface for bacterial capture by neutrophils in the scala tympani adjacent to the mesothelial layer (Fig. 2). Mesothelial cells were seen to proliferate and detach from the basement membrane to float freely in the perilymph and entrap bacteria within the scala tympani, more distant from the RWM (Fig. 3). They also formed attachments between floating cells to develop net-like structures enclosing bacteria within their spaces. Combinations of these methods of entrapment of bacteria by mesothelial cells were also observed (Fig. 4). Inflammatory cells, both polymorphonuclear and mononuclear, were observed in great numbers adjacent to the mesothelial surface, and were frequently seen in association with fibrillar structures, morphologically similar to fibrin. Mesothelial cells lining the bony surface of the scala tympani in close proximity to the RWM showed similar characteristics.

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Discussion

Our data showed that the mesothelial cells of the round window membrane (RWM) provide the last line of defense against the passage of bacteria from the middle ear into the inner ear. Although mesothelial cells were previously believed to be passive cells, studies of the mesothelial cells lining serosal cavities of other organs have shown that they are involved in the: transport and movement of fluid and particulate material across serosal cavities; regulation of leukocyte migration in response to inflammatory

mediators; synthesis of pro-inflammatory cytokines, growth factors and extracellular matrix molecules; control and coagulation of fibrinolysis; and antigen presentation.[7] It has been shown that mesothelial cells of several mammalian species are essentially similar, irrespective of species or anatomical site; however, few studies have compared the functional and biochemical characteristics of these cells between species and anatomical locations.[8]

We observed large numbers of mesothelial cells that were detached and floating freely in the scala tympani. Soluble mediators released from inflammatory cells and injured cells have been shown to induce a 30-80% increase in the mitotic activity of mesothelial cells,[9]and mesothelial cells from other organs have been shown to break their cell-to-cell contacts and detach to become free floating.[10]

Fibrillar fibrin-like structures were observed in the scala tympani of our infected animals. Fibrin deposition is another method for localization and entrapment of bacteria, within its matrix, for removal by phagocytes. Peritoneal mesothelial cells have been shown to have both procoagulant and fibrinolytic activity.[11]

In our study, we observed mesothelial cells of the RWM to not only entrap bacteria for inflammatory cell killing by a number of methods, but we also observed large numbers of polymorphonuclear and mononuclear cells in close apposition to the mesothelial layer. Secretion of chemokines by mesothelial cells has been shown to promote directed and massive transmesothelial migration of neutrophils and monocytes from the vascular compartment to the serosal space of the peritoneal cavity.[12]

A few studies have been done on the mesothelial cells of the basilar membrane surface that faces the scala tympani of the inner ear. Jokay et al[13] detected the monoclonal antibody 27E10. This antibody is believed to play a role in macrophage activation and regulation of inflammatory cell motility. Kwun etal[14] showed mesothelial cells from this same area to be immunoreactive for the renal transporter UT-A3r. Satoh et al[15] found these same cells to produce transforming growth factor. Further functional studies of these dynamic cells are important to better understand their role in transport of solutes and in inflammation.

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Figures

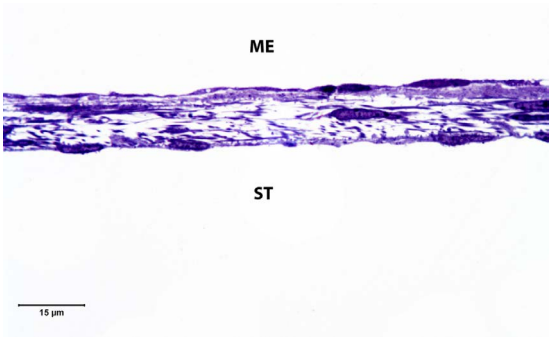

Figure 1a.

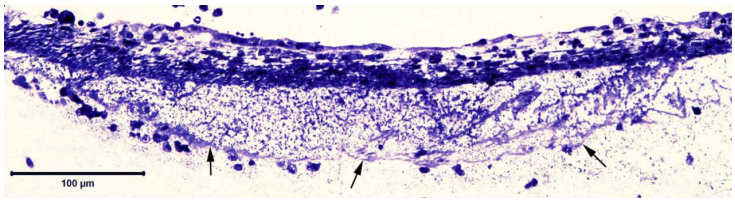

Figure 1b.

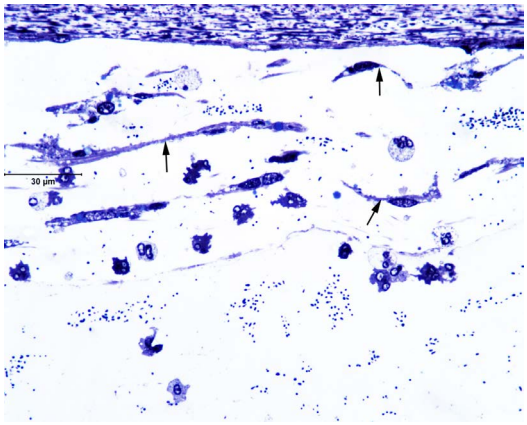

Figure 2

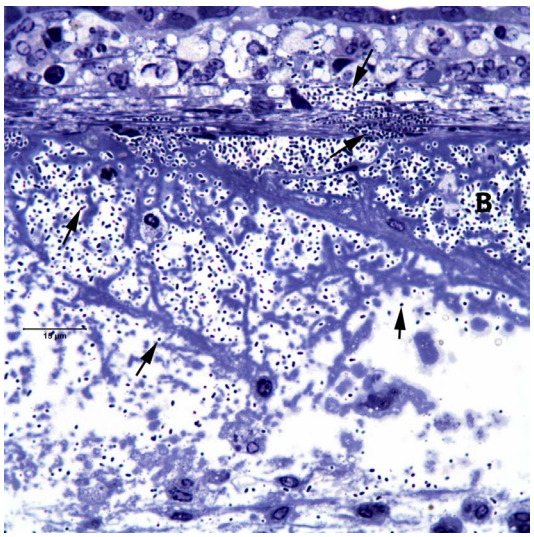

Figure 3

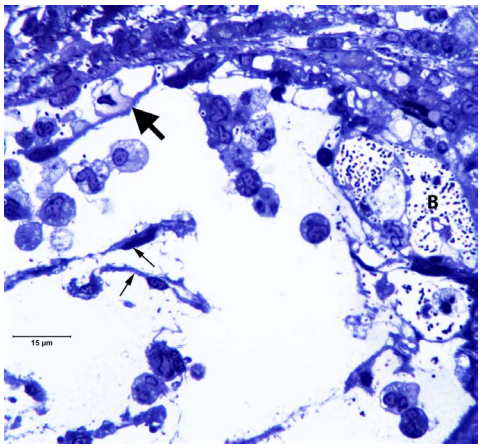

Figure 4.

Fig.1a. Round window membrane from a saline-injected control animal. Note flattened appearance of mesothelial layer. ME = middle ear; ST = scala tympani. Toluidine blue staining

Fig. 1b. Mesothelial cells (arrows) from a chinchilla inoculated with *S. pneumoniae* form a pocket to trap bacteria within the round window membrane. Note fibrin in adjacent scala tympani. ME = middle ear. Toluidine blue staining

Fig. 2. Mesothelial cells extended long cellular processes that seem to provide a surface for bacterial capture by neutrophils in the scala tympani adjacent to the mesothelial layer. Note bacteria within the round window membrane. Arrows = bacteria. Toluidine blue staining

Fig. 3. Mesothelial cells (arrows) detach from the basement membrane and float freely in the perilymph to entrap bacteria within the scala tympani. Toluidine blue staining

Fig. 4. More than one type of entrapment was commonly seen. Balloon-like entrapment (B) within the round window membrane, extension of cellular processes (thick arrow) and free-floating mesothelial cells (thin arrows) can be seen. Toluidine blue staining

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Animal Model

Effect of Topical Glucocorticoid Treatment in Chinchilla Model of Lipopolysaccharide Induced Otitis Media with Effusion

Sang Gyoon Kim, MD[1], Timothy Jung, MD, PhD[1], **Charles Pudrith**[2], You Hyun Kim, MD[2], **Andrew Florea, MD**[2], Seong Kook Park, MD, PhD[2], Michael Wall, PhD[3]
[1]VA Loma Linda Healthcare System, [2]VA Loma Linda Healthcare System, Dr. Jung's otology lab, Loma Linda, CA, [3]Alcon Research, Ltd., Fort Worth, TX

Introduction

Otitis media with effusion (OME) is a common childhood disease. Among manycausative factors of OME,infectionand eustachian tube dysfunction are most common. These twofactorsstimulate middle ear epithelial and inflammatory cells to secrete inflammatory mediators such as cytokines, eicosanoids and nitric oxide which increase vascular permeability and secretory activity resulting inmiddle ear effusion(MEE).[1,2]

Typically, OME is treated with antibiotics, corticosteroids, or antihistamines with and without decongestants.[3] On the other hand, glucocorticosteroids may be a beneficial treatment of OME by stimulating Na(+) transport and fluid clearance in the middle ear epithelia. Dexamethasone has been shown to significantly increase expression levels of mRNAs and proteins of Epithelial Sodium Channel (ENaC)-alpha and -beta subunits.[4] The mineralocorticoids also have anti-inflammatory effects by their fluid transport function(.[5]

Previous studies have shown that a combination of antibiotics and glucocorticoidsare more effective in treating OME than antibiotics alone.[6,7,8] Topical application has also been shown to be more effective than systemic treatment becauseit reduces side effects while achievinghigher local concentration.[6,7,8]

Ciprofloxacin-dexamethasone has not been reported to cause ototoxicity in other animal models. (9) Since topical dexamethasone hinders TM healing by anti-inflammatory action in animal models, caution may be needed.[10]

Currently, ciprofloxacin 0.3%/dexamethasone 0.1% otic suspension (Ciprodex®; Alcon Laboratories, Inc., Fort Worth, Texas; Ciprodex® is a registered trademark of Bayer AG licensed to Alcon) and ofloxacin 0.3% otic solution are the only two ear drops approved by the FDAfor ototopical treatment of the middle ear (table 1).Roland et al. has reported that ciprofloxacin/dexamethasone is superior tofluoroquinolone treatment alone based on time to cessation of otorrhea and clinical cures.[11,12] Though both dexamethasone sodium phosphate (DSP) and hydrocortisone have previously been approved by FDA for use in ear drops (dexamethasone sodium phosphate ear drops and neomycin/polymyxin B/hydrocortisone (NPH) ear drops, respectively), these products have been indicated for external ear canal.Due to the potential aminoglycoside ototoxicity, NPH is contraindicated when middle ear is exposed to the drops.

Rimexolone has been approved for the treatment of non-infectious inflammation of the anterior eye segments because it is much less likely to increase intraocular pressure compared to other corticosteroids.[13] This characteristic has been seen in other corticosteroids that do not have a hydroxyl group in the 21st position.[14] The reduction of pressure may be beneficial to OME treatment due to the otalgia experienced in acute otitis media (AOM).

Fluticasone propionate (FP) is a commonly prescribed glucocorticoid used to treat allergic rhinitis. It is more effective topicallydue to extensive liver metabolism if taken orally and, thus has a low potential for side effects.[15,16] Like dexamethasone, FP is fluorinated, which increases the reductace activity, thereby increasing the anti-inflammatory properties of the glucocorticoid .[17]

Methods

One Hundred and sixty-two chinchillas were divided into 12 groups based on test substances used for treatment. Each chinchilla was injected with 200µl of test substances into both superior bulla after the animal was anesthetized with isofluorane (Baxter, Dearfield, Illinois). Two hours later, animals were injected 300µl of lipopolysaccharide (LPS) at 1mg/ml (Sigma/Aldrich, St. Louis, MO, USA). Formulations of test substances (corticosteroids at 0.1% and 1% and vehicle) Alcon Research, Ltd., Fort Worth, Texas) were re-administerd 24 and 48hrs after LPS injection.Ninty-six hours after LPS injection, animals were euthanized by decapitation following ketamine (Bedford Laboratories, Bedford, Ohio) IM injection (40mg/kg). Middle ear effusion volume were collected and measured. Temporal bones were harvested in 10% formalin. Portions of inferior bullae were dissected out from the temporal bones, decalcified in 5% ethylene diaminetetraacetic acid for 5 days, dehydrated in graded ethanol, and embedded in paraffin. Paraffin blocks were sectioned at 20µm. Every 10th section was stained with hematoxylin and eosin for

microscopic examination. The MT was measured from the slides under an inverted microscope (Zeiss, Goettingen, Germany).Means of MEE and MT samples were compared with vehicle control using the paired t-test to determine significance of each group. Dose dependencies were evaluated by comparing 0.1% and 1.0% measurements of each glucocorticoid with the paired t-test.

Results

Middle Ear Effusion (MEE)

After LPS-induced OME, all glucocorticoids except fluticasone propionate numerically reduced middle ear effusion at0.1% concentration. At 1.0%, all glucocorticoids numerically reduced volume of MEE. Dexamethasone sodium phosphate and rimexolone were most effective in reducing MEE after 0.1% and 1.0% treatments, respectively. Dexamethasone and DSP significantly ($p<0.05$) reduced MEE at both concentrations. Fluticasone propionate and rimexolone only reduced MEE when treated with 1.0% concentrations. Hydrocortisone failed to significantly reduce MEE at either concentration. A dose-dependent correlation for fluticasone propionate and rimexolone was statistically established in reduction of MEE.

Mucosal Thickness (MT)

All glucocorticoids except rimexolonenumerically reduced MTat0.1% concentration. At 1.0% concentration, all samples numerically reduced MT. Dexamethasone sodium phosphate reduced MT to the lowest thickness at both concentrations. Dexamethasone sodium phosphate and hydrocortisone significantly reduced MT at both 0.1% and 1.0% concentrations. Dexamethasone significantly reduced MT only at 1.0% concentration, whereasfluticasone propionate significantly reduced MT at 0.1% concentration for any glucocorticoid. Rimexolone failed to reduce MT at either concentration.Statistically, a dose-dependent MT reduction was not established with any glucocorticoid treatment.

Conclusion

Glucocorticoids reduce MEE volume and MT of lipopolysaccharide induced OME in the chinchilla model. Dexamethasone sodium phosphate was the only drug to significantly reduce both MEE volume and MT at both 0.1% and 1% concentrations. Dexamethasone sodium phosphate reduced MT to the lowest value at both concentrations, and MEE volume to the lowest value at 0.1% concentration. Further study is needed to determine the safety and efficacy of dexamethasone sodium phosphate.

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Measuring Thickness of Middle ear Mucosa Using MRI and CT Imaging vs. Histopathology

Mary Ann Nyc[1,2]**,** Sang Gyoon Kim, MD[1,2]**, Anil Kapoor**[2]**,** Timothy Jung, MD, PhD[1,2]
[1]Division of Otolaryngology-Head & Neck Surgery, Loma Linda University School of Medicine, Loma Linda, CA, [2]Otolaryngology Research, Jerry L. Pettis Veterans Medical Center, Loma Linda, CA

Introduction

Variation exists in the clinical diagnosis of otitis media (OM). For the majority of clinicians, successfully diagnosing OM is achieved from accurate otoscopic examination in which bulging of the tympanic membrane is evidenced along with pervasive erythemia around the ear canal[1]. Traditionally, the causes of OM are classified as eustachian tube dysfunction or infection[2]. Consistent with findings of infection, increased middle ear effusion (MEE) coupled with inflammation of middle ear mucosa and

increase of mucosal thickness (MT) take place as part of the body's immune response to foreign pathogens. Unchecked, misdiagnosed, or inappropriately treated, OM can persist chronically and lead to complications. In order to control and prevent OM progression, some imaging modalities have been used to diagnose and follow-up with treatment progression. Though there are many imaging tools and few guidelines for their use, it does seem that anatomic imaging vis-à-vis CT or MRI is frequently favored.

In the scientific literature, MRI is often used when more accurate views of soft tissue are needed [3, 4]. Conversely, CT is used to show the extent of bony changes [4]. In terms of severe OM cases, CT use is important to identifying early stages of disease and for assessing progression that involves bony regions. Though MRI and CT seem to have clear delineations for their use, they are frequently usedconcurrently and interchangeably.

Therefore, the aim of this study was two fold:

1-To compare two glucocorticoid treatments in order to evaluate drug effectiveness in reducing overall MT.

2-To evaluate and compare the use of radiographic tools (MRI and CT) in visualizing OM changes following treatment.

Methods

Animals

28 chinchillas were divided into three treatment groups: 6 underwent treatment by vehicle control, 11 were treated with Ciprodex®: ciprofloxacin 0.3%/dexamethasone 0.1% (DEX), and 11 received Cipro HC® : ciprofloxacin 0.2%/hydrocortisone 1% (HC).The vehicle control was composed of the sterile, preserved suspension used to make Ciprodex and Cipro HC and was obtained from Alcon, Inc.

Ciprodexand Cipro HC were obtained as a sterile otic suspensions. Both are combination antibiotic (quinolone), steroid treatment commonly used by clinicians to treat acute OM externa and acute OM following tympanostomy tube placement or with tympanic membrane perforation.

Ciprodex and Cipro HC are registered trademarks of Bayer AG licensed to Alcon, Inc and are available by prescription only.

Procedure

Chinchillas were injected via the superior bullae with 0.2ml of their respective treatment. Two hours later, chinchillas were inoculated with 0.3ml of *Salmonella enteric*lipo-polysaccharide (LPS) and additional 0.2ml treatments of respective treatments were injected into the inferior bullae at 24, 48, and 72 hrs post-LPS inoculation. At 120 hrs post inoculation, chinchillas were euthanized using an 80mg/kg ketamine intramuscular injection. Following animal sacrifice, chinchilla temporal bones were preserved in 10% formalin for 72 hrs, then washed in 10X PBS, and finally stored in 10XPBS, 0.1% azide at 3°C.

Radiographic Imaging

MRI: 18 intact chinchilla temporal bones were taken to the Non-Invasive Imaging Laboratory at Loma Linda University and imaged using Brucker MRI 11.7T, which produced T-2 weighted images (Field Of View= 3cm; Slice Thickness=1mm; Interslice Distance=1mm; No of Slices=30; No of Echoes=10; Matrix=256x256; Resolution Read=0.0117cm/pixel; Repetition; Time=3593.9ms; Echo Time=10.2ms; Slice Orientation=sagittal). Temporal bones were dried before imaging processes to reduce effects of water. MRI images were viewed and analyzed using Cheshire© v4.3 software, where MT was measured using the ruler tool which produced measures in pixels. MT was then calculated into mm given that 1 pixel is equivalent to 0.117mm. 10-11 MT measures were calculated per specimen for 36 chinchilla middle ears (18 temporal bones x 2 sides = 36 middle ear cavities), providing an N=380 data points.

CT: 14 middle ear cavities that were MRI imaged were taken to the VMU at the Jerry L. Pettis Veterans Medical Center for imaging using Scanco vivaCT 40 (Slice Thickness=10.5 microns; No of Slices=140). Due to the fact that the specimen were half of the MRI imaged samples, a radiographic pin was inserted into the inferior bullae to facilitate CT imaging and maintain imaging consistency. Once again, specimen were dried before imaging processes to reduce effects of water. Images were analyzed using Scanco software. MT was measured in two ways: bone to mucus ratio, and mucosal volume.

Histology

Following radiographic imaging, all chinchilla specimen were decalcified using 3M EDTA over a four week period with 3 EDTA changes. Following decalcification, specimen were further reduced to a quarter of their original size and placed in a paraffin processor overnight. Paraffin embedded samples were sectioned at 15 microns over approximately the bottom-most 6mm area of the inferior bullae. Sections were later stained by H&E and analyzed using an Olympus biological microscope with digital camera DP72. The DP72 computer software was used for image capture. MT measures were made in Adobe PDF Converter Professional off the images at unit length mm.

Statistics

SAS 9.1 was used to analyze significance of MT values. Microsoft Excel was used to generate bar charts and tables.

Results

MRI MT values were evaluated by treatment groups. The data set consisted of N=380 data points, with an overall and treatment group stratified distribution that appeared normal. ANOVA testing produced statistically different measures of MT among treatment groups ($F=146.0861$, p-value<0.0001). Post-hoc testing by Tukey revealed significant ($\alpha=0.05$) mean differences between treatment groups.

CT MT values were also evaluated by treatment groups. The data set was smaller, with an N=14. The overall and group stratified distribution was grossly nonparametric. Kruskal-Wallis was used to analyze global differences across treatment groups, but did not find any statistical significance when analyzing bone to mucosal thickness ratios ($\chi^2=1.2404$, p-value=0.5378) and total average calculated mucosal volume ($\chi^2=0.9762$, p-value=0.6138). No additional post-hoc testing was conducted since it was not warranted.

Histology MT values were evaluated by treatment groups. The data set consisted of N=45 measures. The overall and group stratified distributions were non-normative. Kruskal-Wallis was used to evaluate difference across treatment groups and revealed a significant finding ($\chi^2=23.4121$, p-value<0.0001). Post-hoc testing with Tukey was conducted on the data and showed statistically significant differences ($\alpha=0.05$) between groups.

For both MRI and histology findings DEX-treated samples exhibited reduced MT compared to HC-treated and vehicle control subjects.

Discussion

Previous research investigating OM found that diagnosis and prognosis using anatomical imaging such as CT and MRI were preferred over nuclear medicine approaches. Furthermore, researchers found that by comparison to CT, MRI was better at exploring soft tissue changes over time [4].Similarly, an investigation into chronic OM affecting the temporo-mandibular joint found the utility of MRI for imaging intra-cranial soft tissue masses and thrombosis of sinus tranversus to evaluate mastoiditis prior to therapeutic surgical techniques[5]. In this same study, investigators also used CT to reveal anatomical changes in bone and surrounding soft tissue mass, but found that MRI provided more detailed analyses of infection severity by imaging soft tissue swelling[5]. Unofficial consensus favors MRI for its cost effectiveness, availability, high resolution, image detail, and absence of radioactivity [6].

In this investigation, we found additional support for MRI use in imaging inflammation and specifically for tracking OM by means of MT. Our MRI data was easy to extract and analyze, producing an adequate sample size estimation of MT outcomes across groups. Our CT data were based on a relatively smaller sample size due to imaging demands and resources. CT results were somewhat harder to evaluate because of the high bone resolution and lower resolution mucosal layer. Given that our MRI and CT data were not exactly comparable, and previous reviews have commented on the inaccuracy of imaging OM [7], histology served as the point of reference. From histology results, we are able to confirm the utility of MRI for imaging MT as an inflammatory marker of OM.For future investigations, however, contrast enhanced MRI-imaging of in-vivo, inoculated specimen would provide greater detail of OM processes and changes over time; in-vivo imaging practices would parallel clinical manifestations more accurately.

This investigation also highlighted the promising role of DEX for improving inflammation of OM. MT measures across treatment groups using all analytic methods (MRI, CT, and histology) showed that DEX was more effective at decreasing MT. The use of DEX in combination with antibiotics seems to be a more effective treatment to reduce inflammation in the middle ear. MRI seems to be a better imaging method of determining stage and severity of acute or chronic OM.

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Therapeutic Tissue Engineering with Peptide Hydrogel for the Promotion of Mucosal Regeneration in the Middle Ear of SD Rat

Naotaro Akiyama, MD[1]**,** Tomomi Yamamoto-Fukuda, MD[1]**,** Yoshitaka Hishikawa, MD[2]**,** Takehiko Koji, PhD[2]**,** Haruo Takahashi, MD[1]
[1]Department of Otolaryngology-Head and Neck Surgery, [2]Department of Histology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Nagasaki
Running head: *in situ* tissue engineering of middle ear mucosa with peptide hydrogel

Introduction
Middle ear mucosa plays an important role in maintaining normal middle-ear pressure. The presence or absence of the mucosa in the mastoid (mastoidectomized or not) has beenwell correlated with whetherthe posterior meatal wall retractsafter surgery; in other words, whetherthe mastoid cavity revives as an aerated cavity.[1] If mucosal cells regenerate from the tympanic cavity to the mastoid after surgery, adhesion within the mastoid caused by scar contraction may be prevented, and in turn prevent retraction of the posterior meatal wall. In a previous study,[2] a sheet of mucosal cells grown on collagen gel was used to promote mucosal regeneration in animal models, and successfully implanted in the middle ear following surgery. However, considering the clinical application, there is a need of an*in situ* tissue engineering model for physiological regeneration. In this study, we examined the usefulness of isolated mucosal epithelial cells mixedwith BD™ PuraMatrix™ Peptide Hydrogel (BD Biosciences, California, USA), a polymer that has been successfully used as a scaffold in tissue engineering.

Materials and Methods
Cell preparation
We prepared donor cells from four SD-TG (CAG-enhanced green fluorescence protein [EGFP]) rats by explant culture,[3]in order to use EGFP-expressed cells as a tracer for transplantation. Middle-ear epithelial cells were obtained from the middle ear mucosa. They were cultured in the collagen-Icoated dish with a culture medium (DMEM:BEGM (Small Airway Epithelial Basal Medium) (Cambrex, New Jersey, USA) =1:1). The outgrowth cells were subculturedup to three passages.

The cultured middle ear epithelial cells from the SD-TG rats were observed under a phase contrast microscope for morphological analysis and EGFP expression confirmed under a fluorescent microscope. To characterize the cells, we performed immunohistochemistry using anti-pancytokeratin as an epithelial cellular marker (Novocastra, Newcastle, UK) and anti-vimentin as a mesenchymal cellular marker (Dako, Glostrup, Denmark).The above primary antibodies were used as previously described.[4]

Transplantation
The cultured mucosal epithelial cells from the SD-TG rats were mixed with the peptide hydrogel; the culture medium was then added at a concentration of $1x10^6$ cells/ml. The SD rats (n=3) were used as recipients; their middle ear mucosa had been eliminated surgically under anesthesia with pentobarbital before transplantation. We demonstrated an immunohistochemical analysis of mucosal elimination models and confirmed that epithelial cells were completely eliminated from the lesion of the middle ear in this model. Donor cells mixed with the peptide hydrogel were transplanted into the middle ears of the SD rats. After transplantation, recipients were immuno-suppressed with FK506 by intraperitoneal injection[5]from Day 0 to Day 5(0.32 mg/kg/day) and administrated penicillin Gvia intramuscular injection each day(22,000 U/kg/day).Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation, Nagasaki University, with approval of the Institute of Animal Care and Use Committee.

Analysis of regeneration after transplantation
At seven days after transplantation, the middle ear bullae with the transplanted cells were collected and frozen immediately on dry ice. Embedded in an optimum cutting temperature[OCT] compound, serial frozen sections of middle ear bullae after transplantation were cut to a 5µm thickness and placed onto glass slides using the film method. EGFP-labeled transplanted cells from the middle ear bullae were analyzed under a laser scanning microscope. Adjacent sections were processed for immunohistochemical analysis of pancytokeratin and vimentin to confirm the formation of mucosal epithelium from the transplanted cells,thenstained with Schiff's Reagent (Periodic Acid Schiff [PAS] staining) to evaluate function.The Schiff's Reagent was used according to the McManus method.

Results

The mucosal epithelial cells from the SD-TG rats showed a cobblestone appearance, and EGFP expression was stable up to the third passage as shown under a phase contrast microscope.The results of immunohistochemistry of the anti-pancytokeratin and anti-vimentin antibodies in the mucosal epithelial cells at the third passage collected by cytospin revealed that the percentage of cells positive for pancytokeratin but not for vimentin was 98.6%.In one of the three cases, EGFPexpressed cells were found on the bone surface of the host middleear bullae (Table1, Fig.1a). These cells were positive for pancytokeratin but not for vimentin (Figs.1b, c). PAS stain-positive cells were detected in some of the epithelial cells (Fig.1d).

Discussion

In this study, we evaluated the effect of peptide hydrogel as scaffolding for tissue engineering using middle ear epithelial cells from CAG-EGFP rats.As a result, the EGFP expression of the cultured cells was found to be stable up to the third passage. Using these cultured cells, we established a new animal model of the transplantation of isolated middle ear epithelial cells mixed with hydrogel. We found the EGFP expressed cells on the surface ofthe subepithelial lesion of the middle ear after elimination, and these cells were positive for pancytokeratin but not vimentin. It suggested that transplanted donor cells had actually migrated into the host tissue. Moreover, the results of PAS staining indicated that the migrated epithelial cells produced some mucus proteins. Finally, we found that peptide hydrogel could promote the proliferation of donor cells in the host tissue, and retain the character of the middle ear epithelial cells.

FK506 was provided by Astellas Pharma Inc. (Tokyo, Japan).

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Table 1. EGFP expression after transplantation

samples	EGFP (+)	EGFP (-)
1.0×10^6 cells/ml + hydrogel + medium (n=3)	1	2
1.0×10^6 cells/ml + medium (n=3)	0	3

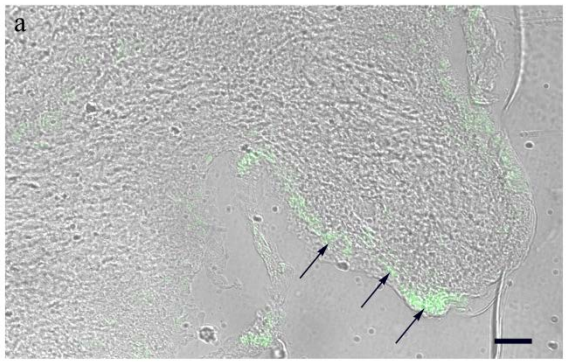

Fig.1a

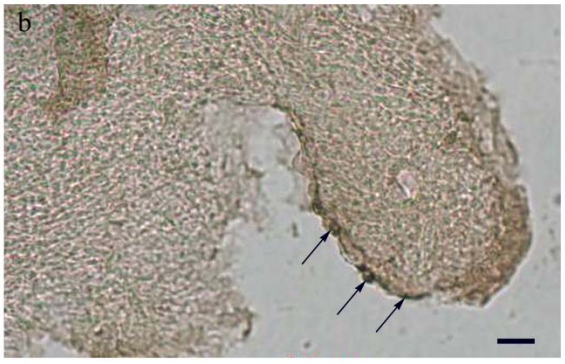

Fig.1b

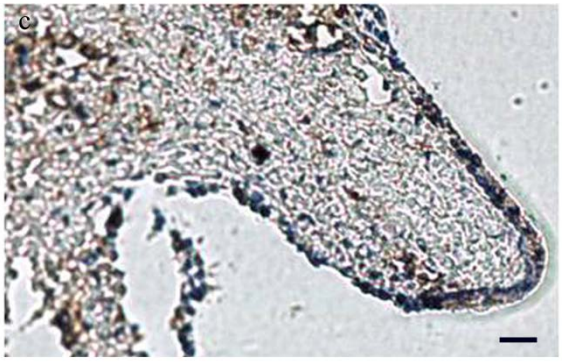

Fig.1c

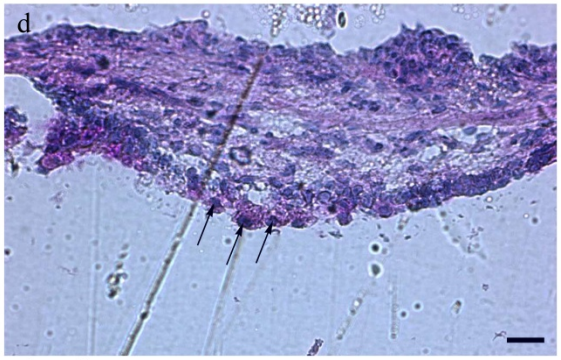

Fig.1d

Fig.1. Analysis of regeneration after transplantation.EGFP-expressed cells were detected on the bone surface of the host middle-ear bulla under a laser scanning microscope (arrows) (a). Intense staining of pancytokeratin was detected in these cells (arrows) (b), but vimentin-positive cells were scarcely found (c). PAS staining-positive cells were detected in some epithelial cells(arrows) (d).Bars=20μm.

