# TRANSLATIONAL RESEARCH IN OTITIS MEDIA IN WESTERN AUSTRALIAN CHILDREN


## HARVEY COATES


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# SUMMARY

Otitis media and its sequelae are a major cause of childhood morbidity and family disruption in Australia, particularly in the Aboriginal and Torres Strait Islander population. The causal pathway in the pathogenesis of childhood otitis media is multi factorial with genetic, immunologic, environmental and social factors impacting on the prevalence and severity of the disease. The management of otitis media can be challenging, especially with bacterial preservation strategies leading to the chronic forms of the disease, otitis media with effusion and chronic suppurative otitis media becoming recalcitrant to conventional antibiotic therapy. This thesis is an effort to show the effect of translational basic and clinical research on component parts of the causal pathways of otitis media in Western Australian children.

# SYNOPSIS

This thesis describes translational basic and clinical research studies in the causal pathways of otitis media in Western Australian children, especially in the Aboriginal population. Translational research requires a team effort, including researchers from a variety of research areas, health professionals and the involvement of community members. The author's contribution in each study is outlined in each chapter and summarised in the final chapter of this thesis.

The translational basic research addressed in this thesis will include studies in epidemiology, microbiology, immunology and genetics of otitis media, whilst translational clinical research will encompass clinical trials in drug treatment and surgical interventions and the author's advocacy for Aboriginal ear health.

The epidemiological studies in Kalgoorlie showed, for the first time, the effects of passive smoking on the ear health in both Aboriginal and non-Aboriginal children in an arid climatic region. The longitudinal urban Aboriginal schoolchildren study showed the persistence of closed middle ear disease throughout the year and beyond age ten years in this population, with a low prevalence of chronic suppurative otitis media compared with those children in rural and remote Western Australia. The swimming pool study in remote communities, which investigated whether introduction of swimming pools could influence ear health, showed a reduction in active middle ear disease, rhinitis, skin diseases, chest infections and the use of antibiotics, as well as reducing truancy with the 'no

school, no pool' rule, whilst the general community cohesiveness and esprit de corps improved.

The cost burden of otitis media in Australia was surveyed with Access Economics and showed, for the first time, the enormous direct and indirect costs of otitis media and its sequelae to the community, with annual net loss of wellbeing being of the order of $ $1.05 billion to $2.6 billion in 2008.

In the microbiological scope, studies in recurrent acute and chronic otitis media reported for the first time in humans, three different forms of bacterial preservation strategies on and within the middle ear mucosa, and in the middle ear fluid of children: bacterial biofilm, intracellular colonisation and neutrophil extra-cellular traps (NETs). This discovery has lead to changes in treatment, including the use of fluoroquinolone and macrolide antibiotics, which penetrate the cell wall in middle ear mucosal cells and the beginning of clinical trials evaluating the potential innovative use of Dornase Alfa to dissolve the thick fluid in ' glue ear ' and the biofilm seen in chronic suppurative otitis media.

Immunological and vaccine studies showed that, contrary to some other research, antibody responses to specific bacteria were not impaired in children with recurrent otitis media. In addition, the author and his team described changes in pneumococcal serotypes following the introduction of the pneumococcal vaccine PCV7.

Genetic studies addressed the effects of various genes in children with recurrent acute otitis media and discovered that FBXO11, a regulator of the TGF beta

pathway, is associated with severe otitis media in Western Australian children. Further studies utilising genome-wide association testing are investigating the genetic determinants of otitis media susceptibility in childhood.

In clinical trials for surgical intervention, the author introduced basic Fibroblast Growth Factor (b-FGF) tissue engineered myringoplasty to Australia and with colleagues published results of this technique in 40 adults and children, the first such study in the English-speaking world. This safe, quick, inexpensive procedure has the potential to quadruple the number of myringoplasties performed on the estimated 106,000 Australians with chronic tympanic membrane perforations. The author has participated in clinical trials for drug treatment of Dornase Alfa at grommet insertion evaluating its efficacy in reducing postoperative ear infections and subsequent need for further ventilation tubes.

The author has advocated for changes in prescribing antibiotic ear drops in chronic otitis media from the previous widely used potentially ototoxic aminoglycoside ear drops to the non-ototoxic fluoroquinolone ear drops, participating in a position statement leading to changes in protocols and Pharmaceutical Benefits Scheme (PBS). An Aboriginal and Torres Strait Islander Ear Health manual principally developed by the author has been widely distributed in Australia and overseas.

Findings of this thesis indicate the need for an integrated and multi-pronged approach to the management of otitis media in Western Australian children. This research has provided insights, which improve our knowledge of risk factors, pathogenesis and treatment of this chronic childhood condition.

# DISCLAIMER

Professor Harvey Coates is an author in all the papers in this thesis; however, his contribution to each paper has varied from being principal author (**P**); to secondary author (**S**); and to minor author (**M**). In line with modern collaborative basic and clinical research, much of the basic scientific research has been conducted by scientists, in concert with or supervised by the principal investigator. The clinical work collaborative research, involving multiple disciplines necessitated a secondary or minor authorship. The author was partially responsible for the conceptualization, design and conduct of the papers and research studies presented in this thesis, including processing and interpretation of data and writing of the papers. Please refer to section 7.1 Author's bibliography for a complete table of papers presented and author's contributions.

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# CHAPTER 1

# INTRODUCTION

## *1.1 Otitis media (OM)*

Otitis media (OM) is characterised by an acute inflammation with infection in the middle ear related to viral and/or bacterial infection in the middle ear and manifests in the acute stage with fluid in the middle ear (bulging eardrum), otalgia, fever, irritability and occasionally, discharge through a perforation. In developed countries OM is one of the most common paediatric illnesses for which medical advice is sought, antibiotics prescribed and surgical interventions are necessary, causing a significant burden on the healthcare system and the economy. OM affects up to 80% of children who will have at least one episode of Acute Otitis Media (AOM) by the age of 3 years [3]. In Australia, an estimate of the number of new cases of OM in 2008 suggested that between 658,006 and 1,615,486 Australians were affected [4].

The terminology used to describe OM and its sequelae has changed over time and the current definitions of the various related clinical phenotypes are summarised in **Table 1** [5].

The aetiologic factors relating to OM are multiple, and in particular are related to the social determinants of health. There are many and complex factors ranging from broad historical, cultural, social and economic factors as well as the most commonly described behavioral and biomedical risk factors. OM is also strongly related to genetic factors [6] and its prevalence is known to be greater in specific populations, such as Aboriginal, Indians and Eskimos.

In children, acute and chronic middle ear disease can lead to severe complications, including mastoiditis, facial nerve paralysis, meningitis, brain abscess, impaired hearing and can occasionally be fatal. Impaired hearing can

seriously affect performance at school, subsequent employment, and social circumstances in adulthood. There are over a 106,000 Australian estimated to have chronic perforations of their tympanic membranes with associated hearing loss, the majority of these being Aboriginal children. OM and its complications are the cause of significant burden in the Aboriginal Australian population.

| SUBTYPE | ACRONYM | DEFINITION |
|---|---|---|
| *Acute otitis media without perforation* | **AOM** | Presence of middle ear fluid with symptomatic or signs of suppurative infection may include, otalgia, fever, irritability, vomiting or diarrhoea |
| *Acute otitis media with perforation* | | Acute suppurative infection with recent discharge from the middle ear (within the last 7 days) |
| *Recurrent acute otitis media* | **rAOM** | Recurrent bouts of AOM with 3 episodes in 6 months or 4-5 in 12 months |
| *Otitis media with effusion* | **OME** | Presence of middle ear fluid without symptoms or signs of suppurative infection |
| *Chronic suppurative otitis media* | **CSOM** | Persistent discharge from the middle ear through a tympanic membrane perforation for more than 6 weeks |
| *Middle ear Effusion* | **MEE** | As a consequence of OM, the air in the middle ear is replaced by serous or mucoid fluid |

**Table 1:** Definitions in otitis media [5]

### 1.1.1 Otitis Media in Aboriginal Children of Western Australia

OM and its multiple forms affects Aboriginal and Torres Strait Islander (ATSI) children more commonly, at an earlier age and with more severity than their non-Aboriginal counterparts. The World Health Organisation (WHO) has described a prevalence of Chronic Suppurative Otitis Media (CSOM) of 4% as being a massive public health problem, requiring urgent attention, yet, in some remote

Indigenous communities in Australia, and in particular in Western Australia, there is a prevalence up to 10 times higher than this figure [7].

In Western Australia (WA), middle ear disease in Aboriginal children can vary in its prevalence from 42% in school aged children in suburban Perth [8] through to levels as high as 90% in some rural and remote Aboriginal communities. On average, the Aboriginal child has thirty two months of hearing loss in their first five years with three months for the non-Aboriginal child [9]. In the Aboriginal population of WA, the long-term social, educational and vocational effects of OM with prolonged or permanent hearing loss may lead to truancy, school failure, lack of vocational attainment and unemployment, which favours interaction with the justice system [10].

In addition to the multiple factors associated with OM, such as genetic, immunologic and gender factors, there are specific risk factors associated with OM related to the WA Aboriginal population, such as crowded housing conditions, poor nutrition, inadequate access to water and functioning sewage systems, poor or limited access to medical care, exposure to passive smoking, lack of breastfeeding and hygiene issues [5]. The association of these risk factors leads to the most severe pattern of OM found especially in children from the remote Western Australian communities, where early colonisation of the nasopharynx and upper airways by one or more of the three common pathogens causing OM (*Streptococcus pneumoniae, Moraxella catarrhalis* and *nontypeable Haemophilus influenzae)* is often reported. Early invasion of the nasophraynx by bacteria can overwhelm the immature innate immune system, resulting in inadequate antibody response [11]. These bacteria may be accompanied by or follow upper respiratory

tract infections by rhinovirus, coronavirus, adenovirus, syncytial virus and parainfluenza virus.

## *1.2 Managing Otitis Media*

The cost of OM to the Australian community is significant and costs accounting only for treatment (excluding the costs of the complications and co-morbidities) were between AUS$100 and $400 million in 2008 [4].

Management of the AOM and its sequelae usually involves administration of oral antibiotics and in some cases antibiotic ear drops together with irrigation or swabbing of ear discharge. In some cases of Recurrent Acute Otitis Media (rAOM) or the persistence of the middle ear effusion (OME) and hearing loss, insertion of ventilation tubes (grommets) is necessary, often in conjunction with adenoidectomy, to ameliorate the condition and prevent recurrence of further infections and correct the hearing loss due to the middle ear fluid.

In children with chronic middle ear perforations, repair of the tympanic membrane perforation is performed utilising a myringoplasty or tympanoplasty procedure, with autograft material such as cartilage or temporalis fascia. Newer, genetically engineered tympanic membrane repair has being pioneered by the author and his colleagues for the first time in the English-speaking world [12,13].

Adjunctive measures to help with the associated hearing loss in chronic middle ear disease include the use of hearing aids and sound amplification in the classroom.

Preventing middle ear disease includes attention to the social determinants of health, environmental modifications addressing overcrowding and environmental

smoke, the use of vaccination, early detection of OM and hearing loss and appropriate medical and surgical treatment for children with persistent middle ear disease in all its forms. In addition, it is important to educate the family, community and health workers about the importance of this condition and support them with both preventive and treatment strategies.

Implicit in the prevention and management of the middle ear disease is a competent workforce and available Aboriginal health workers, both the community health worker and the ear health worker are the critical components in the management of this condition in rural and remote Western Australia, together with ear nurse specialists, nurse practitioners, audiologists, speech pathologists, general practitioners and otolaryngologists.

Inherent in this approach is the interface between medicine and education. It is crucial to educate the pre-school teachers and teachers of Aboriginal and Torres Strait Islander children about the effects of a long-term conductive hearing loss in young children who might have English as a second or third language [14]. It is important that teachers realise the potential lifelong effects of long-term conductive hearing loss on the child's educational, learning, behavioural and vocational outcomes.

The management of OM and its sequelae still requires considerable improvement with regards to its prevention and treatment. Researchers all over the world have been focused on developing and trialling new ways to manage this disease. This type of research is called *Translational Research*.

## *1.3 Translational Research*

Translational Research (TR) is the translation of knowledge into policy and practice and has been known by various terms such as research utilisation, knowledge utilisation, research transfer and more recently, knowledge translation [15]. The term "translational research" has been adopted by the National Health and Medical Research Council [16]. The best definition of TR is "*A comprehensive applied research that strives to translate the available knowledge and render it operational in clinical and public health practice*" [17].

Initially, there were only two translational types: The first (**T1),** focusing on translating new knowledge obtained in the laboratory into the development of new methods for diagnosis, therapy and prevention of diseases and their first trials in humans. The second (**T2),** subsequently focusing on the translation of results from clinical trials into everyday clinical practice [18]. Four translational type models have been proposed both by Khoury (**Figure 1**) and the Harvard Catalyst consortium, including **T3,** which relates to the widespread adoption in clinical practice of the discovery, and **T4** to the application of the practice to derive maximum impact at a population level [2,19]. Researchers, such as Ogilvie and colleagues, believe that TR based on the 4 levels model is incomplete, lacking attention to the complex social, cultural and environmental factors [20]. Subsequently, Neil Thomson introduced a new TR level (**T5)**, which gives attention also to social and other determinants of health, which addresses broader population-level issues, including the health of specific population groups such as the Aboriginal communities of Western Australia [21].

**Table 2:** Translational research: from basic science to clinical, public health and determinants-related interventions applied to specific populations (Adapted from [2,19,20]).

| TYPE | AREA OF TRANSLATION | ATTENTION TO SOCIAL AND OTHER DETERMINANTS OF HEALTH |
|---|---|---|
| **T1** | Basic research to clinical effect | No |
| **T2** | Clinical effect to clinical intervention | No |
| **T3** | Clinical intervention to clinical practice | Some |
| **T4** | Specific public health intervention at a population level | Some |
| **T5** | Clinical, public health and determinants-related interventions applied to the population or specific sub-populations | Yes |

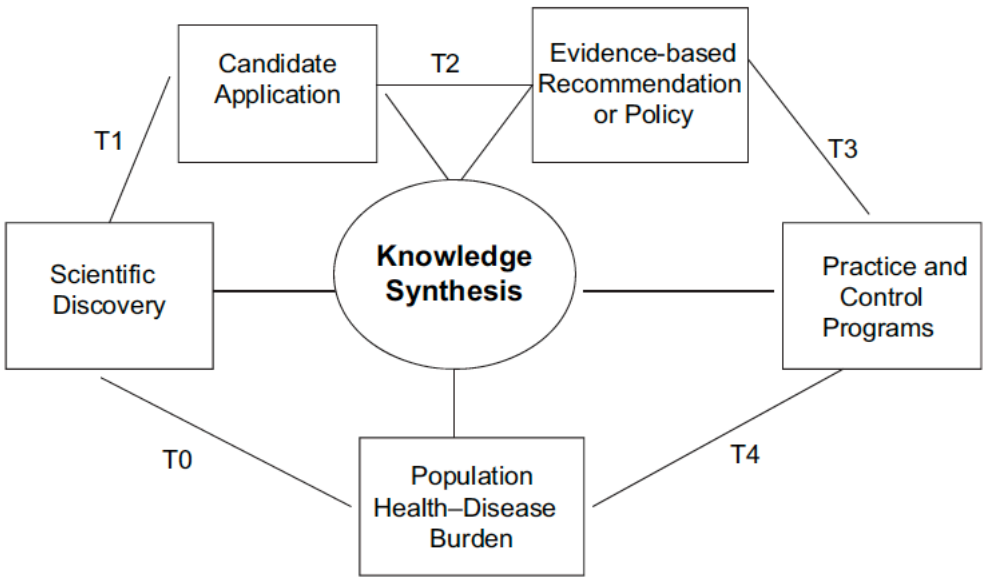
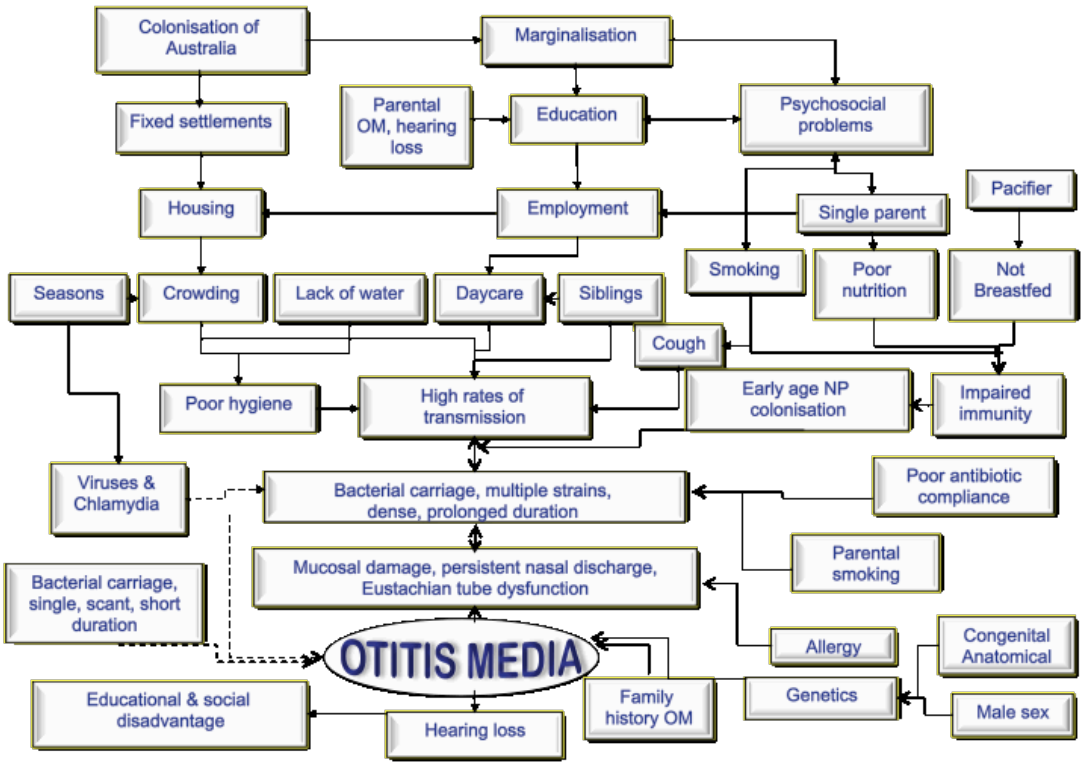



**Figure 1:** Epidemiology and the phases of translational research (TR). **TO**: scientific discovery research; **T1**: TR from discovery to candidate application; **T2**: TR from candidate application to evidence-based recommendation or policy; **T3**: TR from recommendation to practice and control programs; **T4**: TR from practice to population health impact [2].

### 1.3.1 Translational Research in Otitis Media

From the proceeding outline of the presentation and manifestation of this common chronic childhood condition can be seen the need for targeted basic and clinical research, innovative medical and surgical techniques and advocacy.

This thesis will demonstrate a number of translational, basic, and clinical research studies, which the author and his team believe will make a difference in the aetiologic pathway in otitis media. **Figure 2** shows an aetiologic framework for OM [1]. Throughout this thesis the various points at which our TR intersects the causal pathway of OM will be shown.




In 1983 the author established a specialist children's ear clinic at *Derbarl Yerrigan Health Services* in Perth. This was an endeavour to facilitate early detection and



**Figure 2:** Conceptual framework to study causal pathways to OM [1]

intervention for OM in ATSI children. Subsequently, outreach clinics in urban regions near high-density Aboriginal population areas, including Armadale-Kelmscott Community Centre, Midvale primary school, South-West Aboriginal Medical Service, Bunbury as well as outreach visits to Kalgoorlie, Carnarvon, the Kimberley and the Mid-West were established.

**Microbiologic investigations** were conducted into the influence of bacterial biofilm in the chronicity of middle ear disease. Studies in chinchillas had previously shown bacterial biofilm to be associated with acute otitis media (AOM), otitis media with effusion (OME), and chronic suppurative otitis media (CSOM) [22]. The author and his colleagues studied children with OME and CSOM and discovered and reported the first human **bacterial biofilm** found in OM, which was in an Aboriginal child with CSOM [23,24]. Further studies established the presence of bacterial biofilm in AOM, OME, and subsequently mucosal biopsies showed, for the first time, **intracellular bacteria** within the middle ear mucosa in children with OME and recurrent acute otitis media (rAOM) [25]. The presence of both biofilm and intracellular bacteria in the middle ear of children with OME and CSOM explains the recalcitrance to conventional treatment and the chronicity of these conditions. In addition, utilising samples of middle ear effusions obtained by the author and his colleagues, studies of the middle ear fluid in children with chronic OME revealed, for the first time, the presence of ***Neutrophil Extracellular Traps*** **(NETs)**, which is another bacterial method of preservation against antibiotics and the host immune system [26].

These discoveries have led to innovative strategies, including the use of **non-ototoxic topical antibiotics**, the preference for antibiotics that are absorbed intracellularly as opposed to the conventional oral antibiotic therapy for OM, and

the use of **Dornase alfa** to dissolve the NETs in the middle ear fluid of children with chronic OME in a similar methodology to which it is used for management of the excessive mucus in the airways in children with cystic fibrosis. Clinical studies of treatment included the author's participation in the National Aboriginal Community Controlled Health Organization's (NACCHO) **antibiotic eardrop trial** in rural and remote Australia, which demonstrated the improved efficacy of a non-ototoxic ear drop compared with the commonly used potentially ototoxic antibiotic eardrop previously used [11]. Advocacy for a change in the treatment pathway for CSOM, initiated by the author and colleagues, led to changes in the guidelines and protocols for medical treatment of CSOM and eventually changes in the Pharmaceutical Benefits Scheme (PBS), reducing the risk of ototoxic permanent sensori-neural hearing loss in those Australians with tympanic membrane perforations or patent ventilation tubes.

**Epidemiologic studies** with colleagues in the **Kalgoorlie Otitis Media Project** from 1989-2004 showed increased risk factors for otitis media in children exposed to overcrowding and environmental tobacco smoke. This knowledge was translated into practice with clear outcomes of a reduction in middle ear disease when tobacco smoke avoidance in the environs was adopted [27]. This study also showed the predictive value of utilising a relatively simple device detecting oto-acoustic emissions (as used in newborn hearing screening tests) to predict whether or not an Aboriginal child would develop severe OME in the first six months of life [28].

Another epidemiologic study, **the swimming pool project** assessed the benefits of community swimming pools in two remote Aboriginal communities in the Pilbara (Burringurrah and Jigalong, WA) and showed a significant reduction in OM, skin

infections, trachoma, chest infections as well as reduction in the rates of antibiotic prescription. In addition, there was a significant benefit in terms of community cohesiveness and social outcomes [29,30]. The success of this program translated into the subsequent widespread adoption of remote swimming pools in rural and remote Australia with over 20 pools installed since that time.

In 2003 the author saw a widespread need for an **Aboriginal Ear Health Manual** to aid in the teaching of Aboriginal health workers, clinical nurses, medical students and doctors, particularly in the remote and rural regions. With grants from The American Society of Pediatric Otolaryngology and a pharmaceutical company, the first and second editions of the Aboriginal Ear Health Manual were produced with copies being requested from throughout Australia, Papua New Guinea, the Pacific Islands, Canada and New Zealand [31]. The third edition, renamed "*The Aboriginal and Torres Strait Islander Ear Health Manual*" was sponsored by the Telethon Foundation and has been widely distributed [32].

In 2009 the author met Prof. Shin-Ichi Kanemaru from the Department of Otolaryngology-Head and Neck Surgery/School of Medicine (Kyoto University, Japan) who had successfully tissue engineered repair of chronic tympanic membrane perforations in Japanese patients utilising a gelatin foam scaffold and an E. *coli* genetically engineered basic fibroblast growth factor (b-FGF). The author and his colleagues have performed the first **Tissue Engineered Myringoplasty (**Kanemaru technique) in the English speaking world, and are conducting further trials funded by the State Health Research Advisory Council (SHRAC) grant to compare the use of b-FGF alone or in combination with various scaffolds in tissue engineering new tympanic membranes in patients with

chronic tympanic membrane perforations. With over 106,000 patients, principally Aboriginal children in Australia, with chronic tympanic membrane perforations, the benefits of this TR with a 10-minute procedure as opposed to the conventional 1/2 to 1 hour procedure is significant, particularly in terms of cost-benefit and utilisation of operating theatre time.

In summary, the author, together with his collaborators and colleagues, has endeavoured to intersect those risk factors for, and the sequelae of, OM in order to transform the basic research outcomes into clinical benefits to our patients, particularly those in the Aboriginal paediatric population. This thesis explores TR into OM, particularly the prevalence, aetiology, and clinical studies, leading to innovative clinical approaches for treatment of otitis media and its complications. Our studies propose clinical and public health changes to ameliorate this condition. Otitis media and its sequelae continue to be a multi factorial debilitating condition in Australia, particularly in Indigenous children where it is worse than in many third world countries.

Specific points at which our TR intersects the causal pathway of OM are highlighted in **Figure 3** and will be described further in this thesis. Studies have been arranged in chapters and comprise: **Epidemiology and Disease Burden Studies** (Housing, Crowding, Poor Hygiene and Nutrition, Education, Employment and Passive Smoking, addressed by the Kalgoorlie Study and Urban Aboriginal School Children Study), **Translational Studies in OM** (Antibiotic Therapy addressed in the Ototoxicity study, Surgical Treatment, addressed in the Tissue Engineered Myringoplasty Trial and Clinical Trials in Therapy utilising Dornase Alfa), and **Microbiologic, Immunologic and Genetic Studies in OM**

(Early age NP colonization and Bacterial Carriage, addressed in the Biofilm Study and Genetics).

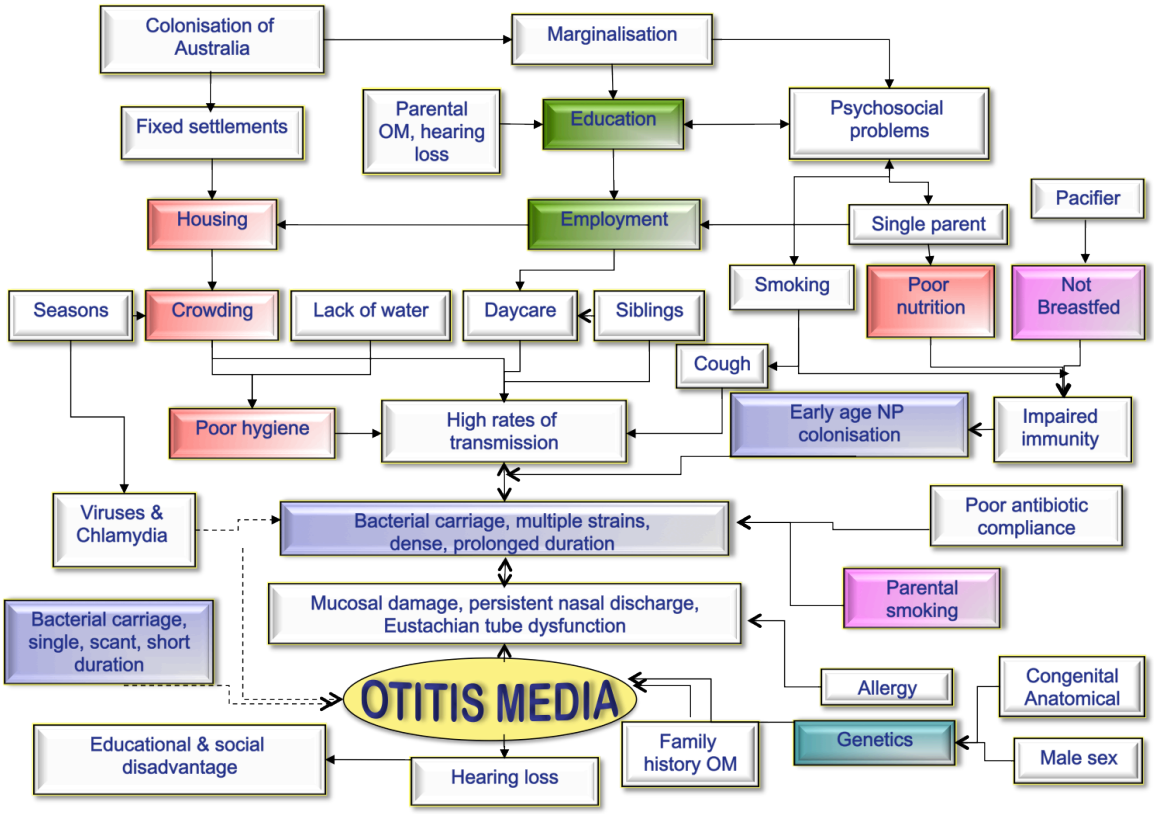



**Figure 3:** Causal pathways to Otitis Media addressed by the author and his colleagues. Figure adapted from [1].

# CHAPTER 2

# EPIDEMIOLOGY AND DISEASE BURDEN STUDIES


| Paper no* | | Supporting Articles | Thesis page no |
|---|---|---|---|
| 1 | [27] | The effect of passive smoking on the risk of otitis media in Aboriginal and non-Aboriginal children on the Kalgoorlie-Boulder region of Western Australia | 24 |
| 2 | [28] | Absent otoacoustic emissions predict otitis media in young Aboriginal children: a birth cohort study in Aboriginal and non-Aboriginal children in an arid zone of Western Australia | 29 |
| 3 | [01] | The Kalgoorlie Otitis Media Research Project: rationale, methods, population characteristic and ethical considerations. | 40 |
| 4 | [33] | Association between early bacterial carriage and otitis media in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia: a cohort study | 52 |
| 5 | [08] | Middle ear disease in Aboriginal children in Perth: analysis of hearing screening data, | 61 |
| 6 | [29] | Benefits of swimming pools in two remote Aboriginal communities in Western Australia: intervention study. | 64 |
| 7 | [05] | Natural history, definitions, risk factors and burden of otitis media. | 69 |
| 8 | [04] | Cost of treating otitis media in Australia. | 74 |


*Superscript numbers correspond to the designated general bibliography numbers – when cited in the text.