Respiratory Syncytial Virus Promotes Ascension of *Moraxella catarrhalis* into the Chinchilla Middle Ear in a Polymicrobial Model of Experimental Otitis Media

Elizabeth Brockson, Joseph Jurcisek**,** Glen McGillivary, PhD**,** Martha Bowers**,** Lauren Bakaletz, PhD
Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital and Ohio State University College of Med, Columbus, Ohio

Otitis media (OM) is a polymicrobial disease wherein prior or concurrent infection with an upper respiratory tract virus is frequently observed. In episodes of acute bacterial OM, respiratory syncytial virus (RSV) is the most commonly isolated virus and thus likely serves as an important co-pathogen with one or more bacterial species. Of the predominant bacterial agents of OM, the pathogenesis of disease due to *Moraxella catarrhalis* is the least well understood. Rigorous study of *M. catarrhalis* in the context of OM has been significantly hindered by lack of a relevant animal model.

To bridge this gap, we assessed whether co-infection of chinchillas with RSV and *M. catarrhalis* would facilitate ascension of *M. catarrhalis* from the nasopharynx into the middle ear. To do so, juvenile chinchillas were challenged intranasally with *M. catarrhalis* strain 7169 followed 48 hrs later by intranasal challenge with RSV strain A2. Within 7 days, 100% of animals were colonized in the nasopharynx with *M. catarrhalis* and these animals were also culture-positive in homogenates of middle ear mucosa. Moreover, within the middle ear space, the mucosa exhibited hemorrhagic foci and serosanguinous middle ear fluid was observed. Thus, these data demonstrated that infection with RSV predisposed to *M. catarrhalis*-induced disease. The continued development of this animal model will facilitate an understanding of *M. catarrhalis* pathogenesis in this polymicrobial disease. Additionally, this model has potential to provide a means to investigate strategies to prevent or treat OM due to *M. catarrhalis*.

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Investigation of the Novel Otitis Media Mouse Mutant, *edison*: Elaborating the Genetic Pathways Involved in Otitis Media

Michael Crompton[1], Steve Brown, PhD[1], Martin Burton, MD[2], Michael Cheeseman, PhD[1]
[1]Mammalian Genetics Unit, MRC Harwell, Harwell, Oxfordshire, [2]Nuffield Department of Sugical Sciences, John Radcliffe Hospital, Oxford, Oxfordshire

Objectives
Edison is a new genetic mouse model of otitis media (OM) recently discovered by the ENU phenotype-driven mutagenesis program at MRC Harwell. On preliminary characterisation,*edison*demonstrates chronic OM andis recessive.Detailed phenotypic characterisation will be carried out along with identification of the underlying gene involved.Other OM mouse mutants, *Jeff* and *Junbo*, have been shown to affect signaling of the TGF-β superfamily and the middle ear of the *Junbo* mutant is hypoxic. So in addition, the hypoxic state of the middle ear and perturbations to the TGF-β pathway will be assessed.

Methods
A longitudinal study has been set up to characterise the onset of OM.Fine mapping and analysis of next generation sequencing data was used to identify potential candidate genes involved in producing the mutant phenotype. Once the gene is identified and verified to be the causative gene, we can investigate expression in whole-body tissue and middle ear tissue.

Results
The mutation has been mapped to chromosome 14 and the mutation lies within a critical region between 17.31Mb and 31.75Mb. Next generation sequencing of this interval has identified a putative functional SNP which may underlie the phenotype, and will be explored further.

Conclusions
Edison could represent a third monogenic non-syndromic mutant mouse that develops chronic OM. Overall, the work aims to elaborate further the underlying genetic aetiology of OM, providing further insight into the pathways involved and the role of TGF-β or hypoxia signaling, and presenting a new candidate gene for human chronic OM.

Regeneration Potential of Tympanic Membranes in Animal Models with Acute or Chronic Perforations

Han Bin Lee, Yun-Hoon Choung, MD, PhD, Seung Won Kim, MD, Yeon Ju Kim, Keehyun Park, MD, PhD, Hun Yi Park, MD, Hye Jin Lim, MD, Oak-Sung Choo, MD
Otolaryngology, Ajou University School of Medicine, Suwon

Introduction
While spontaneous healing is commonly seen in acute perforations of tympanic membranes, most of chronic perforations (CTMPs) are incapable of this process. The purpose of the study was to evaluate the presence of epidermal stem cells in TMs and to analyze regeneration potentials of TMs in the state of acute or chronic perforations in vivo animal models.

Materials and Methods
Sprague-Dawley neonatal rats (P3-5) were used for culture of TM cells. The cultured TM cells were immunostained with cytokeratin 19, integrin ß1, p63 (epidermal stem cell marker), Ki67 (proliferation marker), and DAPI. Rat models for acute or chronic perforations in TMs were prepared. Chronic perforations were produced by a new method using combination techniques of thermal myringotomy, mitomycin C and steroid. The TMs were regularly obtained from rat models with acute or chronic perforations and immunostained with cytokeratin 19, integrin ß1 integrin a6, p63, Ki67, and DAPI. The maintenance rates of chronic perforations were analyzed in animal models according to perforations sites.

Results
TM-derived cells showed the co-expression of cytokeratin 19, integrin ß1, p63 and Ki 67. For immunostaining, normal TM cells showed weak expression of cytokeratin 19 only around malleus handle. TMs with acute perforations revealed highly expression of cytokeratin 19, integrin ß1 and integrin a6 around malleus handle and annulus areas, but not intermediate portions of pars tensa. TMs with chronic perforations showed weak expression of integrin ß1 and cytokeratin 19 around malleus handle and annulus. In animal models for chronic TM perforations, the perforation success rates according to sites were higher in cases involving malleus handle (7/11), lower annulus (3/6), or posterior-inferior quadrant (4/8) than other cases involving upper annulus (2/7) or anterior-inferior quadrant (1/10).

Conclusions
TMs contain progenitor or epidermal stem cells and the most important locations for regeneration may be areas close to malleus handle or annulus. Regeneration ability of TMs is induced in the state of acute perforation, however weakened in the status of chronic perforation.

Tympanic Membrane Perorations- The Early Immunological Answer and Application of Growth Factors

Malou Hultcrantz, MD, PhD[1], Jamel Tahar Aissa[2]
[1]Otorhinolaryngology, CLINTEC, Stockholm, Stockholm, [2]Karolinska Institutet, Clintec, Stockholm, Stockholm

Introduction
Chronic tympanic membrane perforations, frequently seen after recurrent otitis media or after tube insertions, are a sequelae which often needs surgical treatment. It would be of great benefit for the child if the perforation could heal without surgery. An animal model of chronic tympanic membrane perforation will be required for further studies on healing using different agents instead of surgery.

Objectives
10 Sprague Dawley rats were used in the present study as well as 15 edges of human tympanic membrane perforations.

Methods
The rats were anesthetized and the tympanic membrane was perforated with a laser beam of 0.4 mm on both sides. The right eardrum was exposed to a solution of Granulocyte Macrophage Colony Stimulating Factor (CM-CSF). The time for healing and the immunological answer after different time points (1 and 3 h, 3,6 and 12 days) were tested with antibodies against macrophages, T-cells and B cells. A semiquantification of number of cells was performed.
The human specimens were harvested at surgery and prepared for immunohistochemistry with antibodies against macrophages, T-cells and B cells

Result
At the end of the study, the ear drums of the 12 rats treated with CM-CSF showed a slightly better healing time than the controls. Immunohistochemical pattern of antibody label showed a specific pattern in both the animal and human tympanic membrane.

Conclusions
An animal model for chronic tympanic membrane perforations has been established and the immunohistochemical pattern has been calculated.

Genetics

Association of Single Nucleotide Polymorphisms in Surfactant Protein ï¿½A and ï¿½D with Otitis Media

Catherine M. E. Barnett[1], Anthony Cecire, MD[2], Ray Cursons, PhD[3]
[1]School of Medicine, The University of Queensland, Brisbane, Queensland, [2]Department of Otorhinolaryngology, Waikato Hospital, Hamilton, Waikato, [3]Department of Biological Sciences, The University of Waikato, Hamilton, Waikato

Introduction
Pulmonary surfactant is present in the middle ear and pharyngotympanic tube.[1, 2] In the lungs, surfactant is synthesized to reduce the surface tension at the alveolarair-liquid interface and is composed of phospholipids, cholesterol, and four surfactant proteins.[3] Surfactantproteins (SP) A and D are important components of the innate immune system via opsonisation and aggregation of pathogens.[4-6] In addition to the well-known individual and environmental risk factors, there is also a genetic predisposition to developing otitis media[7].The human SP-A locus has two functional, highly homologous genes (*SP-A1* and *SP-A2*) located onchromosome 10q22-q23 in a cluster along with the SP-D gene[8]. Some alterations in *SP-A1*, *SP-A2*, and *SP-D* are known to be associated with a range of respiratory diseases.[9-12] This study proposed that any reduced immuneactivity of SP-A and SP-D may predispose an individual to otitis media. Such a change could occur within the protein's coding DNA, where a single nucleotide substitution may result in an altered protein primary sequence and subsequent function.Single nucleotide polymorphisms (SNPs) in theSP-A and SP-D genes have been well documented.[13] Linkage of the genes allows for all thepolymorphisms to be assembled into haplo types:combinations of alleles of different SNPs along the same chromosome which can be inherited as a unit. Polymorphisms in the SP-A loci have been found to be associated with otitis media in both Finnish and American populations.[14,15]

Objective
Investigate allele and haplo type frequencies for polymorphisms in the genes *SP-A1*, *SP-A2*, and *SP-D*in New Zealand patients with recurrent acute otitismedia (RAOM) or otitis media with effusion (OME)compared to a control population.

Method
Using allele-specific real-time PCR, eleven SNPsin *SP-A1*, *SP-A2*, and *SP-D* were genotyped in 160 unaffected individuals (control population) and in136 individuals diagnosed with RAOM or OME, inorder to identify any associated alleles/haplotypes. The SNP loci investigated were situated at codons19, 50, 62, 133, and 219 of *SP-A1*, and codons 9,91, 140, and 223 of *SP-A2*. Lastly, SNPs at *SP-D*codons 11 and 160 were examined.Simple $\chi 2$-tests were carried out using Haploview©to calculate any significant difference in allele and haplotype frequencies between the case and control populations.Ethical approval was obtained from the University of Waikato Human Research Ethics Committee and the Northern Y Regional Ethics Committee, Ministry of Health (Ethics Reference Number: NTY05/05/030).

Results
Compared to the control population, the population with RAOM/OME had significantly higher frequencies of two *SP-A1* all eles and one *SP-A2* allele (Figure 3). One *SP-A1* haplotype (CGAGC) and three *SP-A2* haplo types (AGTC, AGTA, CCTA), were significantly more frequent in the case population (figure not shown). Haplo types TGAAC and CGCC were significantly less frequent. No significant differences in the *SP-D* haplo type frequencies were seen.

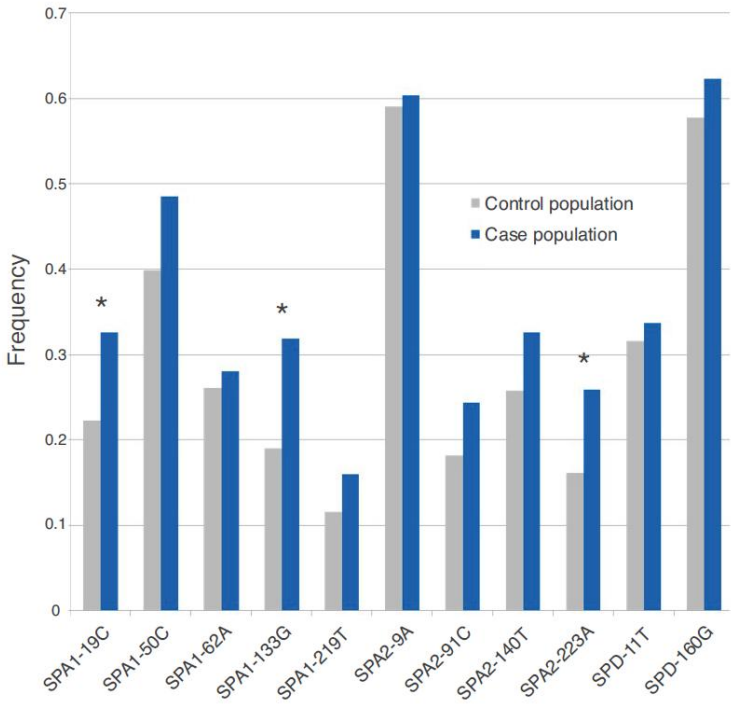

Figure 1. SNP frequencies in the control and case populations. Significant differences are marked by an asterix ($P < 0.05$).

Conclusion

This study showed that in a New Zealand populationwith RAOM/OME, two *SP-A1*alleles (19C, 133G), one *SP-A2* allele (223A), and four haplo types were over represented in comparison to a control population.Conversely, two *SP-A1* haplo types were under represented.No significant difference in *SP-D* variants between the populations was seen. In conclusion, specific genetic variants of the surfactant proteins found in the middle ear appearto be associated with altered risk of developing RAOM and/or OME. However, there are numerous socio-economic and environmental factors that also contribute to OM patho genesis. The immune activity of SP-A and SP-D variants needs further investigation to confirm the association with susceptibility or resistance to otitis media, and most importantly, how this information can be clinically applied.

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Significant Linkage Identified at Chromosome 19q for Chronic Otitis Media with Effusion And/or Recurrent Otitis Media (COME/ROM)

Emma Allen[1], Wei-Min Chen, PhD[1], Josyf Mychaleckyj, PhD[1], Fang Chen, PhD[1], Xuanlin Hou[1], Stephen Rich, PhD[1], Kathleen Daly, PhD[2], Michele Sale, PhD[1]
[1]Center for Public Health Genomics, University of Virginia, Charlottesville, Virginia, [2]Department of Otolaryngology, University of Minnesota, Minneapolis, Minnesota

Significant linkage identified at chromosome 19q for chronic otitis media with effusion and/or recurrent otitis media (COME/ROM)

Objectives
In previous analyses, we identified a region of chromosome 19as harboring a susceptibility locus for chronic otitis media with effusion and/or recurrent otitis media (COME/ROM). Our aim was to further localize the linkage signal and ultimately identify the causative variant or variants.

Methods
We followed up our previous linkage scan with dense SNP genotyping across in a 5-Mb region. A total of 607 individuals from 139 families, including 159 affected sib pairs and 62 second-degree affected relative pairs, were genotyped at 1,233 SNPs. We carried out a nonparametric linkage analysis, modeling marker-to-marker linkage disequilibrium.

Results
The maximum log of odds (LOD) score increased from 2.61 to 3.75 ($P=1.6\times10^{-5}$) at position 63.4Mb, with a 1-LOD support interval between 61.6Mb and 63.8Mb, providing significant evidence of linkage between this region and COME/ROM. The LOD-1 interval contains 90 known genes, including several genes involved in the inflammasome protein complex, a key regulator of the innate immune response to harmful exogenous or endogenous stimuli.

Conclusion
Parametric linkage analysis of our data suggests that for a sib of an affected individual, the recurrence risk of COME/ROM that is due to this linkage region is 2 times higher than the recurrence risk in the population. We are currently sequencing the LOD-1 intervalto identify variants contributing to COME/ROM.

Genetic Analysis of Hypoxia Pathways in Acute and Chronic Otitis Media

Hayley E. Tyrer, Michael T Cheeseman, PhD**,** Paul Potter, PhD**,** Steve D.M. Brown, PhD
Mammalian Genetics Unit, MRC, Harwell, Oxfordshire

Introduction
Development of Otitis Media (OM) is multi-factorial with a significant genetic component. The mouse mutant *Junbo*(*Evi1*$^{Jbo/+}$)discovered in an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen at MRC Harwell spontaneously develops chronic OM (COM). The observed phenotype results from a missense mutation in the transcription factor*Evi1*.*Evi1* acts on TGF-β/SMAD signalling that links with hypoxia pathways. As middleear exudates and mucosa of the *Evi1*$^{Jbo/+}$mouse arehypoxic, this mutant is a useful tool to examine the role of hypoxia in COM.

Methods
We have generated compound mutants of the *Evi1*$^{Jbo/+}$ mousewhere hypoxia inducible factor-1α (*Hif-1α)* and von-Hippel Lindau (*Vhl)* floxed alleles are deleted in a global (ERT2^{Cre}) or myeloid cell (LyzM^{Cre}) specific manner. Consequently,effects ofgain or loss of Hif-1α in the middle earcan be examined. Phenotyping involves auditory brainstem response, gene and protein expression

analysis of middle ear fluids and cellular *in vitro* studies. Mice that do not develop COM but contain*Hif* or *Vhl* deleted alleles will be challenged with nontypeable *Haemophilus influenzae*, a bacteriumcausing acute OM in humans.

Results
Progress concerning compound mutantgeneration and preliminary phenotyping data will be reportedupon. Initial phenotyping of $Hif^{fl/fl}$LyzM$^{Cre/+}$$Evi1^{Jbo/+}$mice reveals OMstill develops, suggesting myeloid cells may not be the main contributor inCOM.

Conclusions
Current treatments for COM utiliseventilation tubes, however their method of action is not well established. This study should helpidentify the role of hypoxia in acute and COM, potentially identifying a new molecular pathway for therapeutic intervention.

Molecular Biology/Immunology

Otitis Media, Childhood Respiratory Infections and Developmental Enamel Disturbances

Kari Kvaerner, MD, PhD[1], Øyvind Asmyhr[2], Jostein Grytten, PhD[2]
[1]Research, Innovation and Education, Oslo University Hospital, Oslo, Norway, [2]Institute of clinical dentistry, University of Oslo, Oslo, Norway

Introduction
There is increasing interest in the causes of developmental enamel disturbances. Although no causal relationship has been found, special attention has been paid to otitis media, infectious childhood illnesses, pre- and perinatal conditions. Tooth development is strictly genetically controlled but sensitive to environmental disturbances. Once formed, any insults are detectable as defects in the mature enamel.

Objective
To assess the relationship between molar incisor hypomineralisation (MIH) and otitis media, childhood infections, asthma and allergy.

Study design
The study is based on the Norwegian recruit study conducted by the Norwegian Defence Forces. Data from the study population of 2273 recruits were collected by clinical and radiological examinations and questionnaire information. Main outcome measure was the clinically estimated molar incisor hypomineralization. Information on otitis media, otitis media surgery and childhood illnesses were collected as questionnaire information. In addition, there is an ongoing application to the medical Birth Registry for the collection of pre- and perinatal birth information.

Results
Of the 2273 recruits, 3% were females and average age at participation was 21 years. The prevalence of molar incisor hypomineralisation was approximately 10%. Recurrent otitis media was experienced by 1.3% (n=347), 15.3% reported lifetime otitis media surgery and 1.8% of the study population had been hospitalised for acute otitis media during childhood years. Corresponding figure for childhood hospitalisation due to infections or allergic disease was 24.5% (565). Associations between hypomineralisation and recurrent otitis media and -surgery, respiratory illnesses, allergic disease is estimated and the final results will be presented.

Comparison of PCR Methods for Differentiation of *Haemophilus influenzae* and *Haemophilus Haemolyticus*

Michael Binks[1], Lea-Ann Kirkham, PhD[2]**,** Selma Wiertsema, PhD[2]**,** Eileen Dunne, PhD[3]**,** Peter Richmond, MD, PhD[2]**,** Amanda Leach, PhD[1]**,** Heidi Smith-Vaughan, PhD[1]
[1]Menzies School of Health Research, Charles Darwin University, Darwin, Northern Territory, [2]School of Paediatrics and Child Health, University of Western Australia, Perth, Western Australia, [3]International Child Health, Murdoch Childrens Research Institute, Melbourne, Victoria

Background
Unambiguous identification of nontypeable *Haemophilus influenzae* (NTHi) is not currently possible. Molecular characterisation of phenotypically defined NTHi isolates has revealed that up to 40% are *Haemophilus haemolyticus* (Hh).

Objectives
To identify an optimal PCR test for differentiation of NTHi from Hh in clinical isolates.

Methods
Seven ATCC reference isolates and 60 phenotypic NTHi isolates classified by 16S PCR as NTHi (22), Hh (27) or equivocal (11) were further characterised by PCR of lgtC, iga, fucK, 16S, omp P6, omp P2 and hpd genes. A novel PCR-HRM (high resolution melt) using omp P6 was also developed. Receiver operator characteristic (ROC) curves were used to assess the sensitivity and specificity of each PCR.

Results
Reference isolates
omp P6, omp P2, hpd1 and hpd3 were positive exclusively for the NTHi reference strains. Other targets lacked sensitivity (lgtC) or specificity (fucK, iga and 16S amplified related species).
Clinical isolates
Differentiation of NTHi, Hh and equivocal isolates varied between PCRs. The ROC curves indicated that the omp P2 (0.80 [0.69, 0.91]) and hpd3 (0.80 [0.68, 0.91]) PCR results are most akin to the 16S confirmed results.

Conclusions
The best candidate target genes for PCR are the omp P2, omp P6, hpd1 and hpd3 for which results were consistent. The omp P2 and hpd3 PCR results were most comparable to the 16S PCR results, however 16S and recA sequence phylogeny is necessary to delineate the equivocals and qualify the best target.

Clinical Evaluation of Enzyme-Linked Immunosorbent Assay (ELISA) (ODK-0902) for Detection of *Haemophilus influenzae* Antigen in Nasopharyngeal Secretion and Middle Ear Fluids

Gen Sugita, MD[1], Muneki Hotomi, PhD[1], Rinya Sugita, MD[2], Akihisa Togawa, PhD[1], Masamitsu Kono, MD, MD[1], Masaki Hayashi, PhD[1], Yorihiko Ikeda, PhD[1], Yuki Tatsumi[1], Shinji Tamura, PhD[1], Noboru Yamanaka, MD[1]
[1]Otolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama-shi, Wakayama-ken, [2]Ear-Nose-Throat, Sugita-ENT-Clinic, Ichikawa, Chiba-ken

Introduction
Haemophilus influenzae is one of the major causative pathogens responsible for acute otitis media (AOM) and acute rhinosinusitis (ARS). There has been an alarming increase in ß-lactamase non-producing ampicillin resistant *H. influenzae* (BLNAR) worldwide with special emphasis on AOM. For treatments of AOM, it is important to determine *H. influenzae* accurately and rapidly as a causative pathogen. We developed a novel immunochromatographic antigen detection kit (ODK-0902) for detecting P6 antigen of *H. influenzae* in middle ear fluids (MEFs) and nasopharyngeal swabs from AOMand ARS.

Method
A total 523 samples, 257 MEFs and 265 nasopharyngeal swabs were obtained from patients with AOM and used in this study. We identified *H. influenzae* in both specimens by immunochromatographic antigen detection, real-time PCR and conventional bacterial culture. Informed consent was obtained by patients themselves, or their guardians in case of infant patients.

Results
The sensitivity and specificity of ODK-0902 were 83.8 % (75/90) and 85.6 % (143/167) in the middle ear fluid or otorrhea sample of otitis media patients, and 71.5 % (113/158) and 92.5 % (99/107) in the nasopharynx secretion sample of rhinosinustis patients, respectively.

Conclusion
We clearly indicated that ODK-0902 would be a noble tool for identifying the pathogen as *H. influenzae* in MEFs and nasopharyngeal secretions. The rapid immunochromatographic test shows high specificity and will be attractive for identifying *H. influenzae* in MEFs and nasopharyngeal swabs in the era of antimicrobial resistance.

Changes in Innate and Adaptive Immunologic and Inflammatory Gene Regulation When Children Develop Acute Otitis Media (AOM) Caused by *Streptococcus pneumoniae* (Spn)

Keyi Liu, PhD, Michael Pichichero, MD
Research Institute, Rochester General Hospital, Rochester, New York

Objective
To define changes in gene expression patterns involving immunologic and inflammatory responses occurring during AOM caused by *S*pn.

Methods
cDNA microarray was performed using the Human Whole Genome OneArray™ on PBMCs from 4 children prior to and at the onset of *Spn* induced AOM. Only changes in gene expression >1.5 fold occurring in all 4 of the studied children were considered in the analysis as significant.

Results
Of 30968 genes profiled, 3949 genes were differentially expressed during AOM compared to the pre-infection stage; 1823 genes were up-regulated and 2126 were down-regulated. Among these we limited our analysis to potentially relevant genes involved in the immune and inflammatory response to infection. Important gene families that had changes in regulation were:

Gene Family	Regulated gene No	% Up-regulated	% Down-regulated
Cytokines & chemokines	21	43%	57%
Complement	7	86%	14%
TLR	5	60%	40%
T cell response	19	26%	74%
Cell adhesion	43	67%	33%
Apoptosis	29	66%	34%
Inflammatory response	23	96%	4.%
Response to bacteria	18	67%	33%

Genes for IgG and IgE were regulated.

Conclusion
Significant changes consistently occur in innate and adaptive systemic immune and inflammatory responses during *Spn* infection in the middle ear. Differences in such changes among child populations such as otitis prone vs. non-prone may provide insight for therapeutic or preventative interventions.

Supported by NIH NIDCD RO1 08671.

Impaired Innate Mucosal Defense in Chd7 Mice Lead to Increased Susceptibility to Otitis Media

Cong Tian[1], Peter Scacheri, PhD[2], Heping Yu[1], Fengchan Han, PhD[1], Bin Yang, MD[1], Cindy Benedict-Alderfer, PhD[1], Qing Yin Zheng, MD[1]
[1]Otolaryngology, [2]Genetics, Case Western Reserve University, Cleveland, Ohio

Heterozygous mutation in Chd7 gene is reported to cause CHARGE syndrome. Patients with CHARGE syndrome showed symptoms such as growth retardation and abnormalities in eye, heart, choana, reproductive organ, and ear/hearing. Among the diseases discovered in the ears of CHARGE syndrome patients, otitis media was seen in near 100% of the patients. Although mouse models for human CHARGE syndrome have been reported and several features were studied on these mice, otitis media and related hearing loss have not been reported and studied yet. Here, we report a mouse model with a spontaneous mutation in the Chd7 gene, which is a deletion of exon 2 and 3, showing chronic otitis media with effusion. Otitis media in the Chd7 mutant mice is characterized by early onset age, tympanic membrane retraction, middle ear effusion, and hearing loss, which is mainly caused by impaired innate mucosal defense and partially by eustachian tube dysfunction. This is the first report of otitis media in the Chd7-deficiency mice and will facilitate the study of Chd7 in auditory system function and development.

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Capsular Switching Enhances Virulence of Emerging *Streptococcus pneumoniae* Serotype 6C in Experimental Otitis Media Model (EOM)

Vishakha Sabharwal, MD, Abbie Stevenson**,** Marisol Figueira, MD**,** Stephen Pelton, MD
Pediatrics Infectious Diseases, Boston Medical Center, Boston, MA

Introduction
Capsular switch events in which a pneumococcal strain is the recipient of the capsule operon from a donor strain of a different capsular serotype have been described with increasing frequency following the introduction of PCV7. These capsular switch events enable pneumococci to acquire new phenotypic traits through natural transformation. Thus, vaccine serotype chassis with acquired non vaccine capsules, creating new combinations with increased virulence compared to the original nonvaccine strain, may be created.

Specific Aim
To compare the capacity of serotypes 6A wt, 6C wt, and 6A-C capsular transformant (6A chassis with 6C capsule) to produce EOM in a chinchilla model.

Methods
A virulent 6A and non virulent 6C strain were used to create a capsular transformant with the 6A chassis and the 6C capsule. The 6A wt, 6C wt and the 6A-C strains were compared by Pulse Field Gel Electrophoresis (PFGE). Complement (C3) binding, using flow cytometry, was measured for each of the three strains. Virulence was determined in the chinchilla EOM model- which employs initial nasopharyngeal colonization followed by barotrauma. The proportion of chinchillas that developed culture positive EOM, density of middle ear infection and proportion of chinchillas with bacteremia were compared.

Results
The 6A-C and the 6A wt were identical on PFGE. The 6A-C bound less complement compared to 6C wt. EOM was found in 15/22 (68%) ears challenged with 6A-C transformant compared to 4/32 (12.5%) with 6Cwt [p=0.00003]. Disease due to 6C wt was characterized by low density infection (~10E2) when compared 6A-C transformant (~10E4) and 6A wt (~ 10E5).

Conclusions
The capsular transformant 6A-C demonstrates reduced C3 binding and increased virulence (both proportion of ear infected and density of infection) compared to 6C wt confirming the potential of capsular switch events to result in new phenotypes.

The Role of the Innate Immune Regulator ASC in a Murine Model of Otitis Media

Stephen Wasserman, MD[1], Jasmine Lee[2], Kwang Pak[2], Bruce Beutler, MD[3], Chelsea Wong[2], Allen F. Ryan, PhD[2]
[1]Medicine, UCSD, La Jolla, California, [2]Surgery, Otolaryngology, University of California, San Diego, La Jolla, CA, [3]Genetics, The Scripps Research Institute, La Jolla, CA

Otitis media (OM) caused by NTHi in the mouse characteristically demonstrates mucosal thickening and cellular infiltration peaking day 2-3 with resolution by day 5-7. Membrane-associated innate immune receptors including TLR-2, TLR-4 and TLR-9, induce TNF production, to activate CCL3 in a cascade critical to OM resolution. An alternative, intracellular innate immune pathway acts to produce the inflammasome, a complex important in generation of activated IL-1 and IL-18, but its role in OM is unknown.

To assess the potential role of this pathway we employed gene array analysis of middle ear (ME) tissue from mice infected with NTHi, and identified marked up-regulation of the IL-1 and IL-18 pathways 24-72 hours after infection: IL-1 (40-72 fold), IL-18 (3.5 fold), IL-1 receptor 1 (7-8 fold), IL-18 binding protein (3 fold), and IL-18 receptor accessory protein (5 fold). ASC (apoptosis-related speck-like protein) is key to this process, binding to the pyrin domain of the family of intracellular innate immune receptors known as NALPS (aka NLRPs) and linking them to caspase-1 forming the inflammasome. ASC mRNA was up-regulated 3 fold during OM.

We then compared ME inflammatory and bactericidal responses to NTHi, between ASC knockout and normal mice. Animals lacking ASC showed prolonged inflammation and delayed bacterial clearance from the ME.

Our results support the role of ASC, and indirectly that of intracellular innate immune recognition mechanisms, in OM. They also provide new insight into potential causes of recurrent and/or persistent OM in children.

Alternative Complement Pathway-Dependent Complement Activation in the Middle Ear During Acute Pneumococcal Otitis Media in Mice

Hua Hua Tong, MD, Qian Li, MD, PhD**, Yong Xing Li, MD**
Otolaryngology, The Ohio State University, Columbus, Ohio

Objectives
We have recently reported that the complement system plays a pivotal role in innate immune defense against *Streptococcus pneumoniae* (*S. pneumoniae*) during acute otitis media (OM) in mice. The purpose of this study was to define complement components synthesis as well as specific complement pathway activation in the middle ear of mice during acute pneumococcal OM.

Methods
Complement gene and protein expression in the middle ear mucosa in a mouse model of pneumococcal OM was examined by real time PCR, ELISA and western blot. Complement activation fragments in the middle ear of wild type and complement deficient mice were analyzed by western blot and Immunofluorescence staining. Opsonophagocytosis of *S. pneumoniae* by neutrophils in the middle ear was evaluated with alaser scanning confocal microscope.

Results
We demonstrated that *S. pneumoniae* induced an increased gene expression of factor B and C3 of the alternative complement pathway in the mouse middle ear epithelium. Additionally, the simultaneous activation of factor B and C3 were more significant in the middle ear lavage fluid samples than its corresponding serum samples. Using mice deficient in complement C1qa, factor B and factor B/C2, We found that complement C3 activation and opsonophagocytosis of *S. pneumonia*in the middle ear were greatly attenuated in factor B and factor B/C2 deficient mice.

Conclusions
Our data indicate that the alternative complement pathway activation plays a central role in controlling the middle ear innate immune response to pneumococcal infection during the early stage of acute OM.

Serum IgG Levels to Pneumococcal and *H. influenzae* Vaccine Candidate Protein Antigens Are Not Impaired in Children with RAOM

Selma Wiertsema, PhD[1], Karli Corscadden[1], Lea-Ann Kirkham, PhD[1], Eva Mowe[1], Shyan Vijayasekaran, MD[2], Harvey Coates, MD[2], Timothy Mitchell, PhD[3], Wayne Thomas, PhD[4], Peter Richmond, MD[1]
[1]School of Paediatrics and Child Health, [2]Department of Otolaryngology, Head and Neck Surgery, University of Western Australia, Perth, Western Australia, [3]Department of Infection and Immunity, Glasgow University, Glasgow, Scotland, [4]Molecular microbiology, Telethon Institute for Child Health Research, Perth, Western Australia

Objective
There are no combined data on the immunogenicity of vaccine candidate protein antigens of S. pneumoniae and nontypeable H. influenzae (NTHi) in children and their role in carriage and acute otitis media.

Methods
Using a bead-based multiplex assay, we investigated serum IgG levels against pneumococcal antigens PspA1, PspA2, CbpA and Ply and NTHi proteins P4, P6 and PD in 247 children under 3 years of age either with or without a history of rAOM. Anti-protein antibody levels were correlated with nasopharyngeal bacterial carriage status.

Results
Children with rAOM had significantly higher geometric mean IgG levels against NTHi proteins P4, P6 and PD compared to healthy controls (P4: 182.0 AU [Arbitrary Units] vs 74.1 AU, p=0.001; P6: 831.8 AU vs 407.4 AU, p=0.01; PD: 871.0 AU vs 398.1 AU, p=0.02). Antibody levels against pneumococcal proteins PspA1, PspA2, CbpA and Ply were similar in both groups of children. NTHi was the predominant pathogen carried and infecting children with rAOM, suggesting a correlation between carriage/AOM and the development of antibodies to conserved protein antigens.

Conclusions
Pneumococcal and NTHi proteins are immunogenic in children with rAOM, suggesting these proteins could be promising candidates for an OM-targeted vaccine. The functionality of these antibodies remains to be investigated.

Effect of Phosphorylcholine on Mucin Production and Gene Expression in the Middle Ear in the Mice

Tarou Iwasaki, MD, Takashi Hirano, MD, MD, Satoru Kodama, MD, MD, Masashi Suzuki, MD, MD
Otolaryngology, Oita University, Yufu city, Oita

Introduction
Phosphorylcholine (PC) is a structural component of a wide variety of pathogens and also possesses immunomodulatory properties. Lipooligosaccharide (LOS) in Nontypeable Haemophilus influenzae (NTHi) undergoes phase variation in expression of the PC epitope. We reported that the expression of PC in the surface of NTHi is an important factor in the pathogenesis of persistent otitis media with effusion (OME) in humans and the expression of PC may have affected the characteristic of MEEs of the mucoid type when tympanostomy was performed. In this study, we examined the effect of PC on mucin production and gene expression in the middle ear in the mice.

Methods
Balb/c mice were used in this study. PC-conjugated bovine serum albumin (PC-BSA) was dissolved with phosphate buffered (PBS) saline to the concentration of 10mg/ml. 10µl of PC-BSA solution was infected into the right tympanic cavity. As a control group, 10µl of BSA solution (10mg/ml) were also injected. At 1, 3, 7 and 14 days after the injection, middle ear washing and middle ear tissues were collected.
Middle ear tissues were used for histological evaluation and mucine gene expression by RT-PCR.

Results
In PC-BSA treated mice, the number of goblet cells in the middle ear tended to increase when compared to BSA control mice. In mucin gene expression, Mac5ac and Mac2 mRNA expressions were higher in PC-BSA treated mice than those in BSA control mice.

Conclusion
PC may be related to change the mucin gene expression and the number of goblet cells in the middle ear.

A Flow Cytometric Analysis of Fab Mediated Inhibition of Adherence of Pneumococcus to Human Nasopharyngeal Cells: Role of Three Surface Proteins as Adhesins

Sharad Sharma, PhD, Nadeem Khan, PhD**,** Laura Filkins**,** Michael Pichichero, MD
Research Institute, Rochester General Hospital, Rochester, New York

Introduction
Streptococcus pneumoniae (*Spn*) is the major cause of acute otitis media and Invasive Pneumococcal Disease (IPD) in children and adults. Adherence of pneumococcus to the human epithelial cells in the upper respiratory tract is a primary step in pneumococcal pathogenesis hence, the development of antibody mediated immunotherapy that blocks *Spn* adherence to the host cells is a prerequisite of the anti-pneumococcal vaccination. Moreover, determining the role of surface proteins as major surface adhesins in pneumococcal adherence is helpful in the vaccine design against pneumococcus. In the present study, we developed a flow cytometry based approach that facilitates evaluation of *Spn* adherence to the human nasopharyngeal epithelial cells (Detroit 562). IgG from an adult human sera having high titers of anti-*Spn* antibodies blocked *Spn* adherence to the D562 cells however, upon further analysis the mechanism of the inhibition of *Spn* adherence to the host cells was found due to aggregation of the *Spn* cells by total IgG used for blocking, as assessed by flow cytometry and microscopic analysis. On the other hand, Fab fragments prepared from total IgG did not aggregate bacterial cells and blocked bacterial adherence to the host cells which indicates a precise blockage of the bacterial cell surface adhesions by used Fab fragments. Moreover, role of three pneumococcal surface proteins (Phtd, PcpA & PhtE) in adhesion to the host cells was observed by depleting individual protein specific Fab fragments from total Fab sample. Among three surface proteins used in the assay for Fab depletion, eliminating PcPA specific Fab fragments demonstrated an increase in the *Spn* adherence to the D562 cells that indicates a major role of PcpA as a *Spn* adhesin. Hence, the present methodology of using the flow cytometry based analysis involving the usage of *Spn* specific Fab fragments for adherence blocking holds promise in evaluating the precise blocking of adherence to the host cells as well as defining the role of surface adhesins.

Materials and Methods
Bacteria, pneumococcal proteins and antibodies:
Non-encapsulated strain of *Streptococcus pneumonia* (*Spn ;* Rx1) grown in Todd Hewitt Broth (THB) supplemented media. *Spn* surface exposed choline binding protein PcpA, autolysin LytB and a derivative of the secreted protein pneumolysin (PlyD1) were procured from Sanofi Pasteur.
Purification of total IgG from serum and Fab Fragments Preparation:
IgG from human adult serum sample was purified using a IgG purification kit (Pierce). Fab fragments were prepared as published earlier (3).
Fluorescent labeling of Streptococcus pneumoniae:
Bacterial cells were labeled with 7.5 μM PKH2 Green Fluorescent Cell Linker Kit (Sigma-Aldrich, PKH2GL) according to manufacturer's instructions at a concentration of about 5 x 109 CFU/ml in the diluent for 5 minutes.
Adherence assays:
PKH2 labeled *Spn* cells were added to D562 cells and incubated for 30 minutes. Finally, cells were re-suspended in 200μL PBS and 10,000-50,000 cells were studied on a LSR II flow cytometry (BD Biosciences).

Results
1. Preparation of Fab fragments from total IgG

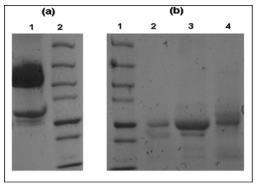

Figure 1: Preparation of Fab fragments from purified IgG: (a) Lane1: Purified IgG; Lane 2: Protein molecular weight marker **(b)** Lane 1: Protein weight marker; Lane 2 & 3: Purified and concentrated Fab fragments respectively; Lane 4: crude papain digested IgG.

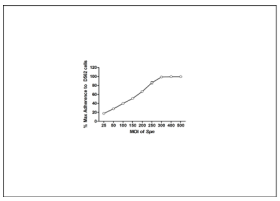

Figure 2: Determination of MOI for assay: D562 cells were incubated with various multiplicity of infection (MOI) of PKH2 labeled *Spn* and samples were analyzed by flow cytometer. Assay demonstrated a dose dependent increase in *Spn* adherence.

2. Human IgG inhibits Spn adherence to D562 cells by aggregation of Spn.

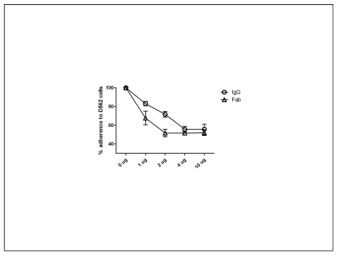

Figure 3: IgG- and Fab-mediated inhibition of adherence: D562 cells were incubated with 200 MOI of labeled *Spn* and adherence observed in the presence of varying concentrations of purified IgG or Fab fragments. Relative adherence for each concentration was calculated based on maximum adherence. Fab treatment showed better adherence inhibition at similar concentration as of IgG.

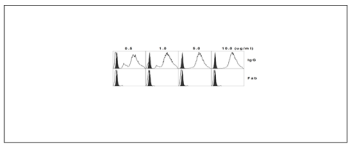

Figure 4: Aggregation of labeled *Streptococcus pneumoniae* in the presence of purified human IgG and Fabs fragments: Forward scatter of histograms: IgG treatment (upper panel, open histograms) caused bacterial aggregation at different concentrations whereas Fab treatment (lower panel, open histograms) did not. Solid (filled) histograms show negative control.

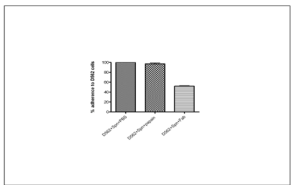

Figure 5: *Spn* adherence to D562 cells with controls: *Spn* adherence with PBS and papain control (a control for the Fab digest method) as well as Fab preparation. A significant decrease in adherence was seen with 1 µg of Fab preparation (p= 0.012).

3.Use of Fab fragments from IgG inhibits Spn adherence to D562 cells without aggregation.

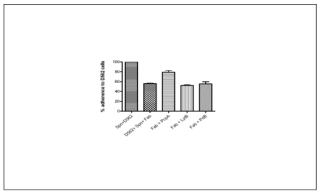

Figure 6: Adherence inhibition with anti-PcpA depleted Fab fragments: PcpA, Lyt B and PlyD1 specific Fab fragments were depleted from the total Fab samples. The depletion of PcpA specific Fab resulted in a significant increase in the adherence (p = 0.023).

4. Pneumococcal surface protein, PcpA, contributes to Spn adherence to D562 host cells.

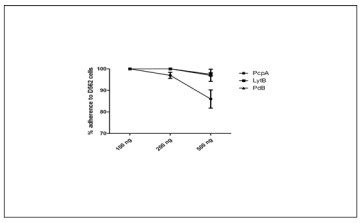

Figure 7: PcpA mediated adherence blocking: *Spn* adherence in the presence of different Spn proteins (PcpA, PlyD1, and LytB). At the concentration used in the adherence assays (100 ng), proteins alone did not inhibit adherence.

Conclusions

Surface adhesin specific IgG blocks bacterial adherence to the D562 cells by causing *Spn* aggregation rather than specific blocking.

Use of adhesin specific Fab preparation is a preferred method for studying bacterial adherence and role of protein adhesins.

Choline binding protein PcpA contributes to adhesion of *Spn* to the D562 cells.

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Induction of Specific Immune Responses Against *Streptococcus pneumoniae* by Maternal Immunization with Pneumococcal Surface Protein a (PspA)

Masamitsu Kono, MD[1], Muneki Hotomi, MD, PhD[1], Yorihiko Ikeda, MD, PhD[1], Susan Hollingshead, PhD[2], David Briles, PhD[2], Noboru Yamanaka, MD, PhD[1]
[1]Otolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama, Wakayama, [2]Microbiology, University of Alabama at Birmingham, Birmingham, Alabama

Introduction
Despite their successes, pneumococcal polysaccharide vaccines must include multiple polysaccharide serotypes and are not protective against strains with capsular types/groups not present in the vaccines. Furthermore, children younger than 2 years old usually have lower levels of pathogen specific IgG antibody in sera due to the age-related immaturity of immune responses. In this study, we investigated the specific immune responses in offspring delivered from mother mice maternal immunized with pneumococcal surface protein A (PspA).

Method
BALB/c bjy mice, 4 wks old female, were intranasally immunized with PspA mixed with cholera toxin B subunit for 2 to 3 weeks and mated with male mice to obtain offspring. We evaluated the protection against pneumococcal nasal carriage, pneumonia, and sepsis in offspring and the production of IL-4, IL-17A and IFN-g by splenocytes of offspring by ELISA.

Results
The levels of anti-PspA specific IgG in sera from offspring breast-fed by PspA-immunized mothers were maintained during day 7 to day 14. The pneumococcal carriage density of nasal tissue and lung homogenate in PspA maternal immunized offspring was significantly lower than those in controls ($p<0.05$). The survival after fatal systemic pneumococcal infections in PspA maternal immunized offspring was significantly extended compared with those of controls. In offspring delivered from PspA-immunized mother and breast-fed by PspA-immunized mother, IL-17A was produced but IL-4 and IFN-g were not produced by splenocytes.

Discussion
Although immunity to pneumococcal diseases has long been assumed to depend mainly on the humoral immune response, recent studies suggested antibody-independent immunity mediated by IL-17A in mice model. IL-17 was also involved in the immune responses induced by maternal immunization. Maternal immunization could effectively protect both pneumococcal nasal carriage and invasive infections. Maternal immunization would be an attractive procedure against pneumococcal infections in early childhood.

Effect of Regulatory T Cells on Bacterial Clearance in Vitro Assay

Takashi Hirano, PhD, MD[1], Satoru Kodama, PhD, MD[2], **Toshiaki Kawano, MD**[2], **Kazuhiko Maeda, PhD, MD**[2], **Masashi Suzuki, PhD, MD**[2]
[1]Faculty of Medicine, Department of Otolaryngology, Oita University, Yuhu, Oita, [2]Faculty of Medicine, Department of Otolaryngology, Oita University, Yufu, Oita

Introduction
Previously, we made a murine model of chronic OME induced by a combination of inoculation with nontypeable *Haemophilus influenzae* (NTHi) into the bullae and eustachian tube blockage via an external surgical approach, and the mice were sacrificed at day 3, 2 weeks, 1, 2 and 6monthes after the inoculation. In this model, we examined the relationship of chronic inflammation in experimental otitis media on changes of mucosal immune competent cells in middle ear mucosa. Bacteria counts from middle ear effusions were detected in 38% of treated mice at 6 months after the inoculation. In the chonic state of otitis media, the ratio of CD4+FoxP3+T cells also increased and maintained up to 6 months after the inoculation (data not shown). These cells mainly belonged to CD4+CD25+ cells. These findings indicated that natural regulartory T cells may affect the bacterial clearance from the middle ear in the chronic state of otitis media. To elucidate the role of regulartory T cell, we isolated the regulartory T cells from spleen and investigated the effects of these cells on innate immune responses by using *in vitro* assay.

Materials and Methods
Animals
Balb/c male mice, 5weeks old, were used in this study. The mice were maintained in the facility for at least 1 week before the study.
The interaction of natural regulatory T cell and neutrophil
To investigate the effect of natural regulatory T cells on the neutrophil phagocytotic function , neurophils (Ly-6G-positive cells) and natural regulatory T cells (CD4+CD25+ cells) isolated from spleen were purified with the anti-Ly-6G MicroBead kit and natural regulatory T cell MicroBead isolation kit by using MACS cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany).

CD4+CD25+ cells, CD4+CD25- cells and Ly-6G-positive cells were divided into three groups as shown in Figure 1. These cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine in 24-well culture plates. NTHi solution (10^6cfu) was added to all wells of the culture plate. At 1 and 6 hours after the incubation, culture mediums were collected and these mediums were diluted serially in PBS, and 10 μl of each dilution then plated on chocolate agar. Bacterial colonies were counted after overnight incubation. After the samples were centrifuged at 120×g for 10 minutes. The precipitations in these samples were examined using real time RT-PCR assay for IL-10, TGF-b and FoxP3 mRNA expression. For the detection of these mRNA, total RNA was extracted from the precipitations . The primers and probes for for 18rb, IL-10, TGF-b and FoxP3 mRNA used in this study are commercially available and were obtained from Applied Biosystems (Foster City, CA). Extraction of total cellular RNA and the first cDNA synthesis were performed with commercially available RNA extraction and reverse transcription kits (Qiagen, Tokyo, Japan). Real-time PCR data were analyzed with Sequence Detection software, version 1.6, included with the 7700 Sequence Detector (Perkin-Elmer Applied Biosystems). Final quantitation was derived by the comparative CT (threshold cycle) method and was reported as the fold difference relative to a calibrator cDNA (spleen cells were used as a standard control) prepared in parallel with the experimental cDNAs.

Result

Effect of Natural regulatory T cells on bactericidal function of neutrophil.

The number of NTHi in the culture mediums in CD4+CD25+ T cell group is significantly higher than that in the other groups at 1 hours incubation, especially that in Ly-6G cell group. This tendency continued at 6 hours incubation, and there is a significant difference between Ly-6G cell and CD4+CD25+ T cell group (Table 1).

IL-10, TGF-b and FoxP3 mRNA expression of the cultured cells

IL-10 mRNA expression in CD4+CD25+ T cell group is significantly higher than that in Ly-6G cell group at 1hour incubation. FoxP3 mRNA expression in CD4+CD25+ T cell group is significantly augmented when compared to in CD4+CD25- T cell and Ly-6G cell group throughout this experiment. TGF-b mRNA expression in CD4+CD25+ T cell group is higher than that in other groups, however there is no significant difference statistically (Table 2).

Conclusion

In this study, we investigated the effect of natural regulatory T cells on neutrophil function. Natural regulatory T cell (CD4+CD25+ T cell) inhibited bactericidal function of neutrophil.CD4+CD25+ T cell inhibited the bactericidal function against NTHi. Real time RT-PCR study indicated that CD4+CD25+ T cell had more inhibitory effect than other cells. On the basis of these results, natural regulatory T cells may play an inhibitory effect on innate and acquired immunity, and may be related to pathological findings of chronic otitis media.

Table 1

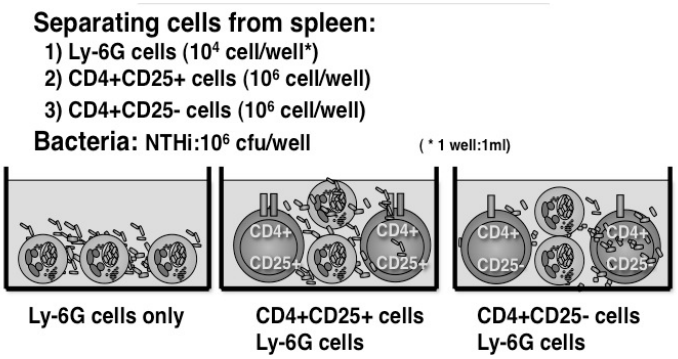
	1 hour	6 hours
Ly-6G only	110±14*	6946±185*
CD4+CD25+	200±31	22680±2762
CD4+CD25-	150±14*	18133±5780

Unit: cfu/ml , *: $p<0.05$, compared to CD4+CD25+ group

Table 2

	1 hour			6 hours		
	IL-10	TGF-b	FoxP3	IL-10	TGF-b	FoxP3
Ly-6G only	7.3±4.3	0.9±0.6	1.4±0.5	114±68	3.3±1.3	2.2±0.3
CD4+CD25+	238±10	2.3±1.5	331±93	92±19	4.2±1.4	171±26
CD4+CD25-	154±31	1.4±0.4	7.3±3.3	159±34	2.9±0.7	1.9±0.3

Figure.1



Innate Immune Response in a Mice of Acute Otitis Media with Moraxella Catarrhalis

Toshiaki Kawano, MD[1], Takashi Hirano, MD, PhD[1], Takahiro Mitsui, PhD[2], Kamruddin Ahmed, PhD[2], Akira Nishizono, PhD, MD[2], Masashi Suzuki, PhD, MD[1]
[1]Department of Otolaryngology, [2]Department of Microbiology, Oita University Faculty of Medicine, Yufu, Oita

Introduction
Moraxella catarrhalis(M. catarrhalis), a gram-negative diplococcus, is the third most common isolate after Streptococcus pneumoniae and nontypeable Haemophilus
influenzae as the causative agent of acute otitis media. Previous our study indicated that pili of M. catarrhalis plays an important role in eliciting an innate immune response via Toll-like receptor(TLR) 5 in mRNA level.

Methods
We used B-88-152 strain:pili(+) and F strain:pili(-) of M. catarrhalis suspension. Middle ear effusions(MEEs) were collected at 6,12,24 hours from mice in each group after the injection with pili(+) or pili(-)of M. catarrhalis. To analyze the TLR5 expression on neutrophil in the MEE responding to M. catarrhalis injection, middle ear washes were collected. Two color staining for inflammatory cells in MEE was performed with FITC-conjugated anti-CD11b monoclonal antibodies and PE-conjugated anti-TLR5 mAb, and flow cytometric analysis was performed.

Results
Neutrophil accumulated rapidly in the middle ear, and this finding was related to the bacterial clearance from the middle ear. TLR5 mRNA and protein level expression induced by M. catarrhalis pili(-) of FCM was weeker compared with M. catarrhalis pili(+) from 6 to72 hours.

Conclusion
TLR5 recognition of pili regulates early neutrophil recruitment to the middle ear membrane and influences persistence of middle ear inflammation up to 72h after injection. These data may address the relation of pili of M. catarrhalis microbial toTLR5 regarding microbial recognition.

Does IL-10 Regulate Intercellular Cell-Adhesion Molecule-1 Level Differently in Otitis Prone Children?

Keyi Liu, PhD, Michael Pichichero, MD
Research Institute, Rochester General Hospital, Rochester, New York

Objective
Intercellular cell-adhesion molecule-1 (ICAM-1) is expressed on endothelial and immune cells, and regulated in response to pro-inflammatory cytokines. Our recent work suggests it is a potential biomarker for acute otitis media (AOM) and may be used as a drug target for systemic immunosuppression in AOM treatment. In this study we sought to identify the ICAM-1 status and its regulation in otitis prone children.

Methods
Serum sICAM-1 was measured with ELISA and gene expression regulation was tested from PBMCs with real-time RT-PCR.

Results
We found that serum sICAM-1 was elevated (350 to 600ng/ml) in 10 *nontypeable Haemophilus influenzae* (*NTHi*) and 8 *Streptococcus pneumoniae* (*Spn*)-infected otitis prone children compared to healthy controls (232ng/ml). However, in the

convalescent stage, the serum sICAM-1 in otitis prone children remained elevated unlike non-otitis prone children,.At mRNA levels, of 3 *NTHi*-infected otitis prone children, two showed modest ICAM-1 expression up-regulated in the AOM stage (1.5 and 1.3 fold, respectively) compared to the pre infection healthy stage whereas in 3 non prone *NTHi*-AOM tested children a significant elevation of ICAM-1 expression was found (6.3, 2.5 and 7.3 fold change, respectively). Interestingly, the gene expression of IL-10 was also up-regulated significantly in all 3 non otitis prone *NTHi*-AOM children (2.1, 1.6, 4.5 fold change, respectively) whereas among 3 *NTHi*-infected otitis prone children, only two showed up-regulation (3.3 and 1.1 fold, respectively).

Conclusion
sICAM-1 level are different in otitis prone and non-prone children. The regulation of sICAM-1 might be controlled by IL-10.
Supported by NIH NIDCD RO1 08671.

Extracellular DNA in a Nontypeable *Haemophilus influenzae*-Induced Biofilm Serves as a Cationic Sink for Host Defense Peptides

Eric Jones, Glen McGillivary, PhD**,** Lauren Bakaletz, PhD
Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital and Ohio State University College of Med, Columbus, Ohio

Biofilms formed by the commensal bacterium nontypeable *Haemophilus influenzae* (NTHI) are frequently associated with chronic infections of the upper airway such as otitis media. The resistant nature of bacterial biofilms to mucosal immune effectors and antibiotic therapy plays an important role in NTHI persistence and disease chronicity. Extracellular DNA (eDNA) contributes to the structure of NTHI biofilms, and has been reported to co-localize with host defense molecules such as antimicrobial peptides (APs) *in vivo*. We hypothesized that eDNA would interact with cationic APs, and that this association would have important consequences for NTHI biofilm formation.

Herein, we demonstrated that the mucosal immune effector human β-defensin-3 (hBD-3) bound eDNA in a dose dependent manner *in vitro*. Furthermore, this binding completely inhibited migration of eDNA in an agarose gel, which indicated that hBD-3 altered the biochemical properties of eDNA and suggested that NTHI biofilms would be impacted as well. As such, we incubated NTHI with sub-lethal concentrations of hBD-3 and showed that NTHI biofilms demonstrated an altered architecture with bacteria arranged along unique structures, compared to medium alone. The concentrations of eDNA isolated from hBD-3-treated and untreated biofilms were equivalent, which indicated that the altered morphology was not due to increased eDNA content. Experiments to determine the functional impact of hBD-3 on biofilms formed in an experimental model of NTHI-induced OM are underway.

Together, our data demonstrated that eDNA bound hBD-3 and that this association influenced the development of NTHI biofilms. These results further suggested that the presence of eDNA in NTHI biofilms could provide a niche in which host defense peptides are sequestered and their activity diminished, which in turn contributes to the chronicity of NTHI-induced diseases.

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Complications/Sequelae

Acute Mastoiditis (AM) in Southern Israel: A 7-Year Retrospective Study (2002-2008)

Alberto Leiberman, MD[1]**, Eugene Leibovitz, MD, MD**[2]**,** E Orgad, MD, MD[2]**,** Dudi Greenberg, MD[2]**,** Marc Puterman, MD, MD[1]**,** Ron Dagan, MD, MD[2]
[1]Otolaryngology, [2]Pediatric Infectious Disease Unit, Ben Gurion University, Beer Sheva
Pediatric Infectious Disease Unit, Department of Otolaryngology, Ben-Gurion University, Beer-Sheva, Israel

Introduction
Pneumococcal conjugate vaccines (PCV) can dramatically reduce and modify pneumococcal disease in children, but their effect on AM epidemiology is unknown yet.

Objectives
To describe the experience accumulated with AM at the Soroka University Medical Center, Beer-Sheva, southern Israel

Methods
Records of all children <15 years with AM were reviewed.

Results
AM (133 episodes) occurred in 124 children (mean age 48±45 months; 30 [23%] <1 year). 94 (76%) were Jewish and 30 (24%) were Bedouin children. Mean yearly AM incidence was 10.2 cases/100,000; a significant increase was recorded in Bedouin children (from 4.2/100,000 in 2002 to 9.8/100,000 in 2008, P=0.03). AM accompanied the 1st acute otitis media (AOM) episode in 83/124 (67%) cases.Complications were recorded in 47 (35%) patients. Cultures data were available in 127 (95%) episodes. 77 pathogens were isolated; the most common were S. pneumoniae (SP) (47%), S. pyogenes (25%), nontypable H. influenzae (NTHI, 13%) and PA (13%). 20 (55%) and 2 (6%) of the SP isolates had penicillin MICs =0.1 and =1.0 µg/mL, respectively. Main SP serotypes were PCV7 serotypes 14 (21%), 19F (18%) and 6B (14%). Surgery was required in 44 (33%) patients

Conclusions
1) AM incidence was higher than reported in the literature; 2) AM following a 1st AOM episode was common, even in patients treated with antibiotics, 3) A significant increase in AM rates was recorded in Bedouin children; 4) Pathogen distribution in AM differed from that of AOM, with significantly higher S. pyogenes and lower NTHI proportions; 5) Penicillin-nonsusceptible SP played only a minor role in the etiology of AM.

Recurrent Acute Mastoiditis in Sweden 1993-2007

Anita Groth, MD, PhD[1]**,** Frida Enoksson, MD[2]**,** Ann Hermansson, MD, PhD[3]**,** Karin Stenfeldt, MD, PhD[4]
[1]Skåne, Strama, Lund, Skåne, [2]Dept of Otorhinolaryngology, Skåne University Hospital, Helsingborg, [3]Dept of Otorhinolaryngology, Skåne University Hospital, Lund, [4]Dept of Otorhinolaryngology, Skåne University Hospital, Malmö

Objective
To retrospectively study the incidence and characteristics of recurrent acute mastoiditis (AM) in Sweden during 15 years.

Methods
Data in records from patients with recurrent AM (a new episode > 4 weeks after an earlier AM episode) were extracted and compared with data on all patients treated for AM during 1993–2007 at all ENT centers in Sweden.

Results
Out of 812 cases, fulfilling the criteria for AM, 37 (5 %) experienced recurrent AM of which 30 had 2 and 7 had three episodes. About 50 % had a new AM episode within one year and the remaining part later on. Those with recurrent AM were younger than the whole group and over 50 % of the patients had their first AM episode before 2 years of age. There was no difference in general illness, signs or duration of ear symptoms, prehospital antibiotic treament or in bacterial cultures between the group of recurrent AM and the whole group. Subperiostal abscess was found in 47 % and mastoidectomy was performed in 78 % of the recurrent AM group at the first episode compared to 20 % subperiostal abscesses and 30 % mastoidectomies in the whole group. Only 3 of the recurrent AM group never underwent mastoidectomy.

Conclusions
Five percent of all cases with AM developed recurrent AM in this study. It seems as if previous mastoidectomy might predispose to recurrent AM due to easier access to the mastoid cavity causing early retroauricular symptoms and sometimes even retroauricular abscess.

Advances in OME Diagnosis by the Novel Use of Noninvasive Doppler Ultrasound

George A. Gates, MD[1], Arne H. Voie, PhD[2], Mark A. Moehring, PhD[3]
[1]Otolaryngology-Head Neck Surgery, University of Washington, Boerne, Texas, [2]Dept of Radiology, UC San Diego, San Diego, California, [3]replace, Spencer Technologies, Seattle, WA

Objective
We introduce a new concept for inspection of the tympanic membrane. Light otoscopy has been the primary method of assessing middle ear contents for over 80 years. The technology has changed little over the past 30 years.

The rationale for this work is that errors in diagnosis of middle ear effusion occur frequently. Even experts are wrong 25% of the time. Criteria for otoscopy techniques and findings vary widely. Uncertainty about the diagnosis of bacterial otitis media leads to antibiotic overuse, a well-known and a substantial contributor to the emergence of resistant bacterial strains.

Neither otoscopy, pneumatic otoscopy, nor tympanometry can determine the nature of the middle ear effusion, only its presence. We propose a new concept for assessing the middle ear contents using ultrasound Doppler technology. We believe that validation of physicians' otoscopic imputation of middle ear effusion with ultrasound will increase diagnostic precision.