The fundamental underlying drivers of poor health, especially poor ear and hearing health in Aboriginal children, are the social determinants of health, including lack of adequate housing, education, poverty and isolation. In this chapter epidemiologic studies conducted by the author and his colleagues will be described as well as its outcomes, which aimed to clarify the relative contribution of modifiable risk factors, such as: **smoking, overcrowding in homes, hygiene and poor nutrition** in the causal pathways of OM (**Figure 4**).

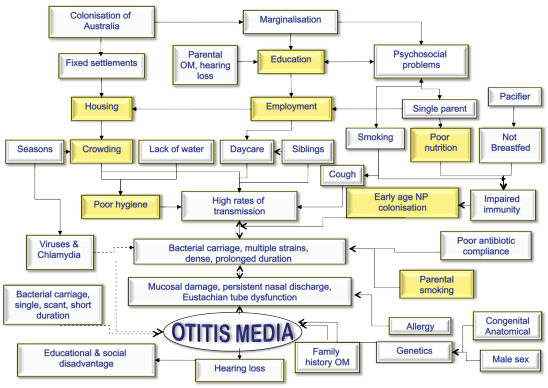



**Figure 4:** Causal pathways to OM addressed by the author and his colleagues through Epidemiologic Studies.

Amongst the epidemiologic studies in which the author participated, is the **Kalgoorlie Otitis Media Research Project,** which was the first study in Australia examining the upper-respiratory tract carriage, burden and risk factor of otitis media in Aboriginal and non-Aboriginal children living in the same geographical area. In addition, the association between "*passive smoking"* and otitis media in a cohort of Australian Aboriginal children was the first such study in Australia. Another epidemiologic study, the **Urban Aboriginal School Children Project,** was the first such longitudinal study of otitis media in an urban Aboriginal population in Australia. The **Swimming Pool Project** was similarly the first such study conducted in the country, which reached important translational outcomes in Western Australian communities and T**he Cost Burden of Otitis Media in Australia Studies**, was the first documented report developed by the author in conjunction with Access Economic Pty Limited to report on the significant impact

of otitis media upon Australian society and the cost burden of the disease in the country. The author also contributed and participated in the first utilisation of an **Earbus** to conduct epidemiologic research in Australia.

## *2.1 Epidemiology*

The author and his team has conducted epidemiologic research in otitis media with one longitudinal study in the semiarid region of Kalgoorlie in Western Australia [1,28] and the other, a retrospective, cross-sectional study of OM in urban Aboriginal school children over a 6 year period [8]. In addition, as part of these studies and other projects, the risk factors for OM in Indigenous children's populations has been addressed looking at early bacterial colonisation [33], passive smoking [27], living conditions [34] and swimming pool use [29].

### 2.1.1 The Kalgoorlie studies

The Kalgoorlie Otitis Media Research Project [1] was an epidemiologic study designed to identify key risk factors and their interaction with complex causal pathways in otitis media in Aboriginal and non-Aboriginal children in the first 2 years of life in the Kalgoorlie region of Western Australia. This was the first study in Australia to examine the upper respiratory tract carriage and burden and risk factors of OM in Aboriginal and non-Aboriginal children living in the same geographic area, which is a semiarid desert region. The influence of risk factors in the causal pathway for OM as shown in **Figure 4** comprises data relating to demographic, socio-economic and environmental, as well as microbiologic and immunologic factors in both populations.

A hundred Aboriginal children and a hundred and eighty non-Aboriginal children followed from birth to age 2 years were studied with assessment of the ear health by clinical examination, audiometry, transient evoked oto-acoustic emission (TEOAE) testing and tympanometry. This was correlated with demographic and social-economic data, as well as microbiologic and immunologic testing.

High rates of OM in Aboriginal children, as expected, were related to the poor socio-economic and environmental conditions, i.e., especially overcrowding, maternal education level, employment status and breastfeeding status.

Another important finding in the Kalgoorlie OM study was the **predictive value of the Transient Evoked Oto-acoustic Emissions (TEOAE) testing for risk of future OM** [28]. This unique use of Oto-acoustic Emission Screening (OAE), which is usually used to identify neonatal sensorineural hearing loss, of children in the first six months of life demonstrated results mimicking the finds of multi-frequency tympanometry. Absent TEOAEs correlated with the presence of otitis media with effusion (OME) and appeared to predict children at risk for OM in the future. Aboriginal children who failed TEOAE at 1 to 2 months of age had 2.6 times greater likelihood of developing future OM compared with babies who had normal TEOAE – findings not replicated in non-Aboriginal babies. This study proposed the use of the relatively simple OAE device in 1 to 2 month old Aboriginal children as it could act as a useful screening tool for OM in Aboriginal babies, being significantly less expensive and easier to use than the multi-frequency tympanometer.

In the same cohort of 100 Aboriginal and 180 non-Aboriginal children from the Kalgoorlie OM project we analysed **early carriage of the three major pathogens in OM:** *S. pneumoniae* (Pnc), *M. catarrhalis* (Mcat) and nontypeable *H. influenzae* (NTHi), utilising aspirates from the nasopharynx of babies between 1 and 3 months of age [33]. Previous studies had indicated that early bacterial carriage in the upper respiratory tract increased the risk of OM in the future [35,36]. The Menzies School of Health study on nasopharyngeal colonisation noted a 33 fold higher risk of OM in babies with both Pnc and NTHi compared with those who had only one or none of these bacteria in the nasopharyngeal aspirates [35]. Studies in Kalgoorlie of nasopharyngeal bacterial carriage showed carriage rates of Pnc (51%), Mcat (67%) and NTHi (42 to 62%) in Indigenous children, but the non-Indigenous carriage rate was 26% for Pnc to 37% for Mcat and 11 to 18% for NTHi [36]. These results are in accordance with the study by the author and colleagues, which showed that the nasopharyngeal bacterial carriage of these bacteria was higher in Indigenous children with fewer rooms in the family home or with an increased number of siblings. This study points to the need to address environmental risk factors for bacterial carriage such as overcrowding and exposure to tobacco smoke and proposes an early administration of pneumococcal-*Haemophilus influenzae* D protein conjugate vaccine to reduce bacterial carriage in infants, which would benefit especially Aboriginal children in Australia. This approach is being evaluated in Australia and Papua-New Guinea by the Vaccines Trial Group from The University of Western Australia and the Menzies School of Health Research in Darwin.

**Passive cigarette smoke** can affect mucociliary function, increase bacterial adherence to the respiratory epithelium and have a deleterious effect on local immune function. High smoking rates in Aboriginal people have been documented and this is the first cohort study of the effects of smoking in OM in an Aboriginal population [27]. Children below the age of 2 years were studied to establish whether relationship existed between OM and passive smoking. Exposure to passive cigarette smoke in the home was significantly higher in Aboriginal population with 23% of Aboriginal compared to 6% of non-Aboriginal children exposed to tobacco smoke. In addition, 49% of Aboriginal mothers smoked during the pregnancy compared to 21% of non-Aboriginal mothers. Non-Aboriginal children who attended day-care had lower risk for OM, perhaps by decreased exposure to cigarettes. To address this issue, the author and his colleagues encouraged the establishment of strong public health campaigns tackling Aboriginal people who smoke, particularly around children at home, in cars and during pregnancy. With a population attributed risk of OM due to passive smoking being 27% the potential for reduction of the burden of OM by cessation of smoking is significant.

### 2.1.2 Urban Aboriginal school children Study

The Urban Aboriginal OM project involved **Ear bus** visits to three schools in suburban Perth from 1998-2004 [8]. This was the first Ear bus utilised to screen school children in Western Australia and was a similar sound-treated bus to those utilised in New Zealand and by the author in Bali/Indonesia, where 5,000 children had their hearing screened. Longitudinal data in a cross-sectional study

comprising 80% of the Aboriginal children population of the three schools was obtained from clinical, audiologic and tympanometric tests. Data on middle ear disease and hearing impairment were collected from 119 and 94 children, respectively. The presence of 19% hearing loss observed during the first hearing testing was significant. Closed middle ear disease (characterised by OM, Eustachian tube dysfunction (ETD) or OME) was common, compared with CSOM, which is a finding quite different from rural and remote Aboriginal populations. Hearing loss was reported in 6.4% of children over the age of 10 years, whilst almost 41% of children belonging to group 4 to 7-year age (the time at which formal education begins) were affected. This is of critical importance as episodes of middle ear disease and associated hearing loss have significant implications in the children's educational progress. These findings highlight the importance of efficient health and educational programs, to reduce the occurrence of OME and CSOM, and to address the learning needs of affected children.

### 2.1.3 The Swimming Pool Study

The effect of swimming pools on a number of medical conditions including OM was studied in two remote communities (Burringurrah and Jigalong, WA) where swimming pools were introduced [29]. These studies showed reduction in the tympanic membrane perforations from 32% to 13-18% and a reduction of 61% of middle ear presentations to the health clinics. In addition to reducing the rate of otorrhoea the dilute hypochlorite solution in the swimming pools reduced the prevalence of trachoma, scabies and other skin conditions, purulent rhinitis and there was a general improvement in chronic coughs and chest conditions in the

community. In addition, with the introduction of the "no school, no pool" rule there was a dramatic increase in class attendance, in particular during the hot weather. Social and psychological improvements to the community were also noted although more difficult to quantify. More recent random controlled trials in South Australia and the Northern Territory on health effects of swimming pools have cast doubt on the efficacy and benefits of community swimming pools on OM [37,38]. It is important to note that health improvements in the Aboriginal children's population is extremely hard to achieve, and because of that, studies shouldn't ignore the overall improvement in skin health as well as the general psychological and social benefits to the community from introduction of the swimming pools [39].

## *2.2 The Cost Burden of Disease*

In Australian Aboriginal and non-Aboriginal children otitis media is the commonest cause for a child being taken to their general practitioner and the commonest reason for antibiotic prescription [40].

Otitis media impacts significantly upon Australian society and the cost burden of the disease in the country was first documented in the report by Access Economics Pty Ltd for GlaxoSmithKline, entitled: "*The Cost Burden of Otitis Media in Australia*" [41]. The author advised and assisted the production of this report and the subsequent journal article on the subject [4].

In 2008 the report estimated that 659,000 Australians suffered otitis media of whom just over half (365,000) were under 15 years of age. Children were the most affected group also in the Indigenous community, where 47,000 Indigenous

children were reported with otitis media. The majority of children with otitis media often develop a temporary hearing impairment of mild-moderate degree with an estimated 15 patients developing permanent hearing loss annually. Complications including CSOM were reported to affect 106,000 from whom an estimated 385 would suffer serious intracranial complications and a similar number would suffer mastoiditis following acute otitis media.

Productivity losses associated with otitis media in Australia amounted to $212 million, most of which was associated with loss of productivity from carers looking after children suffering otitis media. The net cost of lost wellbeing due to otitis media varied between an estimated $1.05 billion to $2.6 billion for that year.

ADDRESSING DISEASES OF DISADVANTAGE — RESEARCH

# The effect of passive smoking on the risk of otitis media in Aboriginal and non-Aboriginal children in the Kalgoorlie–Boulder region of Western Australia

Peter A Jacoby, Harvey L Coates, Ashwini Arumugaswamy, Dimity Elsbury, Annette Stokes, Ruth Monck, Janine M Finucane, Sharon A Weeks and Deborah Lehmann

## ABSTRACT

**Objectives:** To determine the risk of otitis media (OM) associated with passive smoking in young children, and any competing effect between passive smoking and childcare attendance.

**Design, participants and setting:** Prospective cohort study of 100 Aboriginal and 180 non-Aboriginal children born in Kalgoorlie Regional Hospital between 1 April 1999 and 31 January 2003. These children underwent routine clinical examinations by an ear, nose and throat specialist up to three times before the age of 2 years, and tympanometry at routine field follow-up visits from the age of 4 months. Childrens' mothers were interviewed at 1–3 weeks postpartum to provide sociodemographic data.

**Main outcome measures:** Associations between OM and exposure to environmental tobacco smoke (ETS) and childcare attendance.

**Results:** 82 Aboriginal and 157 non-Aboriginal children attended for routine clinical examinations. OM was diagnosed at least once in 74% of Aboriginal children and 45% of non-Aboriginal children; 64% of Aboriginal children and 40% of non-Aboriginal children were exposed to ETS. Exposure to ETS increased the risk of specialist-diagnosed OM in Aboriginal children (OR, 3.54; 95% CI, 1.68–7.47); few attended childcare. Non-Aboriginal children exposed to ETS but not attending childcare were at increased risk of OM (OR, 1.91; 95% CI, 1.07–3.42) while those attending childcare had no increased smoking-related risk. Tympanometry was performed on 87 Aboriginal and 168 non-Aboriginal children; a type B tympanogram (suggesting fluid in the middle ear) was also associated with passive smoking in Aboriginal children.

**Conclusions:** Reducing the exposure of children to ETS is a public health priority, especially for the Aboriginal population. A smoke-free environment will help reduce the burden of OM.

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Otitis media (OM) is the most common paediatric illness for which medical advice is sought;[1] in the general population,10%–20% of children have more than three episodes of OM before the age of 1 year.[2] The resultant hearing loss can affect educational outcomes and social circumstances in adulthood. In Aboriginal children, OM typically starts at a younger age, is much more common and is more likely to result in hearing loss than in non-Aboriginal children.[3-6]

Passive smoking can increase the adherence of bacteria to the respiratory epithelium, depress local immune function and decrease mucociliary action, and thus may be a risk factor for the development of OM.[7,8] While there are conflicting reports on the link between environmental tobacco smoke (ETS) exposure and the development of OM,[2,9-13] several meta-analyses indicate that passive smoking leads to an increased risk of OM in children of 50%–80%.[13-16] Despite high smoking rates in indigenous populations,[10,17-20] no studies investigating an association between smoking and OM in a cohort of Australian Aboriginal people have been undertaken.

Childcare attendance has also been reported to be a risk factor for OM in young children.[9,11,21,22] However, a competing effect may exist between ETS exposure and attendance at childcare. Children who are exposed to ETS in the home environment and who also attend a childcare facility will have less exposure to ETS than children who do not attend childcare and who live with smokers; therefore childcare may be beneficial for some children. A comparable competing effect between ETS exposure and childcare attendance has recently been reported in a study on passive smoking and pneumococcal carriage, which is associated with increased risk of OM.[23,24]

The Kalgoorlie Otitis Media Research Project (KOMRP) in Western Australia collected social, demographic, environmental and biological data to investigate causal pathways to OM in Aboriginal and non-Aboriginal children.[25] To establish priorities for intervention, we looked at the relationship between passive smoking and development of OM before the age of 2 years among participants in the KOMRP, and investigated any competing effect between passive smoking and attendance at childcare.

## METHODS

We undertook this research in the Kalgoorlie–Boulder area of WA, a semi-arid region about 600 kilometres east of Perth. We enrolled 100 Aboriginal and 180 non-Aboriginal children born in Kalgoorlie Regional Hospital between 1 April 1999 and 31 January 2003 into a prospective cohort study. Children were followed regularly to the age of 2 years. Multiple births, children with severe congenital abnormalities and those whose birthweight was less than 2000 g were not eligible. Details of the methods used during the study are reported elsewhere.[25]

Aboriginal and non-Aboriginal research assistants interviewed mothers or guardians of the children enrolled in the study 1–3 weeks postpartum in their homes. They collected information on smoking behaviour of parents and other household members. Mothers or guardians were asked: "Do you smoke now?" and "Do you smoke inside the house, outside only or both?" If the baby's father was part of the family unit, we asked the same questions about the baby's father. Lastly, we asked the mother or guardian whether anybody else smoked inside the house. An answer of "yes" to any of these questions was taken to indicate ETS exposure. Information was also collected about feeding practices, parental education and employment, family history of allergy or atopy, and number, age, and childcare or school attendance of other children in the household. The number of adults and chil-

**1 Otitis media outcome and demographic, social and environmental predictor variables for 82 Aboriginal and 157 non-Aboriginal children***

| Predictor variable | | Aboriginal | Non-Aboriginal |
|---|---|---|---|
| Otitis media at least once | Yes | 61 (74.4%) | 70 (44.6%) |
| | No | 21 (25.6%) | 87 (55.4%) |
| Exposure to environmental tobacco smoke (indoors and outdoors) | Yes | 51 (63.8%) | 63 (40.1%) |
| | No | 29 (36.2%) | 94 (59.9%) |
| Other children in house | Yes | 65 (79.3%) | 79 (50.3%) |
| | No | 17 (20.7%) | 78 (49.7%) |
| Mother worked recently | Yes | 24 (29.6%) | 113 (72.0%) |
| | No | 57 (70.4%) | 44 (28.0%) |
| Mother has post-school education | Yes | 33 (41.3%) | 121 (68.0%) |
| | No | 47 (58.7%) | 36 (32.0%) |
| Sex | Male | 49 (59.8%) | 80 (51.0%) |
| | Female | 33 (40.2%) | 77 (49.0%) |
| Exclusive breastfeeding 6–8 weeks postpartum | Yes | 34 (46.6%) | 98 (64.9%) |
| | No | 39 (53.4%) | 53 (35.1%) |
| Maternal age (years) at delivery | < 25 | 44 (53.7%) | 23 (14.6%) |
| | 25–29 | 20 (24.4%) | 44 (28.0%) |
| | ⩾ 30 | 18 (21.9%) | 90 (57.4%) |
| Crowding (> 1 person/room) | Yes | 59 (72.0%) | 21 (13.4%) |
| | No | 23 (28.0%) | 136 (86.6%) |
| Family history of allergy or atopy | Yes | 52 (64.2%) | 122 (77.7%) |
| | No | 29 (35.8%) | 35 (22.3%) |
| Gestational smoking | Yes | 37 (45.7%) | 31 (19.7%) |
| | No | 44 (54.3%) | 126 (80.3%) |
| Childcare ever attended | Yes | 8 (11.1%) | 52 (34.4%) |
| | No | 74 (88.9%) | 99 (65.6%) |

* Discrepancies between total numbers of children for different variables are the result of missing data. ♦

dren living in the house as well as the number of rooms in the house were documented to obtain a measure of crowding (number of people/room). Research assistants performed further routine follow-up on study participants at ages 6–8 weeks, and 4, 6, 12, 18 and 24 months, when they again enquired about feeding practices and whether the study participant was attending childcare. At these follow-up visits, we also asked the child's mother or guardian whether they smoked, and whether anybody smoked inside the house. However, as preliminary analysis indicated little change from responses at first interview and, in view of potential bias towards children remaining in the study, analyses were based on the first interview.

We scheduled three routine clinical examinations by an ear, nose and throat (ENT) specialist: before the age of 6 months, at 6–11 months and at 12–24 months. A clinical diagnosis was established by otoscopy, pneumatic otoscopy and tympanometry, and was based on national clinical guidelines.[26] Presence of acute otitis media (AOM), otitis media with effusion (OME) or perforation of the tympanic membrane (TM) with or without purulent discharge was recorded.

We performed tympanometry during routine ENT clinics from the age of 4 months onwards. From 1 May 2000, after training by an audiologist (SAW), research assistants also performed tympanometry during their routine field follow-up of children aged 4 months or older; this became routine practice once a dedicated tympanometer became available in July 2002. Interobserver variation was assessed regularly.

The final diagnosis was based on the child's most severely affected ear. If a diagnosis could not be made for one ear (eg, because of wax), then the child's final diagnosis was based on the diagnosis for the other ear.

Ethical approval to conduct this study was given by the WA Aboriginal Health Information and Ethics Committee, Northern Goldfields Health Service and Nursing Education Ethics Committee in Kalgoorlie, Princess Margaret Hospital Ethics Committee and the Confidentiality of Health Information Committee of the Health Department of WA.

## Data analysis

Two different outcomes indicative of OM were considered: diagnosis of OM (ie, AOM, OME, TM perforation with or without discharge) at a routine ENT clinic visit, and a type B tympanogram reading (indicative of fluid in the middle ear or a TM perforation) at routine ENT clinic or at field follow-up visits.

Presence or absence of OM diagnosed by an ENT specialist and tympanometry outcomes were analysed by longitudinal logistic growth modelling on a complete case basis with an underlying quadratic age dependence of disease risk assumed. Generalised estimating equations (GEE) with robust standard errors were used in these models to account for the within-subject dependencies in the data. Missing data assumptions inherent in the modelling approach were examined using Mann–Whitney U tests to compare numbers of clinical examinations and log-rank tests for time-to-study-entry (first examination) and loss-to-follow-up times.

The logistic models give rise to odds ratios (ORs) for disease risk which were assumed to be constant over time. Relative risk for a given OR was estimated on the basis of the modelled disease prevalence at 12 months of age[27] and population-attributable risk was estimated from relative risk and observed exposure prevalence.[28]

In all analyses, the primary independent variable was the presence or absence of exposure to ETS, assessed from interview responses. Indoor and outdoor ETS exposures were initially considered separately, but disease risk was not found to be affected by the reported location of the exposure, so these two categories were combined. Potential confounding factors shown in Box 1 were first assessed in univariate models and were included as covariates in final multivariate models if the univariate effect was significant ($P < 0.10$).

Separate analyses were performed for Aboriginal and non-Aboriginal children. SAS, version 9 (SAS Institute Inc, Cary, NC, USA) was used to perform all the longitudinal modelling while SPSS, version 12 (SPSS Inc, Chicago, Ill, USA) was used to perform the cross-tabulations.

**2 Results of univariate and multivariate analyses* of risk factors for otitis media diagnosed in 82 Aboriginal and 157 non-Aboriginal children at routine ear, nose and throat clinics**

| | Aboriginal | | | | Non-Aboriginal | | | |
|---|---|---|---|---|---|---|---|---|
| | Univariate | | Multivariate† | | Univariate | | Multivariate† | |
| Predictor variable | OR (95% CI) | *P* | OR (95% CI) | *P* | OR (95% CI) | *P* | OR (95% CI) | *P* |
| Environmental tobacco smoke exposure | **4.24 (2.03–8.83)** | **< 0.001** | **3.54 (1.68–7.47)** | **< 0.001** | 1.28 (0.76–2.15) | 0.35 | 1.32 (0.78–2.23) | 0.30 |
| Sex (male v female) | 0.76 (0.36–1.61) | 0.47 | | | **1.72 (1.03–2.89)** | **0.039** | **1.78 (1.07–2.97)** | **0.027** |
| Season (winter v summer) | 0.66 (0.34–1.29) | 0.22 | | | 1.20 (0.73–1.96) | 0.47 | | |
| Mother's age | | | | | | | | |
| 25–29 v < 25 years | 1.01 (0.44–2.33) | 0.98 | | | 1.15 (0.51–2.58) | 0.73 | | |
| ⩾ 30 v < 25 years | 1.45 (0.62–3.41) | 0.39 | | | 1.20 (0.58–2.45) | 0.62 | | |
| Mother with post-school education | 0.62 (0.30–1.29) | 0.20 | | | 1.20 (0.61–2.36) | 0.61 | | |
| Exclusive breastfeeding at 6–8 weeks | 1.30 (0.63–2.69) | 0.48 | | | 1.22 (0.70–2.14) | 0.48 | | |
| Other children in house | **3.06 (1.24–7.58)** | **0.016** | **2.75 (1.09–6.96)** | **0.033** | **1.80 (1.05–3.07)** | **0.032** | **1.98 (1.14–3.43)** | **0.016** |
| Mother employed | 0.46 (0.21–1.02) | 0.05 | 0.68 (0.30–1.54) | 0.35 | 0.74 (0.44–1.24) | 0.25 | | |
| Crowding (> 1 person/room) | **2.67 (1.16–6.18)** | **0.021** | 1.77 (0.74–4.24) | 0.20 | 1.20 (0.57–2.52) | 0.63 | | |
| Family history of allergy or atopy | 0.86 (0.39–1.88) | 0.70 | | | 0.64 (0.35–1.15) | 0.14 | | |
| Gestational smoking | 1.78 (0.86–3.67) | 0.12 | | | 0.87 (0.45–1.67) | 0.68 | | |
| Recent childcare attendance | Not calculated‡ | | | | 1.49 (0.80–2.78) | 0.21 | | |

OR = odds ratio. Comparison for each variable was yes v no unless otherwise indicated. Bold type indicates a statistically significant result ($P < 0.05$).
* All models adjusted for age and age$^2$. † Covariates were included in final multivariate models where the univariate effect was significant ($P < 0.10$).
‡ Very few Aboriginal children attended childcare.

## RESULTS

### Diagnosis of OM at routine clinic

There were 167 successful routine clinic examinations by an ENT specialist on 82 of the 100 enrolled Aboriginal children. Of these examinations, 101 (61%) resulted in a diagnosis of OM (82 OME, 13 AOM and six TM perforations with purulent discharge). OM was diagnosed at least once in 74% of the Aboriginal children; 64% of Aboriginal children were exposed to ETS (Box 1). Of the 180 enrolled non-Aboriginal children, 157 children (87%) had a total of 364 clinic examinations; 102 examinations (28%) resulted in a diagnosis of OM (91 OME and 11 AOM). OM was diagnosed at least once in 45% of non-Aboriginal children, and 40% of non-Aboriginal children were exposed to ETS (Box 1).

Box 2 shows that exposure to ETS was a significant predictor of OM in Aboriginal children (OR, 3.54; 95% CI, 1.68–7.47, after adjustment for confounders), but was not significant in non-Aboriginal children (OR, 1.32; 95% CI, 0.78–2.23). Assuming an OM prevalence of 49% in Aboriginal children who were not exposed to ETS (this is the fitted mean value for age 12 months), the estimated OR equated to a relative risk for ETS of 1.58. The estimated population-attributable risk of OM resulting from passive smoking among Aboriginal children was thus 27%, assuming the prevalence of ETS exposure found in our study. Other significant (at $P < 0.05$) predictors of OM from multivariate modelling were the presence of other children in the household for both Aboriginal and non-Aboriginal children, and being male for non-Aboriginal children only.

We found a significant interaction between exposure to ETS and childcare attendance in non-Aboriginal children, with childcare attendance reducing the impact of ETS exposure in the home on OM. For children who had not attended childcare in the period before a clinic examination (as assessed by the interview response immediately before the examination), ETS was a significant predictor of OM (OR, 1.91; 95% CI, 1.07–3.42, after adjustment for confounders). This equated to a relative risk of 1.47, and assuming an OM prevalence of 33% among children who were not exposed to ETS, a population-attributable risk of 16%. There was no significant ETS effect for non-Aboriginal children who recently attended childcare (OR, 0.39; 95% CI, 0.12–1.27). This interaction was not investigated among Aboriginal children because they rarely attended childcare (Box 1). Interactions between ETS exposure and crowding (number of persons/room) were not significant.

We investigated associations between the key model predictor, ETS exposure, and both the number of ENT examinations for each child and the age at the first and last examination to determine whether relevant missing-completely-at-random model assumptions were violated. No significant associations were found.

### Tympanometry

Tympanometry was performed on 249 occasions on 87 Aboriginal children, either by

research assistants in the field or by audiologists at routine ENT clinics; 148 (59%) were type B tympanograms. Of the Aboriginal children, 73 (84%) had a type B tympanogram on at least one occasion. Tympanometry was performed on 659 occasions on 168 non-Aboriginal children, and 161 (24%) were type B tympanograms; 89 (53%) had a type B tympanogram at least once.

ORs from models of documented type B tympanograms were broadly similar to those for clinical diagnosis of OM. Exposure to ETS was a significant predictor of having a type B tympanogram in Aboriginal children (OR, 2.38; 95% CI, 1.14–4.97, after adjustment for confounders), but was not significant in non-Aboriginal children (OR, 1.12; 95% CI, 0.70–1.79).