Methods
We constructed a middle ear phantom into which fluids of various viscosities were instilled and the ultrasound characteristics were determined. Human temporal bone models were also tested with the same fluids.

Results
The phantom models showed distinct patterns of tympanic membrane oscillation with little overlap when air, thin fluid (water) and thick fluid (honey) filled the artificial middle ear. Similar findings occurred with human temporal bones.

Conclusion
Reliable assessment of middle ear contents will facilitate healthcare.
Doppler ultrasound technology offers improved precision and refinement in otoscopic diagnosis.

The Cavalier King Charles Spaniel: Investigating a Natural Canine Model of Otitis Media with Effusion

Michael Cohen, MD[1], Lynette Cole[2], Kenneth Ong[3], J. Douglas Swarts, PhD[4], Charles Bluestone, MD[1]
[1]Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, [2]Veterinary Clinical Sciences, Ohio State University, College of Veterinary Medicine, Columbus, Ohio, [3]College of Arts and Sciences, [4]Otolaryngology, University of Pittsburgh, Pittsburgh, PA

Objectives
The Cavalier King Charles Spaniel is the one of the only breeds of dog known to commonly develop otitis media with effusion, a condition referred to in the veterinary literature as primary secretory otitis media. One theory for the development of this maladaptive condition is that breeders selected for a short midface, or brachycephaly, which resulted in changes to the anatomy of the skull base, palate, and musculature of the Eustachian tube, with concomitant dysfunction. The objective of this study is to investigate the anatomy and physiology of the middle ear and Eustachian tube of the Cavalier King Charles Spaniel with specimen dissection and cross-sectional imaging.

Methods
The fixed heads of two Cavalier King Charles Spaniels and two mixed breed dogs were examined and imaged with computed tomography and magnetic resonance imaging in order to compare their relative physical characteristics. Dissection of the palate and nasopharynx was performed in one Cavalier King Charles Spaniel.

Results
The Cavalier King Charles Spaniel was found to have a short midface and shallow pharynx in comparison to the mixed breed specimens. Cross-sectional imaging confirmed this relationship.

Conclusions
Physical changes in the musculoskeletal anatomy of the Cavalier King Charles Spaniel, resulting from selective breeding for brachycephalic appearance, may be the cause of primary secretory otitis media in this breed. The Cavalier King Charles Spaniel may serve as a useful model for the study of Eustachian tube dysfunction and the pathogenesis of otitis media with effusion.

Occurrence and Complications of Acute Mastoiditis in Children in Southeastern Norway

Marie Bunne, MD, PhD, Greg Jablonski, MD, PhD**,** Leif-Runar Opheim, MD
ENT Department, Oslo University Hospital Rikshospitalet, Oslo

Introduction
In 2004,an unexpectedly high incidence of severe acute mastoiditis (AM) in small children was noticed at the regional hospital in Southeast Norway, serving 2,5 million people.

Objectives
Toinvestigate the occurrence, treatment and complications of AM following acute otitis media (AOM) in children < 6 years.

Method
Prospective clinical study 2005-2010. Registration of clinical, surgical, bacteriological and CT findings, and patient's history in children hospitalized for AM. In addition to systemic antibiotics and paracenthesis, mastoidectomy was performed when bone destruction was found on CT scan.

Results
In total, 116 children < 6 years were hospitalized because of AM. They represent the most severe cases in the region, thought to require surgical intervention beyond paracenthesis. Mastoidectomy was performed in 91 cases (78%). Bone destruction was found toward the middle fossa in 8 cases, the posterior fossa in 1 case, the sigmoid sinus in 6 cases. Sinus thrombosis was found in 14 cases, 3 of which had Lemierre's syndrome. There were 3 epidural abscesses, one facial paralysis, and one meningitis. Positive bacterial cultures for Streptococcus pneumoniae were achieved in 70%; in 27% cultures were negative.

Conclusions
The high and steady proportion of severe complications of AM, especially sinus thrombosis, in small children is unexpected and worrying. The reason is not obvious. There were no correlations to ethnic or cultural subgroups, nor was there any unexpected bacterial predominance. Reluctance to treat AOM in infants with antibiotics, and failure to follow up untreated cases may be explaining factors. A new prospective study of all AM in Norway, including typing of bacterial findings and focus on AOM treatment is planned.

Subperiosteal Abscess in Children with Acute Mastoiditis; Treatment and Outcome

Frida Enoksson, MD[1]**,** Anita Groth, MD, PhD[2]**,** Karin Stenfeldt, MD, PhD[3]**,** Ann Hermansson, MD, PhD[1]
[1]ENT-department, Clinical Sciences, Lund, Skane, [2]ENT-department, STRAMA, Lund, Skane, [3]ENT-department, Clinical Sciences, Malmo, Skane

Objective
To compare the outcome of different ways of treating children with acute mastoiditis and subperiosteal abscess in a retrospective study.

Methods
In a retrospective study of acute mastoiditis in Sweden during 1993-2007 there were 116 children age 0-16 years with acute mastoiditis and subperiosteal abscess without other complications. They were treated either with intravenous antibiotics, tympanotomy and needle aspiration or incision of the subperiosteal abscess or with intravenous antibiotics and mastoidectomy. Multiple data on symptoms and outcome were compared.

Results
There were no differences between the groups concerning general condition, CRP and WBC at admission to hospital. A total of 34 children were treated conservatively and 82 with a mastoidectomy. The conservatively treated group consisted of younger children, median age 18,5 months who had a median duration of hospitalization of five days while the other group had a median age of 26 months and stayed at hospital for seven days. In the first group three patients were readmitted to hospital with a suspected new episode of acute mastoiditis that resolved without mastoidectomy. In the group who underwent a mastoidectomy primarily ten patients experienced a new episode of acute mastoiditis and three were readmitted due to a new episode of suspected acute mastoiditis.

Conclusions
In this retrospective study based on patient records the children treated with a mastoidectomy had a higher morbidity and a larger need of healthcare than the more conservatively treated children. We found no negative effects of a conservative way of treatment.

Longterm Otoscopic Dynamics in an Unselected Population with High Risk of Otitis Media

Preben Homøe, MD, PhD[1], Ramon Jensen, MD[1], Anders Koch, MD, PhD[2]
[1]Dept. Otolaryngology, Head & Neck Surgery, Rigshospitalet, University Hospital of Copenhagen, Copenhagen, Denmark, [2]Dept. Epidemiology Research, Statens Serum Institut, Copenhagen, Denmark

Introduction
Otitis media (OM) is frequent in Inuit all over the Arctic. The longterm impact and chronicity of OM problems are not known.

Objective
To examine the long-term chronicity of OM in a high-risk population in Greenland.

Methods
Follow-up study of a population-based cohort of > 18 year-olds living in Nuuk and Sisimiut, Greenland who participated in a survey concerning epidemiology of OM in 1993 and 1994. The participants were contacted by letter and phonecalls and examined in 2010 with an otomicroscope. Audiometry was obtained in as silent rooms as possible.

Results
Contact was established with 348 persons out of the original cohort of 591 persons and of these participated 226 (65%). Twenty-seven (12%) were diagnosed with chronic OM (COM). Seventeen of these also had COM in 1993/94. Of those with COM in 1993/94, 39% had healed spontaneously. Any kind of tympanic membrane pathology was present in 101 (45%) participants. This number was 54% in 1993/94.

Conclusions
OM in childhood often results in chronic longterm ear problems still present in early adulthood. Clinical follow-up is important. Hearing loss and educational problems are associated with OM problems. Although tympanic membrane perforations have a quite high tendency to heal spontaneously there is a high need for closure by ear surgery in OM high risk areas.

Long-Term Occurrence of Myringosclerosis Following Tympanostomy Tube Insertion in Secretory Otitis Media: Relation to Age at Treatment and Tube Functioning Time

Jonathan Aavang Petersen, MD, Gita Jørgensen, MD, Dominika Drozdziewic, MD, Sven-Eric Stangerup, MD, Mirko Tos, MD, Per Bonding, MD, Per Caye-Thomasen, MD, PhD
Oto-rhino-laryngology, Head and Neck surgery, Copenhagen University Hospital Gentofte, 2900 Hellerup

Objective
This report relates the long-term occurrence of myringosclerosis to age at treatment and tube functioning time.

Materials and Methods
Two hundred and twenty-four children with bilateral secretory otitis media were treated by bilateral myringotomy and insertion of a ventilation tube on the right side only, at mean 3.9 years of age. The children were re-examined by otomicroscopy after 3, 7, and 25 years.

Results
1) there was a significant correlation between the occurrence of myringosclerosis and age at tubulation. 2) myringosclerosis occurred more frequently when the tube functioning time exceeded 6 months. 3) the extension of myringosclerosis increased with time for children treated at a young age (1-3 years), whereas stability was seen for older children.

Conclusion
The long-term occurrence of myringosclerosis following tympanostomy tube in-sertion is related to age at treatment and tube functioning time. The myringosclerotic lesion ex-tends with time for children treated at a young age, whereas stability is seen for older children.

Tympanostomy Tube Placement and Balance Function in Children

Michael Cohen, MD[1], Ellen Mandel, MD[1], Joseph Furman, MD[2], Margaretha Casselbrant, MD, PhD[1], Patrick Sparto, PhD[2]
[1]Pediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, [2]Otolaryngology, University of Pittsburgh, Pittsburgh, PA

Objectives
Determine the effect of bilateral myringotomy with tube placement (BMT) on vestibular and balance function in children 3 to 9 years old.

Methods
This is a case-control study of children undergoing BMT for persistent otitis media (OME). Controls with normal middle ear status were age- and gender-matched. Data were collected over a 5-year period.

Outcome measurements included gain and phase of the vestibulo-ocular reflex using rotational chair testing (RCT), and sensory organization test (SOT) composite scores and sway velocity using computerized dynamic posturography (CDP). Testing was performed preoperatively, and at 1 and 3 months postoperatively. Controls underwent identical testing at similar time intervals. Analysis of covariance was performed to evaluate the effect of BMT on RCT, SOT, and sway velocity, with age as a covariate.

Results
72 cases and 55 controls were enrolled. Mean age was 69 (SD 12) months for cases and 72 (SD 15) months for controls. No difference was seen between groups on rotational chair outcomes or SOT scores after adjusting scores for age. There was a trend toward higher sway velocity in the BMT group seen both preoperatively ($p=0.041$ to 0.282) and 1 month postoperatively ($p=0.055$ to 0.188). There was no difference between groups 3 months postoperatively ($p=0.468$ to 0.892).

Conclusions
Our data suggest that children with OME have higher sway velocity on CDP than normal controls. This difference normalizes by three months after tympanostomy tube insertion.

Taste Disorders in Middle Ear Disease and after Middle Ear Surgery – a Preliminary Report

Katarina Berling, MD[1], Magnus von Unge, PhD[2]
[1]County Council of Västmanland, Center for Clinical Reserch, Gavle, Gävleborg, [2]Dept of Otorhinolaryngol, Akershus University Hospital, Oslo, Norway

Introduction
Taste sensations are provided by three different nerves of which the chorda tympani nerve (CTN) is the major taste nerve. It innervates taste buds in the anterior two thirds of the tongue. The CTN location in the middle ear predisposes it to trauma.[1] In various forms of middle ear pathology, such as chronic otitis and cholesteatoma the nerve can be affected by the pathologic process per se, since the nerve may become exposed to bacteria toxins, enzymes or mechanical damage.[2,3] During middle ear surgery, it can be cut, stretched, touched or dried out by the heat of the microscope light beam. The nerve function in these situations is not clear. Is it for example better to cut the nerve than to leave it traumatized after surgery.[4,5,6,7] Therefore previous reports on objective CTN function pre- and postoperatively unfortunately suffer from inadequate descriptions of ear disease that were studied. In order to elucidate these questions and to give the surgeon a deeper knowledge about how to handle CTN during otosurgery a prospective study was initiated on patients to be operated on with primary middle ear surgery because of chronic otitis media, cholesteatoma and otosclerosis.

We believe that patients with chronic otitis media and cholesteatoma have taste disturbances before surgery due to the disease itself, of course depending on degree of the disease, and that patients with otosclerosishave normal nerve function before surgery. We also believe that patients with normal taste before surgery are more likely to notice a taste disturbance and that a nerve in continuity after surgery, even if it is maltreated, gives less taste disturbance than a divided nerve.
Two methods of taste measuring are used in the study, Electrogustometry (EGM) and the Filter Paper Disc method (FPD).[8,9,10,11] We have evaluated EGM regarding possible bias and artifacts as well as the correlation regarding results from the two methods. The results indicate that EGM is a reliable method to measure taste with a high degree of reproducibility and that the two methods have a good correlation.[12]

Methods
The study is divided into three substudies.
A clinical prospective trial where 100 patients that undergo primary middle ear surgery because of chronic otitis, cholesteatoma or otosclerosis are evaluated with EGM and FPD before and after surgery. They also answer a questionnaire about subjective taste disturbance and a questionnaire about quality of life. Twenty-seven patients were followed 1 year postoperative.

A clinical multi center study on 200 patients that undergo primary surgery because of otosclerosis. These patients answer a questionnaire about subjective taste disturbance and a questionnaire about quality of life.So far 38 patients have been tested one year postoperatively.

A histological study with electron microscopy of CTN from healthy ears and from ears with chronic otits or cholesteatoma.We have samples from five healthy and five sick ears that we have started to investigate with EM. The samples of healthy nerves were taken from patients undergoing surgery for acusticus neurinoma and the samples of sick nerves were taken from patients undergoing surgery for chronic otitis media and cholesteatoma where the nerve could not be preserved during operation.

Results
Study 1
The results indicate that patients that undergo surgery because of the inflammatory diseases chronic otitis media and cholesteatoma are more likely to notice postoperative disturbance of taste than patients undergoing surgery for otosclerosis and that the symptoms are more likely to be permanent.

Study 2
The preliminary results show that 58 % of patients reporttastedisturbance postoperatively. Ninety-five percent recover during the first year postoperatively and most are improved by 6 weeks after surgery and recovery during the first year postoperatively is 95%.

Study 3
A first evaluation with electron microscopy on CTN from healthy and diseased ears shows significant differences. We have seen signs of disarrangement in nerves from diseased ears, a thickening of the perineural and epineural connective tissues, cellular and myelin degeneration, increased amount of collagen and connective tissue, inflammatory elements and edema.

Conclusion
This preliminary report shows that a large percentage of patients who undergo middle ear surgery experience taste disturbance postoperatively (59% in our population) and that it therefore is important to evaluate this issue to be able to inform the patients about risks and prognosis of taste disturbance and to make clear how otolaryngologistsshould handle the CTN in the different situations that arise during middle ear surgery. The results are based on 1/3 of the planned study population and on the patient's own subjective opinion of the taste disturbance. The subjective symptomswill be compared to objective measurements of taste function and compare the three different diagnosis chronic otitis media, cholesteatoma and otosclerosis. We also hope to prove that there is a histologic difference between nerves from non-inflamed earsand those subject to inflammation.

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Role of Prothrombotic Risk Factors for Lateral Sinus Thrombosis in Otitis Media

Ricardo Vaz, MD[1], Jorge Spratley, PhD, MD[1], Luciana Gonçalves, MD[2], Pedro Marques, MD[1], Margarida Santos, MD[1]
[1]Otorhinolaryngology, [2]Transfusion Medicine and Blood Bank, Centre of Thrombosis, Hemostasis and Vascular Biology, Hospital S. João, E.P.E., Oporto, Oporto

Introduction
With the advent of antibiotics the incidence of intracranial complications of otitis media decreased dramatically.[1-4]
Lateral sinus thrombosis (LST), although rare nowadays, continues to be a potentially devastating intracranial complication with acute and chronic consequences.[2-4]
The anatomic position of the lateral sinus adjacent to the mastoid cavity makes it particularly vulnerable to inflammatory changes of this region. LST formation may result either from direct spread of the infection from the mastoid or from thrombophlebitis of small veins of the mastoid, which communicate with the lateral sinus.[4-6]
Otogenic LST may present with predominantly neurologic, rather than otologic, complaints. In fact, the clinical picture may sometimes be unclear due to the masking effect of the previous use of antibiotics.[1] Typically, the disease is marked by spiking-fever ("picket-fence"), headache and signs of otologic disease. Occasionally, signs and symptoms of sixth or seventh-nerve impairment and raised intracranial pressure may be important clues to the diagnosis.[5]
On clinical grounds alone it is sometime difficult to distinguish LST from other intracranial complications.[7] Imagiologic evaluation plays nowadays a crucial role in establishing the diagnosis and management planning.[2-4,7] Contrast-enhanced computed tomography (CT) and, more recently, magnetic resonance venography (MRI) are the preferred methods. (Figs. 1-3)
Inherited or acquired hypercoagulability states are considered risk factors for intracranial venous thrombosis (IVT).[3,8] Congenital deficiencies of antithrombotic factors may also predispose for IVT in the presence of acquired local hypercoagulabilities such as AOM and mastoiditis.[4] In addition, there is a risk of secondary cerebral or systemic venous thrombosis in children older than 2 years and the presence of the G20210A mutation in factor II.[9]
Some literature supports the anticoagulation in patients with clot burden extending beyond the lateral sinus, neurologic changes, embolic events, or persistent fevers despite adequate surgical intervention.[5]
The current study aimed to evaluate the possible role of prothrombotic risk factors in patients with otogenic LST and its implications for the use of anticoagulant treatment in affected patients.

Methods
Prospective evaluation of cases with acute (AOM) or chronic (COM) associated to LST in our institution between 2005 and 2010.
All patients had a diagnosis of otogenic LST confirmed by the clinical symptoms, otoscopic findings of ongoing OM (either AOM or COM) and imaging techniques (CT scan and/or MRI).
Prothrombotic risk factors such as factor V Leiden mutation, factor II G20210A and metiltetrahydrofolate reductase (677TT) polimorphisms, homocystein levels, antithrombin, protein C and S, activated protein C resistance, lupus anticoagulant and antiphospholipid antibodies (anticardiolipin and anti-b2GPI) were investigated.
Details of the clinical presentation, laboratory and radiological investigations and long term clinical and radiological follow-up were registered.

Results
Seven patients treated for otogenic LST were enrolled in the study. In five cases LST was related to AOM and in the remaining two cases to COM with cholesteatoma.
In the AOM cases mean age was 4,8 years. The COM cases had 16 and 28 years respectively. Two patients also presented ipsilateral paralysis of the abducens nerve and one with subdural empyema and meningitis. Excluding infection, none had other predisposing factors such as trauma, cancer, leukemia, cardiac or autoimmune diseases. None of the studied prothrombotic risk factors was positive in this group of patients.

Every patient was treated with mastoidectomy with exposure of the lateral sinus, ceftriaxone and unfractioned heparin. Warfarin was given for secondary long-term (6 months) prophylaxis All patients recovered uneventfully without any residual neurological sequels and no recurrence was found after discontinuation of anticoagulant therapy. A 6-month MRI imagiologic survey showed signs of recanalization of the diseased lateral sinus in all patients. (Fig. 4)

Conclusions
Although rare nowadays, LST is still a serious complication of OM that warrants immediate attention and care. Not infrequently may coexist with other intracranial complications. Contrast-enhanced CT and MRI play a major role in determining diagnosis and treatment plans. The negativity of the studied prothrombotic risk factors suggests that probably infection by itself is the most

important cause in the pathogenesis of otogenic LST. Laboratory work-up for hypercoagulability should be the routine in patients with IVT secondary to AOM/COM and mastoiditis. Anticoagulant treatment should be considered in these cases. Otogenic LST has a favorable prognosis if timely diagnosed and appropriately managed. Further investigations are needed to identify preventable risk factors for this severe complication of OM.

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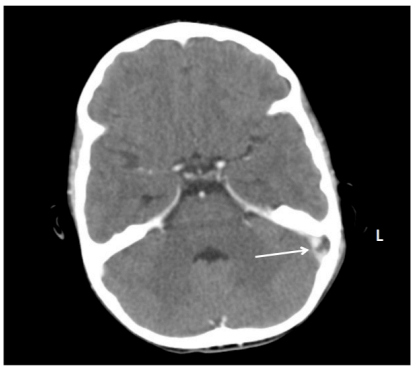

Fig. 1- Contrast enhanced CT showing a filling defect and perisinus halo at the left lateral sinus (arrow)

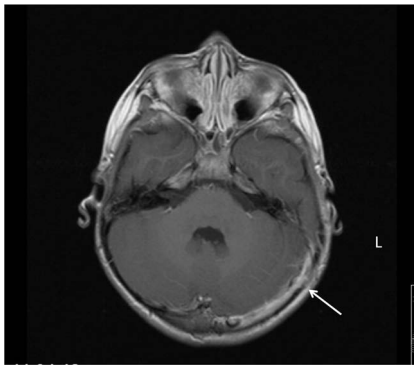

Fig. 2- T1-weighted MR axial image with gadolinium enhancement displaying thrombus extension from the left lateral sinus to horizontal sinus (arrow)

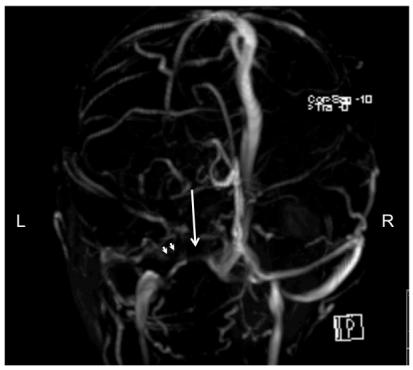

Fig. 3- MR venography image demonstrating loss of signal and the lack of flow in the left lateral (arrowheads) and horizontal sinus (arrow)

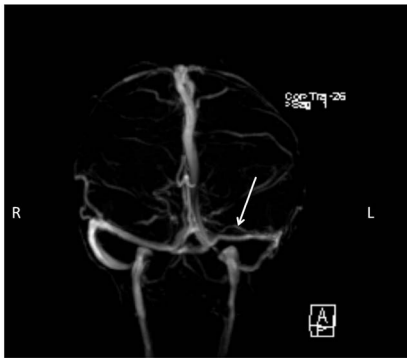

Fig. 4- MR venography 6-months after surgery and hypocoagulation showing recanalization of the left sinuses presented on Fig. 3 (arrow)

Mastoiditis and Gradenigo's Syndrome

Chris Ladefoged Jacobsen[1], Mikkel Attermann Bruhn[1], Mette Madsen[2], Michael Gaihede[1]
[1]Department of Otolaryngology, Head and Neck Surgery, [2]Department of Paediatrics, Aalborg Hospital, Aarhus University Hospital, Aalborg

Gradenigo's syndrome is a rare disease, which is characterized by the triad of suppurative otitis media, pain in the distribution of the first and the second division of trigeminal nerve, and abducens nerve palsy. The syndrome is related to apical petrositis, which in the pre-antibiotic era was a common complication of otitis media or mastoiditis. The full triad may often not be present, but it can develop if the infection is not treated correctly.

Case Report
We report a case of a 3-year old girl, who presented with fever and a left-sided acute otitis media. She developed acute mastoiditis, which initially was treated by intravenous antibiotics, ventilation tube insertion and abscess drainage without cortical mastoidectomy. However, her condition deteriorated and mastoidectomy was performed the next day. Subsequently, general improvement resulted, though the clinical picture was complicated by the development of left-sided abducens palsy combined with the fact that Fusobacterium necrophorum grew in the pus culture. In order to exclude the possibility of septic sinus thrombosis MR-scan was performed, which showed osteomyelitis within the petro-mastoid complex, hyperintense signal of the adjacent meninges, whereas no sign of sinus thrombosis. She was treated successfully and discharged after a total of 20 days of intravenous antibiotic therapy. At the time of 8 weeks follow-up there was no sign of recurrent infection or abducens palsy.

Cholesterol Granuloma in Ear Surgery: Its Prevalence and Co-Incidence with Other Otologic Findings

Yusuf Kemaloglu, MD[1], Metin Yilmaz, MD[1], Nebil Goksu, MD[1], Suat Ozbilen, MD[1], A. Necmettin Akyildiz, MD[1], Mustafa N. Ilhan, MD[2]
[1]Department of Otolaryngology, [2]Department of Public Health Medicine, Gazi University Faculty of Medicine, Ankara, Turkey

Introduction
Cholesterol granuloma (CG) is a nonspecific inflammatory response to the cholesterol crystals (by-products of hemoglobin degradation) accumulated in the pneumatized bones when under-aeration develops[1]. Main et al. (1970) observed CG formation including iron deposits and hemorrhages in the squirrel monkey in which the Eustachian tube was obstructed for 6 to 12 months[2]. The negative pressure in the ME and mastoid cells may cause tears in the blood vessels, and subsequently hemorrhage; the blood in the cavity, since it is unable to drain, is broken down to cholesterol, fibrin and hemosiderin and these products subsequently induce a foreign body reaction for CG formation[3,4]. Nevertheless, Sade (1979) suggests that the negative pressure in the ME and mastoid cells is not high enough to cause such a bleeding[5]. Miura et al (2002) found that a large amount of remaining mesenchyme that was in continuity with the hematopoietic bone marrow in the locations in which CG was present and that chronic effusion and inflammation of the Eustachian tube were observed in all of the reported cases[6].
CG has been reported within the middle ear (ME) and mastoid and petrous portions of the temporal bone. Since the tympanic membrane otoscopically appears to be dark blue (steel-blue) when CG is present within the ME, this entity has been named as blue ear or idiopathic hemotimpanum[1,5,7,8].
It is a generally accepted that "blue ear" is squeal of chronic otitis media with effusion (COME)[1-8]. As said by Sade (1979) and Bluestone and Klein (1995), it is the same disease with SOM, but it points out more severe condition, even sequel[5,8].
CG was also reported in the subjects with chronic suppurative otitis media (OM) with or without cholesteatoma[1,9-11]. Its co-incidence with retraction pockets (RPs) and/or adhesive OMwere pointed-out[1,5,9,10]. Further, it has been suggested that, in the "blue ear" cases, involvement of the mastoid cells is almost invariably the rule[1]. However, in the literature, there is no data on real CG incidence in ear surgery and co-incidence with other ear pathologies.
Purpose of this study is to look for rate of CG in different types of chronic OM (COM) and its associations with other ear pathologies, retrospectively.

Methods and Materials
A retrospective analysis was done for CG incidence in the ear surgery archive of the department (Gazi University, Ankara), between 1985 and 2004. The persons in charge of retrospective data collection, who were both senior residents in the department, did not know real purpose of the study and they were not included to the authors. During this work, they noted localization and extend of CG, and further the following parameters for all ear operations with or without CG: retraction (R), RPs and adhesive OM or severe athelectasis (AA) of the tympanic membrane (TM), cholesteatoma and ossicular lesions.
This retrospective analysis included only the operations with mastoidectomy. Those without mastoid surgery were excluded from the counting, even if they had TM perforations, R, RPs, AA or conductive hearing loss without TM perforations.

All ears with perforation, RPs and AA without cholesteatoma were defined as COM group, while those with cholesteatoma were classified as ChCOM. The revision operations for COM and ChCOM were named as rCOM and rChCOM, respectively. Besides, the mastoidectomy operations done for 'blue ear' or refractory COME (rfCOME, the subjects suffering from COME with severe R or RP, and hearing loss even after 2 or 3 VT insertion) were separately classified.
Statistical analysis was done by x^2 test in Microstat software by the last author of the study.

Results
This survey included 3262 surgery between 4 to 82 years of age, and in 91 (2.79 %) CG was noted in the operation data (Table 1). Mean age of these subjects (M: 53, F: 38) were 25.79±16.63 years, and minimum and maximum ages were 4 and 77 years, respectively. As seen in table 1, the subjects under 40 years of age presented more CG than the older ones (x^2 test, p= 0.073).
Only 7 subjects had blue ear appearance in otoscopy and CG was found in the middle ears of these subjects (Table 2). Of them, 6 also had CG in the attic region. There was only case with CG in both middle ear, antrum and perisinusal cells. The remaining ears presented CG either in the middle ear, antrum or mastoid cells.

In most cases, CG was noted in one or two regions within the mastoid. Main accumulation of CG in the mastoid was inthe retrofacial, perifacial or perisinusal cells. Sinodural angle was the second most common localization (Table 2). Besides, in 8 cases it was noticed that CG occupied majority of the mastoid cells.

In table 3, type of the ear operations and CG incidence were presented. Only 2.81 % of all mastoidectomies were done in the subjects with rfCOME or blue ears, and 15.08 % was done for revision cases. Highest CG incidence (8.2 %) was found to be in rfCOME group among the ears without 'blue ear' appearance in preoperative otoscopy (x^2 test, p= 0.027).In COM group, those with RP/AA had CG more than the others (x^2 test, p = 0.001). However, there was no statistical difference in CG incidence between rfCOME and COM with RP/AA groups (x^2 test, p > 0.05).

RP/AA was noted in 84.89 % of ChCOM group by preoperative otoscopy and no difference was detected between those with RP/AA-subgroup and the others in ChCOM (x^2 test, p > 0.05).

Statistical analysis disclosed that CG incidence was not different in ChCOM from COM with RP/AA (x^2 test, p > 0.05).

In revision operations, similarly, CG incidence was significantly higher in COM group with RP/AA, while it was not different between the subgroups in rChCOM group, in which R/RP/AA was detected in 88.97 % otoscopically.

In total, the ears in which R/RP/AA (including 'blue ear' and rfCOME groups) was detected in preoperative otoscopic examination had significantly higher CG than the remaining ones (5.11 % vs 1.41%; x^2 test, p< 0.0001).

Furthermore, it was found that, in 18 (19.78%) of the ears with CG, ossicular reconstruction was included to the operation, while this rate was about 13.94% in the ears without CG (x^2 test, p = 0.084).

Discussion
Although "blue ear" is known as a childhood disorder[1], our study clearly presented that CG in the antrum and mastoid cells was common in all age groups. Majority of our cases were younger than 41 years of age, and its incidence shows a statistically insignificant decrease by age (3.54% to 1.97%).

Our data also presents that CG is closely associated with COME, and that CG could be detected in the ears without blue ear appearance in otoscopy. Comparison to the ears with perforation without R/RP/AA or cholesteatom, CG incidencewas higher in the ears with rfCOME (1.41 % vs 8.2%) and in both COM and ChCOM groups with R/RP/AA (4.65 % and 4.64 %, respectively). In accordance with our data, Jaisinghani et al. (1999) reported significant correlations of RPs with both CG and cholesteatoma[10].Further, Sade and Teitz (1983) documented close association between CG and cholesteatoma, and even atelectasis[9]. da Costa et al. (1992) discloseda higher rate of CG in intact and perforated tympanic membranes (12% and 21%, respectively)[11].
High incidence of CG in ChCOM group could be explained by the fact that cholesteatoma is mainly related with RPs and/or AA, or speculated that mass of cholesteatoma obstructs the cellular tracts and destroys the aeration and drainage pathways of more posterior cells in the mastoid bone.It has been reported that "blue ear" and CG in the mastoid cells are associated with blockage of the Eustachian tube and/or aeration routes between the mastoid cells and ME, and a chronic granular mastoiditis[1,2,8-10,12-14]. Miura et al. (2002) documented morphological abnormalities of the ET and its associated structures in the temporal bones with CG[6].