## DISCUSSION

We found that children exposed to passive smoking in the home, and who were not attending organised childcare were at increased risk of OM. The relative risk of OM related to passive smoking in our study was consistent with that found in previous studies.[13-15] Our results highlight the importance of reducing childrens' exposure to passive smoking, and that this is particularly important among Aboriginal people in whom rates of both smoking and OM are high. While Australia has been a world leader in reducing ETS exposure in public places and in reducing smoking rates in the general population, public health campaigns have not been as successful in Indigenous populations. European colonisation resulting in marginalisation and social deprivation, the lack of sustained appropriate programs, and smoking being seen as the norm in many communities are some of the reasons for limited success in reducing smoking rates among Indigenous Australians.[29-31] However, with an estimated population-attributable risk of OM due to passive smoking of 27%, such campaigns have the potential to reduce the burden of OM substantially. There is a need for more input by Indigenous Australians in the development of intervention programs, and a need for appropriate levels of funding to ensure programs are sustainable and to improve access to nicotine replacement therapy.[29]

We found that attending childcare reduced the risk of OM among those exposed to passive smoking, presumably because children who attend childcare spend less time among people smoking in the home environment. This is in keeping with studies elsewhere indicating that the greatest exposure of children to tobacco smoke is in the home and in cars, and that interventions aimed at banning smoking in homes can reduce ETS exposure.[32,33] Contrary to findings of other studies,[9,11,21,22] we did not find childcare attendance to be an independent risk factor for OM. Childcare attendance was rare among Aboriginal children in our study, in line with nationwide patterns.[34] A previous study has concluded that appropriate childcare facilities for Aboriginal children are urgently needed to improve early development and educational outcomes,[35] and our findings suggest that, given the high rates of smoking in and around homes, such facilities may offer the additional benefit of reducing the burden of OM in Aboriginal children.

A major strength of our study was its inclusion of both clinic-based and field-based outcome measures for OM. Also, the longitudinal nature of the study ensured that the progress of the study population was closely monitored to the age of 2 years, and all routine clinical assessments for OM were included in the analyses. Our findings appear to conflict with those of a recent study involving indigenous North American children, which found no association between passive smoking and any documented episode of OM in the first 6 months of life.[10] However, when we analysed our data to the age of 6 months, we found that the association did not reach significance and that it was necessary to include all OM diagnoses up to age 2 years to achieve sufficient power to detect the association between passive smoking and OM.

A potential limitation of our study was that exposure to ETS was assessed by interview response, rather than by measurement of urine cotinine levels which can detect the presence and the amount of tobacco smoke exposure. Many Aboriginal children have multiple carers, and it is possible that the person who was interviewed was not always familiar with the smoking habits of all carers and others visiting or living with these other carers. Furthermore, indoor and outdoor exposure may not have been documented reliably. Despite these limitations, the relative risk of OM associated with passive smoking found in our study was consistent with results of a study which used cotinine levels.[36]

In our study, there may have been incomplete assessment of socioeconomic status, as the household income of study participants was not recorded. Instead we relied on indicators such as maternal education, employment and crowding to capture the variation in socioeconomic status which may confound the association between smoking and disease. Indeed, it has been argued elsewhere that smoking may be merely a surrogate marker of socioeconomic disadvantage.[12]

The multidisciplinary nature of the KOMRP provides an ideal opportunity to examine the relative contributions of upper respiratory tract bacterial carriage, mucosal immune function and socioeconomic risk factors to the causal pathways leading to OM. We have shown an association between passive smoking and OM, and a beneficial effect of childcare in this context. We conclude that interventions to reduce the exposure of young children to ETS, especially in the home environment, should be urgently applied and evaluated.

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## COMPETING INTERESTS

None identified.

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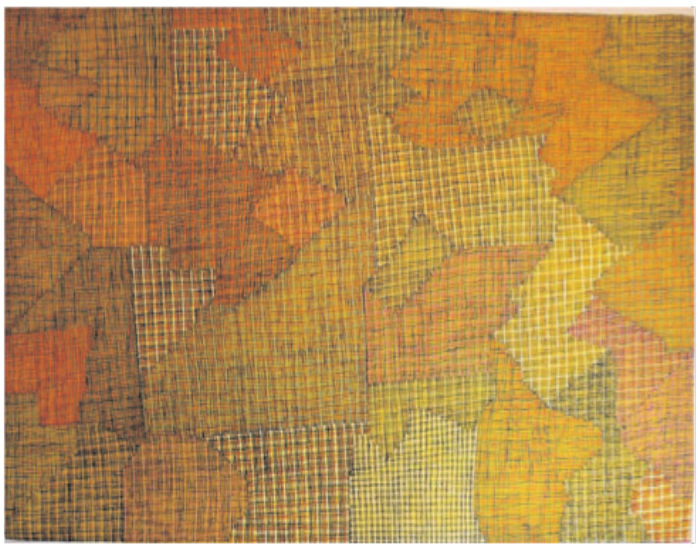

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# Absent otoacoustic emissions predict otitis media in young Aboriginal children: A birth cohort study in Aboriginal and non-Aboriginal children in an arid zone of Western Australia

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## Abstract

**Background:** Otitis media (OM) is the most common paediatric illness for which antibiotics are prescribed. In Australian Aboriginal children OM is frequently asymptomatic and starts at a younger age, is more common and more likely to result in hearing loss than in non-Aboriginal children. Absent transient evoked otoacoustic emissions (TEOAEs) may predict subsequent risk of OM.

**Methods:** 100 Aboriginal and 180 non-Aboriginal children in a semi-arid zone of Western Australia were followed regularly from birth to age 2 years. Tympanometry was conducted at routine field follow-up from age 3 months. Routine clinical examination by an ENT specialist was to be done 3 times and hearing assessment by an audiologist twice. TEOAEs were measured at ages <1 and 1–2 months. Cox proportional hazards model was used to investigate the association between absent TEOAEs and subsequent risk of OM.

**Results:** At routine ENT specialist clinics, OM was detected in 55% of 184 examinations in Aboriginal children and 26% of 392 examinations in non-Aboriginal children; peak prevalence was 72% at age 5–9 months in Aboriginal children and 40% at 10–14 months in non-Aboriginal children. Moderate-severe hearing loss was present in 32% of 47 Aboriginal children and 7% of 120 non-Aboriginal children aged 12 months or more.

TEOAE responses were present in 90% (46/51) of Aboriginal children and 99% (120/121) of non-Aboriginal children aged <1 month and in 62% (21/34) and 93% (108/116), respectively, in Aboriginal and non-Aboriginal children at age 1–2 months. Aboriginal children who failed TEOAE at age 1–2 months were 2.6 times more likely to develop OM subsequently than those who passed.

Overall prevalence of type B tympanograms at field follow-up was 50% (n = 78) in Aboriginal children and 20% (n = 95) in non-Aboriginal children.

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**Conclusion:** The burden of middle ear disease is high in all children, but particularly in Aboriginal children, one-third of whom suffer from moderate-severe hearing loss. In view of the frequently silent nature of OM, every opportunity must be taken to screen for OM. Measurement of TEOAEs at age 1–2 months to identify children at risk of developing OM should be evaluated in a routine health service setting.

## Background

In industrialised countries otitis media (OM) is the most common paediatric illness for which medical advice is sought and antibiotics are prescribed [1]. OM can lead to impaired hearing, which can seriously affect early language development, performance at school, and subsequent employment and social integration in adulthood. Repeated antibiotic treatment for OM contributes to the increasing levels of antibiotic resistance worldwide [2].

In the general population, OM incidence peaks at age 6–18 months and 3–17% of children suffer ≥ 3 attacks of acute OM annually. The prevalence of OM with effusion (OME) is ~12% at age 1 year [3]. In Western Australia (WA), OM is the second most common reason for paediatric hospital admission [4]. There are no population-based data on the burden of OM in the general population of Australia other than a nationwide study conducted 30 years ago [5].

Aboriginal Australians are the most disadvantaged sector of Australian society [6]. The enormous burden of OM in Australian Aboriginal children contributes to lifelong social disadvantage. Compared with non-Aboriginal children, OM in Aboriginal children is more common, starts at a younger age, is more likely to result in hearing loss and is associated with early onset of upper respiratory tract bacterial carriage [7-11]. The disease may be asymptomatic until purulent ear discharge is visible [8] and so treatment may not be sought until late in the disease process [12]. A recent study in remote communities in the Northern Territory (NT) of Australia showed that 91% of Aboriginal children aged 6–30 months had current clinical signs of OM and that tympanic membrane (TM) perforation rates varied between communities from 0% to 60% [11]. Data on the burden of OM in young Aboriginal children living in urban areas are sparse [5,13]. Despite the enormous burden of disease, there is currently no routine screening for ear health in preschool-age children in Western Australia. Thus, many Aboriginal children reach school age having had recurrent or continuous ear infections with serious consequences, in particular hearing loss and impaired language development. This results in poor educational attainment and behavioural problems, perpetuating the cycle of ill health, poverty and social exclusion faced by many Aboriginal people. In view of the early onset of disease which is frequently asymptomatic in Aboriginal children, we need appropriate methods of identifying children at high risk of OM in early infancy. Clinical diagnosis of OM in young children is difficult. Therefore a simple affordable tool that can be used at the primary health care level is needed to identify those in need of prompt treatment to avoid the serious consequences of tympanic membrane perforation and hearing loss.

Tympanometry is a standard method used to detect middle ear effusion and OM. Impedance audiometry (tympanometry) is normally conducted using a 226 Hz probe and is effective in people over the age of 6 months [14,15]. Before age 6 months, a 1000 Hz probe tone improves sensitivity of the test and a screening instrument with 1000 Hz probe tone has recently become available.

Measurement of otoacoustic emissions (OAEs) offers an alternative to high-frequency tympanometry to assess middle ear function in early infancy [16]. OAEs originating in the cochlea are low-level sounds in response to a given stimulus and can be measured in the outer ear. Transient evoked OAEs (TEOAEs) are used widely to identify sensorineural hearing loss in neonates, but OAEs may be absent as a result of fluid in the middle ear of young infants [17-19]. Furthermore, absent OAEs in young children may identify children at risk of OM in the future [20]. However, a recent cohort study of American Indian children followed from birth to age 2 years found no association between absent OAEs in the first month of life and subsequent risk of OM [21]. There are no other data on OAEs for indigenous populations and to our knowledge no studies have investigated the association between presence or absence of TEOAEs in early infancy after the neonatal period (without the use of other assessments of middle ear disease such as otoscopy) and subsequent risk of OM. Measurement of OAEs in the postneonatal period may offer a simple tool for use by primary health care workers to identify a high-risk group of children.

Between 1999 and 2005, we undertook a study in the Kalgoorlie-Boulder area of WA, a semi-arid region approximately 600 km east of the state capital, Perth, to investigate the causal pathways to OM in Aboriginal and non-Aboriginal children. The study aimed to identify the most important, avoidable risk factors in order to develop appropriate interventions [22]. As expected, we found that, compared to non-Aboriginal participants, Aboriginal

mothers were younger, smoked more and had poorer educational outcomes, mother and fathers were less likely to be employed and families lived in more crowded conditions [22]. In this paper we report for the first time the burden of OM in Aboriginal as well as non-Aboriginal children aged <2 years in such a setting and assess the use of TEOAEs in the first three months of life in predicting subsequent risk of OM before age 2 years.

## Methods

Details of the methods used in the study, socioeconomic and demographic characteristics and the completeness of follow-up are described elsewhere [22]. Briefly, between April 1999 and January 2003, children born in Kalgoorlie Regional Hospital to mothers intending to stay in the area for at least 2 years were recruited into the study. Following informed consent from mothers, 100 Aboriginal and 180 non-Aboriginal babies were enrolled. Multiple births, children with severe congenital abnormalities or those whose birthweight was <2000 g were not eligible. An initial evaluation was conducted in the home 1–3 weeks postpartum. Subsequently children were to be seen at ages 6–8 weeks, 4, 6, 12, 18 and 24 months.

### *Assessment of ear health*

Ear health was assessed by a variety of methods at different ages: measurement of TEOAEs in children aged <3 months, tympanometry from age 3 months onwards, clinical examination on at least 3 occasions before age 2 years, and assessment of hearing from age 6 months onwards.

#### *Transient Evoked Otoacoustic Emissions (TEOAEs)*

From April 2000 onwards, following training by the senior audiologist (SW), research assistants (RAs) measured TEOAEs in quiet surroundings during the scheduled visits at ages 1–3 and 6–8 weeks using an Echocheck TEOAE hand-held screener (Otodynamics, Hatfield, UK) [14,23]. Results of the test required no interpretation and were recorded as pass, fail or not valid, the last usually due to excessive environmental or subject noise. Valid TEOAE measurements on each ear were included when investigating the association between TEOAE and subsequent risk of OM. However, to determine the prevalence of failed TEOAEs, if the TEOAE was not valid in one ear the overall TEOAE assessment for a child was documented as not valid. Babies who had two consecutive failed TEOAE responses were referred to an audiologist.

#### *Specialist clinical examination*

ENT/audiology clinics were held 4 times annually in the Audiology Department of the Kalgoorlie Regional Hospital for routine examination of study participants. Children were to have a clinical examination at least once at ages <6, 6–11 and 12–23 months. During the first year of the study, children were to be assessed more frequently by the resident audiologist (KM) during field follow-up visits, but subsequently there was no audiologist permanently resident in Kalgoorlie. We asked parents and guardians whether the child had a cold or had any current ear problems. For convenience, children were occasionally seen by an ENT specialist (FL) at clinics held every 2–3 months at Bega Garnbirringu Aboriginal Health Services Aboriginal Corporation (BEGA).

The ENT specialists established a clinical diagnosis using otoscopy, pneumatic otoscopy and tympanometry. Diagnosis was based on national clinical guidelines [24] and classified as normal, eustachian tube dysfunction, OME, AOM without perforation, AOM with perforation, dry perforation, perforation with purulent discharge, or unknown when complete examination was not possible (e.g. unable to visualise TM). It was not possible to make a diagnosis of chronic suppurative OM given the extended time intervals between clinical examinations. The final overall clinical diagnosis was based on the child's most severely affected ear. If a diagnosis could not be made for one ear (e.g. due to presence of wax), then the final diagnosis was based on the diagnosis for the other ear. However, to examine the association between failed TEOAEs and subsequent risk of OM, we included all available diagnoses on each ear.

Children were followed up, treated or referred by the ENT specialist or audiologist as required. Routine and review visits were clearly differentiated on the database. Here we present only results of routine clinical examinations. But, when reporting on the number of children who had TM perforations, we have included additional information obtained from local medical practitioners, with parental consent obtained to access their medical records [22].

#### *Tympanometry*

Tympanometry with the 226 Hz probe tone is generally not recommended before age 6 months since a compliant ear canal may result in lower sensitivity of the test. However, good specificity and positive or negative predictive values have been reported in young children [25]. In view of irregular examinations of young children by a medical specialist, we did tympanometry from age 3 months during routine clinic and field visits to obtain an estimate of burden of OM in young infants, acknowledging that prevalence rates might, if anything, be underestimated.

Audiologists performed tympanometry (Grayson-Stadler GSI 38, Madison, Wisconsin, USA) on ears without discharge at the ENT clinic. Some children did not attend ENT clinics at all or only infrequently, despite assistance with transport and attempts to make appointments at the most convenient time for families. This might have resulted in a selection bias of those who chose to attend

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the routine ENT clinics. Therefore, to obtain additional, possibly less biased, ear health outcomes more frequently on more children, RAs performed tympanometry, using a Maico I24 screening tympanometer (Maico Diagnostics, Eden Prairie, MN, USA), during routine follow-up visits from May 2000 onwards, following training by an audiologist (SW). Inter-observer variation, involving two RAs doing tympanometry sequentially on the same child, was assessed regularly. All tympanograms were classified by an ENT specialist (HC) or an audiologist (SW) according to standard criteria [15,26]. If tympanometry could not be assessed in one ear, then the classification for tympanometry was based on the result for the other ear.

### *Hearing assessment*

An audiologist performed hearing assessments in children aged 12–23 months at the routine ENT/audiology clinics. From March 2002 onwards, hearing was also assessed when children were aged 6–11 months. Conditioned Orientation Response Audiometry was conducted in a single wall paediatric test booth. Narrow-band filtered noises at 500, 1000, 2000 and 4000 Hz were presented by a GSI 16 Clinical Audiometer (Grayson-Stadler, Madison, Wisconsin, USA) calibrated to ANSI 1980 standards [15]. Responses were categorised by averaging the four frequencies tested and classified as normal (<= 25 dB HL), mild hearing loss (26–40 dB HL), moderate loss (41–60 dB HL), or severe loss (> 60 dB HL).

### *Analysis*

The chi-square test with continuity correction and Fisher's Exact test were used to compare variables of interest in terms of TEOAE outcome. Logistic regression, incorporating Generalised Estimating Equations to account for repeated measures on individuals, was used to compare groups for tympanometry and hearing loss outcomes. The regression models were adjusted for age.

We used the Cox proportional hazards model to investigate progress to OM by computing hazard ratios for TEOAE failure. Survival times were from date of test to first subsequent OM diagnosis. The analysis was conducted on data from individual ears and robust standard errors were used to account for within-person correlation.

### *Ethical clearance*

The study was endorsed by two local Aboriginal organisations in Kalgoorlie, namely BEGA and Ngunytju Tjitji Pirni Inc. Ethical approval for the study was given by the WA Aboriginal Health Information and Ethics Committee, the Ethics Committee of Princess Margaret Hospital in Perth, and that of the Kalgoorlie-Boulder Health and Education Region.

## Results

### *Transient evoked otoacoustic emissions*

180 infants (57 Aboriginal and 123 non-Aboriginal) were screened at age <1 month and 168 infants (45 Aboriginal and 123 non-Aboriginal) were screened at 1–2 months. TEOAE responses were inconclusive for 6 (11%) Aboriginal children and 2 (2%) non-Aboriginal children aged <1 month and 11 (24%) Aboriginal and 7 (6%) non-Aboriginal children aged 1–2 months. Excluding children with non-valid results in one or both ears, TEOAE responses were present in at least one ear in 90% (46/51) of Aboriginal children and 99% (120/121) of non-Aboriginal children aged <1 month. Equivalent figures in the 1–2-month age group were 62% (21/34) and 93% (108/116), respectively. Figure 1 shows the proportion of children in whom TEOAE responses were detected in both ears. Pass rates were significantly lower in Aboriginal than non-Aboriginal children (<1 month 82% vs 97% Fishers Exact test p = 0.003; age 1–2 months 56% vs 90%, Yates chi-square = 17.33, p < 0.0001) (Figure 1). No children had sensorineural hearing loss.

### *Tympanometry*

Overall, 37% (n = 57) of 155 tympanometry readings that RAs conducted on Aboriginal children during routine follow-ups in the field were normal (type A), 50% (n = 78) had evidence of middle ear effusion (type B) and 13% (n = 20) had eustachian tube dysfunction (type C) compared with 62% (n = 296), 20% (n = 95) and 18% (n = 84), respectively, in 475 tympanometry readings in non-Aboriginal children (chi-square = 54.3, 2 df, p < 0.0001).

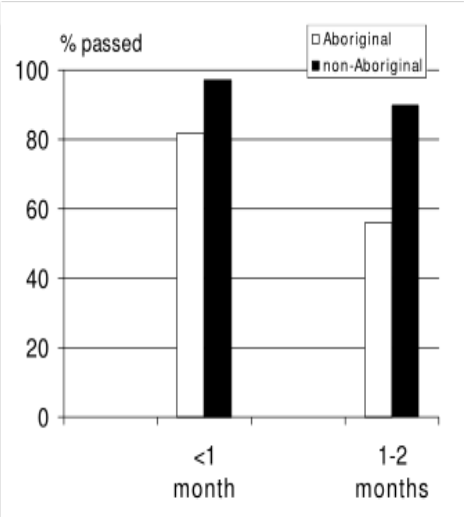


**Figure 1**
Proportion of Aboriginal and non-Aboriginal children with TEOAE responses present in both ears at ages <1 month and 1–2 months.

There was a significantly higher prevalence of type B tympanograms in Aboriginal children than non-Aboriginal children both when done by RAs in the field (odds ratio (OR) = 4.35, 95%CI 2.73–6.95) and when done by audiologists at routine ENT clinic examinations (OR = 5.16, 95%CI 3.12–8.52) (Table 1). Type B tympanograms were recorded more frequently at routine ENT clinics than at routine follow-ups in the field in both Aboriginal (OR = 1.86, 95%CI 1.22–2.86) and non-Aboriginal (OR = 1.47, 95%CI 1.06–2.04) children. When we excluded the 21% of measurements in non-Aboriginal children whose parents reported current symptoms at the routine clinic visit, the prevalence of type B tympanograms was lower (15%, 26%, 22%, 24% and 25% at ages 3–4, 5–9, 10–14, 15–19 and 20–24 months, respectively) and closer to the field visit rates shown in Table 1. There was no such reduction in prevalence of type B tympanograms at routine clinics when we excluded the 14% of measurements in Aboriginal children who were symptomatic at the routine clinic.

The peak prevalence of type B tympanograms in both field and clinic was at age 5–9 months in both groups of children (Table 1). More than two-thirds of readings in Aboriginal children aged 5–19 months attending the routine follow-up clinic and approximately half in the field were type B. In non-Aboriginal children the prevalence of type B tympanograms was 25% between ages 5 and 14 months on examination in the field and 30% at routine ENT examination. Type C tympanograms were generally more common in non-Aboriginal than Aboriginal children (Table 1).

### *ENT specialist examinations*

Among the 83 Aboriginal clinic attenders 59% were male and 51% of the 164 non-Aboriginal attenders were male. Eighty-three percent of Aboriginal children were seen at least once for routine ENT follow-up and 59% were seen at least twice. Equivalent figures for non-Aboriginal children were 91% and 72%, respectively (Table 2).

On 184 routine clinical examinations in Aboriginal children between 8 days and 24 months of age, 55% had signs of OME, AOM or TM perforation (with or without purulent discharge); 27% of examinations were normal (Table 3). In non-Aboriginal children, 26% of 392 clinical examinations between age 6 days and 23 months had evidence of OME or AOM and 57% were normal (Table 3). In Aboriginal children, the prevalence of OM (i.e. OME, AOM, and/or perforations) rose from 44% in the first month of life to 72% at age 5–9 months and remained at 60% or more; in non-Aboriginal children, prevalence rose to 40% at age 10–14 months and was still 28% in those aged 20 months or more (Figure 2). Age-specific prevalence rates of OM were similar when examinations of symptomatic children were excluded.

A total of 21 (21%) Aboriginal children in the study had a TM perforation documented at least once during the study, the earliest being documented in an 8-day-old child. By the age of 6 months 7% of Aboriginal children had had a TM perforation at least once (6 of the 85 children followed up to age 6 months or more) and 19% (15/

**Table 1: Classification of tympanograms in Aboriginal and non-Aboriginal children by age during routine follow-up by research assistants in the field or by audiologists at routine ENT follow-up.**

| Age (months) | Tympanogram | Aboriginal | | Non-Aboriginal | |
|---|---|---|---|---|---|
| | | Field | Clinic | Field | Clinic |
| 3–4 | A | 13 (52%) | 5 (33%) | 65 (77%) | 17(68%) |
| | B | 11 (44%) | 8 (53%) | 9 (11%) | 5(20%) |
| | C | 1 (4%) | 2 (13%) | 11 (13%) | 3(12%) |
| 5–9 | A | 11(36%) | 8(22%) | 66 (61%) | 47(56%) |
| | B | 18 (58%) | 26(72%) | 30 (27%) | 26(31%) |
| | C | 2 (6%) | 2(6%) | 13 (12%) | 11(13%) |
| 10–14 | A | 15 (40%) | 7(19%) | 63 (54%) | 31(49%) |
| | B | 18 (49%) | 25(69%) | 26 (22%) | 18(29%) |
| | C | 4 (11%) | 4(11%) | 27 (23%) | 14(22%) |
| 15–19 | A | 11 (39%) | 5(22%) | 48 (60%) | 36(52%) |
| | B | 13 (46%) | 15(65%) | 16 (20%) | 21(30%) |
| | C | 4 (14%) | 3(13%) | 16 (20%) | 12(17%) |
| 20 + | A | 7 (21%) | 2(20%) | 54 (63%) | 8(44%) |
| | B | 18 (53%) | 6(60%) | 14 (17%) | 5(28%) |
| | C | 9 (26%) | 2(20%) | 17 (20%) | 5(28%) |

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**Table 2: Number of times children attended ENT/audiology clinic for routine follow-up.**

| | Frequency of routine follow-up at ENT clinic (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | Total |
| Aboriginal | 17 (17) | 24 (24) | 34 (34) | 14 (14) | 6 (6) | 4 (4) | 1 (1) | 100 |
| Non-Aboriginal | 16 (9) | 35 (19) | 64 (36) | 42 (23) | 15 (8) | 5 (3) | 3 (2) | 180 |
| Total | 33 (12) | 59 (21) | 98 (35) | 56 (20) | 21 (8) | 9 (3) | 4 (1) | 280 |

Note: During the first year of study, children were seen by the visiting ENT specialist or by an audiologist who was resident in Kalgoorlie and examined children at time of field follow-up. Thereafter, children were to be seen routinely by an ENT 3 times before age 2 years.

80) by the age of 12 months. A perforation was seen in 6 (3%) of the non-Aboriginal children.

ENT specialists recommended insertion of ventilation tubes (for recurrent AOM and/or persistent OME with hearing loss and speech concerns) in 12% (n = 12) of Aboriginal children and 10% (n = 18) of non-Aboriginal children. This provides a further indication of the burden of severe middle ear disease.

### *Hearing assessment*

Between 6 and 24 months of age, 61 routine hearing assessments were performed in Aboriginal children and 169 in non-Aboriginal children. Hearing loss was significantly more common in Aboriginal than non-Aboriginal children (OR = 5.40, 95% CI 2.68–10.89). In Aboriginal children moderate-severe hearing loss was seen in 39% of 13 assessments done at ages 6–11 months and in 32% of the 47 assessments in children aged 12 months or more (Figure 3). In non-Aboriginal children, moderate-severe hearing loss was detected in 10% of 40 assessments at age 6–11 months and in 7% of 120 assessments at age 12 months or more (Figure 3).

**Table 3: Clinical diagnosis by ENT specialists at routine follow-up.**

| Middle ear diagnosis | Aboriginal* | | Non-Aboriginal* | |
|---|---|---|---|---|
| | N | % | N | % |
| Normal | 49 | 26.6 | 225 | 57.4 |
| Eustachian tube dysfunction | 17 | 9.2 | 37 | 9.4 |
| Otitis media with effusion | 82 | 44.6 | 91 | 23.2 |
| Acute otitis media (AOM) | 4 | 2.2 | 9 | 2.3 |
| AOM with TM perforation | 9 | 4.9 | 2 | .5 |
| TM perforation ± discharge** | 6 | 3.3 | - | - |
| Other | 2 | 1.1 | 3 | .8 |
| Unknown | 15 | 8.2 | 25 | 6.4 |
| Total | 184 | 100.0 | 392 | 100.0 |

* 11% and 8% of diagnoses in Aboriginal and non-Aboriginal children, respectively, were based on successful examination of one ear only.
** 2 had dry TM perforation

### *TEOAE responses and subsequent risk of OM*

We had valid TEOAE measurements in the first month of life followed by at least one successful clinical examination for 102 ears in 54 Aboriginal children and for 234 ears in 120 non-Aboriginal children; at age 1–2 months there were valid TEOAEs and subsequent clinical examination on 60 ears in 34 Aboriginal children and on 218 ears in 111 non-Aboriginal children. In Aboriginal children, OM was subsequently diagnosed in 55% (n = 46) of ears for which TEOAE responses were present <1 month of age compared with 72% (n = 13) of ears with no detectable TEOAEs at the same age. Equivalent figures for non-Aboriginal children age <1 month were 31% (n = 70) and 40% (n = 2), respectively. In 1–2-month-old Aboriginal children, 51% (n = 19) of ears with TEOAE responses present had a subsequent diagnosis of OM compared with 87% (n = 20) of those with no TEOAE detected. Equivalent figures for non-Aboriginal children were 37% (n = 73) and 32% (n = 6), respectively.