Thus, it could be considered that CG is not only and directly associated with effusion within the middle ear and mastoid, but rather with under-aeration, inflammation and drainage problem. During the childhood in which OME is very common and ET function is impaired, it occurs in the middle ears in some cases, and points out its refractory and/or severe character. However, in contrary to the previous statement that involvement of the mastoid cells by CG is almost invariably the rule[1], it tends to be limited

within the middle ear and attic, although involvement of the mastoid cells by granulation tissue and effusion were found to be in majority.

In later ages, in the subjects in which R,RP and/or AA are still present, it may occur within the antrum or mastoid, not middle ear. In the later ages,since function of the ET is improved, and/or VT insertion or perforation provides sufficient aeration in the middle ear, the mastoid cells turns to be main accumulation area. Thus, it could be speculated that detection of CG in the antrum and/or mastoid cells leads us that under-aeration or drainage problem could be more severe and/or long-lasting in these cases. Its high co-incidence with cholesteatoma also supports the previous suggestion that it points out more severe condition, even sequel[5,8].

In majority of the cases with CG, it accumulates in one or two cellular regions not whole of the cells in the mastoid.These regions could be speculated as those with the hematopoietic bone marrow in the residual mesenchyme[6]. Or according to earlier hypothesis that the negative pressure in the ME and mastoid cells may cause tears in the blood vessels, and subsequently hemorrhage[1,5-8], it may be speculated that a large under-aerated cellular compartment with poor drainage could be present behind or around the cells in which CG is detected.

Altogether, to provide better prognosis, in case of detection of CG within the mastoid cells and/or antrum during any ear operation, a severe under-aeration and drainage problem together with severe inflammation and possibly granulomatous mastoiditis should be taken into account for subsequent steps of the surgery. Besides, surgery should be enlarged for possibility of an additional large but obstructed and inflamated cellular tract behind the cells in which CG is detected.Nevertheless, it could be kept in my mind that all data about CG is limited to the studies, in which mastoid surgery was done.

Acknowledgment
The authors thank Dr. Yusuf Kizil and Dr. Utku Aydil for collecting data.

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Table 1. Incidence of the Cholesterol Granuloma (CG) According to Age Groups.

Age groups	CG group		All Operations		Rate of CG (%)
	n	%	n	%	
≤16	33	36.26	932	28.57	3.54
17≤ ≤40	40	43.96	1453	44.54	2.75
41≤ ≤60	14	15.05	674	20.66	2.08
60≤	4	4.39	203	6.22	1.97
Total	91	100.00	3262	100.00	2.79

Table 2. Localization of the Cholesterol Granuloma (CG).

Localization	n (%)
Middle ear	7 (7.69)
Attic	5 (5.49)
Antrum	18 (19.78)
Mastoid cells	67 (73.63)
Sinodural angle	22 (24.18)
Presinusal or peri-/ retro-facial	34 (37.36)
Others*	25 (27.47)

* includes unknown locations which were not clearly noted.

Table 3 Relationship of Cholesterol Granuloma (CG) with Ear Pathologies.

Ear pathology	n (%)	CG (%)	Incidence
Blue ear	7 (0.21)	7 (7.69)	100.00
rCOME	61 (1.87)	5 (5.49)	8.20
COM	2093 (64.17)	48 (52.74)	1.64
- RP/AA	452	21	4.65
ChCOM	609 (18.33)	25 (27.47)	4.10
- R/RP/AA	517	24	4.64
rCOM	356 (10.91)	4 (4.40)	1.12
- R/RP/AA	55	3	5.45
rChCOM	136 (4.17)	2 (2.20)	1.47
- R/RP/AA	121	2	1.65-
Total	3262 (100.0)	91 (100.0)	2.79
- R/RP/AA	1213	62	5.11

COM, chronic OM with perforation and/or RP, AA.
ChCOM, COM with cholesteatoma
rCOM, revision surgery done for COM,
rChCOM, revision surgery done for ChCOM
rfCOME, refractory COME= cases suffering from COME with severe R or RP and hearing loss even after 2 or 3 VT insertion.
R/RP/AA, the ears presenting R, RP and/or AA

#, The data in this study was partially presented in Pediatric ORL Study Group of Turkish ORL Society Symposium: Treating Otitis Media with Effusion: Benefits on Developing Child and Society' Symposium, World Congress, Roma, June 25-30 ,2005.

Evaluation of Hearing Status After Cholesteatoma Surgery

Michal Luntz, MD, Noam Yehudai, MD
Otolaryngology, Bnai Zion MC, Technion - Israel Institute of Technology, Haifa, Israel

Introduction
The goal of treatment in cholesteatoma is to achieve a safe ear, a dry ear, and to restore hearing as much as possible. A safe ear is achieved in most patients, but many are left with a significant hearing loss. Factors that determine the severity of post operative hearing loss as well as the chance of successful ossicular chain reconstruction are the amount of ossicular chain destruction, middle ear aeration condition, inflammatory-infectious level, disease duration, previous surgeries, the age of patient and the hearing status in the contra lateral ear.

Aim
To evaluate long term hearing status of patients undergoing cholesteatoma surgery in an otologic referral center.

Design
Data was collected from 250 consecutive cholesteatoma patients. Ossiculoplasty was not performed when the middle ear was not aerated, when the condition of the ossicular chain precluded good results or when the patient/family didn't consent for prosthesis removal in case of protrusion.

Results
Complete audiometric follow up data for both ears was available for 63 patients (41 children and 22 adults). Mean follow up was 4.8±3.4 years. All patients achieved disease control. In 75% of the adults and 70% of the children the ear was dry and stable. Mean post operative hearing thresholds in children who underwent canal wall up procedure remained unchanged (AC = 34.8 dB, BC=12.5 dB). Contra-lateral ear mean hearing thresholds were normal. Mean post operative hearing thresholds in patients who

underwent canal wall down procedure was significantly worse, post operative AC thresholds in the children was 48.4 dB and BC was 16.2 dB with normal contra-lateral ear mean hearing thresholds. In adults who underwent canal wall down procedures hearing was far worse, post operative AC thresholds was 73.3 dB and BC was 38.7 dB, with mean contra-lateral ear AC thresholds of 34 dB. 69 patients agreed to complete a telephone survey, 74% of them were not satisfied with their hearing but only 19% of them were using hearing aids. In most (> 80%) of the patients who were not satisfied with their hearing and were not using hearing aids, the reason for not using a hearing aid was not medical.

Discussion
The goal of disease control is achieved in most cholesteatoma patients. Most patients however are left with a significant hearing loss.

Susceptibility of Inner Ear Ion Homeostasis Genes to Chronic Otitis Media

Dennis Trune, PhD, Fran Hausman**,** Beth Kempton**, Carol MacArthur, MD**
Oregon Hearing Research Center, Oregon Health & Science University, Portland, Oregon

Introduction
Sensorineural hearing loss is a significant problem in chronic middle ear disease, although the inner ear disease processes involved are poorly defined. Temporal bones from patients with chronic otitis media often show significant inflammation in the cochlea,[1,2] but what specific impact this has on cochlear processes is unknown. The inner ear functions by tightly regulating the endolymph ionic composition to maintain the endolymphatic potential. Any disruption of this ionic balance would cause hearing loss. Cochlear lateral wall fibrocytes have been shown to produce monocyte chemoattractant protein during acute middle ear bacterial infections[3,4]and inner ear tissues produce numerous inflammatory cytokines during both acute and chronic otitis media.[5]

These studies demonstrate the inner ear is capable of inducing local inflammation, particularly in the structures responsible for endolymph production. Therefore, it was hypothesized that chronic middle ear inflammation may have a prolonged and detrimental impact on these endolymph homeostatic processes. Studies were designed to ascertain the impact of chronic middle ear infection on the various channels and junctions that control endolymph production and maintenance. These include the numerous claudin tight junction proteins, aquaporin water channels, and ion transporters in the inner ear. Identifying the sensitivity of these ion homeostatic mechanisms to inflammation would provide a greater understanding of disease processes that cause hearing loss. This also could potentially lead to better targeted therapies to protect the ear and restore hearing in cases of immune-mediated hearing loss.

Methods
C3H/HeJ mice suffer gram-negative bacterial infections due to a gene defect in their toll-like receptor 4.[6]This often leads to chronically inflamed middle ears that do not clear, cochlear inflammation, and sensorineural hearing loss.[7] This mouse offers a model in which to characterize the impact of chronic middle ear inflammation on key homeostatic mechanisms in the cochlea. Middle ears and inner ears from C3H/HeJ mice with prolonged middle ear disease were collected and mRNA subjected to qRT-PCR for 8 common inflammatory cytokine genes (Table 1). To assess inflammation on inner ear ion homeostasis, qRT-PCR also was conducted on 24 inner ear genes from the following gene groups: Na^+,K^+-ATPase, tight junction claudins, K^+ transport channels, epithelial Na^+ channels, gap junctions, and aquaporins (Table 1). Clear middle ears and their ipsilateral inner ears were used as controls. Sample sizes ranged from 5-8 mice for each assay.

Results
Chronic Otitis Media
Histology of the C3H/HeJ middle and inner ears showed extensive inflammation. The entire tympanic cavity between the round window and tympanic membrane was filled with fluid and inflammatory cells. The round window membrane was inflamed and inflammatory cells were seen invading the scala tympani of the cochlea.

Middle Ear Cytokine Gene Expression
The chronic middle ear inflammation was demonstrated by increased expression of multiple inflammatory cytokine genes (Fig. 1). MIP-2 and MIP-1a were upregulated at the highest levels, with expression levels ranging from 144- to 377-fold over clear middle ears. All other cytokine genes were significantly upregulated ($p<0.05$) as well, with the exception of IL-6. This confirmed that an extensive inflammatory state was present in the middle ears of these mice that could impact the inner ear.

Inner Ear Cytokine Gene Expression
Chronic middle ear disease caused increased expression of several inflammatory cytokine genes in inner ears ipsilateral to the infected middle ears compared to inner ears ipsilateral to clear middle ears. There was pronounced upregulation of IL-1α, IL-1β, MIP-2 (Cxcl2), MIP-1a (Ccl3), and TNFa (Fig. 2). Other genes showed a trend for upregulation (IL-6, KC), but these did not

reach statistical significance. These results demonstrated that cells within the cochlea are expressing cytokines during chronic middle ear disease.

Inner Ear Ion Homeostasis Gene expression
The prolonged middle ear infection increased expression of several ion homeostasis genes in the inner ear (Fig. 3). Significant upregulation was measured for tight junction claudin 3, aquaporins 3 and 5, and gap junction connexin 31 (Gjb3). The claudin and gap junction genes were expressed several fold greater than in the inner ears ipsilateral to clear middle ears.

Discussion
Chronic OM caused the middle ear and inner ear tissues to express similar cytokine genes. These cytokines are involved in a number of functions, including prolongation of the inflammatory response. This offers one explanation for the cochlear inflammation and remodeling often seen with prolonged middle ear disease.

Another significant finding is that chronic middle ear disease can impact the ion homeostatic and transport functions of the inner ear. Many of these channels and transporters are located in the lateral wall (stria vascularis, spiral ligament) or surround the scala media. This implies these K^+ transport and endolymph producing mechanisms are at risk in prolonged middle ear disease.

Other studies also have shown that the lateral wall fibrocytes react to middle ear bacterial infections.[3,4]We also previously demonstrated immunostaining of the transcription factor NF-*kB* in the C3H/HeJ mouse lateral wall.[8] NF-*kB* activation and induction of inflammatory cytokines in the lateral wall could significantly compromise ion homeostasis of the endolymph and cause hearing loss. This may explain the sensorineural hearing loss often associated with chronic otitis media.

Conclusions
Chronic inflammation of the middle ear can impact ion homeostatic and transport functions of the inner ear. Recognizing these inner ear mechanisms at risk may identify potential therapeutic targets to maintain hearing during prolonged otitis media.

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Table 1: Inner Ear Inflammatory and Homeostatic Genes

Inflammatory Cytokines:
Interleukins: IL-1α,, IL-1β, IL-6, IL-10
Chemokine Ligands: MIP-2 (Cxcl2), MIP-1a (CCL3), KC (Cxcl1)
Tumor necrosis factor: TNFa
Ion Homeostasis Factors:
Na^+,K^+-ATPases: Atp1b1, Atp1b2, Apt1a1
Potassium voltage-gated channels: Kcne1, Kcnq 1, 4
Potassium inwardly-rectifying channels: Kcnj10
Epithelial Na^+ channels: Scnn1a, 1b, 1g , tmprss3
Na-K-2Cl cotransporter: Slc12a2
Tight Junction Claudins: Cldn 3, 4, 14
Aquaporins: Aqp1, 2, 3, 5
Gap junction proteins: Gja1, Gjb2, 3, 6
Chloride channel Ka: Clcnka

Fig. 1: Chronic middle ear inflammation leads to increased expression of multiple inflammatory cytokine genes. MIP-2 and MIP-1a were upregulated at the highest levels, with fold change levels ranging from 144- to 377-fold. Red bars: P < 0.05.

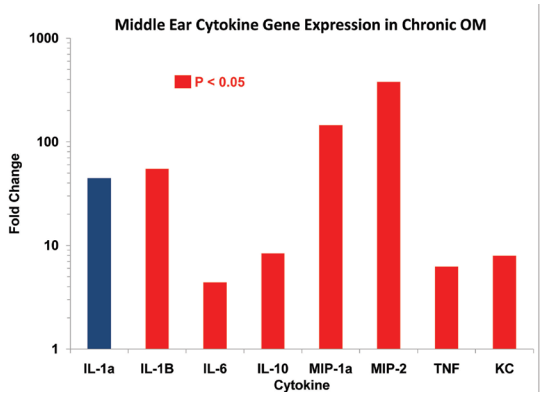


Fig. 2: Chronic OM caused significantly increased expression of several inflammatory cytokine genes in inner ears ipsilateral to the infected middle ears. Control values are gene expression in inner ears contralateral to clear middle ears.

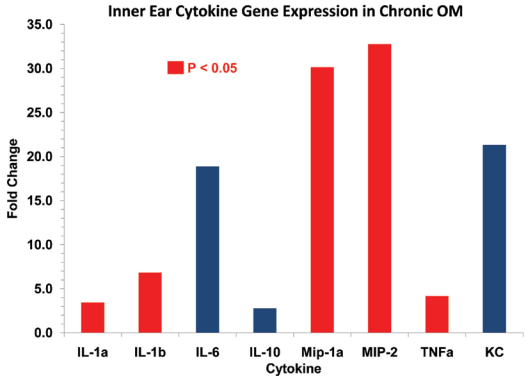
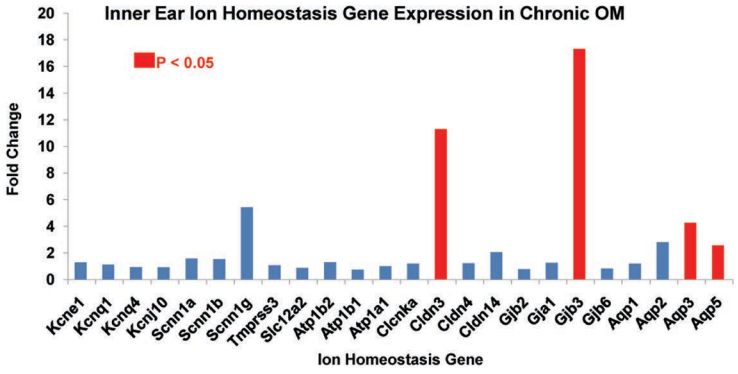


Fig. 3: Increased expression of several ion homeostasis genes occurred due to the chronic middle ear infections and production of inner ear inflammatory genes. These included claudin 3, aquaporins 3 and 5, and gap junction Gjb3, which is connexin 31.



Antibiotic Prophylaxis in ENT Surgery

Eva Westman, MD, PhD[1,2]

[1]ENT-department, Västernorrland County Council, Sundsvall, [2]The Swedish Council on Health Technology Assessment, SBU, Stockholm, Sweden

Introduction

Antibiotic prophylaxis is widely used during surgical procedures. There is inadequate scientific evidence to determine the effect of antibiotic prophylaxis with respect to many of the surgical interventions for which it is applied today. The Swedish Council on Health Technology Assessment (SBU) in august 2010 published a report on antibiotic prophylaxis for surgical procedures in which a review on the literature was performed (SBU. Antibiotikaprofylax vid kirurgiska ingrepp. En systematisk litteraturöversikt. Stockholm: Statens beredning för medicinsk utvärdering (SBU); 2010. SBU-rapport nr 200. ISBN 978-91-85413-36-2). The English version of the summary and the tables can be found at www.sbu.se.

Surgical procedures in the ear nose and throat region (ENT) are of widely varying character, ranging from clean procedures without contamination of the surgical wound to procedures involving areas in which the normal flora have the potential to cause infection. The risk of post-operative infection varies; it is high in cancer surgery where the surgery involves both skin and

mouth/airway. However, the frequency of infection is low in cases of clean head and neck surgery of benign tumours, such as procedures involving the salivary glands and the thyroid gland (13-15).

After tonsillectomy antibiotic prophylaxis is used in varying degrees in the world and reduced pain, decreased inflammation and faster healing has been used as a reason (16). Various clinical parameters have been used to evaluate the efficacy of the prophylaxis.
Questions addressed: Is antibiotic prophylaxis effective in preventing infections after a surgical procedure? How well does the underlying scientific evidence support these effects? Which antibiotic preparations, what doses and treatment times give the best effect?

Method
SBU has established a thorough and systematic method by which available databases are searched, to identify all literature relevant to the issues to be addressed in a project. Each study included in the evaluation has been assessed for quality and tabulated according to a specially developed method. The review comprised screening the studies for relevance to the subject and then for methodological qualities – study design, internal validity (reasonable protection from systematic errors), statistical power and generalisability. Included were only randomised controlled trials (RCT) or systematic reviews of RCTs. Only studies meeting with a high or medium quality score was included and the results graded according to evidence.

Results
The literature search concerning surgical procedures in ENT resulted in 253 abstracts from which 60 studies were evaluated and 20 of these met the criteria from which the results were concluded. The studies from which the evidence-graded results were concluded are presented in more detail in the full report and here they are listed in the references.

Tonsillectomy is one of the most common surgical procedures. One extensive Cochrane review (1) of medium quality examined antibiotic prophylaxis in tonsillectomy. Nine RCTs was examined more closely. In the review the outcomes were defined in primary and secondary. As primary outcomes pain, consumption of analgesics and bleeding was included. Temperature, time to normal diet and activity and adverse effects to antibiotics were considered as secondary. Antibiotic prophylaxis did not decrease significant bleeding after tonsillectomy. One single study could demonstrate reduced analgesic consumption but 3 RCTs could not demonstrate any significant difference. One of 5 RCT could demonstrate difference in pain assessment after antibiotic prophylaxis. When examining the secondary outcomes there was a significant difference where antibiotic prophylaxis reduced fever after tonsillectomy. The included studies are of varying quality and with methodological weaknesses.

Table 1. The effect of antibiotic prophylaxis compared to placebo at tonsillectomy. (From SBU with permission. Antibiotikaprofylax vid kirurgiska ingrepp. En systematisk litteraturöversikt. Stockholm: Statens beredning för medicinsk utvärdering (SBU); 2010. SBU-rapport nr 200. ISBN 978-91-85413-36-2. www.sbu.se)

Effect measures	Number of patients (number of studies)	Average risk in standard group (min – max)	Relative risk (95% CI)	Scientific evidence	Comments
Bleeding	472 (9)	10,7%	RR 0,92 (0,45 to 1,87)	□□□□ Moderately strong	No difference Based on a Cochrane review by Dhiwakar 2008 (1) Studyquality-1
Pain and consumption of analgesics Ab vs placebo	534 (3)		Heterogenous studies, 3 reported no difference	□□□□ Limited	No difference Based on a Cochrane review by Dhiwakar 2008 (1) Studyquality-1 Dataprecision-1

CI=Confidence interval RR= Relative risk
Ear surgery includes a wide variety of procedures. A Cochrane review of moderate quality was identified. In this review article 11 RCTs or quasi-RCTs (20) of patients undergoing clean or clean-contaminated ear surgery were analyzed and systemic, local and systemic plus local antibiotic prophylaxis was compared. The studies differed in choice of antibiotics, dose, when prophylaxis was given and the duration. Definitions of outcomes varied, and the data did not allow subgroup analyzes.

Author Year Reference Country	Study design	Population characteristics	Intervention Method Number individuals	Control Number individuals	Results outcome 1 intervention	Withdrawal Drop outs	Study quality and relevance Comments
Verschuur 2004 [20]	Systematic review Meta-analysis Cochrane Collaboration	11 RCT or quasi-RCT of pts undergoing clean or clean-contaminated types of ear surgery. Skull base surgery was excluded. Follow-up 3 weeks after surgery	I: Any regimen of systematic and/or local prophylaxis administered at or around the time of surgery	C: Placebo, no antibiotic or alternative intervention	Postoperative infection within 2 weeks Systemic antibiotic prophylaxis vs no antibiotics (3 studies) I: 20 pts of 172 C: 14 pts of 127 OR 1.02 (95% CI 0.49–2.15) ns Graft failure rates within 2 weeks Systemic antibiotic prophylaxis vs no antibiotics (1 study) I: 13 pts of 1 158) C: 15 pts of 1 018 OR 0.79 (95% CI 0.37–1.66) ns Postoperative infection within 7 weeks Systemic and/or local antibiotic prophylaxis vs no antibiotics (6 studies) I: 34 pts of 671 C: 38 pts of 620 OR 0.73 (95% CI 0.45–1.20) ns Graft failure rates within 7 weeks Systemic and/or local antibiotic orophylaxis vs no prophylaxis (2 studies) I: 13 pts of 1 189 C: 17 pts of 1 093 OR 0.71 (95% CI 0.35–1.45) ns	In the analyses a "worst-case scenario" was followed	Moderate The information on blinding was often not provided. None of the studies were considered to contain "fatal flaws". The data in the studies were not presented in sufficient detail to make subgroup analyses for children and adults, surgical techniques, or for the distinction between clean and clean-contaminated

C = Control group; CI = Confidence interval; I = Intervention group; pts = Patients; RCT = Randomised controlled trial

Table 2 (From SBU with permission. Antibiotikaprofylax vid kirurgiska ingrepp. En systematisk litteraturöversikt. Stockholm: Statens beredning för medicinsk utvärdering (SBU); 2010. SBU-rapport nr 200. ISBN 978-91-85413-36-2. www.sbu.se)

No prospective randomized study was identified of antibiotic prophylaxis in surgery for cochlear implants. The scientific evidence is insufficient to draw conclusions.

Evidence-graded results:

In tonsillectomies, antibiotic prophylaxis, does not affect postoperative haemorrhage (moderately strong scientific evidence ⊕⊕⊕),pain or consumption of analgesics (limited scientific evidence ⊕⊕), i.e. the substitute measures which are customarily used as indicators of infection in this region. (1)

In cancer surgery in the ENT area with risk of contamination with microbial flora which can give rise to infections, there is strong scientific evidence supporting the administration of antibiotic prophylaxis (strong scientific evidence ⊕⊕⊕⊕) (2-4). Administration of antibiotics for longer than 24 hours does not give a better effect (moderately strong scientific evidence ⊕⊕⊕) (4-8). The prophylactic effect is greater if the antibiotic or combination of antibiotics covers both aerobic and anaerobic bacteria (limited scientific evidence ⊕⊕) (5, 9-12).

Antibiotic prophylaxis does not reduce post-operative infections or complications following surgery of the middle ear or reduce rhinosinusitis symptoms after endoscopic sinus surgery (limited scientific evidence ⊕⊕) (20, 26, 27).

There is insufficient scientific evidence to determine whether antibiotic prophylaxis reduces the risk of meningitis in cases of fractures of the base of the skull (25). The same applies to the preventive effect of antibiotic prophylaxis on post-operative infections after nasal surgery (22-24) or cochlear implants (insufficient scientific evidence ⊕).

Discussion

Although antibiotic prophylaxis is common clinical practice, some aspects have not been clarified and it was therefore considered important to review and evaluate the scientific evidence on which the practice is based. A recurring problem when reviewing the literature in the area is that there are few prospective randomized placebo-controlled trials.

SBUs general conclusions

Correctly used, antibiotic prophylaxis can reduce the total use of antibiotics.

A transition to single-dose prophylaxis would probably reduce the risk of resistant strains of bacteria without increasing the risk of infection.

If all surgical units introduced procedures for registration of post-operative infection, the effectiveness of antibiotic prophylaxis could be documented and applied.

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Immunology/Vaccine

Memory of PspA Specific Immune Responses in Offspring Delivered from Immunized Mother Mice

Masamitsu Kono, MD[1], Muneki Hotomi, MD, PhD[1], Yorihiko Ikeda, MD, PhD[1], Susan Hollingshead, PhD[2], David Briles, PhD[2], Noboru Yamanaka, MD, PhD[1]
[1]Otolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama, Wakayama, [2]Microbiology, University of Alabama at Birmingham, Birmingham, Alabama

Introduction
Streptococcus pneumoniae is responsible for a significant proportion of the upper respiratory infectious diseases. Children younger than 2 years old usually have lower levels of pneumococci specific IgG antibody in sera due to the age-related immaturity of immune responses and are vulnerable to bacterial infections. It is important to induce and maintain effective immune responses against these pathogens during early childhood. We have reported the induction of specific immune responses in offspring by maternal immunization with pneumococcal surface protein A (PspA). In this study, we further evaluated the immunological memory of specific responses in maternal-immunized offspring with PspA.

Method
BALB/c bjy mice, 4 wks old female, were intranasally immunized with PspA mixed with cholera toxin B subunit for 2 to 3 weeks. After immunization, they were mated with male mice, and offspring were obtained. At the 6 weeks old, offspring were stimulated with PspA alone. After the stimulation, we evaluated the development of PspA specific IgG in sera by enzyme linked immunosorbent assay (ELISA).

Results
Anti-PspA specific IgG antibody in offspring′s sera was maintained during the nursing periods and then gradually decreased in the maternal immunized offspring with PspA. After the stimulation with PspA, maternal immunized offspring enhanced anti-PspA specific IgG in sera again.

Discussion
PspA is a surface-exposed antigen and a virulence factor for invasive diseases. Therefore, PspA will be an attractive candidate for future protein based pneumococcal vaccines. Maternal immunization would be a noble procedure against pneumococcal infections among early childhood. The current results suggested that PspA specific immune response was memorized in offspring maternal immunized with PspA for a considerable period of time. The maternal immunization would be one of the most suitable approaches to induce effective immune protections against pneumococcal infections.

Cross Protection Against *S. pneumoniae* Infections by Maternal Immunization with PspA

Yorihiko Ikeda, MD, PhD[1], Muneki Hotomi, MD, PhD[1], Masamitsu Kono, MD[1], Susan Hollingshead, PhD[2], David Briles, PhD[2], Noboru Yamanaka, MD, PhD[1]
[1]Otolaryngology - Head and Neck Surgery, Wakayama Medical University, Wakayama, Wakayama, [2]Microbiology, University of Alabama at Birmingham, Birmingham, Alabama

Introduction
Streptococcus pneumoniae is responsible for a significant proportion of the bacterial upper respiratory infectious diseases. It is important to induce effective protective immune responses against these pathogens during early childhood. Pneumococcal surface protein A (PspA) is a surface-exposed antigen and classifies into 3 families. PspA is one of the attractive candidates for future protein based pneumococcal vaccines. In this study, we evaluated the cross protection against pneumococci with different types of PspA by maternal immunization with single type of PspA.

Method
BALB/c bjy mice, 4 wks old female, were intranasally immunized with PspA family 2 mixed with cholera toxin B subunit (CTB) for 2 to 3 weeks. After the final immunization, they were mated with male mice for two weeks. Approximately 3 weeks after mating, offspring wee obtained. We evaluated the induction of specific immune response in offspring by ELISA and the protection of offspring from systemic lethal infection with pneumococci having different PspA family (PspA family 1).

Results
Maternal immunization with PspA family 1 could induce PspA family 1 specific IgG in sera among offspring. Survival of offspring infected with D39, EF3030 and BG7322 were significantly extended compare to those of controls. However, there were no significant differences in survival times among offspring infected with L82016.

Conclusion
Pneumococcal surface protein A (PspA) is an important virulent factor expressed by all pneumococci. It is highly immunogenic and protective against invasive disease as well as nasal colonization in mice. We clearly indicated that the antibody elicited by maternal immunization was highly cross-protective. Thus, it is hypothesized that a PspA-containing vaccine can protect against pneumococcal infections. Consequently, PspA is an attractive candidate antigen for the development of new effective vaccine.

Lack of Generation of T Helper Cell Memory to Pneumococcal and *Haemophilus influenzae* Proteins in Early Childhood Explains Immunologic Susceptibility to the Otitis-Prone Condition

Sharad Sharma, PhD[1], Ravinder Kaur, PhD[1], Janet Casey, MD[2], Michael Pichichero, MD[1]
[1]Research Institute, Rochester General Hospital, Rochester, New York, [2]Pediatrics, Legacy Pediatrics, Rochester, New York

Introduction
A subpopulation of children experiencing 3 or more episodes of AOM in the first year of their life is considered otitis-prone. Two major pathogens causing AOM are *Streptococcus pneumoniae* (*Spn*) and *Haemophilus influenzae* (NT*Hi*). $CD4^+$ T-cells are considered of prime importance against these extracellular pathogens. Hence, in the present study we enumerated frequencies of pathogen specific functional memory $CD4^+$ T-cells among a cohort of nonotitis-prone and otitis-prone children. Non otitis-prone (n=15) and otitis-prone (n=13) children suffering from *Spn* caused AOM or colonization were compared for the frequencies and functional characteristics of *Spn* specific $CD4^+$ T-cells by stimulating peripheral blood mononuclear cells (PBMCs) using six pneumococcal protein antigens (pHtd, LytB, PcPA, pHtE, PlyD1 and PspA). Additionally, two small cohorts of non otitis-prone (n=8) and otitis-prone (n=5) children having NT*Hi* caused AOM or colonization were stimulated with NT*Hi* antigens P6, OMP26 or Protein D. Detection of rapid accumulation of intracellular cytokines post antigenic stimulation allowed assessment of activation of various $CD4^+$ T-cell subsets using multi-parameter flow cytometry. *Spn* and NT*Hi* specific IgG responses in nonotitis-prone and otitis-prone children to the same protein antigens were also evaluated. To rule out the possibility of generalized intrinsic T- or B-cell defect among otitis-prone children, $CD4^+$T-cell responses to *Staphylococcus* enterotoxin B (SEB) and IgG responses to vaccine antigens - diphtheria, tetanus and pertussis (DTPa) were evaluated in all the cohorts. A deficiency or low percentages of $CD45RA^{Low}$ memory $CD4^+$ T-cells producing *Spn* or NT*Hi* specific cytokines (IFN-γ, IL-2, IL-4 and IL-17a) was observed among *Spn* or NT*Hi* caused otitis-prone children respectively. Similarly, IgG responses to the same proteins were reduced among otitis-prone children. However, upon stimulation with SEB, memory $CD4^+$T-cell in both non otitis-prone and otitis-prone children displayed no significant differences in the cytokine production indicating that otitis-prone children did not lack overall functional memory Th-cells. Moreover, IgG responses to the other vaccine antigens (DTPa) were not different between groups. Hence, a lack of generation of pathogen specific memory $CD4^+$ Th-cells and subsequently reduced B-cell IgG responses among otitis-prone children makes them susceptible to recurrent AOM infections.