There was a non-significant increased risk of subsequent OM in all children with failed TEOAE before age 1 month (Table 4). In contrast, Aboriginal children who failed TEOAE at age 1–2 months were 2.6 times more likely to develop OM subsequently than those who passed TEOAE. Failed TEOAE response did not predict subsequent OM in

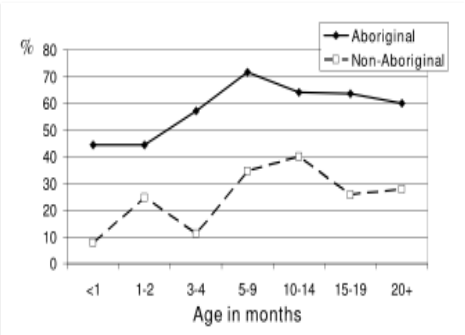


**Figure 2**
Age-specific prevalence of OM in Aboriginal and non-Aboriginal children on routine examination by ENT specialist.

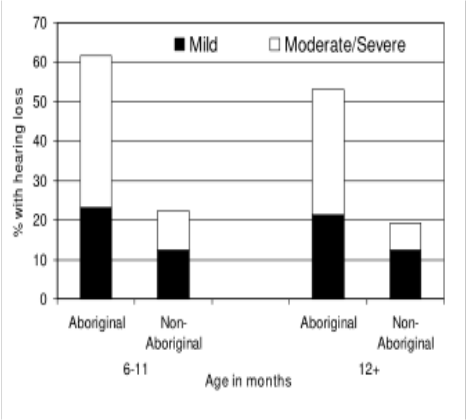


**Figure 3**
Prevalence of moderate-severe and mild hearing loss in Aboriginal and non-Aboriginal children aged 6–11 and 12–24 months of age.

non-Aboriginal children aged 1–2 months (Table 4). Results were very similar when examining TEOAEs in early infancy and subsequent observation of a type B tympanogram either on routine field follow-ups or at routine ENT clinics (data not shown).

## Discussion

To our knowledge this is the first comprehensive investigation of middle ear health (which includes hearing assessment) conducted simultaneously in young indigenous and non-indigenous children living in an urban setting, although an Australia-wide study of Aboriginal and non-Aboriginal people of all ages was conducted 30 years ago [5]. All study participants had high rates of OM but rates were particularly high in Aboriginal children, in whom disease began at a very young age, as has been reported previously [7,9,10]. There was significant hearing loss from age 6 months onwards, particularly in Aboriginal children, one-third of whom had hearing loss >40 dB. The general lack of symptoms with such high disease burden is of concern since families would not be prompted to bring children for medical care.

Aboriginal children were more likely to fail TEOAE measurements than non-Aboriginal children in the first 3 months of life, which is consistent with the earlier onset of OM in Aboriginal children. Of particular interest was the finding that absent TEOAEs in Aboriginal children at age 1–2 months predicted subsequent risk of OM. Not surprisingly, such an association was not seen in non-Aboriginal children, given their high pass rate and later onset of disease.

### *Comparison with other studies*

*Prevalence* The burden of OM and age-specific prevalence in non-Aboriginal children is comparable to that found in studies undertaken elsewhere [1,27,28]. In Aboriginal children, the age-specific prevalence of OM, and specifically the prevalence of TM perforations, in this urban/periurban semi-arid area of WA is lower than in many communities in the NT and WA, though prevalence of disease varies widely between communities [11,29,30]. The lower prevalence of OM in the Kalgoorlie-Boulder region of WA compared with that reported in many NT Aboriginal communities is consistent with lower bacterial carriage rates [9,31]. One cannot exclude the possibility that selection bias may have contributed to the lower rates of disease in our study, i.e. that parents of a healthier group of children chose to participate in our study.

While there are no directly comparable data in WA, the degree of hearing impairment in Aboriginal children in our study is consistent with that reported among children aged <5 years in three Aboriginal communities in 1988–89, where the prevalence of hearing loss ranged between 38% and 63% [29], suggesting that there has been little improvement in ear health in the past 20 years.

In a state-wide population-based study of Aboriginal children in WA 25% of children aged <3 years living in areas of moderate isolation (such as the Kalgoorlie-Boulder region) had a history of ear discharge [13], consistent with our finding that 21% of Aboriginal children had one or more perforations by age 2 years. It is interesting, however, to note that 12% of Aboriginal children in our study were referred for insertion of ventilation tubes. This suggests that, in urban areas at least, closed ear disease (as

**Table 4: Hazard ratios (and 95% confidence intervals) of OM among Aboriginal and non-Aboriginal children with no detectable TEAOE response, compared with those with a TEOAE response, at ages <1 month and 1–<3 months**

| Age | Aboriginal | | Non-Aboriginal | |
|---|---|---|---|---|
| | Hazard ratio | 95% CI | Hazard ratio | 95% CI |
| <1 month | 1.19 | (0.66 – 2.16) | 2.51 | (0.38 – 16.48) |
| 1–<3 months | 2.64* | (1.32 – 5.31) | 0.71 | (0.24 – 2.11) |

* p = 0.006

opposed to perforated ear drums) may now be more common than in the past.

Hunter *et al* conducted a cohort study among American Indians using a similar design to ours, with regular follow-up of 366 children from birth to age 2 years [21]. The prevalence of OM by examination of individual ears was lower (14%, 31%, 47% and 33% in children aged <2, 2–5, 6–12 and 13–24 months, respectively) than in our Aboriginal study population. In the same study, excluding examinations with technical failures, the failure rate of distortion product OAEs (DPOAEs) in American Indian children was of the same order as the TEOAE failure rate in Aboriginal children in our study: 25% at age <2 months and 41% at age 2–5 months compared with 18% < 1 month and 44% age 1–<3 months in Aboriginal children in our study.

***Otoacoustic emissions***

The pass rate for TEOAEs of 97% among non-Aboriginal children in our study is consistent with the pass rate of a newborn hearing screening in Perth, WA, in which the pass rate was 99% following assessment soon after birth and a repeat test if the baby failed the first time [23]. To our knowledge there are no published studies that have measured OAEs with comparable study design in healthy young non-indigenous children after the early neonatal period.

Our TEOAE measurements in Aboriginal children were inconclusive in 11% of children aged <1 month. This is higher than the reported 5% technical failure rate in American Indian children aged <2 months [21], but the difference is not statistically significant. In older Aboriginal children the proportion of TEOAE tests that were inconclusive was 24%, similar to a technical fail rate of 27% in American Indian children aged 2–5 months [21].

*OAE as predictor* Doyle *et al* [20] followed a small number of children with and without middle ear effusion (MEE) at birth. Investigators performed otoscopy in addition to TEOAE measurement and found that early onset of MEE predicted subsequent OME and hearing loss in the first year of life [20]. The only known published study in an indigenous population reported no association between failed TEOAE measurements in newborn American Indians and risk of recurrent OM before age 2 years [21], though the outcome measures differed between the two studies ('recurrent OM' as opposed to time to first diagnosis of OM in our study). We also found no association between failed TEOAE in the first month of life and subsequent risk of OM, but such an association was present for TEOAE measurements after the neonatal period. To determine whether our findings can be generalised to other indigenous populations, the use of TEOAE measurements as a predictor should be evaluated in other settings.

***Strengths and limitations of the study***

The strength of our study is that it provides new information on the burden of OM, including impaired hearing beyond the first year of life and detection of TEOAEs in indigenous and non-indigenous children living in the same region. Furthermore, it is the first study in Australia to investigate TEOAE as a predictor of subsequent disease. The activities around this study also raised awareness about OM and the ENT specialist provided expertise not only to study participants but to others as requested at a time when limited ENT services were available in Kalgoorlie [22].

The principal aim of our cohort study was to investigate microbiological, immunological, demographic and socio-economic factors predisposing to OM. In view of the infrequent assessments of middle ear status, we were unable to follow the natural history of the disease and determine whether the hearing loss and presence of middle ear effusions were continuous or intermittent, as has been described elsewhere [32].

Data are limited for some outcomes. In particular a limited number of Aboriginal children were seen by the ENT specialist on all 3 intended visits. However, tympanometry during routine field follow-up provided important supplementary information on middle ear health and confirms the enormous burden of disease, particularly in Aboriginal children. Associations between failed TEOAEs and presence of type B tympanograms were consistent between field and clinic follow-up measurements.

***Recommendations for surveillance***

Given the high prevalence of asymptomatic OM, particularly in Aboriginal children, regular surveillance for ear disease and hearing loss must be rigidly applied. This implies first and foremost strong financial support for primary health care, as well as training and supervision of primary health care staff.

An optimal screening program in infants should include newborn hearing screening, followed by otoacoustic emission testing or high-frequency tympanometry at age 1–2 months to identify children at increased risk of subsequent OM, and then audiometry and tympanometry (with 226 Hz probe tone) between the ages of 6 and 12 months. Services need to be available so that children can be referred according to national guidelines [24]. Aboriginal Health Workers (AHWs), nurses and doctors need to be encouraged to do otoscopy and/or tympanometry whenever a child presents with URTI and/or fever or irri-

tability in order to initiate treatment at an early stage of disease [12,24].

Following on from our study, an ear health screening program is being introduced in the Goldfields region. The program includes training of community health nurses and AHWs in tympanometry, otoscopy and audiometry. Children are to be assessed at birth, 1–2 months, then 6-monthly from 6–18 months and annually thereafter to 5 years. Treatment and referral procedures are according to local standardised protocols based on national guidelines [24]. This program needs full support and should be formally evaluated. Such an evaluation should consider detection and referral rates, acceptability of the program by health service providers and families and changes in disease rates over a 5-year period as well as an economic evaluation of the program.

***Recommendations for research***

1. The role of health professionals (e.g. nurses and AHWs) with specialist training in ear health should be formally evaluated as anecdotal evidence suggests that such models have been successful in identifying and treating children at Aboriginal Medical Services (Derbarl Yerrigan Aboriginal Medical Service in Perth) and in New Zealand (Variety Ear Bus Program).

2. Children who participated in our study are now aged 5 – 9 years and of school age. It would be worthwhile identifying those still in the Kalgoorlie-Boulder region to determine the long-term outcomes with regard to hearing, speech, language and education, based on our initial clinical assessments of middle ear health.

3. Measurement of TEOAEs at age 1–2 months to identify those at risk of developing OM should be evaluated in a routine health service setting (e.g. through an Aboriginal Medical Service and/or Ngunytju Tjitji Pirni Inc, a Kalgoorlie-based Aboriginal maternal and child health service provider). This could coincide with the two-month immunisation visit. Audiological services must, however, be available for referral of children who fail TEOAE on two occasions for appropriate management. Such a study is currently in the planning stage.

4. As part of the ear health program in the Goldfields, we propose a study comparing performance of TEOAE measurement with that of high-frequency tympanometry at age 1–2 months in predicting risk of subsequent OM. The study will include an economic component and we will ask primary health care workers to comment on the practicality of the different techniques.

5. Given the high rates of OM in the Aboriginal population, evaluation of an intervention addressing hygiene practices to reduce upper respiratory bacterial carriage and hence OM is urgently needed. This must not deter from the need to increase availability of appropriate housing to reduce transmission of respiratory pathogens in crowded homes.

6. The currently available 7-valent pneumococcal conjugate vaccine has not reduced the burden of OM in Aboriginal children in NT [33,34]. There is, however, another conjugate pneumococcal vaccine linked to *Haemophilus influenzae* protein D, which has been found to be efficacious in preventing episodes of acute OM in Czech Republic [35] and merits evaluation in the Aboriginal population. Maternal immunisation with 23-valent pneumococcal polysaccharide vaccine for prevention of OM in their offspring is currently being evaluated in the NT. Other protein-based vaccines to prevent OM due to the pneumococcus, *H. influenzae* and *Moraxella catarrhalis* are under investigation.

## Conclusion

In summary we have found high rates of OM in generally asymptomatic Aboriginal and non-Aboriginal children in an urban/periurban setting in a semi-arid zone of Australia, the rates being particularly high in Aboriginal children, though lower than reported in more remote settings. One-third of Aboriginal children over the age of 6 months had significant hearing loss. The absence of OAEs after the neonatal period in Aboriginal children confirms the early onset of middle ear disease and OAE measurement may be used to identify children at risk of developing OM. Given the silent nature of the disease, regular surveillance for OM and hearing loss must be applied frequently from a young age. The technology to measure TEOAEs using the Echocheck is less expensive than the alternative multi-frequency tympanometer in children under the age of 6 months. However, the recent availability of a screening tympanometer with a 1000 Hz probe tone option may now make tympanometry a more viable alternative option as, unlike OAE measurements, it does not require a quiet environment and settled child. The Echocheck is a simple screening tool and its use should be evaluated in a primary health care setting.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

DL established the study, designed questionnaires and supervised all aspects of the study and wrote the article for submission. PJ undertook data analysis and assisted in preparation of the manuscript. HC is an ENT specialist who conducted almost all the routine clinical examinations on study participants. SW conducted tympanometry and audiometry at routine ENT clinics, trained research

assistants in tympanometry and wrote an early draft of the manuscript. HC and SW classified all tympanometry readings. DE oversaw all data management and field work. DE, AS, RM, JF provided input into development of questionnaires, conducted interviews with study participants, assisted in ensuring attendance at routine ENT clinics and undertook tympanometry during routine field follow-up. All authors have seen and approved the manuscript prior to submission.

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Study methodology

# The Kalgoorlie Otitis Media Research Project: rationale, methods, population characteristics and ethical considerations

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## Summary

Lehmann D, Arumugaswamy A, Elsbury D, Finucane J, Stokes A, Monck R, Jeffries-Stokes C, McAullay D, Coates H, Stanley FJ for the Kalgoorlie Otitis Media Research Project Team. The Kalgoorlie Otitis Media Research Project: Rationale, methods, population characteristics and ethical considerations. *Paediatric and Perinatal Epidemiology* 2008; **22**: 60–71.

Otitis media (OM) is one of the most common paediatric illnesses for which medical advice is sought in developed countries. Australian Aboriginal children suffer high rates of OM from early infancy. The resultant hearing loss can affect education and quality of life. As numerous factors contribute to the burden of OM, interventions aimed at reducing the impact of single risk factors are likely to fail. To identify key risk factors and understand how they interact in complex causal pathways, we followed 100 Aboriginal and 180 non-Aboriginal children from birth to age 2 years in a semi-arid zone of Western Australia. We collected demographic, obstetric, socio-economic and environmental data, breast milk once, and nasopharyngeal samples and saliva on seven occasions. Ear health was assessed by clinical examination, tympanometry, transient evoked otoacoustic emissions and audiometry. We considered the conduct of our study in relation to national ethical guidelines for research in Aboriginal and Torres Strait Islander health. After 1 year of community consultation, the study was endorsed by local committees and ethical approval granted. Fieldwork was tailored to minimise disruption to people's lives and we provided regular feedback to the community.

We saw 81% of non-Aboriginal and 65% of Aboriginal children at age 12 months. OM was diagnosed on 55% and 26% of routine clinical examinations in Aboriginal and non-Aboriginal children respectively. Aboriginal mothers were younger and less educated, fewer were employed and they lived in more crowded conditions than non-Aboriginal mothers. Sixty-four per cent of Aboriginal and 40% of non-Aboriginal babies were exposed to environmental tobacco smoke. Early consultation, provision of a service while undertaking research, inclusion of Aboriginal people as active members of a research team and appropriate acknowledgement will assist in ensuring successful completion of the research.

**Keywords:** *otitis media, Indigenous population, cohort study, ethical guidelines, follow-up bias.*

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## Introduction and background

### *Burden of otitis media*

In industrialised countries, otitis media (OM) is the most common paediatric illness for which medical advice is sought and antibiotics are prescribed.[1] OM can lead to impaired hearing, which can affect performance at school and social circumstances in adulthood. In industrialised countries, 10–20% of children suffer >3 episodes of OM before age 1 year.[2] In Aboriginal Australian children not only are the rates of OM and its sequelae much higher than in non-Aboriginal children[3] but the disease starts at a much younger age and is frequently asymptomatic until a purulent ear discharge is visible.[4–6] A recent study in the Northern Territory of Australia showed that 91% of Aboriginal children aged 6–30 months had clinical signs of OM and by age 18 months 40% had had a tympanic membrane perforation.[7]

### *Risk factors for OM*

A wide variety of demographic, social, environmental, immunological and microbiological risk factors for OM have been identified in studies in industrialised countries, although findings have not always been consistent across studies.[8–15] In Indigenous populations, poverty, crowding, personal hygiene and parental smoking are considered important antecedents to OM.[16,17] *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, rhinovirus, adenovirus, influenza viruses, respiratory syncytial virus and coronavirus are the pathogens most commonly associated with OM.[4,6,8,18]

### *Causal pathways to OM*

Figure 1 shows the hypothesised causal network we have developed on which to base our investigations; it illustrates the complexity of causal pathways to OM. Prevention of the more distal factors on causal

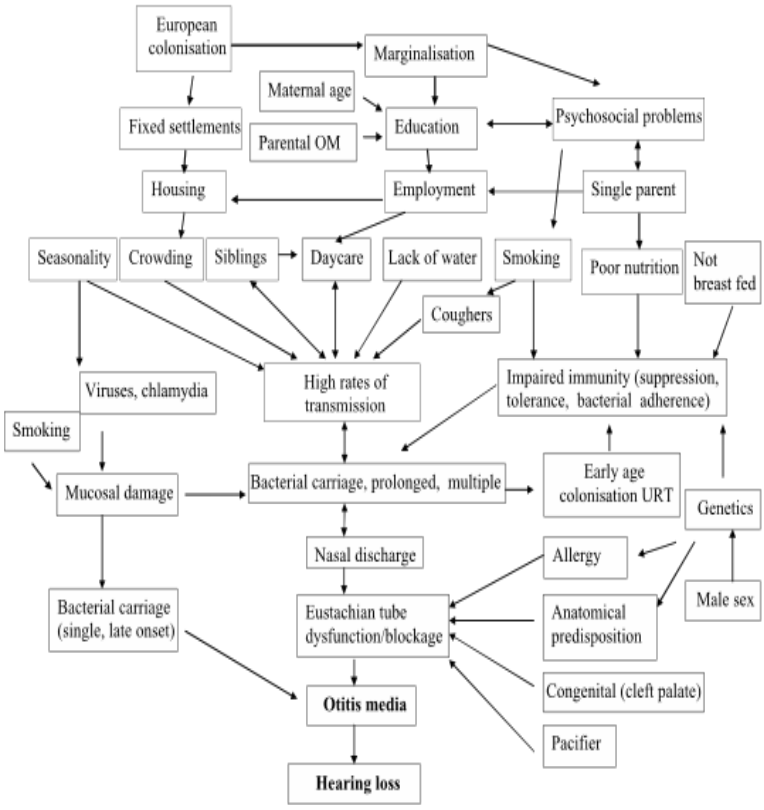


**Figure 1.** A causal network for otitis media (OM). URT, upper respiratory tract.

pathways to OM may be not only more effective and cheaper, but essential in reducing the overwhelming burden of disease.[19] It is also clear that interventions aimed at reducing the impact of single risk factors in isolation are likely to fail.[20] In order to design appropriate interventions to reduce the burden of OM in Aboriginal and non-Aboriginal children, the most important risk factors need to be identified, particularly for children residing in urban arid environments for whom data are limited. We therefore conducted a multidisciplinary longitudinal study in such an environment.

### *Ethical considerations*

Aboriginal peoples have had negative experiences of research which are closely linked to the history of colonisation. Hence, they have been critical of research undertaken in their communities.[21,22] Researchers tended to collect data for their own rather than Aboriginal peoples' benefit, at times they drew false conclusions – often to the detriment of Aboriginal peoples – and they provided limited feedback to communities. By the 1980s there was increasing concern about the conduct of research on Aboriginal peoples who were also becoming increasingly resistant to conventional research practices.[22] Following a series of meetings, a national set of guidelines was developed and endorsed by the National Health and Medical Research Council (NHMRC) in 1991.[23] These guidelines were in place when we developed our study protocol, but were replaced in 2003 by *Values and Ethics: Guidelines for Ethical Conduct in Aboriginal and Torres Strait Islander Health Research*.[24]

The 1991 guidelines emphasised the need for consultation with communities participating in research, community involvement in research, and consideration of ownership and publication of data.[23] The current guidelines are less prescriptive with a move away from mere compliance with legal requirements to the acknowledgement of cultural differences and consideration of values. The values which form the basis of the new guidelines are: 'spirit and integrity, reciprocity, respect, equality, survival and protection', and 'responsibility'.[24]

An important issue for us was how best to conduct a demanding study as a true partnership with local communities, ensure optimal data collection related to multiple risk factors, including the collection of biological specimens, and maintain an acceptable level of follow-up.

### *Aims*

This study aimed to: (1) identify important, avoidable risk factors for OM in Aboriginal and non-Aboriginal children in the Kalgoorlie-Boulder area of Western Australia, and (2) to understand how these risk factors arise and interact in the complex causal pathways in order to develop effective intervention strategies.

In this paper we give an overview of methods used in the study and describe the socio-economic, environmental and demographic characteristics of the study population. We specifically address ways in which we sought to engage the Aboriginal and non-Aboriginal communities and describe the planning and progress of our study in relation to NHMRC ethical guidelines.[23]

## Methods

### *Study setting*

Kalgoorlie-Boulder is located in a semi-arid zone 600 kilometres east of Perth, the capital city of Western Australia. It is the major centre for the Goldfields region and for the mining industry in the area. Approximately 32 500 people live in Kalgoorlie-Boulder and the surrounding areas, 2380 of whom are Aboriginal.[25]

### *Engaging the community*

In order to undertake this complex study requiring regular follow-up of children from birth to age 2 years, our first priority was to obtain the support of the proposed participating communities and the health service providers. There had been a call from the community for such a study: people were concerned about the potentially negative impact of OM on children's well-being, in particular on their school performance. The coordinator (Elsie Edwards) of Ngunytju Tjitji Pirni Inc (NTP, an Aboriginal Health Service) was chief investigator on our first grant application in 1997 and Aboriginal investigators were part of subsequent funding applications. Formal collaboration with Bega Garnbirringu Aboriginal Health Services Aboriginal Corporation (BEGA) was established in 1999. We learnt that BEGA had limited access to ear, nose and throat (ENT) specialists and, therefore, our study ENT specialist (H.C.) offered his services at no cost until another specialist became available. D.L. also assisted BEGA in

obtaining funding for an Ear Health Worker and we offered parents and children transport to ENT clinics.

People were informed about the study through radio and television interviews, newspaper articles and face-to-face discussions. Aboriginal and non-Aboriginal people provided input into the study design and content of information sheets, consent forms and questionnaires, which were developed in line with routine data collection instruments used by NTP. All research staff pre-tested all forms and piloted specimen collection among themselves. This meant they could explain to future participants what discomfort children might experience.

We held meetings with local medical practitioners to explain the purpose of the study, to ask them to encourage their clients to take part in the study and to seek their approval to access participants' medical records.

After 12 months of developing partnerships with local organisations and health professionals, the study was endorsed by the relevant local Aboriginal organisations, leading to approval from the Western Australia Aboriginal Health Information and Ethics Committee (WAAHIEC) to conduct the study.

The research assistants were Aboriginal Health Workers (AHW) and nurses who were well known to the local community. Nurses and AHWs gained experience in research and played an active role in the local Ear Health Committee which aims to increase awareness about OM and develop programmes to improve ear health. Being enthusiastic about the project themselves, research assistants were able to encourage others to participate in the study.

### *Recruitment of study participants*

Between April 1999 and January 2003, Aboriginal and non-Aboriginal children born in Kalgoorlie Regional Hospital to mothers intending to reside for at least 2 years within 1-h drive of Kalgoorlie were recruited into this prospective cohort study in order to enrol 100 Aboriginal and 180 non-Aboriginal babies. Multiple births, children with severe congenital abnormalities or those whose birthweight was <2000 g were not eligible.

### *Enrolment and follow-up of study participants*

Research assistants visited women postpartum in hospital to provide them with information about the study with a view to enrolling four babies each week. If mothers were interested, research staff contacted them at home and, if still interested, they arranged a home visit 1–3 weeks post-partum to obtain consent and collect demographic, socio-economic, environmental and obstetric data. Information was only obtained for those fathers living with the enrolled child. Type of employment was classified using the Australian Standard Classification of Occupations.[26] The research assistants collected nasopharyngeal samples for microbiological studies and breast milk and saliva for studies of mucosal immunity.

Research staff scheduled follow-up visits when children were aged 6–8 weeks, 4, 6, 12, 18 and 24 months at a convenient time and place for study participants (in the home, project office or a public place). Information on time-dependent variables (e.g. feeding practices, smoking, day-care attendance and use of pacifier) and saliva and nasopharyngeal samples were collected. Research assistants noted any ear discharge but did not routinely perform otoscopy as reliable examination is difficult in very young children. We documented all immunisations given to study participants.

### *Morbidity data collection*

In order to obtain as complete information as possible on any OM episodes, once children reached 2 years of age we collected morbidity data from medical practitioners and Kalgoorlie Regional Hospital, the only hospital in the region. Diagnoses were coded according to the ICD10 classification system.[27]

### *Assessment of ear health*

At 1–3 and 6–8 weeks of age we screened for sensorineural hearing loss by measuring transient evoked otoacoustic emissions (TEOAE). A failed TEOAE screen may also indicate fluid in the middle ear of young infants[28,29] and may be useful in identifying children at risk of developing OM in the future.[30]

Clinical diagnosis of OM by a paediatric ENT specialist was the primary outcome for this study. ENT/audiology clinics were held four times annually, specifically for routine examination of study participants, once before 6 months of age and again at 6–11 and 12–23 months of age. An audiologist assessed hearing at age 12–23 months and from March 2002 onwards also at age 6–11 months.

To obtain additional, possibly less biased, ear health outcomes more frequently on more children, research assistants performed tympanometry from May 2000 onwards during routine follow-ups of children aged 4 months or more.

### *Analysis*

To determine how representative our study population was of the general population in the area, data on the characteristics of births in the general population residing in the Kalgoorlie-Boulder region were obtained from the birth records on the Midwives' database of the Western Australia Data Linkage System.[31] In this paper, the chi-square test with continuity correction, Fisher's Exact test and Student's *t*-test were used to compare groups of interest.

### *Ethical approval*

Ethical approval to conduct this study was given by WAAHIEC, the Northern Goldfields Health Service and Nursing Education Ethics Committee in Kalgoorlie, Princess Margaret Hospital Ethics Committee and the Confidentiality of Health Information Committee of the Health Department of Western Australia.

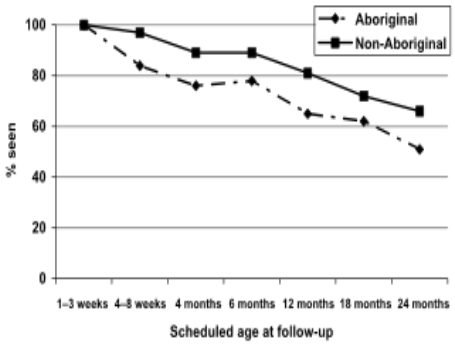


**Figure 2.** Proportion of Aboriginal and non-Aboriginal children seen at each scheduled routine follow-up visit.

## Results

Enrolment of non-Aboriginal babies was completed in September 2002, while the last Aboriginal child was enrolled in January 2003. Approximately two-thirds of Aboriginal children (65%) and 81% of non-Aboriginal children were seen at age 12 months (Fig. 2). Nineteen Aboriginal (19%) and 38 (21%) non-Aboriginal children moved away from the area before age 2 years; parents of six (6%) Aboriginal children and one (0.5%) non-Aboriginal child chose to leave the study and one child died. An ENT specialist saw 83% of Aboriginal children at least once and 59% at least twice, while 91% of non-Aboriginal children were seen at least once and 72% twice. OM was diagnosed in 55% of 184 routine examinations in Aboriginal children and 26% of 392 examinations in non-Aboriginal children.

We collected 436 saliva and 509 nasopharyngeal samples from Aboriginal babies and 878 saliva and 1050 nasopharyngeal samples from non-Aboriginal babies. Breast milk was collected from 79 Aboriginal mothers (99% of breast-feeding mothers) and 144 (92%) non-Aboriginal mothers.

### *Comparison of characteristics of study population with general population of the Kalgoorlie-Boulder Region*

The characteristics of the study population and births in the total population of Kalgoorlie-Boulder in 2000 were similar, except that in our study sample there were more teenage Aboriginal mothers, non-Aboriginal study mothers and fathers were older and non-Aboriginal study mothers were less likely to have smoked during pregnancy (Table 1).

### *Characteristics of mothers*

Five non-Aboriginal mothers had Aboriginal children. One-quarter of Aboriginal mothers were teenagers and one-third were single parents (Table 1), but usually lived with their extended family. Almost half the Aboriginal mothers and 16% of non-Aboriginal mothers were brought up in families that did not include both mother and father (Table 2).

While Aboriginal mothers had a lower level of education and were more likely to be unemployed than non-Aboriginal mothers, 41% of Aboriginal mothers had been employed recently (Table 2). Almost half the Aboriginal mothers had received further training after completing high school (Table 2), but only 58% of those for whom we have data (22/38) had completed such courses compared with 86% (113/132) of the non-Aboriginal mothers.