Materials and Methods
Antigens and T-cell stimulation
Six different pneumococcal protein antigens were used in this study: pneumococcal histidine triad proteins D (PhtD) and E (PhtE), LytB, PcpA, PlyD1 (a detoxified derivative of pneumolysin which has three point mutations that do not interfere with anti-pneumolysin antibody responses) and PspA. *Haemophilus influenzae* protein antigens used were P6, OMP26, and Protein D. PBMCs were stimulated using a standardized protocol in our laboratory. Briefly, cells were counted and placed in a 96-well flat bottom culture plate and were stimulated with either 1μg/ml of various protein antigens or with 1μg/ml of Staphylococcal enterotoxin B (SEB). Cells were then incubated for 2h at 37° C in the presence of 5% CO_2 for antigen processing. After 2 hours, Golgi transport inhibitors (BD Biosciences) were added to preserve cytokines intracellularly and incubation was then continued for an additional 4 hours.

Cytokine profilingand humoral responses
An intracellular cytokine staining assay (ICCS) was used to evaluate antigen specific $CD4^+$ T-cell subsets (Th-1, Th-2 and Th-17). A custom made BD LSR II flow cytometer equipped for the detection of 12 fluorescent parameters was used to collect 2-5 x 10^5 events for each sample and data was analyzed using FLOW JO (Tree Star) software.
For measuring IgG antibody levels in the samples, ELISA was performed as described previously. The levels of IgG in the reference serum were quantitatively measured by using a human IgG ELISA quantitation kit (Bethyl laboratories). A Four-

parameter logistic-log function was used to form the reference and sample curves. This ELISA was fully validated according to ICH Guidance.

Statistics

All data was analyzed using Graph Pad Prism software. Two tailed *P* values for the data were calculated using Mann Whitney Test.

Results

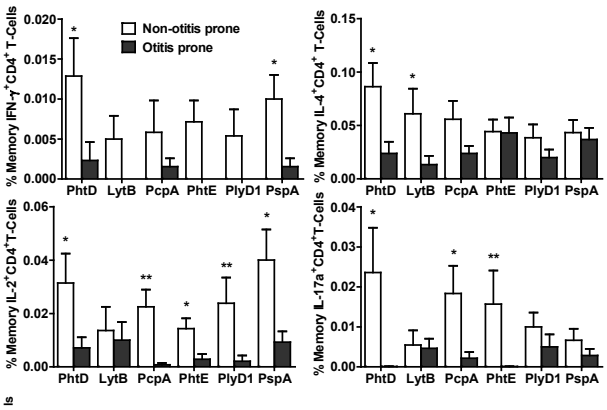

Figure 1. Percent frequencies of CD45RA^{Low} memory CD4^{+} T-cell subsets producing various cytokines against six pneumococcal antigens (a) IFN-γ, (b) IL-4, (c) IL-2 & (d) IL-17a, in the circulation of non-otitis prone and otitis prone children against various pneumococcal antigens. Bar graphs represent mean percentage values of CD69^{+} CD4^{+} T-cells, following antigen stimulations. Error bars represent SEM, P values were calculated using Mann Whitney test. *P <0.05; **P <0.005.

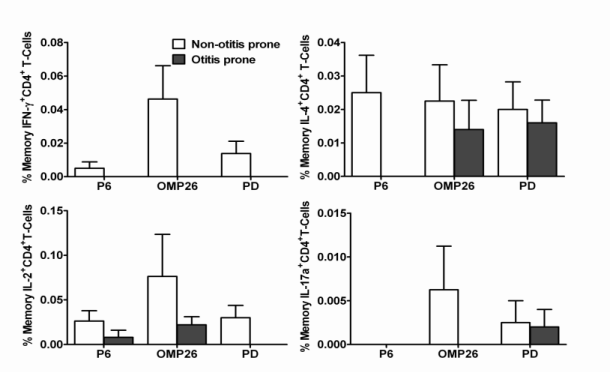

Figure 2. Frequencies of CD45RA^{Low} memory CD4^{+} T-cell subsets producing various cytokines against three NT*Hi* antigens (a) IFN-γ, (b) IL-4, (c) IL-2 & (d) IL-17a, in the circulation of non-otitis prone and otitis prone children against various NT*Hi* antigens (P6, OMP26 and Protein D). Bar graphs represent mean percentage values of CD69^{+} CD4^{+} T-cells, following antigen stimulations. Error bars represents SEM, P values were calculated using Mann Whitney test. *P <0.05.

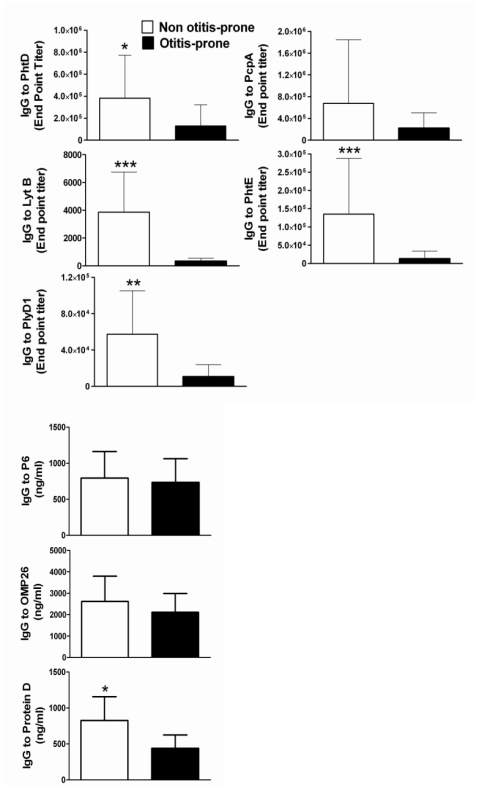

Figure 3. Comparison of IgG responses to five pneumococcal protein antigens (Phtd, LytB, PcpA, PhtE and PlyD1) and NT*Hi* protein antigens (P6, OMP26 and Protein D) in the serum samples of two cohorts of non-otitis prone and otitis prone children. *P <0.05; **P <0.005; ***P <0.0005. Y-axis represents Geometric mean titers and error bars are upper 95% confidence intervals.

Conclusions

Otitis prone children fail to generate a robust memory CD4^{+} T-cell response to *Spn* and NT*Hi* protein antigens.
Otitis prone children have subsequent reduction of memory B-cells and subsequent IgG production.
No difference in the T-cell response to SEB stimulation (data not shown).

Otitis prone children may represent a vulnerable group of children that fail to induce optimal immune response to many antigens.

Supported by NIH NIDCD RO1 08671, Thrasher Research Fund and Sanofi Pasteur.

Functional Disparity in CD4+ T-Cells Comparing Young Children and Adults Using Six Pneumococcal Vaccine Candidate Antigens for Acute Otitis-Media (AOM)

Sharad Sharma, PhD[1], Timothy Mosmann, MD[2], Michael Pichichero, MD[1]
[1]Research Institute, Rochester General Hospital, Rochester, New York, [2]Center for Vaccine Immunology, University of Rochester, Rochester, New York

Introduction

Streptococcus pneumoniae (*Spn*) is a common cause of otitis-media in children and is considered vaccine preventable. $CD4^+$ T-cells display a critical role in immune protection against *Streptococcus pneumonia*. In children, an imbalance of T helper (h)-1: Th-2 responses, could contribute to higher risk of infection than adults. The frequencies and functional characteristics of *S. pneumonia* specific $CD4^+$ T-cells were compared by stimulating peripheral blood mononuclear cells (PBMCs) with pneumococcal vaccine candidate antigens PspA, pHtd, LytB, PcpA, pHtE and PdB using multi-parameter flow cytometry. Detection of intracellular cytokines post antigenic stimulation allowed assessment of activation of various $CD4^+$ T-cell subsets. A striking deficiency of Th-1 cells producing IFN-γ or IL-2 was found among children. Among adults compared to children lower IL-4 frequencies were observed. PspA was the most potent stimulator of $CD4^+$ T-cells. Frequencies of $CD4^+$ T-cells producing IL-13 and IL-17 were low in adults and lower in children. Comparison of up-regulation of the early T-cell activation marker CD69 demonstrated that $CD4^+$ T-cells of children exhibited higher levels of CD69 expression under unstimulated conditions compared to adults. More precisely, $CCR7^{HIGH}CD45RA^{LOW}$ (central memory) $CD4^+$ T-cells among children displayed significantly higher levels of CD69 than adults in their resting phase. Furthermore, occurrence of $CD69^{LOW}$ $CD4^+$ T-cells producing IL-4 were also significantly higher among children compared to adults. Response to pneumococcal protein antigens among adults were primarily Th-1 (IL-2 & IFN-γ) while young children were devoid of *S. pneumonia* antigen specific CD4+ Th-1 responses. However, young children were capable of producing IL-4 responses by $CD69^+CD4^+$ T-cells, indicating enhanced Th-2 activity to pathogen specific antigens.

Materials and Methods

1. Antigens and PBMC stimulations

Pneumococcal protein antigens used for T-cell stimulation included: surface protein, PspA (EF5668), two pneumococcal histidine triad proteins (iPhtD, PhtE), an autolysin (LytB), a choline binding protein (PcpA) and a detoxified derivative of pneumolysin (PlyD1). All the pneumococcal antigens were provided as a gift by Sanofi-Pasteur. NT*Hi* antigens used were Protein D and OMP26 and were gifts from GlaxoSmithKline, UK and Dr. Jenelle Kyd, University of Canberra Australia respectively. PBMCs were stimulated using a standardized protocol in our laboratory. Briefly, cells were counted and placed in a 96-well flat bottom culture plate and were stimulated with either 1μg/ml of various protein antigens or with 1μg/ml of Staphylococcal enterotoxin B (SEB). Cells were then incubated for 2h at 37° C in the presence of 5% CO_2 for antigen processing. After 2 hours, Golgi transport inhibitors (BD Biosciences) were added to preserve cytokines intracellularly and incubation was then continued for an additional 4 hours.

2. Cytokine profilingand humoral responses

An intracellular cytokine staining assay (ICCS) was used to evaluate antigen specific $CD4^+$ T-cell subsets (Th-1, Th-2 and Th-17). A custom made BD LSR II flow cytometer equipped for the detection of 12 fluorescent parameters was used to collect 2-5 x 10^5 events for each sample and data was analyzed using FLOW JO (Tree Star) software.

For measuring IgG antibody levels in the samples, ELISA was performed as described previously. The levels of IgG in the reference serum were quantitatively measured by using a human IgG ELISA quantitation kit (Bethyl laboratories). A Four-parameter logistic-log function was used to form the reference and sample curves. This ELISA was fully validated according to ICH Guidance.

3. Statistics

All data was analyzed using Graph Pad Prism software. Two tailed *P* values for the data were calculated using Mann Whitney Test.

Results

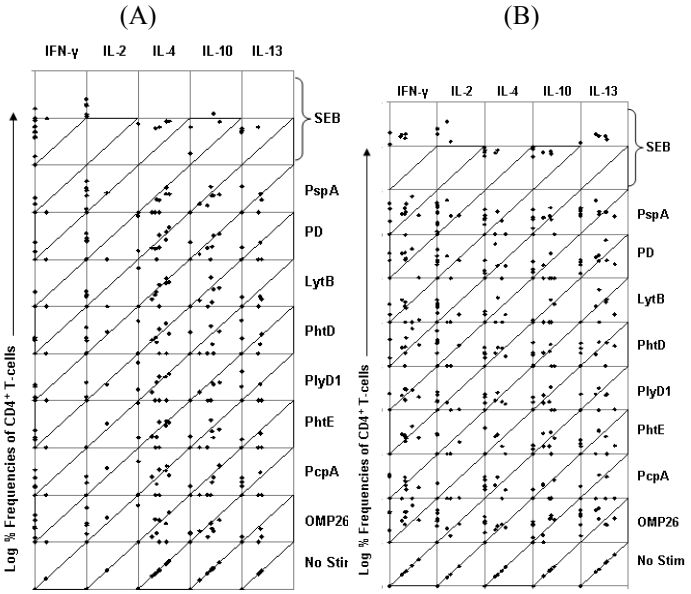

*Figure 1: Characteristics of antigen specific CD4$^+$ T-cells among young children and adults.*CD4$^+$ T-cells cytokine pattern diversity in responses to 6-pneumococcal (PspA, Phtd, LytB, PcpA, PhtE and PlyD1) and 2- NT*Hi* (OMP26 and Protein D) antigens among children (A) and adults (B). After antigenic stimulations, PBMC were analyzed as shown in Fig 1. The numbers of cells displaying each pattern were measured by flow cytometry, and the Log percentages of CD4 T cell frequencies producing each of the 6-single cytokines (IFN-g, IL-2, IL-4, IL-10, IL-13 and IL-17a) were enumerated. Each point in the graph represents a different individual.

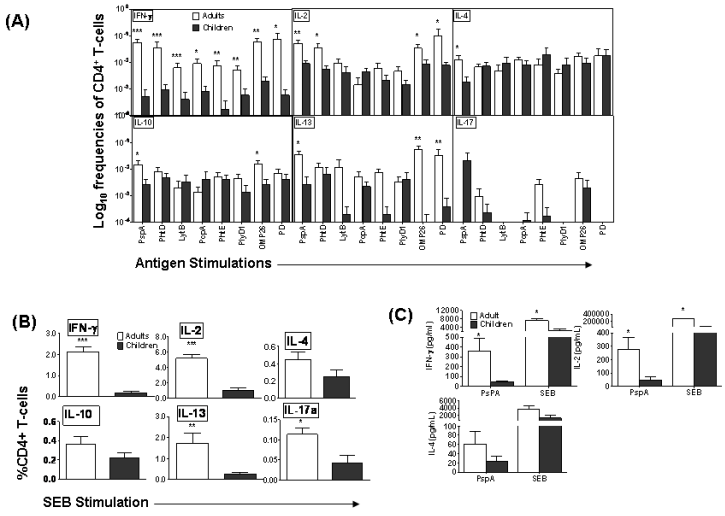

Figure 2: Differences in the frequencies of Spn and NTHi specific CD4$^+$ T-cells among children and adults. Frequencies of CD4$^+$ T-cells among adults and children for each antigen stimulation and individual cytokine production was compared by plotting responding cells after normalization with unstimulated cultures. (A) Bar graphs for each cytokine mentioned in the upper left corner was plotted and compared for adults and children, (B) The pattern of cytokine production in children and adults after SEB stimulation (C) The pattern of cytokine expression in the culture supernatant was also confirmed with cytometric bead array (CBA) method after stimulation with PspA and SEB as described in M&M and are plotted as bar graph after normalization with unstimulated cultures. A two-tailed Mann Whitney test was used to calculate P values. P *<0.05, **<0.005.

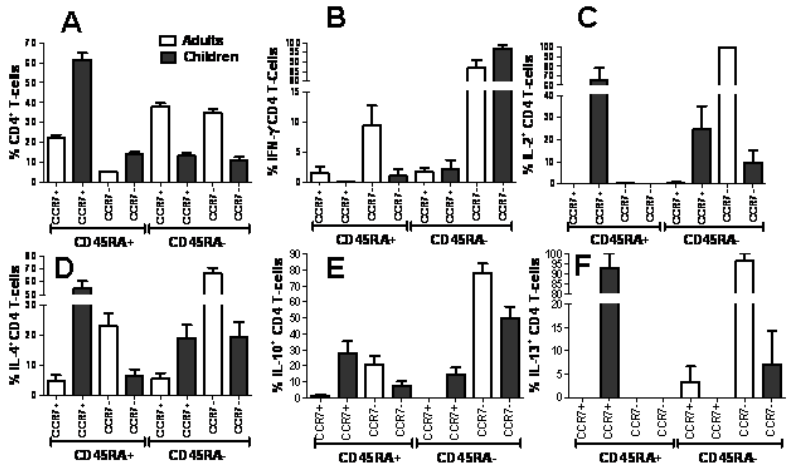

Figure 3: Heterogeneity in the memory phenotypes of responding CD4$^+$ T-cells frequencies among children and adults. Cells were sequentially gated on the individual cytokine positive cells as described in the material and methods for various cytokines. With the help of positive control (SEB) the antigen-specific CD4$^+$ T-cells were divided into four different phenotypes - naive (CD45RA$^+$CCR7$^+$), effector (CD45RA$^+$CCR7$^-$), central memory (CD45RA$^-$CCR7$^+$) and effector memory (CD45RA$^-$CCR7$^-$) and the percentages were calculated for each cytokine (A) IFN-g, (B) IL-2, (C) IL-4, (D) IL-10, (E) IL-13.

Conclusions

CD4$^+$T-cells in the circulation of young children were capable of producing antigen-specific cytokine responses against the two respiratory pathogens.

Distribution of various cytokine producing CD4$^+$ T-cells was divergent between young children and adults.

Memory phenotypic profiles of antigen-induced CD4$^+$T-cells were divergent among young children and adults.

CD69 expression on CD4$^+$T-cells differed under unstimulated and in response to antigenic stimulation in young children compared to adults (data not shown).

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Naturally Acquired and Vaccine Induced IgG, IgG1 and IgG2 Antibody Levels Against Pneumococcal Polysaccharides in Children with Recurrent Acute Otitis Media

Karli Corscadden[1], Lea-Ann Kirkham, PhD[1], Eva Mowe[1], Shyan Vijayasekaran, MD[2], Harvey Coates, MD[2], Peter Richmond, MD[1], Selma Wiertsema, PhD[1]

[1]School of Paediatrics and Child Health, [2]Department of Otolaryngology, Head and Neck Surgery, University of Western Australia, Perth, Western Australia

Objective

S. pneumoniae is one of the most common pathogens associated with recurrent acute otitis media (rAOM). Immunisation with the 7- valent pneumococcal conjugate vaccine (PCV-7) has had a limited impact on rAOM. Second generation pneumococcal conjugate vaccines including additional serotypes have recently been developed (PCV10, PCV13). The aim of this study was to investigate antibody levels against pneumococcal polysaccharides included in PCV7, PCV10 and PCV13 in children vaccinated with PVC7 either with or without a history of rAOM.

Methods

Serum samples were collected from 173 children aged between 6 and 36 months with a history of rAOM and 77 aged-matched healthy controls. Using multiplex fluorescent bead technology, serotype specific serum IgG, IgG1 and IgG2 levels were measured against the PCV-7 serotypes 4, 6B, 9V, 14, 18C, 19F and 23F and additional pneumococcal serotypes 1, 3, 5, 7F and 19A which are included in PCV10 and PCV13.

Results

Non-PCV7 serotype specific IgG, IgG1 and IgG2 titres were higher in rAOM children compared to healthy controls, with serotypes 5 being significant. In contrast, the vaccine induced IgG, IgG1 and IgG2 levels against PCV7 serotypes were similar in rAOM and healthy controls.

Conclusions
Children with rAOM do not have an impaired antibody response to pneumococcal conjugate vaccine, suggesting that vaccines including additional pneumococcal serotypes may have an impact on rAOM. The higher naturally acquired antibody titres to non-PCV7 vaccine serotypes may be due to increased pneumococcal exposure in children with rAOM. Functionality of these antibodies remains to be determined.

Preventative and Therapeutic Application of Transcutaneous Immunization Against Experimental Nontypeable *Haemophilus influenzae*- Induced Otitis Media

Laura Novotny[1], John Clements, PhD[2], Lauren Bakaletz, PhD[1]
[1]Center for Microbial Pathogenesis, The Research Institute at Nationwide Childrens Hospital & The Ohio State University College of Med, Columbus, Ohio, [2]Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, Louisiana

Transcutaneous immunization (TCI) is a noninvasive immunization strategy that utilizes the immunocompetence of the skin to induce systemic and mucosal immune protection. As otitis media (OM) is a disease of the respiratory mucosa, we examined TCI as a means to prevent and treat nontypeable *Haemophilus influenzae* (NTHI)-induced OM. Immunogens that target NTHI OMP P5 and the Type IV pilin adhesins admixed with the potent adjuvant LT(R192G-L211A) were applied to the pinnae of chinchillas followed by NTHI challenge. Immunogen-specific IgG and IgA were detected in serum and nasopharyngeal lavage fluids, which demonstrated the induction of both systemic and mucosal immune responses. Moreover, receipt of the immunogens resulted in 2.5-4 log fewer NTHI within the nasopharynges and middle ears ($p< 0.001$) compared to adjuvant only. The mechanism for this response was attributed to the migration of DC-SIGN$^+$ dermal dendritic cells from the pinnae to local lymphoid tissues. Therefore, TCI with the NTHI adhesin-directed immunogens was efficacious against experimental NTHI-induced OM.
As the chronic nature of OM is attributed to the formation of biofilms within the middle ear, we next evaluated the therapeutic potential of TCI. Chinchillas were first challenged transbullarly to induce biofilm formation, then immunized by TCI. Within 1 week after receipt of adhesin-directed immunogens, pre-established biofilms were markedly reduced ($p< 0.05$) compared to adjuvant alone. Immunogen-specific IgG and IgA were detected in middle ear fluids, in addition to 2-5-times more of the inflammatory mediators IL-4, IFN-γ and IL-17A. These data advocate TCI as a noninvasive and inexpensive immunization regimen. Moreover, TCI could expand the use of vaccines to both protect against as well as treat OM, in addition to other diseases of the respiratory tract due to NTHI.

Support NIDCD/NIH R01DC03915 to LOB

Immune Targeting of Integration Host Factor Disrupts the Architecture of Biofilms Formed by Nontypeable *Haemophilus influenzaein Vivo*

Joseph Jurcisek[1], Kyle Obergfell[2], Steven Goodman, PhD[2], Lauren Bakaletz, PhD[1]
[1]Center for microbial pathogenesis, The Research institute at Nationwide children's Hospital & The Ohio State University College of Med, Columbus, Oh, [2]Herman Ostrow School of Dentistry, University of Southern California, Los Angeles, CA

Nontypeable *Haemophilus influenzae* (NTHI) is an important causative agent of multiple infections of the airway, including otitis media. Biofilms play an important role in the chronicity of NTHI-induced diseases and thereby understanding the biochemistry of these biofilms will likely allow for the development of more effective therapeutic or preventative approaches for the management of these highly prevalent diseases. DNA is an abundant component of the NTHI biofilm matrix, and these DNA strands assemble into a web-like meshwork of DNA which provides structure and architecture to the matrix as evidenced by the loss of biofilm after exposure to DNaseI.
It is known that integration host factor (IHF) binds in the minor groove of DNA and can both bend DNA or bind to pre-bent DNA with high specificity. We hypothesized that IHF is critical for the structural integrity of this web-like meshwork and thereby provides a scaffold for bacterial biofilm formation. We first demonstrated the presence of IHF in the biofilm matrix by immunohistochemistry, with ≥95% of all bends and vertices of adjoining DNA strands labeled for IHF. These data suggested that IHF could play a role in the formation of the meshwork or be critical to the stabilization of the biofilm matrix. We therefore were interested to determine if immune targeting of IHF could destabilize a biofilm formed by NTHI. To investigate the role of IHF in biofilm formation, we used an animal model of active immunization and biofilm reversal. We found a decrease in overall biofilm volume within the middle ears and any biofilm present appeared to have a dense, flattened structure suggestive of a collapsed biofilm. Collectively, our data suggested that IHF plays a role in the structural stability of a biofilm formed by NTHI and that targeting this protein may have significant therapeutic potential for many diseases associated with NTHI biofilms.
This work was funded by discretionary funds to LOB

Haemophilus influenzae P6 Vaccine Candidate May Not Be a Surface Exposed Outer Membrane Protein

Lea Michel, PhD[1], Jennifer Milillo[1], Joy Snyder[1], Breanna Kalmeta[1], Sharad Sharma, PhD[2], M. Nadeem Khan, PhD[2], Paul Craig, PhD[1], Michael Pichichero, MD[2]
[1]Department of Chemistry, Rochester Institute of Technology, Rochester, NY, [2]Rochester General Research Institute, Rochester General Hospital, Rochester, NY

Objective
The outer membrane protein (Omp) P6 has been a leading vaccine candidate against Nontypable *Haemophilus influenzae* (NTHi) for over twenty years. The objective of this study was to elucidate the orientation of P6 in the NTHi outer membrane.

Methods
We utilized the Nuclear Magnetic Resonance structure (solved by Parsons et al) and a combination of molecular, computational and immunological methods.

Results
We have determined the location of an epitope in P6 to monoclonal antibodies 7F3 and 4G4. This finding, along with computational analysis of the P6 structure, has led us to determine that P6 is not, as originally thought, a transmembrane protein.

Conclusions
We propose that NTHi P6, like its homologue (Pal) in *Escherichia coli*, is inserted into the inner leaflet of the outer membrane where it interacts with the peptidoglycan layer (inside the cell), but does not span the cellular membrane. This hypothesis, however, requires that P6 is not surface exposed, which contradicts immunological experiments that have demonstrated several P6 monoclonal antibodies bound to surface exposed epitopes. Therefore, we propose that P6 is not a transmembrane protein or surface exposed, but there is a putative P6-like protein which shares sequence similarity to P6, is surface exposed, and can interact with P6 monoclonal antibodies. One candidate, Omp P5 (also known as OmpA), has a homologue in *Escherichia coli* which contains a P6-like domain that is partially surface exposed at elevated temperatures. This study was supported by NIH NIDCD RO1 08671 (to M. Pichichero) and the Rochester Institute of Technology.

Serum Antibody Response to Five Vaccine Candidate Proteins of *S. pneumoniae* During Acute Otitis Media in Otitis Prone and Non-Otitis Prone Children

Ravinder Kaur, PhD[1], Janet Casey, MD[2], Michael Pichichero, MD[1]
[1]Research Institute, Rochester General Hospital, Rochester, New York, [2]Pediatrics, Legacy Pediatrics, Rochester, New York

Summary
Streptococcus pneumoniae (Spn) is one of the common bacteria responsible for episodic acute otitis media (AOM; non-otitis prone), recurrent AOM (otitis-prone) and AOM treatment failure (AOMTF) in children. In this study, we measured the serum IgG antibody response to vaccine candidate proteins PhtD, LytB, PcpA, LytB and Ply of *Spn* in children with episodic AOM (n=34), in otitis prone (n=35), and in AOMTF (n=25). At their acute AOM visit, the geometric mean titers (GMTs) of anti-PhtD, -LytB, -PhtE and –Ply IgG antibody levels in otitis-prone children were significantly lower compared to non-otitis prone children (p <0.05) and children with AOMTF (p <0.05). Comparing acute to convalescent titers after AOM, otitis-prone, AOMTF and non-otitis prone children had no significant change in total IgG antibody against the five proteins except for PhtE in children with AOMTF. While non-otitis prone children had significant increases (p <0.001) in anti-PhtD, PcpA, PhtE and Ply IgG antibody levels measured longitudinally over time between 6 and 24 months of age, otitis-prone children either failed to show rises or the rises were significantly less than the non-otitis prone children. We conclude that otitis-prone and AOMTF children mount less of an IgG serum antibody response to 5 *Spn* proteins; the lack of an adaptive immune response may account for recurrent infections.

Introduction
Streptococcus pneumoniae (*Spn*) is the most common bacterial pathogen causing acute otitis media (AOM) in children. It also causes pneumonia, bacteremia, and meningitis. Currently available pneumococcal vaccines is although safe and efficacious but induce only serotype-specific immunity; therefore several pneumococcal proteins considered to be potential vaccine candidates that contribute to virulence and are common to all serotypes. This study focuses on five such proteins: PhtD and PhtE (pneumococcal histidine triad proteins), PcpA (a choline binding protein), LytB (a murein hydrolases) and PlyD1 (a non-toxic pneumolysin derivative). In the present study, we compared the development of serum IgG antibodies to PhtD, PhtE, LytB, PcpA and Ply among three groups of 6 to 36 month old children with AOM:
1) Otitis prone group that included children who had 3 or more episodes of AOM in 6 months or 4 or more episodes in a 12 month period;

2) AOM treatment failure (AOMTF) group that included children who failed to achieve bacterial eradication and resolution of symptoms after at least 48 hours of appropriate antibiotic therapy
3) Non-otitis prone group that included children who had only one or two episodes of AOM.

Results

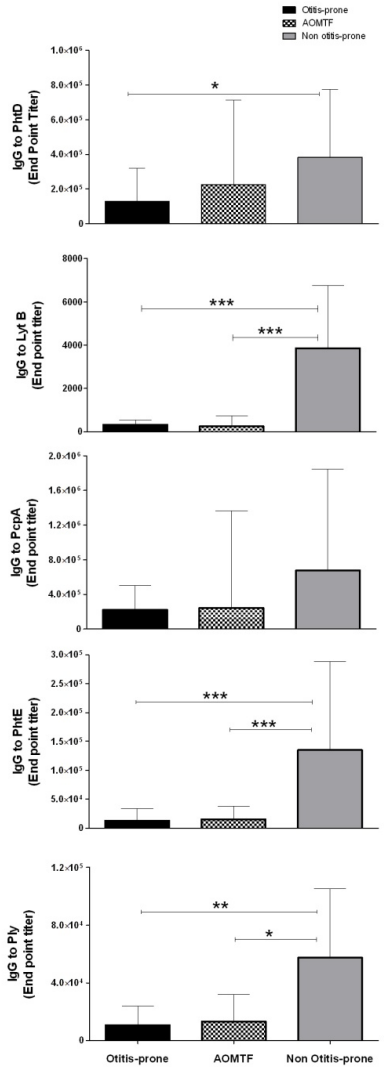

Figure 1: Comparison of IgG antibody in the serum samples of children at their acute visit of AOM in 35 otitis prone, 25 AOMTF and 34 Non-otitis prone children.

Proteins	Group (#) of children	Acute	Convalescence	>2 fold increase in antibody at convalescence stage
		IgG titers (95% Upper & lower confidence interval)		% of children
PhtD	Otitis-prone	$1.8x10^5$ ($4.1x10^4$-$7.92x10^5$)	$1.4x10^5$ ($3.9x10^4$-$5.1x10^5$)	24%
	AOMTF	$7.9x10^5$ ($6.3x10^4$-$1.0x10^7$)	$8.2x10^5$ ($7.7x10^4$-$8.7x10^6$)	15%
	Non otitis-prone	$3.9x10^5$ ($1.2x10^5$-$1.3x10^6$)	$6.1x10^5$ ($1.8x10^5$-$2.0x10^6$)	35%
LytB	Otitis-prone	[a]327 (157-682)	[a]275 (115-658)	20%
	AOMTF	[b]260 (30-2275)	[b]803 (137-4686)	33%
	Non otitis-prone	[a,b]4487 (1711-$1.1x10^4$)	[a,b]5451 (2105-$1.4x10^4$)	33%
PcpA	Otitis-prone	$6.6x10^5$ ($1.39x10^5$-$3.16x10^6$)	$6.8x10^5$ ($1.11x10^5$-$4.21x10^6$)	29%
	AOMTF	$5.1x10^5$ ($3.9x10^4$-$1.1x10^7$)	$6.9x10^5$ ($8.7x10^4$-$2.3x10^7$)	36%
	Non otitis-prone	$4.8x10^5$ ($1.2x10^5$-$1.9x10^6$)	$4.6x10^5$ ($1.2x10^5$-$1.7x10^6$)	25%
PhtE	Otitis-prone	[a]$1.3x10^4$ (3315-$5.8x10^4$)	[a]$1.4x10^4$ (3474-$6.3x10^4$)	32%
	AOMTF	[b, c]$1.8x10^4$ (3974-$8.6x10^4$)	[c]$2.2x10^4$ (3374-$1.4x10^5$)	23%
	Non otitis-prone	[a,b]$1.5x10^5$ ($5.2x10^4$-$4.5x10^5$)	[a]$1.1x10^5$ ($3.2x10^4$-$4.3x10^5$)	19%
Ply	Otitis-prone	[a]$1.6X10^4$ (5861-$4.4x10^4$)	8578 (1852-$3.9x10^4$)	40%
	AOMTF	$1.1x10^4$ (2140-$6.0x10^4$)	8534 (1675-$4.3x10^4$)	18%
	Non otitis-prone	[a]$6.45x10^4$ ($3.4x10^4$-$1.2x10^5$)	$5.46x10^4$ ($3.0x10^4$-$9.6x10^4$)	0%

Table 1: Comparison of geometric mean titer of IgG antibody in the serum samples of 22 otitis prone, 13 AOMTF and 20 non-otitis prone children at their acute vs. convalescence stage.
Significant difference (*p value*<0.05) found:
a: Otitis prone vs Non-otitis prone b: AOMTF vs Non-otitis prone
c: Acute vs. convalescence serum

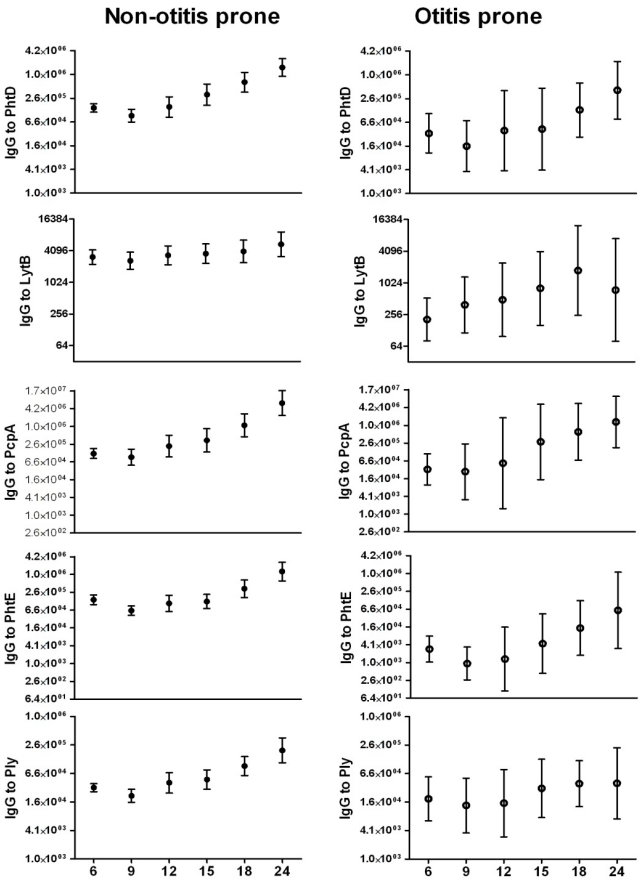

Figure 2: Comparison of IgG antibody titers with age (6-24 months) against five proteins of *S. pneumoniae* in Non-otitis prone and otitis prone children. The numbers of sera included at 6, 9, 12, 15, 18 and 24 months time points were 107, 88, 65, 61, 55, and 44 respectively for the non-otitis prone children 10, 10, 9, 10, 10 and 4 respectively for the otitis prone children. Significant difference for all the five proteins except LytB (p<0.07), comparing relative rise in IgG serum antibody between 6 to 24 months was found in non-otitis prone children while the difference was not significant in otitis prone children (p= 0.40 for protein PhtD, p = 0.39 for LytB, p = 0.11 for PcpA, p = 0.09 for PhtE and p = 0.42 for Ply).

Conclusions

We observed immunologic hyporesponsiveness in otitis prone children against *Spn* antigens. Otitis prone children failed to demonstrate or had a significantly slower age related rise in antibody to all five *Spn* proteins.

Children with AOMTF behave immunologically similar to otitis prone children.

The administration of a vaccine composition comprising at least one or more of PhtD, PhtE, PcpA, LytB and detoxified pneumolysin (e.g., PlyD1) by the parenteral route may prove useful to mitigate the immunological hyporesponsiveness noted following natural exposure to *Spn*.

Supported by NIH NIDCD RO1 08671, Thrasher Research Fund and Sanofi Pasteur

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Sublingual Immunization as a Novel and Noninvasive Method to Prevent Experimental Nontypeable *Haemophilus influenzae*-Induced Otitis Media

Rachel Balder[1], Amanda Dickson[1], Dana Staffen[1], Laura Novotny[1], John Clements, PhD[2], Lauren Bakaletz, PhD[1]
[1]Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital & The Ohio State University College of Med, Columbus, Ohio, [2]Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, LA

Nontypeable *Haemophilus influenzae* (NTHI) is a commensal in children which can cause otitis media (OM) as well as many other respiratory tract diseases. A vaccine against OM due to NTHI is greatly needed and the noninvasive method of sublingual immunization could reduce the discomfort and costs associated with injectable vaccines. Parenteral immunization with NTHI OMP P5- and Type IV pilus-derived immunogens called LB1 and rsPilA, respectively, and a LB1-rsPilA chimeric immunogen called chimV4 demonstrated efficacy against experimental NTHI-induced OM. We examined the character and efficacy of the immune response elicited by sublingual immunization of these formulations as a novel immunization strategy.
Chinchillas were immunized with either LB1, rsPilA, or chimV4 admixed with the adjuvant LT (R192G-L211A), or adjuvant alone. A minimal volume of 20 µl of each formulation was pipetted under the tongue of each animal to facilitate delivery of antigen to the sublingual caruncle and to prevent dose loss by swallowing. Two doses were delivered at weekly intervals, followed by intranasal and transbullar challenge with NTHI. Receipt of rsPilA or LB1 with adjuvant resulted in a 20 and 60% reduction, respectively, of culture-positive middle ears compared to animals that received adjuvant alone. Further, receipt of LB1+ adjuvant resulted in a 40% reduction of culture-positive nasopharynges. Strikingly, animals that received chimV4 + adjuvant eradicated NTHI from the middle ears and nasopharynges within 14 days of direct challenge. Sublingual immunization also induced increased levels of immunogen-specific IgG within serum compared to preimmune titers.
These data are the first to demonstrate efficacy of sublingual immunization to prevent experimental NTHI-induced OM. Furthermore, sublingual immunization has the potential to expand vaccine delivery to underserved populations and serve as an alternative immunization strategy.

Support: NIDCD/NIH R01DC03915

Bactericidal Antibody Response Against P6, Protein D and OMP26 of Non-Typeable *Haemophilus influenzae* After Acute Otitis Media in Otitis Prone Children

M. Nadeem Khan, PhD, Ravinder Kaur, PhD**,** Michael Pichichero, MD
Research Institute, Rochester General Hospital, Rochester, New York

Objective
To study the bactericidal antibody response to 3 nontypeable *Haemophilus influenzae* (NTHi) outer membrane proteins (D, P6 and OMP26) in otitis prone children (age 7-28 months) after an acute otitis media (AOM) caused by NTHi.

Methods
Samples were selected from a repository generated during a prospective, longitudinal study of AOM caused by NTHi. A standard bactericidal assay was used; results were determined based on the minimum dilution of sera producing 50% killing of the target strain.

Results
25 children were studied. Among 17 acute sera available, 4 (24%) versus 18 of 25 convalescent sera (72%)had detectable bactericidal activity to the homologous strain isolated from middle ear fluid by tympanocentesis. 11 sera (58%) had bactericidal activity against a heterologous NTHi strain but titers were lower (p= 0.002) as compared to the homologous strains. Levels of protein D (p 0.002) and P6 (p = 0.003) but not OMP26 antibodies were higher in bactericidal compared to non-bactericidal sera. Serum IgG and bactericidal titers correlated for protein D (r= 0.44) and P6 (r=0.48; p= 0.07 and 0.002, respectively). For 3 (14%) and 18 (86%) of 21 bactericidal sera tested, removal of anti-protein D and P6 antibody, respectively, resulted in a drop in bactericidal antibody (p <0.005 for P6). P6 specific bactericidal antibody accounted for almost 50% of the total bactericidal activity measured.

Conclusion
Otitis prone children develop bactericidal antibody to NTHi protein D and vaccine candidate P6 but not OMP26. This study was supported by NIH NIDCD RO1 08671.

Construction of Adenoviral Vectors Expressing Chimeric Hia-HMW1/HMW2 Adhesion Proteins of Nontypeable *Haemophilus influenzae*

Linda Winter**, Stephen Barenkamp, MD**
Pediatrics, St. Louis University School of Medicine, St. Louis, MO

Introduction
The Hia and HMW1/HMW2 proteins arecritical adhesins and potential protective antigens of NTHi. Recombinant adenoviruses (Ads) are promising vaccine vectors that can stimulate mucosal immunity in the upper respiratory tract. Our objective was to construct recombinant Ads expressing chimericHia-HMW1/HMW2 proteins.

Methods
pDC316 is an E1 shuttle plasmid derived from the Ad5 genome that contains a human CMV promoter, a polycloning site, and SV40 polyadenylation signals. pBHGloxΔE1,3Cre is a complementary plasmid derived from the nearly full-length Ad5 genome containing deletions in the Ad5 E1 and E3 regions. Co-transfection of HEK 293 cells withpDC316containing an *hia-hmw2* or *hia-hmw2* gene insert and pBHGloxΔE1,3CregeneratedrAds by *in vivo* recombination.

Results
A segment of the *hia* gene that encodes the surface-exposed portion of the Hia protein was cloned into pGEMEX-2 to generate pGEMEX-Hia. Segments of the *hmw1A*or*hmw2A*structural genes encoding B-cell epitopescommon to HMW1/HMW2 proteinsof most NTHi were then cloned into pGEMEX-Hia to generate translational fusions encoding chimeric Hia-HMW1 or Hia-HMW2 proteins.The *hia-hmw1* or *hia-hmw2*fusions were excised from the respective plasmids and cloned into pDC316. Co-transfection of 293 cells with recombinant pDC316 Hia-HMW1 or pDC316 Hia-HMW2 and pBHGloxΔE1,3Cregenerated viral plaques from which rAds expressing chimericHia-HMW1/HMW2fusion proteins were recovered. Transfection of non-permissive A549 respiratory epithelial cells with the recombinant Ads resulted in high-level expression of chimeric fusion proteins for up to ten days.

Conclusion
Recombinant Ads expressing chimeric Hia-HMW1/HMW2 proteins will be important new tools in NTHimucosal vaccine development efforts.

Antibodies to HMW1/HMW2-Like and Hia-Like Adhesins Are Opsonophagocytic for Both Homologous and Heterologous Nontypeable *Haemophilus influenzae*

Linda Winter**, Stephen Barenkamp, MD**
Pediatrics, St. Louis University School of Medicine, St. Louis, MO

Introduction
The HMW1/HMW2 and Hia proteins are highly immunogenic NTHi surface adhesins that are promising vaccine candidates. Almost all NTHi express either an HMW/HMW2 or Hia adhesin. Our objective was to assess the opsonophagocytic activity of antibodiesagainstnative and recombinant HMW1/HMW2 proteins and recombinant Hia proteinsagainst a large panel of unrelated NTHi.

Methods
Native HMW1/HMW2 proteins were purified from three HMW1/HMW2-expressing NTHi.Recombinant HMW1 and HMW2 fusion proteins expressing the distal halves of mature HMW1andHMW2 were purified from *E. coli* transformed with HMW1 and HMW2 expression plasmids.Recombinant Hia fusion proteins expressing the surface-exposed portions of mature Hia from two Hia-expressing NTHi were purified from *E. coli*transformed with Hia expression plasmids. Immune sera were raised in guinea pigs and assessed for their ability to mediateopsonophagocytic killing.

Results
No preimmune serum mediated killing of NTHi. The three HMW1/HMW2 native protein immune sera mediated killing, respectively,of 11/26, 22/26, and 15/26 unrelated HMW/HMW2-expressing NTHi. As a group, they mediated killing of 25/26 HMW1/HMW2-expressing strains. The recombinant HMW1 and HMW2 immune sera mediated killing, respectively, of 14/26 and 4/26HMW1/HMW2-expressing strains. The two Hia immune sera mediated killing, respectively, of 14/23 and 15/23 unrelated Hia-expressing NTHi. Together they mediated killing of 17/23 Hia-expressing strains.

Conclusions
Antibodiesagainstnative HMW1/HMW2 proteins and recombinant Hia proteins are capable of mediating broad-based opsonophagocytic killing of homologous and heterologous NTHi. A vaccine formulated with a limited number of HMW1/HMW2 and Hia proteins might provide protection against disease caused by nearlyall NTHi.

Efficacy of Monophospholipid a Via TLR4 on Eliciting Nontypeable *Haemophilus influenzae* Clearance from Nasopharynx in Mice

Takashi Hirano, PhD, MD, Satoru Kodama, PhD, MD**, Toshiaki Kawano, MD, Masashi Suzuki, PhD, MD**
Faculty of Medicine, Department of Otolaryngology, Oita University, Yuhu, Oita

Introduction
Induction of mucosal immunity has been focused on vaccination via mucosal route. However, these vaccinations elicited the acquired immune responses. We investigated the effectiveness of monophosphoryl lipid A (MPL) for inducing innate immune responses on bacterial clearance from nasopharynx and the kinetics of cellular responses against nontypeable *Haemophilus Influenzae* (NTHi) when MPL was inoculated intranasally prior to NTHi inoculation. The aim of the present study was to explore the possibility of clinical new strategy, which induces the innate immune responses via TLR4.

Methods
Mice were administrated intranasally with 1~40μg MPL prior to the bacterial challenge. At 12, 24 hours, days 3 and 7 after the administration with MPL, the NTHi challenge was performed intranasally. Nasal washes were collected at 6 and 12 hours after the challenge, and The number of bacterial colonies were counted.

Result
Depending on dose manner, MPL inoculation enhanced a significant reduction in bacterial recovery from nasopharynx at 6 and/or 12 hours after the bacterial challenge when compared to control mice.

Conclusion
MPL is effective on NTHi clearance from nasopharynx and the kinetics of cellular responses against NTHi when MPL is inoculated intranasally prior to NTHi inoculation. These findings may lead clinical new strategy, which induces the innate immune responses via TLR4.

Immunological Differences in Upper Respiratory Tracts Between Sublingual and Intranasal Immunizations

Yoshiko Hayamizu, MD
Otolaryngology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

Introduction
As the immunization routes to induce mucosal immune responses in upper respiratory tract, oral, intranasal, and sublingual immunizations have been investigated. Among those mucosal immunizations, intranasal and sublingual immunizations have been already applied for clinical use against influenza and allergic diseases. However, the differences between intranasal and sublingual immunization are not fully understood and it is not yet clarified which immunization is more effective and safer in order to prevent upper respiratory infection such as acute otitis media. In the present study, mice were immunized with phosphorylcholine (PC), a structural component of a wide variety of Gram-positive and –negative bacterium including *S.pneumoniae* as well as *Haemophilus influenzae.*, intranasallyorsublinguallyand the immune responses were compared.

Methods
BALB/c 6week-age-female mice were 3 times immunized intranasally or sublingually, with PC-KLH and cholera toxin (CT) as a mucosal adjuvant. Seven days after the final immunization,nasal wash, saliva, vaginal wash, serum,and mucosal specimens were collected. PC specific antibody titers in those samples and IgE level were examined by ELISA and ELISPOT assay.Further, $CD4^+$ T cells were isolated from spleen cells, and the production of IL-4 and IFN-□ was examined.

Results
Saliva, nasal wash and vaginal wash samples obtained from sublingual group as well as intranasal group contained high level of PC-specific IgA compared with control. In addition, sublingual group of vaginal wash samples is higher than intranasal that.Serum levels of PC-specific IgM, IgG, and IgA antibodies were elevated in both.The levels of PC-specific IgG subclass antibodies in serum were higher in both as compared to the control. Furthermore, the level of IgG2a antibody response was significantly higher in the sublingual group than in the intranasal group.The production of IFN-γ and IL-4 from CD4+ T cells was significantly higherin both as compared to the control. Sublingual group produced a greater amount than the intranasal group.T15

reactivity and the IgA antibody reactivity with cell membrane lysates isolated from several different strains of *S. pneumoniae* and NTHi were compared. The reactivity of the nasal wash IgA was positively correlated with that of T15. The correlation was statistically significant for both *S.pneumonia*.IgE levels were remarkably increased when CT was intranasally administrated, but not when administrated sublingually.

Conclusions

PC-KLH sublingual administration induced the same levels of PC-specific immune response as intranasal administration in the mice. Moreover, in vaginal mucosa, sublingual administration caused a strong immune induction.Type Th2 and Th1 responses were more strongly induced by PC sublingual administration than PC intranasal administration. These findings suggest that the mechanism in the induction phase might be different between intranasal and sublingual administration.PC-specific IgA induced in the nasal mucosa by PC sublingual administration responded to multiple strains of pneumococcus. It was considered that sublingual administration effectively regulated immunity in mice.

Senior Lecture/Plenary Session

Manipulation of Innate Immunity by Commensal Bacteria

Jeffrey Weiser, PhD[1]
[1]Pediatrics and Microbiology, University Pennsylvania School of Medicine, Philadelphia, PA

The Weiser laboratory examines host and bacterial factors in colonization of the respiratory tract. The focus of this seminar is the innate immune response of this mucosal surface. It is well established that innate immunity affects the size and composition of microbial populations. This seminar will demonstrate how bacteria subvert this host response for their competitive advantage and how this within-host microbial competition selects for virulence characteristics. This work has also shown how microbial populations provide signals that systemically prime innate immune responses.

Evolutionary Adaptations and Side Effects: Implications for Otitis Media

Paul Ewald, MD[1]
University of Louisville, Louisville, KY

Evolutionary principles provide a basis for understanding why diseases have the characteristics they have and options for long-term control. One insight from evolutionary principles is the distinction between evolutionary adaptations and side effects of evolutionary processes. When a manifestation of disease is a side effect, options for treatment and long term control are broadened. Important aspects of the infectious processes that lead to otitis media appear to be side effects. This conclusion suggests alternative explanations of epidemiological evidence and novel options for controlling otitis media.

Chinchilla to Human and Personalized Medicine

Howard Jacob, PhD[1]
[1]Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin

Ten years ago the human genomic sequence was released, followed by the mouse, then rat, and practically the entire zoo. Many species were selected to bridge evolutionary niches to provide important information about speciation and offering the ability to look at gene conservation to help identify which nucleotides were critical (unchanged across species). Other species were selected because they had key phenotypes that reflect a human disease, symptom or trait. The chinchilla was selected in part because it is an important model of otitis media. This infrastructure of genome sequence has prompted several years of speculation that it will change the practice of medicine.

In July of 2009, the Medical College of Wisconsin embarked on personalized medicine to try and save the life of a young boy with severe Crohn's-like symptoms that had resulted in over 100 surgeries and the loss of his colon. All standard therapeutic interventions had been deployed by the clinical care team. As a last ditch effort, a request was made to have the patient's genome sequenced. A mutation was found in the gene XIAP, previously not identified with the gastrointestinal (GI) tract. This gene's association with XLP2, a disease that is lethal without a bone marrow transplant (BMT), prompted recommendation for BMT in this patient. The BMT was done, and 7 months later, the patient is doing remarkably well with no GI symptoms. We have since developed a clinical protocol that is being used to end diagnostic odysseys and will be discussed during this seminar.

RSV: Implications for Otitis Media

Robert Welliver, PhD
Division of Infectious Diseases, Women and Children's Hospital of Buffalo, Buffalo, NY

It is well accepted that viral respiratory infections are the major predisposing factor for episodes of acute otitis media (AOM) in children. Viral infections are known to impair Eustachian tube dysfunction, but whether other mechanisms contribute to the association of viral infections with AOM is not known. Possibilities include exaggerated cytokine responses to viral infection, enhancement of other inflammatory responses in the middle ear (particularly allergic responses), exfoliation of epithelial cells with obstruction of the tube, release of chemical mediators of narrowing of the tube diameter, formation of mucus plugs, and suppression of innate immune responses with facilitation of bacterial growth.

Innate Immune Response of the Inner Ear Induced by Otitis Media

David Lim, MD[1,2], Sung Moon, MD, PhD[2]
[1]Laboratory of Pathogenesis of Ear Diseases, House Ear Institute, Los Angeles, CA, [2]Department of Cell & Neurobiology, Keck School of Medicine, University of Southern California, Los Angeles, CA

Otitis media(OM) can affect inner ear function, which include sensorineural hearing loss (SNHL)[1, 2]and disequilibrium.[3-5]Although the incidence of SNHL secondary to OM is not high (1 to 3%)[6-8], the incidence can be underestimated because ultrahigh-frequency SNHL and transient hearing threshold shifts are hard to detect with routine hearing tests[9], and vestibular function test is not routinely done for children with history of otitis media.

These inner ear dysfunctionsare believed to be caused by entry of OM pathogen components or cytokines from the middle ear into the inner ear fluid compartments, however, the underlying mechanisms are poorly understood.[10-15] It is believed that the round window membrane is permeable for bacterial molecules of OM pathogens (e.g. endotoxins and peptidoglycans) and inflammatory mediators.[16-20] Once in the inner ear, these molecules induce pathologic changes including sterile inflammation of the perilymphatic space and the spiral ligament, as well as stria vascularis swelling and sensory cell degeneration.[13, 14]

Our laboratory demonstratedearlier, that,the major cell type involved in innate immune response is the spiral ligament fibrocytes (SLFs).Moon et al[21]showed that OM pathogens, such as nontypeable H. influenzae (NTHi) and S. pneumonia, can induce up-regulation of expression of chemokines involved in attracting proinflammatory monocytes and leukocytes by the spiral ligament fibrocytes. It has been known that there are several subtypes of spiral ligament fibrocytes, which are involved in the transporting ions such as K^+ and Ca^{++}, and other molecules necessary for the functional maintenance of the sensory cells.[22, 23]Previously, we reported that SLFs recognize NTHi via TLR2-dependent NF-κB activation and release monocyte-attracting molecules.[24]Our laboratory established a rat spiral ligament cell line and used it for characterizing the cell signaling pathways involved in inner ear inflammation secondary to OM.[25]Our recent study reported in this Symposium (Oh et al, Podium Presentation) that SLFs up-regulateneutrophil-attracting molecule, Cxcl2, in response to NTHi through an ERK/AP-1 signaling pathway. Altogether, we suggest that SLFs plays a critical role in the innate immune response in the inner ear through recognizing of OM pathogens and releasing of chemokines attracting inflammatory cells (Fig. 1).

In summary, OM can impact on the function of the inner ear, includestransient sterile inflammatory response resulting in high frequency hearing loss and subtle balance dysfunction. Disturbance of the inner ear function during the critical period of their sensory and motor development will have significant consequences in the child development. Higher incidences of clumsiness and accident-prone in children with history of OM illustrate such subtle consequence of OM that has been overlooked by clinicians and researchers alike.Clinical implication of these findings envisioned that in the future the assessments for such consequences, and prevention/management of such consequence should be a part of comprehensive OM management. Potential translational research possibility includes novel approach to prevent OM-induced inner ear damage by manipulating cell signaling pathways.

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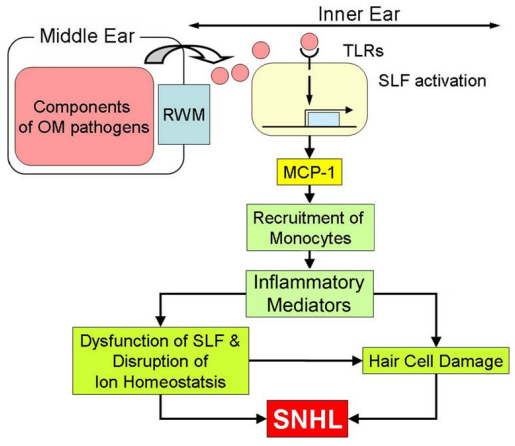

Fig. 1. Schematic illustration of the hypothetical mechanism involved in inner ear dysfunction secondary to OM.

Otitis Media: The Case for Human Evolution in Its Pathogenesis

Charles Bluestone, MD
Division ofPediatric Otolaryngology, Children's Hospital of Pittsburgh of UPMC, Pittburgh, Pennsylvania

The pathogenesis of otitis media is multifactorial, but the role of evolution is one potential factor that has not been addressed. I have posited that middle-ear disease most likely occurs only in humans, in contrast to other species in the wild, since the associated hearing loss would have selected it out during evolution as a result of predation. I present here three possible consequences of adaptations in Homo sapiens during evolution that may have resulted in otitis media: bipedalism and our big brain, speech, and loss of prognathism (facial flattening). As a consequence of our adaptation to bipedalism with the relatively constricted female pelvic outlet and rapid enlargement of the brain, humans are born 12 months too soon. This early birth resulted in an immature structure and function of the Eustachian tube, and, along with an immature immune system, helps explain the high incidence of otitis media in the first year of life. But, middle-ear disease can occur throughout life. As a consequence of adaptations of speech and facial flattening, the morphology of the palate changed, which altered the palatal muscles related to Eustachian tube function. Thus, the impact of evolution has resulted in humans having relatively poor physiologic tubal function in comparison to the non-human primate, which is related to the pathogenesis of otitis media.

Prevention of Otitis Media by Immunoprophylaxis: Past, Present and Future

Jerome O. Klein, MD
Department of Pediatrics, Boston University School of Medicine, Boston, MA

Vaccines and immunoglobulins have demonsrated variable efficacy in prevention of acute otitis media (AOM). Serotype specific pneumococcal serums reduced the mortality of bacteremic pneumococcal pneumonia in the 1930s but were not evaluated for efficacy in prevention of AOM. Later studies of immune globulins identified some benefit for reducing the incidence of AOM in selected groups of children. The first vaccine to be evaluated for prevention of AOM was a pneumococcal polysaccharide vaccine; efficacy was limited because the vaccine was not immunogenic in the infants at greatest risk of AOM. In contrast, the conjugate pneumococcal vaccines have been effective in reducing the burden of AOM when administered beginning at 2 months of age. Inactivated and live Influenza virus vaccines have also been effective in reducing the incidence of AOM complicating influenza virus infections. Nevertheless, AOM persists as one of the most frequent illnesses of childhood. Future studies will focus on the potential of an otitis media vaccine, viral vaccines, efficacy of monoglonal antibody preparations, products (eg oligosaccharides) that prevent attachment of microbial pathogens to mucosa and global distribution of preventive products.

Otitis Media – The Application of Personalized Medicine

Robert Ruben, MD
Departments of Otorhinolaryngology – Head and Neck Surgery and Paediatrics, Montefiore Medical Center, Bronx, NY

The application of knowledge to the care of individual patients has evolved. During most of recorded history it was based on experience -- case reports; not until the middle of the 20th Century did randomized control trials (RCT) come to be the "gold standard." But by the beginning of the 21st Century, the limitations of the RCT and their synthesis, the meta analysis, have been recognized with regard to their applicability to an individual patient. With the intense increase in our knowledge base, medicine now can be personalized beyond the possibilities of earlier periods. The approach of personalized medicine requires evaluation of four parameters: the individual patient's intrinsic susceptibility; intrinsic morbidity, extrinsic susceptibility; and extrinsic morbidity. The characteristics of the disease agent -- how much and its virulence -- must also be factored in. The application of the knowledge of intrinsic and extrinsic risk factors and their effect on susceptibility and morbidity for an individual with otitis media will be presented. These individualized data define the intervention which is instituted based upon whether there is a high susceptibility and/or morbidity or a low susceptibility and/or morbidity from the aggregate of the intrinsic and extrinsic risk factors.

Workshops

Otitis Media Diagnosis

Peggy Kelley, MD
Department of Pediatric Otolaryngology, The Children's Hospital, Aurora, CO

This is THE Otoscopy workshop developed by the late Dr. Sylvan Stool delivered personally throughout Central and South America as well as Malaysia until his passing in 2005 and now continued through the IAPO (InterAmerican Pediatric Otolaryngology). It emphasizes visualizing the entire tympanic membrane and using the pneumatic otoscopy. It is a useful training and review course for many types of health care providers including clinicians and audiologists. The workshop is divided into 3 stations. At the first station, 50 video clips of pneumatic endoscopic eardrum examinations are reviewed. The first viewing of the slides is proctored and the slides are labeled. This is followed with a "test" of unlabeled videos. The second station has a videoendscope where, after being instructed in its use, participants scope each other's ear and discuss findings and pertinent clinical information for each examination. The third station teaches technical points of pediatric otoscopy by practicing on silicone baby heads that have ear canals and different findings on pneumatic otoscopy. Participants rotate through each of the 3 stations. This workshop teaches how to perform pneumatic otoscopy and what to look for which serves to meet the new otitis media diagnosis requirement that in order to diagnose acute otitis media, signs of inflammation as well as the presence of fluid must be present on physical examination of the eardrum.

Quality of Life and Otitis Media Management

Richard Rosenfeld, MD
Department of Otolaryngology, SUNY Downstate and Long Island College Hospital, Brooklyn, NY

This workshop will promote a lively, interactive, and informal discussion (or perhaps, debate) about how clinicians can incorporate quality of life considerations into managing children with otitis media. Quality of life (QoL) refers to the patient's perception of how a disease affects their physical, social, and emotional function. In the context of otitis media the child's parent or caregiver is often used as a proxy in assessing QoL, which therefore includes the impact of illness not only on the child's QoL but also that of the proxy. After a brief review of QoL concepts, the presentation will shift to specific concerns for children wtih recurrent acute otitis media and/or chronic otitis media with effusion. Last, a series of brief, clinical vignettes will be used to engage the audience in a spirited discussion of how QoL considerations may influence management decisions.

Guidelines

Anne Schilder, MD, PhD[1], Maroeska Rovers[2]
[1]Department of Otorhinolaryngology - Head and Neck Surgery, Wilhelmina Children's Hospital, University Medical Center Utrecht, The Netherlands, [2]Department of Epidemiology, Biostatistits & HTA and operating rooms, Radboud University Medical Center Nijmegen, The Netherlands

Clinical practice guidelines are crucial in supporting and promoting good clinical practice, making patient care more effective and achieving better outcomes for patients. The systems used to grade evidence and recommendations however vary accross countries and organizations. This is confusing and impedes effective communication. All participants of this workshop are asked to bring their own (national) guideline on otitis media with effusion. During this workshop we will look at similarities and differences between the guidelines. The workshop will use interactive techniques to facilitate training in the use of GRADE (Grading of Recommendations Assessment, Development and Evaluation)and AGREE (Appraisal of Guidelines for Research and Evaluation in Europe).

New Guidelines Demands New Guidings

Ann Hermansson, MD, PhD
ENT, University of Lund, Lund, Skåne

Objectives
All around the world new guidelines for treatment of Acute Otitis Media and Secretory Otitis Media are made. To make these work it is important to provide a better knowledge of diagnostic skills and basic knowledge of the clinical background. Unfortunately very much time is spent writing these guidelines but less time making them work.

Methods
Theoretical and practical "discussions" from different angles.

Workshop Description
Relevance and purpose: To better the skills and knowledge of diagnozing AOM and SOM and choose the appropriate treatment and follow up. Not only focusing on antibiotic treatment but also targeting other important aspects such as epidemiology, surgical treatment and general counselling.

Comments
Discussions between different specialists(ENT, Pediatricians, Family doctors ..) from different parts of the world would benifit us all! We have just agreed on new guidelines in Sweden and are preparing study material to make implementation better!This is often unfortunately not given enough attention thus making good guidelines less effective.