A high proportion of mothers breast fed their babies during the first month of life (Table 2), but by the age of 4 months, fewer than half the babies were being exclusively breast fed. Six-to-eight weeks postpartum, 9% of

**Table 1.** Comparison of sociodemographic characteristics of the study population with those of characteristics of all births in 2000 in the Kalgoorlie-Boulder Region

| Characteristic | Aboriginal[a] Study *n* (%) | Aboriginal[a] K-B[b] *n* (%) | Aboriginal[a] *P*-value[c] | Non-Aboriginal[a] Study *n* (%) | Non-Aboriginal[a] K-B *n* (%) | Non-Aboriginal[a] *P*-value[c] |
|---|---|---|---|---|---|---|
| Child's gender | | | | | | |
| Male | 60 (60) | 46 (54) | 0.5 | 89 (49) | 309 (51) | 0.84 |
| Female | 40 (40) | 39 (46) | | 91 (51) | 301 (49) | |
| Maternal age (years) | | | | | | |
| <20 | 24 (25) | 13 (15) | 0.02 | 7 (4) | 32 (5) | 0.03 |
| 20–24 | 26 (27) | 39 (46) | | 25 (14) | 107 (18) | |
| 25–29 | 26 (27) | 25 (29) | | 56 (30) | 226 (37) | |
| 30+ | 19 (20) | 8 (9) | | 97 (52) | 245 (40) | |
| Single mother | 31 (34) | 33 (31) | 0.8 | 5 (3) | 33 (5) | 0.4 |
| Mothers' place of birth | | | | | | |
| Western Australia | 88 (94) | 99 (92) | 0.4 | 92 (50) | 302 (50) | 0.96 |
| Elsewhere Australia | 5 (5) | 9 (8) | | 58 (32) | 189 (31) | |
| Outside Australia | 1 (1) | – | | 34 (18.5) | 118 (20) | |
| Paternal age (years)[d] | | | | | | |
| <25 | 22 (34) | 15 (35) | 1.0 | 22 (12) | 72 (13) | 0.03 |
| 25 to 29 | 21 (33) | 13 (30) | | 40 (22) | 168 (30) | |
| 30+ | 21 (33) | 15 (35) | | 124 (67) | 312 (57) | |
| Smoking in pregnancy | | | | | | |
| Yes | 46 (49) | 47 (55) | 0.53 | 39 (21) | 195 (32) | 0.006 |
| No | 47 (51) | 38 (45) | | 146 (79) | 414 (68) | |

[a]Discrepancies in denominators due to missing data.
[b]K-B = Kalgoorlie-Boulder all births in 2000.
[c]*P*-values refer to differences between general and study population (Aboriginal and non-Aboriginal separately).
[d]Paternal information for general population pertains to all fathers for whom information was entered on questionnaires. Some fathers in the general population may not have been residing with child's mother. For the study, paternal information relates to fathers living at same address.

Aboriginal mothers and 4% of non-Aboriginal mothers had introduced solids into their baby's diet, increasing to 67% (48/72) and 56% (93/165), respectively, 4 months postpartum. Non-Aboriginal mothers generally used pacifiers more frequently than Aboriginal mothers (Table 2).

### *Characteristics of fathers*

Demographic data were available for 64 Aboriginal and 186 non-Aboriginal fathers. Sixty Aboriginal and 182 non-Aboriginal fathers were living with the study participant. Forty per cent (19/47) of Aboriginal fathers had received some training after high school, though none had gone to university, compared with 81% (145/180) of non-Aboriginal fathers, 21% of whom had received university education ($\chi^2 = 34.2$, 3 d.f., $P < 0.001$). Aboriginal fathers were less likely to be employed (64%) than non-Aboriginal fathers (98%) ($\chi^2 = 57.0$, 2 d.f., $P < 0.001$). If employed, only 11% of Aboriginal fathers had highly skilled jobs (Australian Bureau of Statistics levels 1 and 2) compared with 29% of non-Aboriginal fathers ($\chi^2 = 78.0$, 2 d.f., $P < 0.001$).

### *Household characteristics*

Compared with non-Aboriginal mothers, Aboriginal mothers were more likely to live in rented accommodation, in houses with fewer rooms and with more people than non-Aboriginal families; they were less likely to have heating in their homes and less likely to have more than one type of cooking facility (Table 3).

### *Characteristics of study children*

Fewer Aboriginal study participants attended day care in the first 6 months of life than non-Aboriginal children: of the 59 Aboriginal children seen at each

**Table 2.** Sociodemographic characteristics of 95 Aboriginal and 185 non-Aboriginal mothers[a]

| Characteristic | Aboriginal[b] *n* (%) | Non-Aboriginal[b] *n* (%) | *P*-value[c] |
|---|---|---|---|
| Brought up by mother and father | 49 (53) | 156 (84) | <0.001 |
| Highest level of education | | | <0.001 |
| Bachelor/postgraduate degree | 1 (1) | 50 (27) | |
| Other post-school | 42 (45) | 87 (47) | |
| Years 11–12 | 17 (18) | 29 (16) | |
| ≤Year 10 | 33 (35) | 18 (10) | |
| Any post-school training | 43 (46) | 137 (74) | <0.001 |
| Employment history | | | <0.001 |
| Works now/recently | 31 (41) | 127 (71) | |
| Worked in past but not recently | 26 (35) | 48 (27) | |
| Has never worked | 18 (24) | 4 (2) | |
| Current employment type[26] | | | <0.001 |
| ABS skill level 1–2 (higher) | 6 (6) | 49 (26) | |
| ABS skill level 3–5 (lower) | 25 (27) | 78 (42) | |
| Unemployed | 62 (67) | 58 (31) | |
| Pacifier use in children aged: | | | |
| 1–3 weeks | 41 (44) | 95 (51) | 0.3 |
| 6–8 weeks | 38 (49) | 120 (68) | <0.01 |
| 4 months | 31 (43) | 96 (58) | <0.05 |
| 6 months | 26 (36) | 93 (56) | <0.01 |
| Any breast milk given at age[d]: | | | |
| 1–3 weeks | 80 (84) | 157 (85) | 1 |
| 6–8 weeks | 57 (72) | 128 (72) | 0.9 |
| 4 months | 45 (62) | 103 (65) | 0.9 |
| 6 months | 43 (59) | 93 (56) | 0.8 |

[a]Five non-Aboriginal mothers had Aboriginal children (i.e. fathers were Aboriginal).
[b]Discrepancies in denominators due to missing data.
[c]*P*-values for comparison between Aboriginal and non-Aboriginal mothers.
[d]Follow-up was incomplete after age 1–3 weeks resulting in different denominators in each age group.
ABS, Australian Bureau of Statistics.

follow-up visit to age 6 months, 8.5% had attended day care compared with 21% of 143 non-Aboriginal children ($\chi^2 = 3.73$, $P = 0.05$). However, 63% of Aboriginal study participants were living with older children who attended day care, playgroup or school, compared with 39% of non-Aboriginal children ($\chi^2 = 12.9$, $P < 0.001$).

### *Exposure to cigarette smoke*

Approximately half the Aboriginal mothers and one-fifth of non-Aboriginal mothers smoked during pregnancy (Table 1, $\chi^2 = 22.2$, $P < 0.001$). Sixty-four per cent of Aboriginal babies and 40% of non-Aboriginal babies were exposed to environmental tobacco smoke in the first month of life ($\chi^2 = 13.35$, $P < 0.001$), while 23% of Aboriginal children and 6% of non-Aboriginal children were exposed to tobacco smoke inside the home.

### *Characteristics of children with incomplete follow-up compared with those followed to age 2 years*

A total of 47 (50%) Aboriginal mothers and 64 (35%) non-Aboriginal mothers did not complete 2 years of follow-up. To validate our findings it was important to compare characteristics of children who did not have complete follow-up with those who did. We found no significant differences with regard to education, employment, parity, maternal age, marital status, number of adults or children in the household and maternal smoking (data not shown).

## Discussion

To our knowledge, this is the first study to investigate causal pathways to OM by examining microbiological, social, environmental, demographic and immunological factors simultaneously in Aboriginal and non-

**Table 3.** Characteristics of households of 95 Aboriginal and 185 non-Aboriginal mothers

| Characteristic | Aboriginal[a] *n* (%) or mean ± SD | Non-Aboriginal[a] *n* (%) or mean ± SD | *P*-value[b] |
|---|---|---|---|
| Number of adults in house | | | |
| Mean ± SD | 2.60 ± 1.04 | 2.10 ± 0.51 | <0.001 |
| Number of other children in house | | | <0.001 |
| 0 | 18 (19) | 93 (50) | |
| 1 | 24 (26) | 45 (24) | |
| 2 | 19 (20) | 34 (18) | |
| 3+ | 33 (35) | 13 (7) | |
| Lives in: | | | <0.001 |
| Rented accommodation | 69 (73) | 45 (24) | |
| Own house | 7 (7) | 136 (74) | |
| Other accommodation | 18 (19) | 4 (2) | |
| Number of rooms in house | 4.00 ± 1.2 | 4.96 ± 1.36 | <0.001 |
| Number of bedrooms | 2.97 ± 0.85 | 3.36 ± 0.83 | <0.001 |
| Number of people/room | 1.48 ± 0.59 | 0.83 ± 0.27 | <0.001 |
| Number of children/room | 0.76 ± 0.38 | 0.38 ± 0.20 | <0.001 |
| Use only disposable nappies in first month | 35 (37) | 105 (57) | <0.001 |
| Cooking facilities in household | | | <0.001 |
| Gas | 58 (62) | 33 (18) | |
| Electricity | 14 (15) | 15 (8) | |
| Gas + microwave | 10 (11) | 72 (39) | |
| Electricity + microwave | 6 (6) | 29 (16) | |
| Gas + electricity | 4 (4) | 6 (4) | |
| Gas + electricity + microwave | 1 (1) | 28 (15) | |

[a]Discrepancies in denominators due to missing data.
[b]*P*-values for comparison between Aboriginal and non-Aboriginal mothers.
SD, standard deviation.

Aboriginal children living in the same geographical environment. As expected, all indicators point to poorer social and economic status of the Aboriginal participants compared with non-Aboriginal participants, putting Aboriginal babies at particular risk of developing OM (Fig. 1).[17]

The study sample was similar to the general population in Kalgoorlie-Boulder except for the higher proportion of teenage Aboriginal mothers (who may have been encouraged to participate by the younger Aboriginal research assistants), the older age of non-Aboriginal study parents and lower proportion of non-Aboriginal study mothers who smoked compared with those in the general population. The apparent difference in smoking rate is likely to reflect the older age of study mothers as older study mothers smoked less than younger ones (manuscript in preparation). Older parents may have been more enthusiastic about taking part in the study than younger parents, some of whom were uncertain how long they would be staying in the area. Social, environmental and demographic characteristics of the Aboriginal study population are consistent with those reported for the region in the Western Australian Aboriginal Child Health Survey.[32]

### *Comparison with an urban study of Aboriginal children*

It is of interest to compare some of the characteristics of our Aboriginal study population with those of 272 Aboriginal children who were enrolled in a longitudinal study in Perth, the Bibbulung Gnarneep Project.[33] These comparisons emphasise the similarities and differences even between two urban Aboriginal communities, one being a metropolitan area and the other a rural mining town in the same state of Australia. Successful follow-up at age 6 months was somewhat better in Kalgoorlie than in the Perth study (78% compared with 61%) but the same at age 12 months (62–65%). Feeding practices around age 2 months were

similar.[34] In Perth, 65% of mothers smoked and 80% of the infants were regularly exposed to tobacco smoke, somewhat higher than in the Kalgoorlie study (Table 1).[35]

In the Perth study, mothers were more likely to have completed post-school training, to be employed and to live in rented accommodation than in Kalgoorlie, but in Kalgoorlie, more households included at least three adults than in Perth.

### *The research process*

How successful were we in conducting this study in line with the ethical guidelines that were available during the planning and recruitment phases of the study and did we meet up to the more recent criteria and values specified in the 2003 guidelines (italicised below)?[23,24]

We believe that in general we were successful in following these guidelines. We developed good partnerships with local communities and Aboriginal people actively participated in the research from the outset. We closely adhered to guidelines regarding consent, confidentiality and acknowledgement of investigators and participants. But there were several areas which could have been done better; in particular, we should have had wider consultation with members of the communities when planning the study.

The 1991 guidelines indicate that 'research should be potentially useful to the community'.[23] This was clearly the case for our study as members of local communities had expressed their concerns about OM and its consequences. Reduction in the burden of OM would improve *survival* of Aboriginal people as improved hearing could lead to better educational outcomes and quality of life. However, initial *consultation* with members of the community was limited. The senior investigator (D.L.) was new to the community and she had to rely on new friends and colleagues to introduce her to gain the community's support and trust. Dr S Eades, an Aboriginal doctor, provided guidance initially and, subsequently, Aboriginal members of ICHR's Kulunga Research Network were investigators on the project.

While members of the Aboriginal community were *participants* from the start, there should have been more widespread discussion when developing the protocol. As a consequence, the consultation process took place over 1 year following the award of the first grant. We learnt that it may take longer than anticipated for Aboriginal organisations to consider all aspects of the study, but their input contributed to its success and gave the local community a sense of ownership. For example, the Aboriginal organisations assigned health workers to the project, their members participated in focus group discussions for an adjunct qualitative study, and men and women alike wore the T-shirts we designed as part of a health awareness campaign aimed at reducing smoking and enhancing breast feeding.

We did not consider it necessary to form a separate reference group as has been done for other studies[21,35–37] as our study was being monitored by the Board of BEGA and members of NTP and we had frequent informal contacts with members of the local Aboriginal community.

In order to develop strong partnerships, *respectful research relationships* and achieve *equality*, we included all stakeholders in the planning process and updated them regularly on progress of the study. We planned follow-up visits so that they would not be too disruptive to people's lives. We sought *reciprocity* and deemed important that there be no research without service. We therefore offered hearing screening to all newborns and pneumococcal conjugate vaccine (PCV, Prevenar™) to non-Aboriginal children, neither of which was generally available at the time (PCV was being given routinely to Aboriginal children). We provided regular ENT follow-up to study participants and their families and arranged prompt surgical intervention if required. PCV is now offered to all Australian children but newborn hearing screening is not available in Kalgoorlie, because there is no resident audiologist. ENT specialist services continue and an exciting hearing screening programme is being developed.

In the spirit of developing true partnerships, AHWs were initially seconded from NTP or BEGA to work part-time on the project. However, they frequently had other commitments and it was only when there were sufficient funds to employ a research assistant (A.S.) to work solely on the research project that recruitment of Aboriginal babies gained momentum and follow-up was more successful. She was asked by members of the community to work on the project to ensure the success of the project and she took on the *cultural and spiritual responsibility* for the project. The Aboriginal staff provided *cultural security* for the Aboriginal aspect of the study, they were the link between non-

Aboriginal investigators and the Aboriginal community and they offered an insight into the broader picture of how children were living with OM.

We made it our *responsibility* to maintain confidentiality and to present results to the local community before submitting manuscripts for publication or presenting results to people outside the local community. We provided feedback to the community in a number of ways. Activities during National Aboriginal and Islander Day Observance Committee week celebrations and newsletters kept study participants and health service providers up-to-date on progress and results emerging from the study. Participating in local social activities also provided an opportunity for people to meet the research team informally. To transfer research into action, community-based awareness projects have already been undertaken[38] and more are planned. We must now continue to report results of our study to relevant local committees, so that they can initiate appropriate interventions.

All members of the research team had equal training opportunities and were acknowledged for their contribution to the study. Aboriginal and non-Aboriginal investigators have presented findings at national and international conferences. In 2002 and 2005, investigators contributed to local ear health conferences which were well attended by a wide range of health professionals. Further feedback was provided to medical practitioners and NTP staff in 2006.

In summary, our experiences and comments with regard to conduct of studies in Aboriginal communities concur with reports from other studies [21,36,37,39] and should be considered by future investigators. It is possible to conduct rigorous research despite constraints.[21,40] Common themes emerging from our study and the studies cited above include the need for early consultation, time to develop partnerships (which requires adequate funding), consideration of establishing a reference group, Aboriginal participation in the research, the development of trust and ensuring confidentiality, Aboriginal control of results, and appropriate acknowledgement. It takes time to gain people's confidence, but then there is the flow-on effect if people have had a positive experience of research activities. Recently published guidelines for Aboriginal and Torres Strait Islander peoples about health research ethics can also assist Aboriginal people and non-Aboriginal investigators in the appropriate conduct of research in Aboriginal health.[41]

### *Limitations of the study*

The main limitation of our study will be the incomplete follow-up of many participants to age 2 years. However, given a peak prevalence of OM well before age 2 years,[4,8] we will have a principal outcome based on OM for most of the study population. We knew that the Kalgoorlie population was mobile but were not aware of the extent of mobility.

Despite every effort, ENT follow-up at a central clinic was difficult for some children (as the NTP coordinator had forewarned us). Medical practitioners' records and tympanometry during routine follow-up in the field will provide additional outcome measures. Ideally, future studies should include clinical examination of ears at every follow-up visit as has been done elsewhere.[6] The statistical power of our study to investigate interactions in detail will be low. However, causal pathway analysis uses all available knowledge to construct causal diagrams, then examines the data 'to see if they are consistent with the diagrams' and utilises 'changes in magnitude of . . . estimates between different models' to determine how interactions between factors may influence risk of disease.[19]

Despite the limitations of our study, we have a wealth of information on microbiological, immunological and sociodemographic factors that have not been investigated previously in a single study. Our findings will provide essential information to develop appropriate interventions to limit the serious consequences of OM in Aboriginal and non-Aboriginal children.

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RESEARCH ARTICLE Open Access

# Association between early bacterial carriage and otitis media in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia: a cohort study

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**Abstract**

**Background:** *Streptococcus pneumoniae* (Pnc), nontypeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* (Mcat) are the most important bacterial pathogens associated with otitis media (OM). Previous studies have suggested that early upper respiratory tract (URT) bacterial carriage may increase risk of subsequent OM. We investigated associations between early onset of URT bacterial carriage and subsequent diagnosis of OM in Aboriginal and non-Aboriginal children living in the Kalgoorlie-Boulder region located in a semi-arid zone of Western Australia.

**Methods:** Aboriginal and non-Aboriginal children who had nasopharyngeal aspirates collected at age 1- < 3 months and at least one clinical examination for OM by an ear, nose and throat specialist before age 2 years were included in this analysis. Tympanometry to detect middle ear effusion was also performed at 2- to 6-monthly scheduled field visits from age 3 months. Multivariate regression models were used to investigate the relationship between early carriage and subsequent diagnosis of OM controlling for environmental factors.

**Results:** Carriage rates of Pnc, NTHi and Mcat at age 1- < 3 months were 45%, 29% and 48%, respectively, in 66 Aboriginal children and 14%, 5% and 18% in 146 non-Aboriginal children. OM was diagnosed at least once in 71% of Aboriginal children and 43% of non-Aboriginal children. After controlling for age, sex, presence of other bacteria and environmental factors, early nasopharyngeal carriage of NTHi increased the risk of subsequent OM (odds ratio = 3.70, 95% CI 1.22-11.23) in Aboriginal children, while Mcat increased the risk of OM in non-Aboriginal children (odds ratio = 2.63, 95% CI 1.32-5.23). Early carriage of Pnc was not associated with increased risk of OM.

**Conclusion:** Early NTHi carriage in Aboriginal children and Mcat in non-Aboriginal children is associated with increased risk of OM independent of environmental factors. In addition to addressing environmental risk factors for carriage such as overcrowding and exposure to environmental tobacco smoke, early administration of pneumococcal-*Haemophilus influenzae* D protein conjugate vaccine to reduce bacterial carriage in infants, may be beneficial for Aboriginal children; such an approach is currently being evaluated in Australia.

**Keywords:** Otitis media, Aboriginal, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*

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## Background

Otitis media (OM) is one of the most common diseases of childhood [1]. Otitis media with effusion (OME), recurrent acute otitis media (AOM) and chronic suppurative otitis media (CSOM) can lead to impaired hearing which in turn can affect school performance and hence social circumstances in adulthood [2]. An estimated 10-20% of children suffer ≥ 3 attacks of OM in the first year of life in first world settings [3]. Among Aboriginal Australians, OM begins at a younger age and the prevalence of OM and serious complications (in particular CSOM) is higher than in non-Aboriginal children [4,5]. By 14 years of age Aboriginal Australians can expect to have suffered an average of 24 months of OM in contrast to 3 months in non-Aboriginal children [6]. Our own study conducted in the Kalgoorlie-Boulder region of Western Australia (WA) found the peak prevalence of OM was 72% at age 5–9 months in generally asymptomatic Aboriginal children and 40% at age 10–14 months in non-Aboriginal children [5]. Furthermore, on routine examination, 60% of generally asymptomatic Aboriginal children had hearing loss >25 decibels at 6–24 months of age compared with 22% of non-Aboriginal children [5].

*Streptococcus pneumoniae* (Pnc), nontypeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis* (Mcat) are the most important bacterial pathogens associated with OM [4,7-9]. Upper respiratory tract (URT) carriage rates of these pathogens are higher in Aboriginal children than in non-Aboriginal children and onset of bacterial colonization occurs soon after birth in Aboriginal children [4,9,10]. In our Kalgoorlie study, carriage rates of Pnc, NTHi and Mcat at 1–3 weeks of age were 18%, 12% and 17%, respectively, in Aboriginal children and 3%, 2% and 6% in non-Aboriginal children; carriage rates of *Staphylococcus aureus* (Sa) were high in both Aboriginal (55%) and non-Aboriginal (61%) children [9].

Several studies have reported that early onset of URT bacterial carriage increases the risk of subsequent OM. In the USA, children colonized with any of the 3 major respiratory pathogens at age ≤ 3 months were approximately twice as likely to suffer their first episode of OM before the age of 6 months than if colonized later [11]. In a study conducted in a tropical region of the Northern Territory (NT) of Australia involving 2-4-weekly nasopharyngeal carriage and ear assessments in a birth cohort of Aboriginal infants living in a remote area and a birth cohort of non-Aboriginal infants living in an urban area, early dual carriage of Pnc and NTHi was associated with a 33-fold higher risk of OM compared to that in children with early carriage of only one or none of these pathogens [4]. Our Kalgoorlie Otitis Media Research Project (KOMRP) was conducted between 1999 and 2005 to investigate causal pathways to OM [12]. In this study we investigated not only microbiological factors, but also environmental, demographic and socioeconomic factors that might predispose children to OM in order to identify appropriate interventions to reduce the burden of OM [13,14]. Compared with the NT study, we enrolled a larger birth cohort of Aboriginal and non-Aboriginal children, all living within the same broad urban and peri-urban areas in a semi-arid climate and we followed them for 2 years. The availability of information on a broad range of risk factors enabled the investigation of associations between early onset of carriage and subsequent OM over the first two years of life while controlling for potential socioeconomic and environmental confounders. Here we present results on the subset of children participating in the KOMRP who had a nasopharyngeal sample collected during the first 3 months of life and at least one subsequent clinical examination of their ears [12].

## Methods

### Setting and study population

Kalgoorlie-Boulder is a mining town located 600 km east of WA's capital, Perth. The population is approximately 32,000 with an estimated 8% being of Aboriginal descent [15]. The climate is dry with minimum and maximum average temperatures ranging from 5°C to 18°C and 17°C to 34°C, respectively, and an average annual rainfall of 265 mm [16].

### Study design

A detailed description of methods used in this study has been reported elsewhere [12]. Briefly, 100 Aboriginal and 180 non-Aboriginal children born in Kalgoorlie Regional Hospital between April 1999 and January 2003 and living within an hour's drive of Kalgoorlie-Boulder were followed from birth to age 2 years. Multiple births, children with severe congenital abnormalities or birth weight < 2000 g and mothers not intending to live in the area for 2 years were excluded.

An initial evaluation was conducted 1–3 weeks postpartum in potential participants' homes when we sought informed consent and collected a nasopharyngeal aspirate (NPA) to identify bacteria and viruses, demographic data and information on potential risk factors for OM (e.g. smoking in the household, feeding practices, number of people living in the household, number of rooms in the house) using standardized structured interview questionnaires [9,12]. Subsequent follow-ups by research assistants were scheduled at ages 6–8 weeks, and 4, 6, 12, 18 and 24 months and conducted in the project office or in the participants' homes. During these visits NPAs were collected and we enquired again about time-dependent factors such as feeding practices. Following NPA collection, 1 ml of saline was added to the specimen. A 0.5 ml volume of mucus plug, or, if no visible

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plug the gently mixed specimen, was pipetted into 1 ml of skim milk-tryptone-glucose-glycerol broth and kept at −20°C for up to 72 hours before transporting to Perth where they were stored at −70°C until processed.

From July 2001 onwards, 7-valent pneumococcal conjugate vaccine (PCV) was offered at ages 2, 4 and 6 months to Aboriginal participants through a national immunization program and we also offered it to our non-Aboriginal study participants.

Details of clinical assessments of children's ears are described elsewhere [5]. Briefly, an ear, nose and throat (ENT) specialist conducted a clinical examination once before 6 months of age, and again at ages 6–11 and 12–23 months. Diagnosis was established using otoscopy, pneumatic otoscopy and tympanometry, was based on the child's most severely affected ear, and was classified according to national clinical guidelines at the time [17]. From May 2000 onwards, research assistants also performed tympanometry in children aged 3 months or more at every scheduled routine follow-up visit conducted in the project office or in participants' homes. All tympanograms were classified by an ENT specialist (HC) or an audiologist (SW) according to standard criteria [2,18]. A type A tympanogram indicates an aerated middle ear, type B the presence of middle ear effusion or a perforation and type C eustachian tube dysfunction. If tympanometry could not be assessed in one ear, then the classification for tympanometry was based on the result for the other ear.

### Laboratory methods

Full details of laboratory methods, including those used to identify bacteria, have been reported previously [9]. Ten microL of each sample was inoculated onto the following media: horse blood agar, chocolate agar containing bacitracin (300 mg/L), vancomycin (5 mg/L) and clindamycin (0.96 mg/L) and colistin nalidixic acid blood agar plates (Oxoid, Australia) [9].

### Data analysis

Data analyses were performed using SPSS (version 15.0 for Windows). Logistic regression models, incorporating generalised estimating equations to account for a loss of independence due to repeated measures on individual children, were used to generate odds ratios to describe the relative likelihood of OM (diagnosed from day of specimen collection onwards) in children who experienced early bacterial carriage compared to children with no early carriage. Limited sample size precluded investigation according to severity of OM. The same analytical methods were used with type B tympanogram as the outcome of interest. All analyses were adjusted for age, age-squared and sex. The age-squared term is included to better capture the observed non-linearity of OM incidence with age in our models. Potential confounders were divided into 2 groups. The first group comprised breast-feeding (exclusive breast-feeding at age 6–8 weeks) and environmental factors i.e. exposure to environmental tobacco smoke (ETS, inside or outside the house), a crowding index (number of people/room) and presence of other children in the house, whilst the second group consisted of simultaneous carriage of other bacteria (Pnc, NTHi, Mcat and Sa as appropriate). Analyses were adjusted for carriage of Sa in view of the high colonization rates with Sa in this population [9] and a possible role of Sa in OM [10,19]. The two groups of confounders were first entered separately into the regression models followed by a final model containing all variables. The crowding index was divided into quintiles for Aboriginal and non-Aboriginal children separately. PCV vaccination was not included as a potential confounder because the first dose of three in the PCV schedule is given at 2 months of age which is too early to have any influence on pneumococcal carriage under 3 months of age. Separate analyses were conducted for Aboriginal and non-Aboriginal children.

The number of otopathogens (Pnc, Mcat, NTHi) identified in children aged <1 month was too sparse to investigate risk of OM associated with carriage in the first month of life. We therefore conducted analyses based on identification of pathogens (a) in one or other or both of the two NPAs collected before the age of 3 months and (b) only in NPAs collected at age 1-<3 months. As the findings were broadly similar, we present results using bacteriology data obtained at 1-<3 months.

### Ethical approval

Ethical approval to conduct this study was given by the WA Aboriginal Health Information and Ethics Committee, the Northern Goldfields Health Service and Nursing Education Ethics Committee in Kalgoorlie, Princess Margaret Hospital for Children Ethics Committee and the Confidentiality of Health Information Committee of the Health Department of WA.

## Results

### Study participants

Eighty Aboriginal children had NPAs collected at age 1-<3 months of whom 66 (83%) had at least one clinical examination by an ENT specialist between the time of NPA collection and age 2 years; 171 non-Aboriginal children had NPAs collected of whom 146 (85%) had at least one clinical examination. A total of 133 clinical examinations were performed between the NPA collection at 1-<3 months and age 2 years in Aboriginal children (37% before age 6 months, 27% at 6–11 months and 35% at age 12–24 months) and 310 in non-Aboriginal children

(32% <6 months, 27% at 6–11 months and 41% at 12–24 months), with a median of two clinical examinations in both Aboriginal and non-Aboriginal children.

### Upper respiratory tract carriage rates

Among members of the KOMRP study population who had NPAs collected at 1- < 3 months and at least one subsequent clinical examination, 60% of Aboriginal children and 14% of non-Aboriginal children carried at least one of the otopathogens (Pnc, NTHi or Mcat) at age 1- < 3 months. The carriage rates of individual pathogens were similar to those reported previously [9]: in Aboriginal children carriage rates of Pnc, NTHi and Mcat were 45%, 29% and 48%, respectively, and in non-Aboriginal children 14%, 5% and 18%, respectively. 17% of Aboriginal children carried both Pnc and NTHi, 30% both Pnc and Mcat, and 18% NTHi and Mcat; 12% carried all 3 pathogens; corresponding figures in non-Aboriginal children were 2%, 6%, 2% and 1% (Table 1). At age 1- < 3 months, Sa was isolated from 39% of NPAs collected from Aboriginal children and 47% of NPAs from non-Aboriginal children.

### Diagnosis of otitis media

OM was diagnosed at least once in 71% (47/66) of the Aboriginal children and 43% (63/146) of the non-Aboriginal children who had an NPA collected and at least one clinical examination (Table 2). Of the 133 clinical examinations recorded in Aboriginal children 40 (30%) were normal, 15 (11%) had a diagnosis of eustachian tube dysfunction (ETD), 63 (47%) had OME, 9 (7%) AOM with or without perforation and 6 (5%) CSOM. The corresponding figures among 310 events documented in non-Aboriginal children were 188 (61%), 31 (10%), 80 (25%), 11 (4%) and 0 (0%). As reported previously for all the KOMRP study participants [5,14], compared with non-Aboriginal children, more Aboriginal children were exposed to ETS, fewer were breast-fed at 6–8 weeks postpartum and they lived in more crowded households (Table 2).

**Table 1 Carriage status at age 1- < 3 months of *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* and/or *Moraxella catarrhalis* and presence or absence of otitis media (OM) in Aboriginal and non-Aboriginal children who had at least one clinical ear examination**

| Micro-organism | | | OM diagnosis[a] | | | |
|---|---|---|---|---|---|---|
| | | | Aboriginal n = 66 | | Non-Aboriginal n = 146 | |
| Pnc[b] | NTHi[c] | Mcat[d] | Yes (%) | No (%) | Yes (%) | No (%) |
| + | + | + | 7 (87) | 1 (13) | 2 (100) | 0 (0) |
| + | + | - | 3 (100) | 0 (0) | 1 (100) | 0 (0) |
| + | - | + | 9 (75) | 3 (25) | 4 (57) | 3 (43) |
| - | + | + | 4 (100) | 0 (0) | 1 (100) | 0 (0) |
| + | - | - | 4 (57) | 3 (43) | 2 (20) | 8 (80) |
| - | + | - | 4 (80) | 1 (20) | 0 (0) | 4 (100) |
| - | - | + | 7 (87) | 1 (13) | 11 (65) | 6 (35) |
| - | - | - | 9 (47) | 10 (53) | 42 (40) | 62 (60) |

[a] Otitis media (OM) diagnosed once or more from time of specimen collection at age 1- < 3 months until last clinical examination before 2 years of age.
[b] *Streptococcus pneumoniae*.
[c] *Haemophilus influenzae*.
[d] *Moraxella catarrhalis*.
+ pathogen present.
- pathogen absent.

### Bacterial colonization and risk of OM

Table 1 shows the carriage of Pnc, NTHi and/or Mcat individually or concurrently at 1- < 3 months of age in children with and without a diagnosis of OM during the study period. Simultaneous carriage of two or more of these pathogens was very common in Aboriginal children, such that only 23% (n = 7) of all Pnc, 25% (n = 8) of Mcat and 25% NTHi (n = 5) were isolated in the absence of the other otopathogens, in contrast to 50% (n = 10) of all Pnc, 63% of Mcat (n = 17) and 50% (n = 4) of NTHi in non-Aboriginal children. In those who carried Pnc, NTHi or Mcat at age 1- < 3 months, OM was diagnosed respectively at least once in 77%, 90% and 84% of Aboriginal children and 45%, 50% and 67% of non-Aboriginal children. Nine of the 10 children carrying all three otopathogens simultaneously at age 1- < 3 months developed OM, all nine children who simultaneously carried either Pnc and NTHi or NTHi and Mcat developed OM, while 13 (68%) of the 19 children who simultaneously carried Pnc and Mcat developed OM. In contrast, if none of the three otopathogens was isolated at 1- < 3 months of age, 47% of Aboriginal and 40% of non-Aboriginal children were diagnosed with OM on one or more occasions. None of the four non-Aboriginal children who carried NTHi alone developed OM.

Table 3 shows the associations between early carriage of Pnc, NTHi, Mcat or Sa and subsequent risk of OM, first adjusted only for age and sex, then for the presence of the other bacterial pathogens of interest, then for environmental factors, and finally for all potential confounders. When comparing children with early onset of carriage to those without early carriage, the early carriage of any of the otopathogens (Pnc, NTHi and Mcat), either alone or in conjunction with one or both of the others, was associated with an increased risk of subsequent OM in Aboriginal children after adjusting for age, sex and the presence of the other bacterial pathogens. This increased risk declined after adjustment for environmental factors (odds ratio (OR) from 3.68 to 2.44). In non-Aboriginal children, there was a small positive association between early carriage of any otopathogen and subsequent risk of OM, with no difference in odds ratios

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**Table 2 Demographic and environmental characteristics, and proportion of children with otitis media diagnosed at least once in Aboriginal and non-Aboriginal children who had NPA collected at 1- < 3 months and at least one subsequent clinical examination for otitis media before age 2 years**

| Predictor variable | Aboriginal | non-Aboriginal | p value * |
|---|---|---|---|
| | n = 66 | n = 146 | |
| Male | 39 (59.1%) | 75 (51.4%) | 0.30 |
| OM diagnosed at least once | 47 (71.2%) | 63 (43.2%) | 0.0002 |
| Exposure to environmental tobacco smoke | 38 (59.4%) | 58 (39.7%) | 0.009 |
| Exclusive breastfeeding 6–8 weeks postpartum | 31 (47.0%) | 94 (64.8%) | 0.13 |
| Crowding index (>1 person per room) | 46 (69.7%) | 19 (13.0%) | <0.0001 |
| Other children living in the house | 49 (74.2%) | 72 (49.3%) | 0.0007 |

Discrepancies between total numbers of children for different variables are the result of missing data.
* P values are for comparisons between Aboriginal and non-Aboriginal children.

between the different models; the fully adjusted OR for early carriage and risk of OM was 1.42 (95% confidence interval (CI) 0.78-2.56).

We then considered individual bacterial pathogens. There was no association between early carriage of Pnc and OM in either Aboriginal or non-Aboriginal children. In non-Aboriginal children, isolation of Mcat at age 1- < 3 months increased the risk of developing OM and there was little change in odds ratios after adjusting for confounders (OR =2.63, 95% CI 1.32-5.23). We found no association between early Mcat carriage and risk of OM in Aboriginal children. In contrast, early NTHi carriage increased the risk of developing OM in Aboriginal children (OR = 3.71, 95%CI 1.22-11.23 after adjustment for all potential confounders) but not in non-Aboriginal children. The OR for increased risk of OM associated with NTHi carriage in Aboriginal children fell from 5.32 (when adjusted for age, sex and presence of the other three pathogens) to 3.71 after adjusting further for environmental factors. Finally, there was a suggestion of a protective effect of Sa carriage in Aboriginal children (OR = 0.52, 95%CI 0.20-1.33 after adjusting for all confounders).

### Tympanometry

Seventy-one Aboriginal children and 160 non-Aboriginal children had had NPAs collected at age 1- < 3 months and tympanometry assessments from age 3 months onwards; 583 assessments (131 in Aboriginal and 452 in non-Aboriginal children) were performed by research assistants at routine field follow-up visits and 342 assessments (99 in Aboriginal children and 243 in non-Aboriginal children) were performed by audiologists at routine ENT clinics. In Aboriginal children 73 (32%), 125 (54%) and 32 (14%) were types A, B and C, respectively; corresponding figures in non-Aboriginal children were 418 (60%), 158 (23%) and 119 (17%). Of the Aboriginal children, 58 (82%) had a type B tympanogram on at least one occasion while 88 (55%) non-Aboriginal children had a type B tympanogram at least once. The

**Table 3 Association between carriage of *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* individually or any of the otopathogens at age 1- < 3 months and subsequent diagnosis of otitis media adjusting for age and sex, presence of other bacteria, and environmental factors**

| Adjusted factors | Aboriginality | Any otopathogen[a] | *S. pneumoniae* | *M. catarrhalis* | *H. influenzae* | *S. aureus* |
|---|---|---|---|---|---|---|
| | | OR (95%CI)[b] | OR (95%CI) | OR (95%CI) | OR (95%CI) | OR (95%CI) |
| **Sex and Age only** | Aboriginal | **3.86 (1.61-9.24)** | 1.39 (0.64-3.01) | 1.97 (0.89-4.39) | **5.32 (2.12-13.35)** | 0.64 (0.26-1.56) |
| | Non-Aboriginal | 1.47 (0.80-2.71) | 1.12 (0.50-2.47) | **2.57 (1.35-4.91)** | 1.1 (0.36-3.42) | 1.02 (0.58-1.77) |
| **Presence of other bacteria** | Aboriginal | **3.68 (1.51-8.96)** | 0.86 (0.39-1.93) | 1.72 (0.74-3.96) | **5.32 (1.95-14.49)** | 0.6 (0.25-1.47) |
| | Non-Aboriginal | 1.48 (0.80-2.72) | 0.77 (0.32-1.88) | **2.81 (1.40-5.61)** | 0.96 (0.33-2.83 ) | 1.06 (0.61-1.86) |
| **Environmental factors[c]** | Aboriginal | 2.54 (0.93-6.93) | 1.14 (0.49-2.65) | 0.81 (0.29-2.27) | **3.66 (1.31-10.25)** | 0.54 (0.22-1.33) |
| | Non-Aboriginal | 1.41 (0.78-2.55) | 1.07 (0.51-2.28) | **2.42 (1.31-4.48)** | 0.87 (0.32-2.39) | 1.06 (0.58-1.93) |
| **All confounders** | Aboriginal | 2.44 (0.88-6.78) | 0.84 (0.32-2.17) | 1.02 (0.34-3.10) | **3.71 (1.22-11.23)** | 0.52 (0.20-1.33) |
| | Non-Aboriginal | 1.42 (0.78-2.56) | 0.81 (0.33-2.00) | **2.63 (1.32-5.23)** | 0.80 (0.30-2.09) | 1.05 (0.58-1.91) |

All associations adjusted for sex, age and age$^2$.
[a] Any otopathogen includes one or more of *S. pneumoniae*, nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis*.
[b] Odds ratio and 95% confidence interval comparing the odds of diagnosis of OM between those who carried the respective organisms at age 1- < 3 months and those who did not.
[c] Environmental factors are: passive smoking, crowding, other children living in the house and also exclusive breast-feeding at 6–8 weeks.

median number of tympanometry assessments was three for Aboriginal children and four for non-Aboriginal children.

The same analyses as for a clinical endpoint of OM (using generalised estimating equation regression models) were performed using type B tympanogram as the endpoint, except that we also controlled for the location where tympanometry was done (at field follow-up visit or at the ENT specialist clinic). ORs assessing early carriage and subsequent risk of a type B tympanogram were broadly similar to those found for a clinical outcome of OM, although generally lower (Table 4). In Aboriginal children nasopharyngeal colonization with NTHi at age 1- < 3 months was associated with subsequent type B tympanogram (OR = 5.35, 95% CI = 1.97-14.87) after adjusting for all confounders. In non-Aboriginal children, in contrast to our findings with clinical OM as the endpoint, there was no association between early colonization with Mcat and subsequent type B tympanogram (OR = 1.09, 95% CI = 0.60-2.00).

## Discussion

To our knowledge, this is the first investigation of early bacterial carriage conducted simultaneously in young Aboriginal and non-Aboriginal children living in the same setting, in this case in an urban and peri-urban rural area of Western Australia. It is one of very few studies investigating associations between early carriage and subsequent OM, despite evidence to suggest that early URT carriage may affect development of long-term humoral and cellular immunity, which in turn can increase susceptibility to disease [20,21].

We have reaffirmed the high rates of single as well as multiple carriage of Pnc, Mcat and NTHi from a young age in Aboriginal children and high rates of OM in both Aboriginal and non-Aboriginal children. Early nasopharyngeal carriage of one or more of the 3 otopathogens, and specifically NTHi, increased the risk of OM in Aboriginal children, while in non-Aboriginal children Mcat was associated with an increased risk of OM as determined by full clinical examination but not by tympanometry alone. The important contribution of environmental factors to the high rates of OM in Aboriginal children [13,14] is emphasized by the observation of reductions in ORs for early carriage (NTHi or any otopathogen) and subsequent risk of OM when controlling for environmental factors (Table 3).

We found no association between early carriage of either Pnc or Mcat and risk of OM in Aboriginal children. This may be because it is only their carriage during the first month (rather than the first 3 months of life) that increases risk of OM, but our data were too sparse to restrict analysis to carriage during the first month of life. Pnc and Mcat carriage begins at a younger age than NTHi: by age 2 months, ~40% of Aboriginal children had carried Pnc and/or Mcat at least once compared with 27% for NTHi [9].

There were similarities and differences between the current study and the only other comparable study which was conducted among Aboriginal children in the NT [4]. Both studies identified the importance of early onset of URT carriage and risk of OM. However, in the NT study Aboriginal children were seen 2-4-weekly from birth and both carriage and ear health status were assessed at each visit. The primary endpoint in the NT study was time to first documented episode of OM (which occurred in all Aboriginal children in the NT before the age of 3 months), while our primary endpoint was any diagnosis of OM up to age 2 years. Carriage

**Table 4 Association between carriage of *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* individually or any of the otopathogens at age 1- < 3 months and subsequent type B tympanogram adjusting for age and sex, presence of other bacteria, and environmental factors**

| Adjusted factors | Aboriginality | Any otopathogen[a] | *S. pneumoniae* | *M. catarrhalis* | *H. influenzae* | *S. aureus* |
|---|---|---|---|---|---|---|
| | | OR (95%CI)[b] | OR (95%CI) | OR (95%CI) | OR (95%CI) | OR (95%CI) |
| Sex and Age only | Aboriginal | 2.21 (0.91-5.42) | 1.16 (0.55-2.17) | 1.60 (0.74-3.48) | **4.58 (1.98–10.59)** | 0.61 (0.27-1.39) |
| | Non-Aboriginal | 1.36 (0.84-2.22) | 1.59 (0.87-2.91) | 1.42 (0.86-2.34) | 0.97 (0.32–2.97) | 0.92 (0.57-1.48) |
| Presence of other bacteria | Aboriginal | 2.07 (0.82-5.22) | 0.67 (0.33-1.38) | 1.63 (0.79-3.36) | **5.51 (2.41-12.58)** | 0.51 (0.24-1.09) |
| | Non-Aboriginal | 1.36 (0.83-2.21) | 0.86 (0.29-2.58) | 1.28 (0.73-2.26) | 1.48 (0.75-2.93) | 0.97 (0.60-1.55) |
| Environmental factors[c] | Aboriginal | 1.56 (0.51-4.74) | 0.99 (0.43-2.27) | 0.68 (0.23-1.98) | **4.31 (1.64-11.32)** | 0.61 (0.25-1.54) |
| | Non-Aboriginal | 1.12 (0.66-1.88) | 1.33 (0.75-2.37) | 1.16 (0.66-2.05) | 0.90 (0.27-3.02) | 0.90 (0.52-1.55) |
| All confounders | Aboriginal | 1.56 (0.52-4.68) | 0.66 (0.32-1.36) | 0.99 (0.33-2.93) | **5.35 (1.97-14.87)** | 0.45 (0.18-1.13) |
| | Non-Aboriginal | 1.11 (0.60-1.87) | 1.31 (0.70-2.43) | 1.09 (0.60-2.00) | 0.83 (0.24-2.90) | 0.92 (0.54-1.58) |

All associations adjusted for sex, age, age$^2$ and where tympanometry was conducted (field or clinic).

[a] Any otopathogen includes one or more of *S. pneumoniae*, nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis*.

[b] Odds ratio and 95% confidence interval comparing the odds of having a type B tympanograms between those who carried the respective organisms at age 1- < 3 months and those who did not.

[c] Environmental factors are: passive smoking, crowding, other children living in the house and also exclusive breast-feeding at 6–8 weeks.

rates of single and multiple pathogens were even higher in the NT study than in ours; hence, in the NT study it was more difficult to investigate the contribution of individual otopathogens to subsequent disease than in our present study. Our lower carriage rates are unlikely to be due to differences in laboratory methods as our laboratory scientist (JB) was trained by colleagues in the NT and used the same methods throughout. The most likely explanation for differences in carriage rates between the two study populations is the somewhat better socioeconomic status in our urban/peri-urban WA community than that on a remote island in the NT, also reflected in the higher prevalence of OM and CSOM in the NT community than in our study population [4,5].

The positive association between early carriage of Mcat, but not Pnc or NTHi, and OM in the non-Aboriginal children in our study is consistent with findings in non-indigenous North American children [11]. However, we did not find an association between early Mcat carriage and presence of middle ear effusion as determined by type B tympanogram, possibly because we were identifying milder forms of OM using tympanometry at routine follow-up in the field than in the clinic setting. Of the three otopathogens, Faden *et al.* found that early carriage (defined as <3 months) of Mcat was the most strongly associated with early onset of OM, though the authors also reported that early carriage of any pathogen resulted in earlier onset of OM [11]. In our study, there was a small positive association between early carriage of any pathogen and risk of clinical OM in non-Aboriginal children. The lack of association in non-Aboriginal children between early NTHi carriage and subsequent OM is most likely due to the small number of NTHi isolated before age 3 months (total of 8 of which only 4 were isolated in absence of other otopathogens). Similarly, the lack of association between early Mcat carriage and subsequent OM in Aboriginal children in our study is likely to be the small number (8) of Mcat isolated alone in this population (37% of all Mcat were isolated together with Pncs, 13% with NTHi and 25% with both Pnc and NTHi). This is in contrast to non-Aboriginal children in whom 63% of all Mcat (17/27) were the only otopathogen isolated from an NPA sample. To our knowledge there are no data available to explain in biological terms why one pathogen is more likely to predispose to OM than another.

Our findings suggest that carriage of more than one bacterial pathogen is more likely to result in OM than carriage of a single pathogen (Table 1). However, in view of the small number of isolates detected at an early age and the limited number of subsequent clinical examinations, we were unable to conduct formal statistical analyses to investigate this further. Nevertheless, a strength of our study was that we were able to use type B tympanogram to confirm our analyses using clinical diagnosis of OM as an endpoint.

In addition to the limited sample size, there are several other limitations or potential biases in this study. Firstly, we were not able to conduct a clinical examination every time an NPA was collected due to lack of available specialist personnel, though we were able to conduct tympanometry at the same time as NPA collection from age 3 months onwards. Secondly, in view of the long period of follow-up required, it is possible that we did not enroll children at greatest risk of OM, since we did not enroll children whose families were not intending to stay in the area for 2 years. We previously looked at how representative our KOMRP study population (of which our present data form a subset) was of the general population in the area: there were more teenage Aboriginal mothers, non-Aboriginal parents were older and non-Aboriginal mothers were less likely to smoke in pregnancy than the general population [12].Thirdly, while all children were asked to attend a clinic 3 times over 2 years for routine examination, it is possible that those who attended were sicker children seeking medical attention. Conducting tympanometry during routine field follow-up visits helped address this potential bias. Finally, we cannot exclude the possibility of residual confounding.

The results reported here were part of a broader study investigating causal pathways to OM, allowing us to control for important environmental factors. It would now be important to consider the role that high bacterial load and/or mixed infections (bacterial-bacterial and viral-bacterial) at a young age may play in subsequent risk of OM [10] in a larger cohort of both Aboriginal and non-Aboriginal children [4,22-24].

## Conclusion

This study provides further evidence that early onset of bacterial carriage, and specifically NTHi carriage, increases risk of OM during the first two years of life in Aboriginal children. Furthermore, the study has shown for the first time the importance of early carriage of Mcat as a potential risk factor for OM in non-indigenous Australian children. The results of our study reinforce the need for early interventions to reduce early onset of carriage in order to reduce the burden of OM. Given the association of early NTHi carriage and subsequent OM in Aboriginal children, early administration of pneumococcal-*H. influenzae* D protein conjugate vaccine may offer additional benefit. A trial investigating the safety and immunogenicity of such an approach in Aboriginal children, with carriage and OM as secondary outcomes, has recently begun in the NT (http://www.anzctr.org.au/trial_view.aspx?ID=335348). Promotion of hand hygiene among those handling young infants will

help reduce URT carriage and hence OM. Improved housing for Aboriginal people to reduce overcrowding is essential as we have previously shown crowding to be the single most important and consistent risk factor for URT carriage [13] which in turn leads to high rates of OM.

## Appendix

### List of investigators and participating institutions

Telethon Institute for Child Health Research (ICHR), Centre for Child Health Research, the University of Western Australia (UWA):

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The University of Western Australia:

• Harvey Coates, Paediatric Otolaryngologist; J Spencer, C Jeffries-Stokes (also ICHR), P Richmond, paediatricians

Department of Microbiology and Infectious Diseases, PathWest Laboratory Medicine (WA):

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Audiologists:

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Menzies School of Health Research, Darwin, NT:

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• D Murphy

### Abbreviations

CSOM: Chronic suppurative otitis media; ENT: Ear, Nose and Throat; KOMRP: Kalgoorlie Otitis Media Research Project; Mcat: *Moraxella catarrhalis*; NTHi: Nontypeable *Haemophilus influenzae*; NT: Northern Territory; OM: Otitis media; Pnc: *Streptococcus pneumoniae*; Sa: *Staphylococcus aureus*; URT: Upper respiratory tract; WA: Western Australia.

### Competing interests

D Lehmann, A Cripps and H Coates have been members of the GSK Australia Pneumococcal-*Haemophilus influenzae*-Protein D conjugate vaccine ("PHiD-CV") Advisory Panel. D Lehmann has received support from Pfizer Australia and GSK Australia to attend conferences. A Cripps and H Coates have been members of the GSK nontypeable *Haemophilus influenzae* Global Advisory Board. A Cripps has received support to attend conferences from GSK Australia and GSK Biologicals. A Leach has received financial support for research and for conference attendance from Pfizer and GSK. A Leach has participated in a GSK-funded Advisory group on CSOM and has undertaken a short term consultancy for GSK.

### Authors' contributions

WS performed the statistical analysis and drafted the manuscript. PJ provided statistical advice and helped to draft the manuscript. TVR and AJL provided microbiological expertise, contributed to design of study, interpretation of results and preparation of the manuscript. JB carried out primary culture of NPAs. AC participated in the design of the KOMRP, interpretation of the results and preparation of the manuscript. HC and SW carried out clinical examinations, classified the tympanograms and had clinical input into the manuscript. DL conceived and coordinated the KOMRP study and helped to draft the manuscript. All authors have read and approved the final manuscript.

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# Middle ear disease in Aboriginal children in Perth: analysis of hearing screening data, 1998–2004

Corinne J Williams, Harvey L Coates, Elaine M Pascoe, Yvonne Axford and Irene Nannup

ABSTRACT

**Objective:** To describe diagnoses and correlates of middle ear disease in Aboriginal primary school children in a targeted school-testing program in Perth, Western Australia.

**Design and setting:** Analysis of records of ear testing carried out over a 6-year period in three primary schools in Perth.

**Participants:** Aboriginal children of primary school age (4–12 years) who attended the schools on the day of testing. Data on middle ear disease and hearing impairment were available for 119 and 94 children, respectively, from their first test.

**Main outcome measures:** Proportions of children with middle ear disease and hearing loss.

**Results:** Middle ear disease was diagnosed in 50 children (42.0%; 95% CI, 33.0%–51.4%). Rates were lower in older children ($P = 0.002$) but did not differ according to season of testing. Hearing loss (mild or moderate) was detected in 18 children (19.1%; 95% CI, 11.8%–28.6%). Hearing impairment was also less prevalent in older children ($P = 0.007$) and had no association with season of testing.

**Conclusions:** Middle ear disease is a significant problem for Aboriginal children in Perth, and is associated with mild–moderate hearing loss. Health authorities must continue to focus on appropriate identification and management of the disease in this population.

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Around 80% of children experience at least one episode of otitis media before 4 years of age.[1] Australian Aboriginal children are more likely than their non-Aboriginal peers to suffer otitis media, to experience the first episode earlier in life, to continue to experience episodes over a longer period of time, and to suffer permanent auricular damage and hearing loss.[2-6] It has been reported that up to 40% of children in some Aboriginal communities present with chronic suppurative otitis media (CSOM).[2] In the Pilbara region of Western Australia, otitis media was found to be present in 25.6% of Aboriginal children up to the age of 10, but in only 3.2% of non-Aboriginal children in the same age range.[3] Another study found that 91% of children in remote communities in northern and central Australia were affected.[4] The disease is common in preschool and early school-aged Aboriginal children[5] and is likely to persist into adolescence.[6]

High rates of otitis media are associated with high rates of hearing loss in the Aboriginal population. Estimates of prevalence vary but are consistently reported to be higher than in the non-Aboriginal population.[7] Otitis media and its accompanying hearing loss are thought to have an impact on the developmental outcomes of Aboriginal children, with particular effects on the development of language and social skills, and ultimately on educational outcomes.[8]

Most of the research into otitis media in Aboriginal populations has reported the situation in rural or remote areas, but some 30% of the Australian Aboriginal population live in major cities.[9] There have been few studies of otitis media in this population.[10]

Here, we present a retrospective report of results of ear testing carried out in Aboriginal children from three primary schools in metropolitan Perth, WA. Both middle ear disease and hearing loss are considered.

## METHODS

### Ethical considerations

Parent or caregiver permission was obtained before testing. Approval to access patient files and to publish de-identified data was granted by the Princess Margaret Hospital for Children (PMH) Ethics Committee and the Western Australian Aboriginal Health Information and Ethics Committee.

### Data collection

Data were collected in three urban primary schools with high Aboriginal enrolments during the period November 1998 – November 2004. The schools were located in areas with high numbers of Aboriginal residents, and had established, positive relationships with Derbarl Yerrigan Aboriginal Medical Service.

At the start of each year, a letter explaining the project and offering hearing screening was sent home with all children whose parents had identified the family as Aboriginal. Only children for whom parent or caregiver permission was obtained were included. Screening visits took place a number of times each year, and most children were tested on more than one occasion. The number of visits to each school in a particular year varied, as did the timing of the visit.

Testing was carried out on the school premises by trained Aboriginal health workers and nurses under an outreach program of PMH and Derbarl Yerrigan Aboriginal Medical Service. Ear health of participating children was screened using otoscopic examination, pure tone audiometry at 1000 and 4000 Hz, tympanometry and acoustic reflectometry. Treatment was prescribed by one of us (HLC), and children who showed evidence of middle ear disease were prioritised for follow-up at subsequent visits to the school. Data were recorded and stored in patient files at PMH.

The records of testing held at PMH were accessed by one of us (CJW) and recorded in a single database. Results for the left and right ears were recorded separately. Middle ear disease was determined separately for each ear using the diagnostic categories defined in Box 1. Hearing impairment was determined using World Health Organization criteria (Box 2).[11]

### Statistical analysis

Data from each child's first assessment visit were used to determine the percentage of children with middle ear disease and hearing impairment. Exact binomial confidence limits (95%) were calculated for all percentages. Variation in percentages by age and season was assessed using univariate exact logistic regression models. Results are reported as percentages and 95% confidence intervals. All statistical analyses were performed using Stata, version 10.0 (StataCorp, College Station, Tex, USA).