Update on NIDCD Support of Otitis Media Research and Funding Opportunities

Bracie Watson, PhD
Division of Scientific Programs, National Institute on Deafness and Other Communication Disorders, NIH, Bethesda, MD

This session will provide an update on NIDCD support of otitis media research. There will be an update on OM study section assignments since the 9th International Otitis Media Symposium in 2007. Funding opportunities for new investigators will be discussed.

Chronic Suppurative Otitis Media: A Systematic Review and Summary of the Evidence on the Effects of Treatment

Peter Morris, MD, PhD[1], Amanda Leach, PhD[2]
[1]Paediatrician, NT Clinical School, Flinders University, Darwin, NT, [2]Child Health Division, Menzies School of Health Research, Darwin, NT

Objectives
To update the 2007 Clinical Evidence summary on treatments for chronic suppurative otitis media (CSOM) by reviewing the evidence from systematic reviews (SRs) and randomised controlled trials (RCTs) in children and adults.

Methods
Medline, Embase, and the Cochrane Library were searched. The search was expanded to include high quality studies of CSOM associated with cholesteatoma and ototoxicity associated with topical treatments. Data on treatment effects were extracted for the following: i) improving symptoms of otorrhoea; ii) healing perforations; iii) improving hearing; iv) reducing complications; and v) any adverse effects. Overall summaries were prepared without meta-analysis using the the "Grading of Recommendations Assessment, Development and Evaluation" (GRADE) approach.

Results
The review update included 1 new SR and 3 new RCTs. We summarised the effects of 8 core treatments: cleansing, topical antiseptics, topical antibiotics, topical antibiotics plus corticosteroids, topical corticosteroids, systemic antibiotics, systemic antibiotics plus topical antibiotics, and surgery. Within these categories, most studies were small, susceptible to bias, and used a range of medications. Only topical antibiotics and topical antibiotics plus corticosteroids had sufficient evidence to be considered "likely to be beneficial". The number of potential comparative studies and the lack of standardisation mean that comparisons may be misleading or difficult to generalise.

Conclusions
Globally, CSOM is the most important cause of moderate hearing loss. It is the type of OM most likely to be associated with severe complications. While a range of treatments have been studied, uncertainty about the beneficial effects and harms of nearly all remain.

Mini Symposia

Sonotubometry With Perfect Sequences in the Assessment of Eustachian Tube Function- Current State-

Ercole Di Martino, MD, PhD[1], Deyan Asenov[1], Viorel Nath, MD, MD[1], Aulis Telle[2], Christiane Antweiler[3], Peter Vary, PhD, PhD[3], Leif-Erik Walther, MD, PhD[4]
[1]ENT, Diako Ev. Krhs, Bremen, [2]Engeneering, IND Aachen, Aachen, [3]Engeneering, IND, Aachen, [4]ENT, Centre of Otorhinolaryngology Sulzbach, Sulzbach

Objectives
The Eustachian Tube (ET) plays an important role in middle ear pathology. Its function and mechanics are not fully understood yet. A great number of methods have been employed to assess ET function but none proved to give detailed insights in all aspects of ET physiology and pathology.
The aim of our studies was to establish a reliable, non-invasive approach to monitor ET-function under physiological conditions based on the principles of sontotubometry.
The authors give an overview on their results and experiences in various studies they undertook since 2002.

Methods
The authors used an acoustic approachand developed various custom made sonotubometric devices. Initial studies were undertakenwith sonotubometry (STM) employing an 8 kHz pure tone signal. Since physiological noise pollution by pharyngeal activity is a major obstacle in the use of acoustic signals a novel generation of signals, so called Perfect Sequences (PSEQ) was introduced to overcome these problems.

PSEQ are a periodic and deterministic random noise broadband signal with high energy efficiency and an ideally flat spectrum. Its main current use is for system identification and acoustic echo control in mobile communication systems. In contrast to other broadband signals, all frequencies greater 0 Hz up to half of the sampling rate (24 kHz in case of a sampling rate of 48 kHz) are stimulated equally and simultaneously with every period. Its features allow for an efficient noise reduction and, in combination with a system identification algorithm and the estimation of impulse responses for further investigations. Furthermore these impulse responses can be transformed by Fast Fourier Transformation (FFT) into frequency responses thus utilizing a much broader frequency spetrum for signal identification. This gives PSEQ a theoretical advantage over all other signals used up to now for sonotubometry. In the PSEQ studies the signal was used with a loud ness of 60-71 dB and a sample rate of 32-48 kHz (1-4).

For improvement of the measurement quality so-called levels were introduced. The aim was to identity noise pollution and to reduce false positive and false negative findings (5).

To trigger ET openings (ETO) four manoeuvres were employed: swallowing water and dry, Valsalva, yawning. Measurements were performed with and without the application of decongestive nasal drops (xylometazoline drops 0.1% in adults or 0.5% in children) and in some studies uni-and contralateraly.

Patients
Five major prospective clinical studies were completed: I) A study with an 8 kHz pure tone signal in healthy young probands (n=40);
II) A study with PSEQ in healthy young probands (n=25);
III) A comparative study between 8kHz signal and PSEQ in the same healthy population (n=20)
IV) PSEQ studies in healthy children up to an age of 10 yrs. (n= 14) and juveniles up to 16 yrs (n=31)
V) PSEQ studies in pathologic ears (currently n=62)
VI) Currently there are two more studies ongoing. One study is about PSEQ in patients with subjective middle ear complaints such as fullness and pressure equilibration problems but normal objective findings in tymanometry and otoscopy (VIa). The second trial is a comparative study about tympanometry, sonotubometry and tubomanometry (VIb).

Results
Study I)STM with 8 kHz could detect ETO in 39/40 patients but only 40% manoeuvres and 55% measurements were found to have triggered an ETO. Increase of loudness was a mean 14.0 dB, ETO duration was 459 ms (median). Four characteristic ETO patterns could be identified. These were spikes (63%, Fig. 1), plateau curves (23%), double spikes (9%) and descendant curves (5%). The curves were characteristic but not specific for a certain manoeuvre (5)

Study II) healthy probands reported in 92% subjective ETO that could be verified in 82% by PSEQ. Sound increase in the outer ear canal was a mean 16 dB. Duration of ETO was 468 ms.
Five characteristic opening patterns were found (Fig.2). Sensitivity of STM-PSEQ was 91%, specifity 72% (6-8).
Study III) the comparison of STM-8kHz vs. PSEQ demonstrated an increase of valid measurements from 57% (8kHz) to 74% (PSEQ). The use of levels increased this result to 82% in PSEQ (9). In 19% ETO could be detected by PSEQ only ($p < .05$). Measurement quality was with PSEQ in 46% better ($p < .005$).

Study IV)normal children and juveniles showed in 75-83% ETO. Loudness was 7.4-9.8 dB. Valsalva was not a feasible ETO manoeuvre in the children group ($p < .0001$). There was a significant difference in loudness in the various manoeuvres ($p<.001$). Other opening manoeuvres were effective than in adults. Decongestives did not improve these results (10,11).

Study V) ETO were found in 36% acute otitis media and 40% COM. Normals had 74% ETO ($p<.005$). Signal amplitude was 9.7 dB duration 231 ms in pathologic ears ($p<.001$/ $p<.05$). Multispike curves (Fig.3) were a characteristic finding inpathologic ears (12,13).
VI) the ongoing studies showed both mixed results. In trialVIa) there was a tendency towards a very favourable result for PSEQ as compared to tympanometry. But yet the number of patients recruited is too low at the moment to come to final conclusions.

Discussion
There are numerous methods for the assessment of ET function but none can cover all aspects (14,15). The use of an acoustic approach allows to examine ET function under physiological conditions. Up to now mostly signals between 6-8 kHz have been employed (16-19). The introduction of a novel type of signal for sonotubometry, the so called PSEQproved to be a useful improvement of this method. Itdemonstrated clinical significance when compared directly to a conventional 8 kHz signal for ETO detection (9). The main advantage lies in the extensive information that can be obtained from the PSEQ thus improving the S/N ratio. Further improvement could be obtained by the introduction of the so-called levels. This mean significantly reduced the number of false results (20).

In children and juveniles ET dysfunction occurs more often than in adults due to various reasons. The results of our studies demonstrated that ETO is found more oftenin juveniles than in children. The same is true for the increase of loudness during ETO as well as for its duration. Both increase with the age of the proband. Computer simulation models could demonstrate that the ET opening mechanism considerably changes with age and skull growth. Also surfactant may play a role (21,22). The examination of patients with acute and chronic pathology in the middle ear showed that this method is feasible also for these patients. Almost all ears had ETO but that function was significantly impaired. The number, the duration and the amplitude of ETO was found to be significantly reduced as compared to normals. PSEQ were able to show if there was an (early) recovery of the ET function in ears with effusion (12,13).

The ongoing studies have brought mixed results mainly due to the fact of a low number of individuals enroled up to now. There is a strong tendency that PSEQ is superior to tympanometry in patients with subjective complaints but no effusion.

Conclusion
Sonotubometry with PSEQ proved to be sensitive method for ETO detection in normal and pathologic ears. It is superior to tympanometry and the 8 kHz signal. PSEQ were feasible for all groups of patients tested. Since PSEQ are able to reflect physiologic changes with a high sensitivity and good specifity they allow a forecast on the recovery of ET function in pathologic ears. Further technical refinements are useful to establish this method in clinical routine. PSEQ are a new and promising concept for research and clinical application in patients with ET function problems.

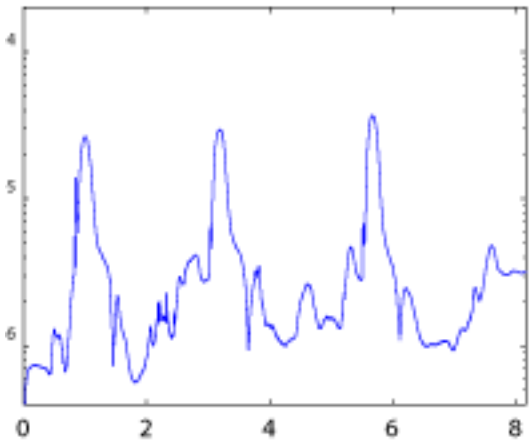

Fig 1: STM- 8kHz curves with spike-type openings

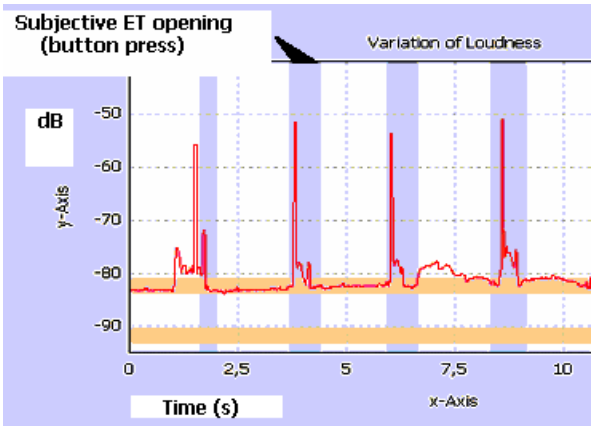

Fig 2: STM-PSEQ curves with spike-type openings

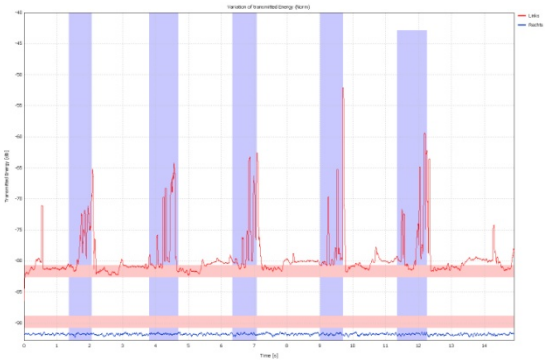

Fig. 3: STM-PSEQ curves with multi-spike openings

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Primary Secretory Otitis Media in the Cavalier King Charles Spaniel Dog

Lynette Cole[1], Valerie Samii[1], **Susan Wagner**[1], **Paivi Rajala-Schultz**[2]
[1]Department of Veterinary Clinical Sciences, [2]Department of Veterinary Preventive Medicine, The Ohio State University, College of Veterinary Medicine, Columbus, Ohio

Primary secretory otitis media (PSOM) is a disease reported in the Cavalier King Charles spaniel (CKCS). In a previous study, diagnosis was made based on visualization of a bulging tympanic membrane and mucus in the middle ear post-myringotomy. No additional tests were performed and CKCS with early disease may have been missed.
The purpose of this study was to compare three diagnostic tests (tympanometry, pneumotoscopy, tympanic bulla ultrasonography [TBU]) using computed tomography (CT) as the gold standard for diagnosis of PSOM. Sixty CKCS (31 females, 29 males) with signs suggestive of PSOM (e.g. hearing loss, neck scratching, pruritic ears) were enrolled. Mean age at presentation was 54 months (range 5-151 months). Forty-three (72%) CKCS had PSOM (30 bilateral, 13 unilateral). A bulging pars flaccida (PF) was identified in CKCS with PSOM (100% specificity); however, only 21/73 ears with PSOM had a bulging PF (29% sensitivity). Sensitivities and specificities for tympanometry, pneumotoscopy, and TBU were (84%, 47%), (75%, 79%), (67%, 47%), respectively. A myringotomy was performed in 69/73 ears with PSOM. Visible mucus was flushed from 61/69 ears. In the 8 ears where no visibile mucus was removed, post-flush CT scan identified a gas-filled bulla in 6 ears.
Based on this study, a bulging PF indicates PSOM, while a flat PF may be present in CKCS that have PSOM and those that do not. In CKCS with a flat PF, none of the above diagnostic tests can be recommended in place of CT scan for the diagnosis of PSOM.

Panel Discussion

The Role of Outer Membrane Vesicles in Bacterial Biofilms and Infection

Sarah Schooling, PhD[1], Christopher Bandoro[2], Cezar Khurisgara, PhD[1]
[1]Dept of Molecular and Cellular Biology, University of Guelph, Guelph, ON, [2]Dept of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario

In the last three decades there has been a growing awareness and appreciation of the membranous vesicles secreted by and shed from cells. This process is conserved across all three domains of life and, despite the tremendous variation in cellular origins, many of the functions fulfilled by vesicles are strikingly similar[1-7].This is likely due to similarities in the forces that drive vesicle biogenesis, where cellular components, such as proteins and lipids, are localized and concentrated into discrete compartments. This packaging of cellular materialsserves several important functions,which are determined by the molecular composition of the vesicle.These functions include the elimination of harmful or unwanted cellular contents, the protection of excreted cellular materials from degradation, inactivation or loss, and the transport of materials since vesicles are shed away from the cell surface. The latter scenario supports membranous vesicles as a vehicle for transport of cellular components from the progenitor cell or population with the ultimate purpose of transfer and delivery of the vesicular contents to distant sites. Our goal is to understand how vesicles derived from Gram-negative bacterial cells are involved in establishing and maintaining bacterial biofilm communities and how vesicles are used by biofilms to promote pathogenesis.

Gram-negative bacteria produce outer membrane vesicles

Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Moraxella catarrhalis*produce outer membrane vesicles (OMVs; Figure 1), nanoparticulate structures between 20-250 nm in diameter[1,2,5,6]. The Gram-negative bacterial cell is enclosed by a peptidoglycan-containing cell wall sandwiched between an inner and outer membrane (OM). Together, these three structures form the cell envelope and provide cellular structure and protection from the external milieu[2].OMVs are derived from the OM and the periplasmic compartment, which lies between both the inner and outer membranes;OMV composition is determined by the progenitor cells and the cellular location from which they arise[1-3,5,6]. Recent research demonstrates that OMVs are shed by over 40 different genera of Gram-negative bacteria and include species found in natural environments, as well as those associated in either commensal or pathogenic host relationships[1-3,5,6,8,9]. For this reason, OMV biogenesis appears to be conserved amongst Gram-negative bacteria.

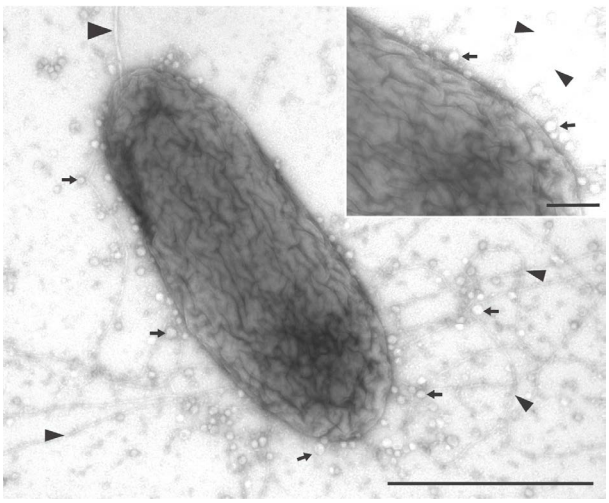

Figure 1 The release of OMVs and cells from *P. aeruginosa* biofilms.*Effluent from a biofilm reactor was prepared for whole mount negative staining with 2 % (w/v) uranyl acetate and viewed using a CM-10 transmission electron microscope at an accelerating voltage of 80 kV. Note the cell bears a polarly-located flagellum (large black arrowhead) and pili (small black arrowheads).*Surrounding the cell are numerous OMVs (black arrows).
Scale bar: 1 μm (main); 200 nm (inset).

To date, the study of OMVs has been largely restricted to those produced by free-swimming planktonic populations yet OMVs are a common feature of sedentary bacterial communitiesas well[1,10]. The ability of bacterial cells to persist and become recalcitrant is greatly enhanced through the development of structured cellular associations commonly known as biofilms[11]. Thus biofilm formation is considered vital to the growth, survival and persistence of microbiota in almost all natural habitats. This includes host-pathogen interactions, where microbial resilience usually manifests as a chronic infection. These persistent bacterial communities display phenotypic alterations such as a higher tolerance to antimicrobial therapies and to the myriadhost immune defences[11]. These factors continue to drive a strong research effort into understanding the mechanisms underlying biofilm formation, organization and function.

Understanding the contributions of OMVs to bacterial biofilms

Planktonic OMVs (p-OMVs) have been extensively studied and their extracellular interactions and activities reveal fundamental roles in bacterial physiology, communication and disease[1-3,5,6,8,9]. In contrast, little is known about OMVs derived from biofilm populations (b-OMVs), which have emerged as a fascinating yet poorly defined component of both the biofilm matrix and the factors secreted by biofilms[1,10]. The fact that b-OMVs are found both within bacterial biofilms and are exported out of the

community suggests a multiplicity of functions. It is tempting to propose that b-OMVs that reside within the biofilm matrix act locally, contributing to matrix metabolism and structure as our earlier studies have already established that b-OMVs contribute to matrix chemistry[10,12]. However, the concept of b-OMVs as vehicles for cellular secretion is intriguing and suggests they may play a significant role in allowing sessile bacterial communities to interact and communicate with the external environment. For example, b-OMVs shed from within the protective confines of an infection-related biofilm may exert effects that have been described for p-OMVs[3,5,8,9] such as cell toxicityand interference with host inflammatory responses. However, it is not yet clearly established whether different b-OMV populations are produced by progenitor cells to be retained within or exported from the biofilm.

Despite our poor knowledge of b-OMVs, the demonstrated diversity of p-OMVs and their various capabilities strongly suggest significant and varied purposes for these membrane vesicles within biofilms. Our current working hypothesis is that b-OMVs produced by Gram-negative bacteria such as *P. aeruginosa* are critical for bacterial biofilm formation and persistence. Specifically, we propose that b-OMVs comprise a tailored vesicle system that contributes to the robustness of biofilm populations within the matrix and which allows the biofilm to communicate and interact with the external environment. Both of these functions are likely critical for maintaining bacterial infections within the host and may play a crucial role in the pathogenic process. OMV formation and composition

Although a common mechanism for OMV biogenesis has yet to be elucidated[5,6], studies of Gram-negative bacteria have highlighted the intimate relationship between OMV production and cell chemistry, ultrastructure and physiology. It is possible that OMV formation is simply a common result of several different cellular processes that physically manifest as blebbing of the OM with concomitant entrapment of the underlying periplasmic content within the lumen of the newly formed vesicle. Indeed, studies reveal that OMV composition is largely representative of these two cellular compartments[2,5,13], although materials originating from other cellular locations or even acellular sources, such as DNA or gentamicin,respectively, may be associated with the OMV[13]. As the OMV forms, the asymmetry of the OM is maintained; the outer leaflet of both the OM and OMV membrane contains a high concentration of lipopolysaccharide (LPS), whereas the inner leaflet is high in phospholipids[2]. This outward-facing LPS is a critical molecule that contributes to OM integrity and permeability, and to the bacterial cell's reactivity and interactivity with its environment. LPS chemistry can affect a wide range of bacterial processes such as cellular interactions and adhesion events, biofilm formation, susceptibility to antimicrobial agents, as well as interfacing with the host immune system[14]. Indeed, p-OMV LPS has been shown to direct specific immune responses[15]. Currently, the full contributions of b-OMV LPS are unknown but our previous work[10]has demonstrated b-OMVs contain significantly more LPS than p-OMVS which, given the established importance of the LPS molecule, must surely have ramifications upon function.

Outer membrane proteins and lipoproteins are also derived from the cell envelope and form a significant portion of OMV composition. Interestingly, certain OM proteins and lipoproteins have been shown to be enriched or absent from OMVs[5,13]. These specific proteins and lipoproteins may be important to OMV function as many of the identified p-OMV proteins and lipoproteins[3,5,8,9,13,16,17] play important roles in cellular processes.For example, the aminopeptidase PepB/PaAP has beendemonstrated to influence OMV interactions with host cell surfaces and is found in abundance within the p-OMVs of pathogenic strains of *P. aeruginosa*[17]. Likewise, UspA1, a known component of *M. catarrhalis* OMVs, has been shown to enhance pro-inflammatory responses[18]. Interestingly, the protein composition of p- and b-OMVs are substantively different in both abundance and composition[10], which raises the question of whetherthese differences in OMV proteins affect OMV-based processes. It is well known that OMVs serve to compartmentalize cellular components and p-OMVs serve to segregate certain secreted proteins[13,19]. Similarly, b-OMVs may provide a method for biofilms to compartmentalize proteins within the matrix.

In addition to the lipid and protein components that are typically found in OMVs, nucleic acids are also commonly incorporated into these membranous vesicles[12,13,20-22]. It has been shown that p-OMVs serve to carry and protect DNA[20,21], and can even enable transformation of recipient bacterial cells[22]. Extracellular DNA can also interact with the surface of both p- and b-OMVs, and it has been hypothesised that OMVs serve as nucleation sites for extracellular polymers within biofilms, thereby influencing matrix dynamics[12]. Finally, unmethylated CpG DNA motifs on OMVs can also interact with the host immune system[23]and modulate responses to bacteria.

Structure and function of OMVs

OMVs bring together lipids, proteins, and nucleic acids in a tightly packed structure and this arrangement can affect the presentation of components and their interactions with the external environment. OMV composition therefore not only plays an important role in OMV structure, but also influences OMV function. In addition to this general description of OMV molecular composition, organism- and strain-specific variations do occur. For example, *P. aeruginosa* OMVs shuttle the hydrophobic quorum sensing *Pseudomonas* quinolone signal (PQS) as well as delivering quinolones that have antibacterial properties[24]. Environmental parameters and growth conditions also elicit changes in OMV composition and function *via* the responsive changes in the molecular chemistry and physiology of the progenitor cells that give rise to the OMVs[1-3,5,13]. One can predict how

the diverse microenvironments shown to exist within a biofilm and that are known to generate a range of cellular phenotypes, could similarly drive a varied population of OMV phenotypes with distinct compositions, structures and functions. These unique local conditions could in turn lead to OMV 'hotspots' with distinct location-specific functions.

To date, many diverse functions have been described for p-OMVs[1-3,5,6,8,9], and some of these functions are shared by b-OMVs[10,12]. But do b-OMVs contribute to the survival mechanisms of biofilm populations and what properties or functions promote this? As mentioned earlier, one fundamental featureof OMVs is to gather a selection of components from the OM and periplasmic compartments[1-3,5,6,13,21], package and liberate them from the cell. Many researchers now view OMVs as a unique secretory mechanism, whereby little packets of cellular content are secreted from the cell, as opposed to single components secreted by more traditional secretion systems[8,13,19]. For example, proteases derived from *P. aeruginosa*that are implicated in tissue damage and the degradation of host-associated factors are found in both p- and b-OMVs[3, 8, 9, 10, 13].Likewise, p-OMV associated PaAP/PepB and Cif are involved in specific binding to host tissue[17]and regulation of host cell physiology[25]. Similarly, the mixture of proteins found within OMVs, which includes OM proteins known to elicit host responses[3], directs the upregulation of inflammatory responses[15,19]. Note that this complex series of events, which are all vital for successful colonization, infection and subversion of the host and host defences, can be mediated through OMVs. Therefore, in addition to the traditional secretion systems that deliver proteases and lipases one at a time to host cells, OMVs, and in particular those shed from biofilms, represent a potentially potent addition to a pathogen's arsenal.

As described previously, this secretion and delivery function is also important for keeping the OMV contents concentrated and protecting them from inactivation or degradation.Since it is known that environmental stressors can also provoke changes in the production, composition and function of p-OMVs [5,13], biofilmsmay respond to the signals they receive within the host. These may include such signals as the physiological environment, administered antibiotics, antimicrobial peptides or cytokines. Interestingly, p- and b-OMVs can detoxify both cells and the external milieu in a number of ways, thereby removing stressors such as antimicrobials or phage[5,13]. The presence of enzymes in association with OMVs enables catalytic conversion of various antimicrobials(*e.g.* β-lactamase incorporated into p-OMVs degrades β-lactam antibiotics[26,27]), thereby reducing the antimicrobial load and diminishing therapeutic efficacy. Since OMVs are chemically similar to the OM of Gram-negative bacteria, b-OMVs may serve as decoys or 'molecular sponges' for antimicrobial agents, mopping up antibiotics[10] or,as has been shown for p-OMVs, components produced as part of the innate or acquired immune responses[3,8]. OMVs can also serve as a vehicle to selectively remove unwanted material from the cell. These include cell-derived components such as misfolded proteins[28], or those that become associated with the cell surface *e.g.* gentamicin[13]. We propose that b-OMVs are generated in response to such a stress and can serve to dispose of unwanted substances from the biofilm. This represents a further potential facet to the diverse mechanisms biofilms employ to tolerate antimicrobials.

OMVs in bacterial communicationand nutrient scavenging

Planktonically-derived OMVs are an established mechanism of intercellular delivery. As mentioned previously, *P. aeruginosa* shuttles the hydrophobic quorum sensing signal PQS *via*OMVs. PQS actually 'self-packages' as its incorporation into the OM leads to the generation of OMVs and the inclusion of PQS[24,29]. This is extremely relevant within the context of bacterial populations such as biofilms since PQS is a signal that drives many cellular processes important for biofilm formation and development, virulence factor production, acquisition of iron, regulation of surfactant production and swarming motility[30]. The inclusion of PQS in OMVs corroborates that OMVs then serve to generate a population-based response. This function also supports the notion that OMVs promote cell-cell interactions within the same organism. It is possible that PQS-containing OMVs shed from biofilms may be delivered to both planktonic and distant biofilm communities. This would be seen as a mechanism for cell-to-cellinteraction and communication.

OMVs can also interact with non-self bacterial cells, with a range of outcomes. For example, p-OMVs can facilitate protective community-based behaviours; OMVs produced by *M. catarrhalis*have been shown to assist in the survival of *H. influenzae*[31]. Yet many descriptions of OMV interactions with non-self bacterial cells often reveal that OMVs participate in a competitive survival process. In certain circumstances OMV-cell interactions initiate a series of events culminating in cell lysis, liberating nutrient-rich cellular contents[2,24]. Thus, these so-called 'predatory' OMVs drive mechanisms for nutrient recycling and scavenging. Predatory OMVs in bacterial biofilms have been hypothesised to be important for cryptic growth within biofilms[1]. Nutrient recycling and scavenging is also mediated through OMV-associated enzymes such asproteases, phospholipases, and lipases, which degrade extracellular substances[5,13] making them available for nutrient assimilation. These enzymes, together with the unique surface properties of OMVs, can facilitate redox processes of metals and minerals[32], making these sometimes-scarce materials available for cellular metabolism. Crucially, iron, which is a vital requirement for most bacteria, and in many cases a biologically unavailable metal, undergoes OMV-mediated catalysis to increase its bioavailability[33]. Since OMVs interact with nanowires[32], this could permit the transportation of electrons within stratified populations such as biofilms and potentially employ OMVs as catalysis centres and the nanowires as electrical conduits.

Outer membrane vesicles, bacterial infections and disease

The most well studied aspect of OMVs is their role in bacterial-driven disease processes[3,5,8,9]. Some Gram-negative bacteria infect organisms, causing acute disease with the goal of colonizing the host to progress to a more insidious chronic infection.The progression to chronic infection usually relies on the formation of bacterial biofilms[11]. The role of OMVs in this progression can be non-specific or directed by specific molecules on the surface or contained within the OMVs. The *Pseudomonas* aminopeptidase PaAP/PepB[17] is an example of how OMV surface proteins aid OMV adhesion to host cell surfaces and facilitate a specific and focussed interaction. This directed delivery approach allows the bacteria to package a variety of virulence factors, as discussed above. The OMVs protect these molecules from diffusion and/or degradation/inactivation, and deliver this pathogenic payload directly to the host cells, resulting in tissue damage and toxicity. In addition to tissue-directed damage, OMVs are known to also interact with components of the innate and acquired immune response. For example, p-OMVs can titrate anti-bacterial serum components andcomplement factors[31]. They can also elicit inflammatory responses[15,18] and generate adaptive immune memory[23]. In the latter context, p-OMVs have been recognized and developed as vaccine candidates, with particular success against *Neisseria meningitidis*. p-OMVs also contribute to biofilm formation by infectious organisms. OMVs affect early events in biofilm formation by contributing to conditioning films that form on surfaces thereby influencing subsequent cell interactions[34] and also by causing aggregation of cells[35]. Thus p-OMVs fuel the transition from acute to chronic infection state, not simply through the hostile functions as described above, but by also effectively paving the way for biofilm formation.

Pseudomonas aeruginosa and cystic fibrosis

At present, very little is known about b-OMVs in the context of infectious disease processes. Our current research focus aims to establish a comprehensive and holistic approach to understanding the complex interplay between biofilm-forming pathogenic bacteria, the OMVs they produce and the host. Our model system is the cystic fibrosis (CF) airway, which is effectively a breeding ground for select bacteria such as *P. aeruginosa*. By establishing biofilms in the lungs of CF patients, *P. aeruginosa* is protected from the host's natural defences and antimicrobial therapies; this leads to aggressive infections with adverse patient outcomes. Our goal is to understand OMV contributions to CF infection, by assessing how mimicking CF airway conditions and biofilm formation affect OMV production, composition and function. By using correlative light and electron microscopy techniques, we are assessing the distribution and migration of OMVs in biofilms to gain knowledge of biofilm formation and development. Finally, we will test OMVs against laboratory cell lines to evaluate how OMV composition changes their ability to alter immune defences and interact with host cells. The long-term goals of this work are to better understand mechanisms bacteria employ to circumvent host defences and antimicrobial agents, and to advance novel treatments by tailoring therapies and identifying design features for multivalent vaccines or drug delivery systems.Given the established importance of biofilms to infectious disease, and in particular to the chronicity of infections, the known diversity of function ascribed to OMVs will undoubtedly be of high relevance with regards to both bacterial populations and the host, to infectious disease processes and biofilm behaviour in general.

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