## RESULTS

### Sample characteristics

An estimated 80% of Aboriginal children at the three schools participated. The informa-

**1 Diagnostic categories of middle ear disease**

| Label | Description | Diagnostic details |
|---|---|---|
| WNL | Within normal limits | Type A tympanogram, average threshold 25 dB or less |
| ETD | Appearance suggestive of eustachian tube dysfunction | Type C, As, Ac or Ad tympanogram. On examination, the ear drum appears retracted or dull or shows increased or decreased mobility |
| OME | Otitis media with effusion | Type B tympanogram (low volume) |
| CSOM | Chronic suppurative otitis media | Type B tympanogram, perforation of the tympanic membrane (high volume) |

**2 Hearing impairment criteria[11]**

| Label | Two-frequency average |
|---|---|
| Pass (hearing within normal range) | 25 dB or less |
| Mild hearing loss | 26–40 dB |
| Moderate hearing loss | 41–60 dB |
| Severe hearing loss | 61–80 dB |
| Profound hearing loss | 81 dB or greater |

tion needed to diagnose unilateral and bilateral middle ear disease was available from the first visit for 119 children, and 94 children could be classified in terms of unilateral, bilateral or no hearing impairment.

Median age for the 119 children with data relevant to the analysis of middle ear disease at first visit was 8.5 years (interquartile range [IQR], 6.4–10.5 years). For the 94 children who contributed hearing loss data, median age at first visit was 8.5 years (IQR, 6.8–10.6 years).

### Middle ear disease

Middle ear disease (defined as appearance suggestive of eustachian tube dysfunction [ETD], otitis media with effusion [OME], or CSOM, in one or both ears) was diagnosed in 50/119 children (42.0%; 95% CI, 33.0%–51.4%). Bilateral middle ear disease was evident in 29 children (24.4%; 95% CI, 17.0%–33.1%) and unilateral disease in 21 (17.6%; 95% CI, 11.3%–25.7%). Excluding ETD from the definition reduced the number of children with middle ear disease to 23 (19.3%; 95% CI, 12.7%–27.6%), of whom 14 had bilateral disease (11.8%; 95% CI, 6.6%–19.0%) and nine had unilateral disease (7.6%; 95% CI, 3.5%–13.9%).

Results for middle ear disease (including ETD) by age and season of testing are given in Box 3. The proportion of children with middle ear disease decreased with increasing age ($P = 0.002$), but there was little evidence to suggest an association with season ($P = 0.52$).

CSOM and OME were also examined separately. Overall, CSOM was diagnosed in 2/119 children (1.7%; 95% CI, 0.2%–5.9%). Both cases of CSOM were instances of unilateral disease. OME was diagnosed in 22/119 children (18.5%; 95% CI, 12.0%–26.6%), 13 of whom had bilateral OME (10.9%; 95% CI, 5.9%–18.0%) and nine had unilateral OME (7.6%; 95% CI, 3.5%–13.9%). There was no evidence of an association between the diagnosis of OME and age ($P = 0.22$) or season ($P = 0.56$) (Box 3).

### Hearing impairment

Of the 94 children with hearing data at first visit, 18 (19.1%; 95% CI, 11.8%–28.6%) had some form (unilateral or bilateral) of mild or moderate hearing loss. Mild hearing impairment was detected in 13 children (13.8%; 95% CI, 7.6%–22.5%), with four cases of mild bilateral loss and nine cases of mild unilateral loss. Moderate hearing impairment was detected in five children (5.3%; 95% CI, 1.7%–12.0%), all of whom had unilateral moderate hearing loss, with either mild loss (two) or normal hearing (three) in the other ear.

Results for hearing impairment by age and season of testing are shown in Box 3. The proportion of children with impaired hearing decreased with age ($P = 0.007$), but there was no evidence of an association with season of testing ($P = 0.44$).

## DISCUSSION

This study indicates alarming rates of occurrence of middle ear disease in an urban Aboriginal population over a 6-year period. Middle ear disease was evident in just over 40% of children at first screening visit. Although this rate is lower than those reported in many studies in rural and remote areas, where rates of more than 90% have been recorded,[7] it is considerably higher than those reported for non-Aboriginal populations. For example, a study of ear disease in New South Wales indicates the prevalence of acute otitis media in children aged 0–14 years is 7.17%. The same study showed prevalence rates of serous otitis media to be 0.56%.[12]

Our rate is also higher than rates of recurrent ear problems found in the Western Australian Aboriginal Child Health Survey,[10] which used parent report to investigate a range of health conditions in children aged 0–17 years. The study found that recurrent ear infections were more common in areas classified as extremely isolated (22.8%), as were recurrent ear infections with at least one episode of discharge (18.4%). Areas classified as no isolation (Perth metropolitan area) and low isolation had the lowest reported prevalence of both recurrent otitis media (16.9% and

**3 Children with middle ear disease and hearing impairment, by age and season of testing**

| | Middle ear disease ($n = 119$) | | | Hearing impairment ($n = 94$) | |
|---|---|---|---|---|---|
| | Any* | | OME | | |
| Predictor variable | No.† | Per cent (95% CI) | Per cent (95% CI) | No.† | Per cent (95% CI) |
| Age (years) | | | | | |
| 4 to < 7 | 33 | 66.7 (48.2–82.0) | 27.3 (13.3–45.5) | 22 | 40.9 (20.7–63.6) |
| 7 to < 10 | 47 | 36.2 (22.7–51.5) | 14.9 (6.2–28.3) | 37 | 16.2 (6.2–32.0) |
| ⩾ 10 | 35 | 25.7 (12.5–43.3) | 11.4 (3.2–26.7) | 31 | 6.4 (0.8–21.4) |
| Season | | | | | |
| Autumn | 37 | 46.0 (29.5–63.1) | 21.6 (9.8–38.2) | 19 | 15.8 (3.4–39.6) |
| Winter | 42 | 47.6 (32.0–63.6) | 19.1 (8.6–34.1) | 37 | 18.9 (8.0–35.2) |
| Spring | 37 | 32.4 (18.0–49.8) | 13.5 (4.5–28.8) | 34 | 17.6 (6.8–34.5) |
| Summer | 3 | 33.3 (0.8–90.6) | 33.3 (0.8–90.6) | 4 | 50.0 (6.8–93.2) |

OME = otitis media with effusion. * Appearance suggestive of eustachian tube dysfunction, OME, or chronic suppurative otitis media, in one or both ears. † Numbers of children may not sum to totals because date of birth was not recorded for four children. ♦

17.6%, respectively) and recurrent infections with at least one episode of discharge (11.2% and 11.6%).

Eustachian tube dysfunction is associated with otitis media[13] and was therefore included in our analysis, contributing to the high rate. However, the rate remained unacceptably high (19.3%) when it was removed from calculations. The proportion of children with CSOM (1.7%) was lower than rates reported for remote communities, but exceeded the 1% seen to constitute an avoidable disease burden.[14]

Hearing loss was recorded in over 19% of the children at first testing. As is typical with otitis media,[15] all recorded hearing loss fell into the categories of mild or moderate loss. The highest percentage was recorded for unilateral mild loss.

The data analysed in this study demonstrate the continuing occurrence of middle ear disease and hearing loss in Aboriginal children across the age range sampled. Consistent with findings reported elsewhere, there was a significant reduction in OME with increasing age. However, the proportion of children in the ⩾10-years age group with OME (11.4%) is higher than the 3%–8% reported for non-Aboriginal children after the age of 7 years.[16] We also found a significant difference in the occurrence of hearing loss according to age, with older children less likely than younger children to suffer mild or moderate hearing loss. In the ⩾10-years age group, 6.4% of children were affected, while hearing loss was evident in 40.9% of children in the 4 to <7-years age range — the time at which formal education begins. This finding has significant implications for the educational progress of affected children. Episodes of middle ear disease and associated hearing loss can be expected to have an adverse impact on learning.

In contrast to previous reports,[17,18] we found no significant difference in rates of middle ear disease according to season, indicating a need for year-round vigilance in monitoring its occurrence.

The results of this study may have been influenced by selection bias if parents were more likely to provide screening permission for children known to have middle ear problems. However, we estimate that 80% of Aboriginal children in the schools participated in the study, suggesting that selection bias was not an issue.

This study highlights the problem presented by otitis media in Aboriginal children. Middle ear disease and mild to moderate hearing loss have been shown to affect large numbers of Aboriginal children attending schools in metropolitan Perth. The effects of these factors on the children's learning remain to be demonstrated, but associated adverse outcomes can be predicted. The findings present challenges for health authorities — to reduce the occurrence and expedite the treatment of OME and CSOM — and for educational authorities — to address the learning needs of the affected children.

## COMPETING INTERESTS

None identified.

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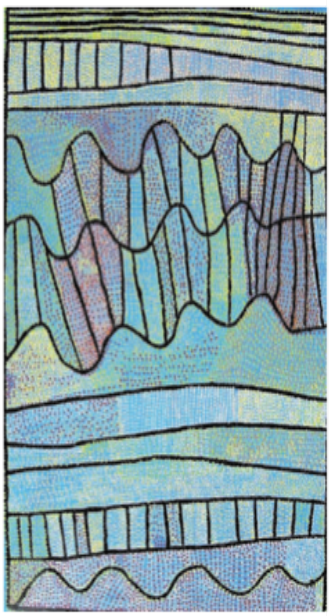

# Papers

## Benefits of swimming pools in two remote Aboriginal communities in Western Australia: intervention study

Deborah Lehmann, Mary T Tennant, Desiree T Silva, Daniel McAullay, Francis Lannigan, Harvey Coates, Fiona J Stanley

### Abstract

**Objective** To determine the health impact of swimming pools built with the aim of improving quality of life and reducing high rates of pyoderma and otitis media.
**Design** Intervention study assessing prevalence of ear disease and skin infections before and at six monthly intervals after opening of swimming pools.
**Setting** Two remote Aboriginal communities in Western Australia.
**Participants** 84 boys and 78 girls aged < 17 years.
**Main outcome measures** Changes in prevalence and severity of pyoderma and perforation of tympanic membranes with or without otorrhoea over 18 months after opening of pools.
**Results** In community A, 61 children were seen before the pool was opened, and 41, 46, and 33 children were seen at the second, third, and fourth surveys. Equivalent figures for community B were 60, 35, 39, and 45. Prevalence of pyoderma declined significantly from 62% to 18% in community A and from 70% to 20% in community B during the 18 months after the pools opened. Over the same period, prevalence of severe pyoderma fell from 30% to 15% in community A and from 48% to 0% in community B. Prevalence of perforations of the tympanic membrane fell from 32% in both communities to 13% in community A and 18% in community B. School attendance improved in community A.
**Conclusion** Swimming pools in remote communities were associated with reduction in prevalence of pyoderma and tympanic membrane perforations, which could result in long term benefits through reduction in chronic disease burden and improved educational and social outcomes.

### Introduction

Indigenous Australian children have very high rates of pyoderma (known locally as "skin sores") and otitis media. Prevalences as high as 70% have been reported for pyoderma in remote Aboriginal communities.[1] The major pathogen of pyoderma, group A streptococcus, is associated with chronic renal failure, which is very prevalent in Aboriginal communities.[2,3] The high prevalence of pyoderma is also the most likely reason for some Aboriginal communities having the highest reported incidence of acute rheumatic fever worldwide.[4]

Impaired hearing can seriously affect performance at school, subsequent employment, and social circumstances in adulthood. Between 10% and 67% of Aboriginal school age children have perforated tympanic membranes, and 14-67% have some degree of hearing loss; remote communities have the highest prevalence.[5]

Some reduction in skin infections was reported after a swimming pool opened in an Aboriginal community in the Northern Territory.[6] An audit of Aboriginal communities in central Australia has also found health and social benefits of pools.[7] Furthermore, communities within easy access of places to swim have been reported to have lower prevalence of otitis media than those further away.[8] These reports are in contrast to concerns about pool related infections in areas with low prevalence of infectious diseases.[9]

After publication of these reports suggesting that Aboriginal children may benefit from access to swimming pools, the Western Australian government built 25 metre, part shaded, salt water pools in three remote Aboriginal communities. In view of possible beneficial or potentially deleterious effects on health and the lack of previous evaluation of such an intervention, we assessed the health impact of swimming pools in the first two communities to have a functioning pool, particularly with regard to skin infections and middle ear disease in children. A secondary objective was to determine whether social benefits might result from pools in these remote communities. Financial constraints precluded inclusion of the third community.

### Methods

#### Setting

We compared disease prevalences before and after swimming pools were opened in two isolated Aboriginal communities (total population about 180 in community A and 250 in community B) located in a semi-arid environment more than 1000 km north of Perth. Daily temperatures range from 15°C to 45°C during the summer, falling to freezing at night in winter. Health services in community A have been erratic, with one nurse or Aboriginal health worker present

*Editorial* by Hall and Sibthorpe

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most of the time and a physician visiting every two to four weeks. Community B has two nurses, two doctors, and two Aboriginal health workers. The swimming pools were opened in September 2000 and are open annually from September to April.

### Surveys

We examined children four times at approximately six month intervals between July 2000 and March 2002. All children aged under 17 years present in the community were eligible for inclusion in the study if informed consent was obtained. At each survey a paediatrician examined all enrolled children who could be located, and children who moved into the communities were also given the opportunity to enrol.

We graded pyoderma according to severity (the "sore score"), on the basis of the total number and severity of sores (crusty, pussy, or dry flat lesions).[10] We classified a sore score of 1-4 as non-severe and ≥5 as severe. We also recorded the presence and severity of scabies, abscesses, and fungal lesions. Experienced paediatricians did otoscopic examinations. If otorrhoea was present the ear was irrigated with 1:20 betadine solution to obtain a good view of the tympanic membrane.[11] The paediatricians recorded the colour and position (retracted, bulging, or normal) of the tympanic membrane, as well as the presence of wax, cholesteatoma, perforations, and otorrhoea. They also noted pathology indicative of past disease (for example, healed perforations and tympanosclerosis). During the first survey two ear, nose, and throat specialists examined children with serious ear disease or to confirm a paediatrician's diagnosis.

To identify any pool related injuries or disease outbreaks, the research team examined data held at the local clinic and obtained information from pool managers. Pool managers keep a daily record of any incidents occurring at the pools.

As parents were concerned about truancy, the communities introduced a "no school, no pool" policy as an incentive for children to attend school. In addition to having swimming lessons at school, children attending school are given passes permitting them to use the pool after school. We collected school attendance records for the school term immediately before each of our surveys. School attendance rate was the number of days attended/number of days enrolled.

During the fourth visit to community A, in a house to house survey, we asked family members a series of questions to document their impressions of the pool and any perceived benefits and concerns they may have had regarding the pool. Time did not permit interviews in community B.

### Analysis

We entered all data into Filemaker Pro version 5 and exported them to SPSS version 10.1 for Windows. We used the $\chi^2$ test and Fisher's exact test to compare groups of interest and the $\chi^2$ for trend to investigate trends in prevalence over time. Small numbers precluded more formal statistical analysis.

## Results

In community A we saw 83 children (48% boys) at least once (61 enrolled during the first survey), 51 (61%) twice, 36 (43%) three times, and 12 (14%) four times. During the first survey, 20% were aged under 5 years, 66% were aged 5-11, and 15% were 12-16 years old. In community B, we saw 79 children (56% boys) at least once (60 enrolled during the first survey), 48 (61%) twice, 30 (38%) three times, and 23 (29%) four times. During the first survey, 37% were aged less than 5 years, 42% were aged 5-11, and 22% were 12-16 years old.

### Skin infections

In community A, during the winter before the pool opened (July 2000), 38 (62%) children had pyoderma; 18 (30%) had severe pyoderma. Children aged 12-16 years had significantly less pyoderma (2/9, 22%) than younger children (36/52, 69%, $\chi^2=7.4$, 2df, $P=0.02$). Figure 1 shows a marked reduction in pyoderma during the 18 months of follow up ($\chi^2$ for trend = 24.88, 1df, $P<0.0001$). The result was almost exactly the same for children aged under 12. The prevalence of severe pyoderma also fell significantly over time (in children aged under 12, $\chi^2$ for trend = 8.46, 1df, $P=0.004$). During the first survey, other skin infections included abscesses in 5 (8%) children, fungal infections in 15 (25%) children, and uncomplicated scabies in 3 (5%) children. The prevalences of any skin infection at the four consecutive surveys were 64% (39), 51% (21), 43% (20), and 18% (6) ($\chi^2$ for trend = 17.32, 1df, $P<0.0001$).

In community B, a scabies epidemic was occurring at the time of the first survey (August 2000); 42 (70%) of 60 children had pyoderma, and 29 (48%) had severe pyoderma. Prevalence and severity of pyoderma did not vary with age. The swimming pool was closed for six weeks before the second survey and for a total of three months during that summer owing to a structural fault. Prevalence of pyoderma fell significantly over time ($\chi^2$ for trend = 17.03, 1df, $P<0.0001$, fig 1). A reduction in severity also occurred, with 29 (48%) children having severe pyoderma before the pool opened, 12 (31%) one year later, and none at the fourth survey. The prevalences of any skin infection at the four consecutive surveys were 78% (47), 43% (15), 69% (27), and 20% (9) ($\chi^2$ for trend = 25.16, 1df, $P<0.0001$).

We saw 29 children in community A and 25 children in community B at all of the first three surveys. No significant difference in prevalence or severity of sores existed during successive surveys between children seen three times and those seen less often.

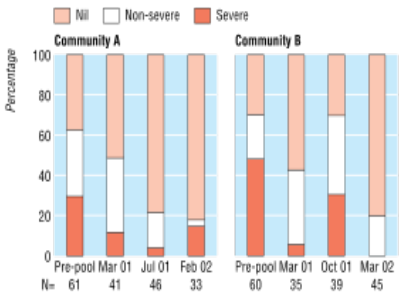


**Fig 1** Proportion of children with severe, non-severe, or no pyoderma during four surveys before and after installation of swimming pools in two communities (N=number of children examined)

### Middle ear disease

Table 1 shows the otoscopic findings during the first survey for those children in whom both tympanic membranes were fully visualised (54 in each community). Only five children in community A and 12 children in community B had no evidence of past or current disease—that is, bilateral intact tympanic membranes, normal position of the tympanic membranes, and no tympanosclerosis or healed perforations. We saw no cholesteatoma throughout the study.

Figure 2 shows the prevalence of perforations of the tympanic membrane and otorrhoea through tympanic membrane perforations during the four surveys. Although no reduction in prevalence of tympanic membrane perforations occurred during the first summer after the pools opened (March 2001), a gradual decline occurred thereafter to 13% (8/44) in community A ($\chi^2$ for trend = 4.32, 1df, P = 0.04) and 18% (4/31) in community B ($\chi^2$ for trend = 2.18, 1df, P = 0.14) in the fourth survey. No significant change in prevalence of otorrhoea through perforated tympanic membranes occurred in either community. Results were similar when we considered all ears that were successfully examined (total of 113, 81, 88, and 62 ears in community A and 112, 66, 76, and 91 ears in community B, during the four surveys): during the 18 months of follow up, tympanic membrane perforation rates declined from 19% (21/113) before the pools were opened to 8% (5/62) ($\chi^2$ for trend = 3.88, 1df, P = 0.05) in community A and from 23% (26/112) to 15% (14/91) ($\chi^2$ for trend = 2.5, 1df, P = 0.11) in community B. Stratification by age made no difference to our findings in either community.

In community A, the prevalence of tympanic membrane perforations declined more rapidly in children seen on each of the first three surveys than in those seen less often. After the second survey, the prevalence of tympanic membrane perforation in children seen regularly was 21% (6/29) compared with 64% (7/11) in those seen less frequently (Fisher's exact test, P = 0.02); equivalent figures during the third survey were 10% (3/29) and 43% (6/14) (P = 0.04). No such differences existed in community B.

### School attendance

In community A, 50, 47, 57, and 61 children were enrolled during the school terms preceding surveys 1, 2, 3, and 4. The proportion of children with attendance rates of at least 70% rose from 42% (21) during the term before the pool opened to 51% (24), 65% (37), and 67% (41) during the terms preceding the second, third, and fourth surveys ($\chi^2$ for trend = 8.70, 1df, P = 0.003). In community B, 79, 60, 58, and 85 children were enrolled during the terms preceding the four consecutive surveys; 52% (41), 40% (24), 33% (19), and 54% (46) of children had attendance rates of at least 70% during the terms preceding consecutive surveys, significantly lower during the terms preceding the second and third surveys than during those preceding surveys 1 and 4 ($\chi^2$ = 8.32, 3df, P = 0.04).

Otoscopic findings during first survey before pools were opened. Values are numbers (percentages)

| | Community A (n=54) | Community B (n=54) |
|---|---|---|
| Bilateral normal tympanic membranes | 5 (9) | 12 (22) |
| Abnormal signs: | | |
| Unilateral or bilateral dry perforation of tympanic membrane | 12 (22) | 8 (15) |
| Otorrhoea through a tympanic membrane perforation with or without contralateral dry perforation | 5 (9) | 9 (17) |
| Bulging tympanic membrane with no contralateral perforation | 2 (4) | 3 (6) |
| Healed perforations or tympanosclerosis with none of above | 5 (9) | 10 (19) |
| Retracted tympanic membrane with none of above | 25 (46) | 12 (22) |
| Total abnormal tympanic membranes | 49 (91) | 42 (78) |

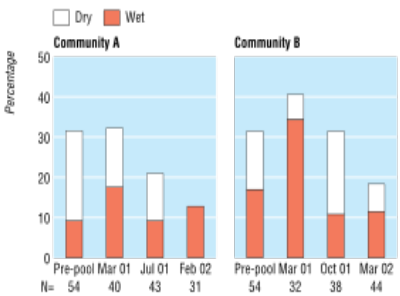


**Fig 2** Prevalence of dry perforations and otorrhoea through a perforation (wet) during four surveys before and after installation of swimming pools in two communities (N=number of children with both tympanic membranes examined)

### Pool related incidents and social impact of pools

No disease outbreaks have been reported, and only two reports of faecal contamination of the pools were made. One pool related trauma (broken arm or mild concussion) occurred in each community.

The 12 extended family groups interviewed in community A during the last survey were almost unanimous in their support of the swimming pool. People generally thought that children looked happier and healthier and were learning to swim. Some comments were: "Fun, enjoy, stop getting bored, health improved," "Keeps kids out of mischief, some place for kids to go, no other pools around, it's clean," and "Not breaking in and being a mischief at home." One person felt that there had been no benefit: "Kids have broken into the pool, still play money hole [gamble] and use gings [slingshots]."

Parents were pleased that their children were encouraged to attend school through the "no school, no pool" policy. We contacted the local police by telephone, and they commented that they had noted a marked decline in petty crime.

## Discussion

We have found significant improvements in health after the introduction of swimming pools in two remote Aboriginal communities. Swimming in a salt water pool provides the equivalent of a nasal and ear washout and cleans the skin. Prevalence of pyoderma declined from over 60% to around 20% during the study period. In the short term this is likely to reduce the clinical workload and prescription of antibiotics; in the longer term it may help to reduce the enormous burden of chronic renal and cardiac diseases in Aboriginal communities.[3-4]

Chronic suppurative otitis media is very uncommon in first world countries, and a prevalence over 4% in children is considered to be a massive public health

problem.[12] During our first survey, 9% of children in community A and 17% in community B had otorrhoea through large perforations of the tympanic membrane (well known to be chronic from a young age in these Aboriginal communities[5 11]). Despite intervention and published guidelines, ear health among Australian Aboriginal children has not improved for over two decades.[5 11 13] In 1986 a tympanic membrane perforation rate of 31% was reported in community B, with 17% otorrhoea (S Weeks, unpublished report, 1986). These rates remained unchanged over 14 years, as reflected in our pre-pool data. Thus the reduction in tympanic membrane perforation rates in our study from 32% to 18% in community B, although not statistically significant, is the first documented successful preventive intervention for otitis media in the past 15 years. This should lead to improvements in language development and literacy.

A limitation of this study was the poor follow up of individual children due to the high mobility of the population. Nevertheless, at each survey, virtually all enrolled children attending school had a clinical examination. Of interest was the more rapid decline in prevalence of tympanic membrane perforations in community A among resident children compared with those who were more mobile. Whether this is because more permanent residents used the pool more or because of differences in social circumstances between the more and less mobile groups is unknown. We did not see a similar finding in community B, which may be owing to closure of the pool for a total of three months during the first summer. Children in community B tend to move between the local school and a boarding school 500 km away, which in part explains the lower attendance rate we found in the middle of the study period.

A further limitation was the lack of a control community. This was neither logistically nor ethically possible: no comparable communities were nearby, and a control community would have been appropriate only if it had been possible to offer them a pool if the intervention proved to be effective. To our knowledge no other activities were taking place during the study period that might have reduced the burden of skin infections and ear disease. However, we cannot exclude the possibility that our four very brief visits to the communities over 18 months may have had an impact on the burden of ear disease through paediatric consultation and training of health and education staff, including dissemination of best practice guidelines. Ongoing monitoring of the impact of these pools is necessary, and funding for this has been secured.

The pools are being used for educational and recreational functions. Virtually all school age children use the pools, thus benefiting from regular exercise and instruction in swimming and resuscitation techniques, which are important to reduce rates of drowning in this population. In the Northern Territory, 93% of drowning in Aboriginal children occurs in open waterways.[14] Drowning rates are higher in Aboriginal than in non-Aboriginal children in Western Australia,[15] but until now supervised swimming lessons have not been available for children in remote Aboriginal communities, which is in contrast to other areas of Western Australia where access to swimming pools and lessons is taken for granted.

### What is already known on this topic

The very high prevalence of otitis media and its long term sequelae can seriously affect quality of life of Aboriginal children and adults

Pyoderma is endemic in many Aboriginal communities and contributes to the high prevalence of chronic renal disease and rheumatic heart disease

Rates of drowning are high in Aboriginal communities

### What this study adds

Introduction of swimming pools in two remote Aboriginal communities was associated with reduced prevalence of pyoderma and tympanic membrane perforations

Swimming pools provide important social capital in disadvantaged communities

The pools have been well managed by the Royal Life Saving Association. Water quality is regularly monitored according to government regulations. To date no outbreaks of infectious disease have occurred, but the ongoing success of the pools will depend on adequate funding to maintain high safety and environmental standards.

In conclusion, the pools have resulted in significant health and social gains. The community's enthusiasm for the intervention and support for the study have been key to the success of the project. Programmes to improve the health of Aboriginal Australians living in isolated communities should certainly focus on better housing, sanitation, nutrition, education, and access to health care,[16] but consideration should also be given to building and maintaining swimming pools. The costs involved will be a small price to pay for the reduction in severe chronic disease and improved health, educational, and social outcomes in this seriously disadvantaged segment of Australian society.

We thank Peter Richmond and John Stuart, who each participated in one survey in which they did all the paediatric examinations; Sharon Weeks for audiological expertise; and Irene Nannup, Jacinta Johnston, Kate Butler, and Claire Gordon, who assisted in the field work. We thank all members of the two communities participating in the study for their support and the teachers and health staff for assistance and hospitality. Kim Hames, previously minister for housing, Aboriginal affairs and water resources, had the vision to install swimming pools in remote communities. Kerryn Sivwright established the databases, and Peta Serna and Norries Pomat assisted in database management. Anne Read and Sandra Eades provided valuable guidance in establishing the project. We thank Marilyn Lyford, Mark Middleton, and Lester Ball from the Royal Life Saving Association and Peter Smith, department of housing and works, for their continuing support and Michael Alpers and Carol Bower for their critical review of the manuscript.

Contributors: FJS and DL initiated the project. DL, MTT, DMcA, and DTS developed the study design. MTT, DTS, DMcA, and DL conducted the field work. HC and FL provided ear, nose, and throat examination, training, and advice. MTT and DL did the data analysis. All authors contributed to writing the paper. DL is the guarantor.

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way). The guarantor accepts full responsibility for the conduct of the study, had access to the data, and controlled the decision to publish.

Competing interests: None declared.

Ethical approval: The ethics committee of Princess Margaret Hospital for Children and the Western Australian Aboriginal health information ethics committee approved the study.


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# Review of research on aboriginal populations in Canada: relevance to their health needs

T Kue Young


## Abstract

**Objective** To determine if research has adequately examined the health needs of the aboriginal population of Canada.

**Design** Review.

**Study selection** Medline search of journal articles published during 1992-2001. The search terms used were "Canada" and various synonyms and categories for Canadian aboriginal people. Each paper was categorised according to the aboriginal group, age-sex group, comparison group, geographic location, and type of research topic (health determinant, health status, or health care).

**Results** Of 352 citations found, 254 were selected after elimination of those without abstracts, not containing data on Canada, or not focusing on health issues. The proportion of papers does not reflect the demographic composition of aboriginal people in Canada, with severe under-representation of Métis, urban aboriginal people, and First Nations people not living on reserves and over-representation of the Inuit. Children and women received less attention proportional to their share of the population. A few prolific research groups have generated a disproportionate amount of publications from a few communities and regions. 174 papers dealt with health determinants (for example, genetics, diet, and contaminants), 173 with health status, and 75 with health care. Injuries, which account for a third of all deaths, were studied in only 8 papers. None of the health care papers examined rehabilitation.

**Conclusion** Researchers have not adequately examined several important health needs of the aboriginal population.


## Introduction

Despite tremendous progress, the health of the aboriginal population of Canada continues to lag behind that of the national population.[1] Health research may hold the key to why such disparities exist and to solutions for eliminating them. In 1990 the Commission on Health Research for Development recognised the close links between health research and equity in development and promoted the concept of "essential national health research."[2] Since then the "10/90 gap" (less than 10% of global spending on health research is devoted to diseases that account for 90% of the global disease burden) has become recognised.[3] The Global Forum for Health Research advocates setting priorities on health research on the basis of analyses of disease burden, health determinants, current knowledge, cost effectiveness, and available resources.

This paper aims to review health research conducted among Canadian aboriginal people and to determine if research has examined the health needs of the population. While recent international studies have used a common "currency" (such as disability adjusted life years) to measure disease burden and impact,[4] which would facilitate considerably the priority setting process, such an approach cannot yet be used for the Canadian aboriginal population.

According to the 2001 Canadian census, just under one million (3.3%) Canadians identified themselves as

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*Sixty six of the 254 references appear on bmj.com as examples of the articles reviewed*

OTITIS MEDIA 2009: AN UPDATE

# Natural history, definitions, risk factors and burden of otitis media

Kelvin Kong and Harvey L C Coates

In this review, we describe the various forms of otitis media seen in Australia in the two diverse populations of Indigenous and non-Indigenous people, the natural history in these two populations, and the risk factors for otitis media. In addition, we address the social and cost burden of otitis media and its complications in Australia. We have reviewed the major literature on otitis media, especially in the Australian context, to support this discussion.

## ABSTRACT

- Otitis media remains a major health problem in Australia, with an unacceptably great dichotomy of incidence and severity of otitis media and its complications between Indigenous and non-Indigenous Australians.
- Among most children with acute otitis media, infection resolves rapidly with or without antibiotics, with ongoing middle ear effusion the only sequela.
- Overcrowding, poor living conditions, exposure to cigarette smoke, and lack of access to medical care are all major risk factors for otitis media.
- Estimates of the number of cases of otitis media in 2008 vary between 992 000 and 2 430 000 Australians, with a total estimated cost of $100–$400 million.

MJA 2009; 191: S39–S43

## Definitions

The terminology used to describe otitis media and its sequelae has changed over the years. Current terminology is consistent with present understanding of the pathogenesis and pathology of the disease.

Acute otitis media (AOM) without perforation involves acute suppurative inflammation with local and systemic symptoms and signs, including otalgia, otorrhoea, fever, irritability, anorexia, vomiting or diarrhoea. The tympanic membrane bulges and is usually opaque, and pneumatic otoscopy reveals little mobility. AOM with perforation refers either to AOM that has perforated the tympanic membrane and caused discharge or otorrhoea from the middle ear within 7 days of infection, or to the presence of discharge through a tympanostomy tube (also known as a ventilation tube or "grommet") on an acute basis.

Recurrent AOM (RAOM) refers to separate bouts of AOM — three episodes in 6 months, or four to five in 12 months. There are two distinct types of RAOM: that accompanying an upper respiratory tract infection, with total resolution of middle ear fluid between attacks; or recurrent infection with an underlying persistent otitis media with effusion (OME).

OME is the presence of middle ear fluid without symptoms or signs of suppurative infection. It supersedes previous terms, such as serous otitis media, as the effusion may not be serous, and an asymptomatic middle ear effusion can contain bacteria. OME has myriad appearances, including air fluid levels, bubbles or translucent tympanic membrane, but pneumatic otoscopy almost always shows a retracted tympanic membrane with impaired mobility. Despite the lack of acute suppuration, a child with OME may still have discomfort, pressure, shooting pains in the ear, or balance disorder — OME is the commonest cause of balance disorder in childhood.

Chronic suppurative otitis media (CSOM) is defined as a persistent discharge from the middle ear through a tympanic membrane perforation for more than 6 weeks. CSOM may include a chronic perforation with or without acute or chronic otorrhoea.

These definitions are summarised in Box 1.

**1 Otitis media definitions**

**Acute otitis media (AOM) without perforation:** presence of middle ear fluid with symptoms or signs of suppurative infection, which may include otalgia, fever, irritability, vomiting or diarrhoea.

**AOM with perforation:** acute suppurative infection with recent discharge from the middle ear or through a tympanostomy tube (within the past 7 days).

**Recurrent AOM (RAOM):** recurrent bouts of AOM — three episodes in 6 months or four to five in 12 months.

**Otitis media with effusion (OME):** presence of middle ear fluid without symptoms or signs of suppurative infection.

**Chronic suppurative otitis media (CSOM):** a persistent discharge from the middle ear through a tympanic membrane perforation for more than 6 weeks. CSOM may include a chronic perforation with or without acute or chronic otorrhoea. ♦

## Natural history

Otitis media is one of the commonest childhood illnesses, affecting 80% of Australian children overall by the age of 3 years.[1] The proportion of Indigenous children suffering otitis media and its complications is disproportionately high; up to 73% by the age of 12 months.[2] Non-Indigenous children may be treated expectantly and without antibiotics and 80% will be symptom-free in 3 days (although middle ear effusion may remain), whereas Indigenous children may suffer RAOM that leads to chronic otorrhoea and CSOM. This dichotomy necessitates different perspectives on the aetiology, pathogenesis and management in the two groups.[3]

Among non-Indigenous children, the general improvement in socioeconomic conditions and the advent of routine antibiotic therapy for otitis media have led to a decrease in the more serious extracranial and intracranial complications that were seen in the pre-antibiotic era.[4] Despite a recrudescence in the incidence of otitis media in the past three decades, related to the increasing use of daycare centres for infants and children, there has been a general tendency in the past 10 years to reduce immediate antibiotic treatment for AOM for children aged over 2 years, exercising a "wait and see" policy.[5,6] Among children aged under 2 years, antibiotic therapy results in more rapid resolution of clinical signs (bulging, erythematous tympanic membrane) and

symptoms (fever, otalgia, irritability), although even this guideline is being questioned.[7]

In Australia, surgical management (insertion of grommets or ventilation tubes) for RAOM and OME is the second most common reason for admission to hospital for a procedure in childhood, with 30 126 such procedures in the 2006–07 financial year.[8] However, among non-Indigenous children, resolution of AOM (with or without antibiotic treatment) is the most common outcome, with the prevalence of middle ear effusion declining from 65% at 2 weeks after onset of infection to 25% at 3 months after onset.[9]

Indigenous children in both remote and urban settings tend to suffer otitis media earlier, more frequently and severely, and with more serious complications than non-Indigenous children.[2,10] In developed countries, Australian Aboriginal and Torres Strait Islander children remain, arguably, the most vulnerable in terms of otitis media prevalence and severity.[11,12] There has been no decrease in otitis media complications in this population, in which infective complications — such as CSOM — occur in up to 30% of children in some communities.[13] The end result of RAOM, OME and CSOM, when not treated adequately, is significant conductive hearing loss with a resultant speech and language delay, especially where English may be a child's second or third language. This results in educational problems, social isolation, truancy and ultimately early school-leaving and difficulties gaining employment. Although hearing aids and school sound field systems (where an infrared signal transmits the teacher's amplified voice through loudspeakers) are theoretically suitable approaches to this problem, in reality, they are of limited help to infants and young children with draining ears. Indigenous children suffer a mean of 32 months of hearing loss in childhood, compared with 3 months among non-Indigenous children. Among Indigenous children, symptoms of otitis media include otalgia, ear rubbing, fever, excessive crying, restless sleep and poor appetite. Traditionally, management regimens have tended to centre on symptoms and symptom relief. This is compounded by the poor access to health care and socioeconomic standards experienced by many Aboriginal and Torres Strait Islander populations, and consequently poses a significant impact and burden of disease, not just for this population but for the Australian population in general.

Why is there such variation in the incidence, onset, severity and complication rates of otitis media between these two populations? Although much of this will be covered in other articles in this supplement, in brief, early invasion of the nasopharynx by encapsulated bacteria overwhelms the immature immune system, leading to a failure to elicit an adequate antibody response. Nasopharyngeal carriage, sustained by biofilm formation and intracellular bacterial colonisation, leads to chronic adenoiditis, rhinitis and middle ear infection via the eustachian tube.[14-16] Social conditions, such as poverty, overcrowding, poor nutrition, exposure to cigarette smoke and lack of running water, all have a role to play in perpetuating the milieu conducive to ongoing carriage of pathogenic bacteria throughout the upper respiratory tract.[17] These socioeconomic issues must be addressed, but innovative preventive treatments to diminish bacterial carriage also need to be implemented.

## Risk factors for otitis media

Otitis media is, in essence, an infectious disease with bacterial and viral infection in an environment where the host immune response counters infection. The main factors that influence the risk of otitis media developing can be host-related factors or environmental factors (summarised in Box 2).These factors primarily interact in the nasopharynx and eustachian tube. Two systematic reviews of risk factors for AOM and RAOM noted that daycare centre attendance and dummy (pacifier) use were modifiable factors, whereas breastfeeding, presence of siblings, passive smoking, craniofacial abnormalities and the presence of adenoids were probably risk factors.[19,20]

### Host-related factors

Several host-related factors for otitis media have been noted.

- Incidence of otitis media peaks at two age groups: between the ages of 6 and 24 months, when infants are weaned and exposed to environmental conditions, and at age 4–5 years, when children enter kindergarten. Among Indigenous children, infection occurs as early as 2 months of age.[21]
- Otitis media, in particular middle ear effusion, has a higher incidence among boys; reasons for this are unknown.[1]
- In Australia, Indigenous status significantly increases the risk of middle ear disease in urban, rural and remote locations. This is also the case for other ethnic groups, including Inuit, Native American and Maori children.[22]
- Individual factors such as age, sex and ethnicity may be secondary to eustachian tube size differences.[23]
- Premature birth has been variously reported as either having no effect or increasing the risk of otitis media.[24]
- The influence of allergy as a risk factor for otitis media is contentious. An association is noted, with supportive epidemiological and therapeutic evidence in some studies, whereas other studies show no association.[25,26]
- Immune deficiencies associated with RAOM may include deficiencies in secretory IgA, which is implicated in bacterial and viral adherence and has been shown to reduce bacterial colonisation of the nasopharynx. RAOM that is non-responsive to conventional medical and surgical therapy may reveal low serum IgG2 levels, poor response to *Haemophilus influenzae* polysaccharide–protein conjugate vaccination and low levels of specific IgG antibodies against pneumococcal capsular polysaccharides 6A and 19F.[27]
- Genetic factors may play a part in susceptibility to otitis media. A Norwegian study of 2750 twin pairs concluded that heritability of otitis media susceptibility was 74% among girls and 45% among boys.[28] The *HLA-A2* gene has been noted to be associated with RAOM but not OME.[29]
- OME is over-represented among children with Down syndrome and unrepaired cleft palate and craniofacial disorders. The high incidence of disease is related to the dysfunction of the eustachian tube together with lack of functional resistance to aspiration of nasopharyngeal content.[30]
- The current weight of expert opinion is that the presence of infected adenoids or tissue, with biofilm and intracellular bacteria identified in the tissue, is a greater contributor to AOM and OME than the physical bulk or size of the adenoids. A large Western Australian study of 50 000 children who underwent ventilation tube insertion showed a 50% reduction in subsequent grommet insertion following adenoidectomy.[31]
- Despite studies showing pepsinogen carried by gastro-oesophageal reflux into the middle ear fluid in OME and grommet otorrhoea, further definitive studies are required.[32,33]

**2 Risk factors for otitis media**

| Risk factor | Comment | NHMRC level of evidence*[18] |
|---|---|---|
| **Host-related** | | |
| Age | Highest incidence between 6 and 11 months | A |
| Sex | Slightly higher preponderance among males | C |
| Ethnicity | Indigenous children are at increased risk of earlier and more severe disease | A |
| Premature birth | Increased risk | C |
| Allergy | Link noted, but pathways unclear | D |
| Immunosuppression | Subtle immune deficiencies often noted in recurrent acute otitis media | A |
| Genetic predisposition | Familial clustering noted | A |
| Craniofacial abnormalities | Increased incidence in children with cleft palate, Down syndrome and craniofacial anomalies | C |
| Adenoids | Infected adenoids or tissue increases risk more than size of adenoids | C |
| Gastro-oesophageal reflux | Link noted, but further study required | D |
| **Environmental** | | |
| Daycare or overcrowding | Higher incidence with daycare attendance | B |
| Siblings | Increased risk with older siblings | B |
| Upper respiratory tract infection | Viruses predispose to otitis media | B |
| Seasonality | Increased incidence in winter months | D |
| Cigarette smoke exposure | Increased risk | B |
| Breastfeeding | Has a protective effect | C |
| Socioeconomic status | Variable but generally increased risk with lower status | C |
| Dummy (pacifier) use | Increased risk after age 11 months | B |

NHMRC = National Health and Medical Research Council.
* NHMRC levels of evidence: A = body of evidence can be trusted to guide practice. B = body of evidence can be trusted to guide practice in most situations. C = body of evidence provides some support for recommendation(s) but care should be taken in its application. D = body of evidence is weak and recommendation must be applied with caution. ♦

## Environmental factors

There is much epidemiological evidence indicating that otitis media and middle ear effusions have a higher incidence in winter and lower incidence in summer in both hemispheres.

By far the largest environmental factor for the "mini-epidemic" of otitis media and OME in the past three decades has been the increase in daycare attendance by children; one in five children in full-time daycare may have ventilation tubes in situ.[34] This is directly related to the number of children in each daycare centre. There is a threefold increased risk of upper respiratory tract infection with daycare attendance.[34] The same factors hold for overcrowding in homes and families with a number of older siblings.[26]

Upper respiratory tract infections occur most frequently in the winter months, and respiratory viruses can be isolated from the middle ear fluid in 19% of children with AOM; these may include respiratory syncytial virus, rhinovirus, adenovirus and coronavirus.[35]

Passive cigarette smoking increases the risk of otitis media among Aboriginal and non-Aboriginal children. In the Kalgoorlie Otitis Media Research Project, 64% of Aboriginal children and 40% of non-Aboriginal children were exposed to environmental tobacco smoke.[17] A meta-analysis demonstrated a significant increase in risk (66%) of RAOM and chronic OME in the presence of parental smoking (risk ratio [RR], 1.66; 95% CI, 1.33–2.06).[20]

Breastfeeding has a protective effect on the development of middle ear disease related to the immunological properties of breast milk; a meta-analysis showed that breastfeeding for at least 3 months reduced the risk of AOM by 13% (RR, 0.87; 95% CI, 0.79–0.95).[20]

Low socioeconomic status with limited access to health care may be a factor affecting the association with otitis media. There is great variability, however, in the association between otitis media and low socioeconomic status.

The use of a dummy after the age of 11 months is significantly associated with otitis media. In a meta-analysis, the use of a dummy increased the risk of AOM by 24%.[20]

## Modifying risk factors

The authors of a 2-year population-based study of 2512 children, 825 of whom experienced otitis media, estimated that one in five children could have avoided otitis media, and two in five affected could have avoided RAOM, if they had been moved from full-time daycare to home care.[36] They concluded that about 14% of all episodes of otitis media could have been avoided if all of these children had been cared for at home. The other modifiable risk factors of significance are cessation of dummy use after the age of 11 months and reduction in passive cigarette exposure.

## The burden of disease in Australia

Otitis media is a common and debilitating condition that affects up to 73% of children by the age of 12 months. In Australia, the difficulty in establishing the incidence and prevalence rates of

otitis media in children is due to definitional and diagnostic differences and uncertainty. An estimate of the number of new cases of otitis media in 2008 among all Australians suggested that between 658 006 and 1 615 486 Australians were affected, with 12.8% of these accounted for by Indigenous children.[37]

Otitis media is one of the most common conditions diagnosed in the primary health care setting, is one of the most common diagnoses resulting in antibiotic prescription for children, and is a major contributor to childhood morbidity. Few children escape experiencing a form of otitis media before they reach school.

Of Australian children aged 14 years and under experiencing otitis media each year, 15 will suffer permanent hearing loss and over 250 000 will experience temporary hearing loss. Further, 60 263 will experience CSOM, of whom 217 will have intracranial complications and 237 will have mastoiditis. A cross-sectional survey of remote communities in 2001 showed a prevalence of 16.5% for CSOM.[2] In 2008, treatment costs (mainly general practitioner visits and prescriptions) accounted for between $100 million and $400 million. The estimated net cost of lost wellbeing due to otitis media in 2008 varied between $1.05 billion and $2.6 billion.[37]

The estimated number of Indigenous Australians with otitis media in 2008 was between 98 097 and 240 841; 12.8% of these cases occurred before the age of 14 years (47 000 children).[37] Aboriginal and Torres Strait Islander children are more likely to experience recurrent disease that sets in at a much earlier age than non-Indigenous children.

Aboriginal and Torres Strait Islander children are at particular risk, enduring living standards equivalent to those in developing nations. In Australia, where non-Indigenous populations generally experience high standards of living and health care, Aboriginal and Torres Strait Islander children continue to suffer an unacceptable incidence of ear disease. This inequality has a significant impact on growth, development and education, and the negative social implications remain difficult to measure.

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## Competing interests

Harvey Coates has been a consultant for GlaxoSmithKline and Alcon Laboratories.

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Research Article

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EXPERT REVIEWS

# Cost of treating otitis media in Australia

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**Objective:** Otitis media (OM) is an inflammation of the middle ear. It is very common and associated with serious complications, including hearing loss. This study aimed to estimate the treatment costs of OM in Australia and the associated burden of disease (in disability-adjusted life-years). **Methods:** Little Australia-wide epidemiological information is available, so international studies in the main were used to estimate the incidence and prevalence by age and gender. These were triangulated against the available Australian data. Australian health data sets and data collected from the emergency department of a tertiary pediatric hospital were used to estimate the costs of primary care, pharmaceuticals, pathology and imaging, emergency department presentations, specialists, and admitted hospital care. **Results:** Excluding the costs of the complications and comorbidities associated with OM, treatment costs for the disease in 2008 were between AUS$100 and 400 million. Visits to general practitioners and medicines constituted a high proportion of these costs. Antibiotic prescribing rates remain high despite clear evidence for a limited benefit from antibiotics for most OM cases and concerns regarding bacterial resistance. **Conclusion:** Treatment costs of OM in Australia are high and can only be estimated within a broad range. Further research on the links between antibiotics for OM and antibiotic resistance, and on the cost–effectiveness of prevention or amelioration of OM would be useful.

**Keywords:** antibiotic prescribing • Australia • disease costing • incidence • otitis media • prevalence • treatment cost

Otitis media (OM) is an inflammation of the middle ear and is one of the most common afflictions of childhood. Acute OM (AOM) can cause severe pain. Types of OM are given in Box 1.

Complications of OM and associated illnesses include otorrhea, hearing loss (and potential consequent language and developmental delay in children), tympanic membrane perforation, chronic suppurative OM and mastoiditis. Some complications, such as brain abscess and meningitis, are potentially fatal.

Otitis media often resolves without medical intervention. There is no universally agreed treatment algorithm for OM in Australia. Drawing on evidence from the Office for Aboriginal and Torres Strait Islander Health [1], Gunasekera *et al.* [2] and Kadhim *et al.* [3], for AOM, those at low risk (non-indigenous children aged 12 months or older) with uncomplicated illness may at first be observed. Treatment guidelines differ on the length of time that observation is safe. If there is no improvement after a 'safe' period, antibiotics are recommended. However, antibiotic use is contentious in the management of OM [4,5], particularly given bacterial resistance. Treatment was once the routine use of antibiotics, but treatment guidelines in various countries have tempered recommendations for antibiotic use in light of evidence of their limited benefit for most OM and concerns regarding bacterial resistance [4,5]. Australian guidelines also differ on management of OM with effusion. The general approach is watchful waiting for 3 months for children not at risk. For children at risk or those with persistent OM with effusion, hearing, speech and language checks are necessary. Tympanostomy tubes may be inserted depending on factors including hearing status, developmental risk and/or structural damage to the tympanic membrane or middle ear. For indigenous children especially (although this also applies more generally), the availability of supportive pre- and post-operative care is crucial. Further surgery may include adenoidectomy or adenotonsillectomy [3]. Two pneumoccocal vaccines are currently used in populations in Australia that may prevent a proportion of OM cases (7 valent pneumococcal conjugate vaccine and 23 valent pneumococcal polysaccharide vaccine) [101].

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**Box 1. Types of otitis media.**

Terminology and definitions for OM are not universally agreed upon, but types of OM include:

- AOM, characterized by rapid onset and short duration of signs and symptoms that may be local and/or systemic, such as otalgia, irritability, fever, vomiting or diarrhea
- OM with effusion, which occurs when a blockage in the Eustachian tube leads to negative pressure in the middle ear and a transudate of fluid drawn from middle ear mucosa. OM with effusion may follow AOM or occur without prior AOM. OM with effusion is often longer lasting and asymptomatic
- Chronic suppurative OM is a chronic inflammation of the middle ear cleft. In the literature, chronic suppurative OM is sometimes referred to as chronic OM (active and inactive), suppurative OM or chronic perforation. Chronic suppurative OM is a sequel of AOM or unresolved OM [30], particularly in children with poor socioeconomic circumstances, and usually presents within the first 5 years of life [31]

AOM: Acute otitis media; OM: Otitis media.

The main aim of this study was to derive treatment costs for OM in Australia and to estimate the associated burden of disease. Little is known of the costs of OM in Australia despite epidemiological evidence among small communities of high incidence and prevalence rates, consistent with findings from a substantial number of studies internationally. Such information is a necessary prerequisite for determining the cost–effectiveness of health interventions for the disease, and for government decisions regarding prioritizing research and funding of prevention and treatment, including in disadvantaged indigenous communities where studies have found high prevalence rates. Furthermore, information on antibiotic prescribing feeds into a broader discussion of the net benefits of interventions to address antibiotic resistance.

## Methods

### *Prevalence of otitis media episodes*

Incidence and prevalence rates for Australia were derived from a combination of Australian and international epidemiological research and population surveys. In Australia, while there are a number of useful studies of indigenous children (e.g,. Morris *et al.* [6]), there is a paucity of epidemiological research among the broader community.

Two types of estimates of the number of episodes of OM occurring were derived for the purposes of the analysis. Annual prevalence rates by age and gender (i.e., the number of episodes per year, noting that the same individual might experience more than one episode of illness in any given year) were applied to a projection of the 2008 Australian population by single year of age and gender. (Population projections were created based on Australian Bureau of Statistics [ABS] information on fertility, mortality and life expectancy). Given the diagnostic uncertainty associated with OM and the uncertainty resulting from the large range of estimates of international incidence and prevalence rates available (see 'Expert commentary' section), upper and lower bounds were calculated for annual prevalence (the upper- and lower-bound estimates of annual prevalence for 2008 are in given in Table 1). For the lower bound, annual prevalence rates for 2003 from Begg *et al.* were used [7]. These draw on data from the Bettering the Evaluation and Care of Health (BEACH) program (described later in the 'Health service utilization' section) and the ABS National Health Survey, and are adjusted for higher prevalence among indigenous communities. To update the Begg *et al.* rates to 2008 [7], a downward trend was applied, reflecting BEACH data that suggest a 4% per year fall in AOM cases seen by general practitioners (GPs) between 2002–2003 and 2006–2007 [8]. While this may reflect a decrease in the proportion of children aged less than 15 years at encounter in the BEACH data, it is consistent with the fall in hospital separation rates with a principal diagnosis of OM during the period 1998–1999 and 2006–2007 [102]. No adjustments were made for diagnostic uncertainty because the extent to which diagnosis is inaccurate is not clear – particularly in Australia; given that the BEACH data are not stratified by patient characteristics, adjustments would not necessarily improve the accuracy of the estimates here. The

**Table 1. Prevalence of otitis media (episodes) in 2008.**

| Gender | Age (years) | | | | | Total episodes |
|---|---|---|---|---|---|---|
| | *0–14* | *15–24* | *25–64* | *65–74* | *75+* | |
| *Lower-bound estimate* | | | | | | |
| Males | 378,280 | 50,983 | 91,799 | 2970 | 4764 | 528,795 |
| Females | 279,725 | 45,630 | 117,043 | 14,076 | 6932 | 463,406 |
| People | 658,006 | 96,613 | 208,842 | 17,046 | 11,695 | 992,202 |
| *Upper-bound estimate* | | | | | | |
| Males | 928,725 | 125,169 | 225,378 | 7292 | 11,695 | 1,298,259 |
| Females | 686,761 | 112,027 | 287,355 | 34,560 | 17,018 | 1,137,720 |
| People | 1,615,486 | 237,196 | 512,733 | 41,851 | 28,713 | 2,435,979 |

Data from [8,11,16] and population projections using Australian Bureau of Statistics information on fertility, mortality and life expectancy by single year of age.

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upper-bound estimates are based on the highest rates cited in the set of studies described by Casselbrant and Mandel [9] and Pirozzo and Del Mar [10].

In order to estimate the treatment costs that might be avoided if a targeted vaccine were introduced at the beginning of 2008 that prevented OM, the number of episodes of OM occurring in the cohort born in 2008 and for the first 5 years of life to the year 2012 was estimated. As noted previously, population projections were created based on ABS information on fertility, mortality and life expectancy. ABS age/gender mortality rates were used to project the size of the 2008 birth cohort annually to 2012. The proportion of children experiencing OM by single year of age was multiplied by the average number of episodes per child per year to derive the number of episodes of OM experienced by children in the cohort annually (Table 2). Both parameters were drawn from Butler *et al.* [11], who derived them from Teele *et al.* [12].

### Health service utilization

Management of OM by GPs (including prescription and over-the-counter medications, clinical and procedural treatments, pathology, imaging and referrals) was based on BEACH data, a continuous national study of general practice activity, supplied by the Australian GP Statistics and Classification Centre, University of Sydney. BEACH data were specially requested for this study and reflected encounters (i.e., any professional interchanges between a patient and a GP) at which AOM, serous OM or chronic OM (the relevant International Classification of Primary Care version 2 codes are H71, H72 and H74) were registered as a problem for children aged 0–14 years and covered the period from April 2000 to March 2007.

There are no national Australian data sets summarizing the illnesses or demographic characteristics of people who present to hospital emergency departments (EDs). ED data were therefore drawn from the Royal Children's Hospital (RCH), Melbourne, Australia, a tertiary pediatric hospital. Data were for the period from 1 June 2007 to 31 of May 2008, for presentations to the ED leaving with a diagnosis of OM/ear infection, discharging/otorrhea, AOM with perforation or glue ear. While attendances to the RCH Melbourne ED include children living locally in the surrounding area, other patients may be referred and therefore have more severe illness than might be expected at a nontertiary ED. In addition, only one diagnosis is recorded at RCH Melbourne, so these estimates exclude patients who may have had OM as a comorbid condition.

Admitted patient data were from the Australian Institute of Health and Welfare (AIHW) National Hospital Morbidity database, which has in-patient data from a census of Australian hospitals. The International Classification of Diseases version 10 (ICD-10) codes used were H65 and H66 [102].

### Estimates of treatment costs

Two methods were used to calculate treatment costs, reflecting the different sources available in Australia for estimating the treatment costs of disease.

The first AIHW method simply used AIHW estimates of direct health system costs by disease for 2000–2001 [13] – a special request for OM. The AIHW advises that expenditure able to be allocated by disease included only 87.5% of total recurrent health expenditures [13]. The AIHW estimates were therefore factored up to incorporate the 'unallocated' remainder (mainly capital expenditures). The estimates were then inflated to 2008 prices using Australian health inflation estimates from recent history [14].

The alternative 'bottom-up' method used the health service utilization rates from the BEACH, RCH and National Hospital Morbidity Database data, outlined earlier, and applied unit costs to these rates from the following data sets: the Medicare Benefits Schedule 1 November 2007, the Australian Ambulatory Classification codes for ED visits for ear, nose and throat [103], the Australian Department of Health and Ageing National Hospital Cost Data Collection, which surveys a sample of hospitals (the most recent estimates available were inflated to 2007–2008 using AIHW [14]), and the Pharmaceutical Benefits Schedule 1 November 2007 [15], with patient copayments applied as per Department of Health and Ageing guidelines [103]. The method used to calculate the bottom-up treatment costs is illustrated by example in Box 2.

#### *Discount rate*

The discount rate used to calculate net present values was 3%, based on recent history for long-term nominal bond rates [104] and the Reserve Bank mandate for monetary policy (target: 2–3% inflation).

**Box 2. Example of the method of calculating the cost of visits to general practitioners.**

The total cost of GP visits was calculated by adding together the costs of short, standard, long and prolonged surgery consultations, and other types of GP visits (e.g., home visits) in which OM was the reason for encounter. In more detail, to calculate the cost of long surgery consultations, the following were multiplied together:

- The number of GP encounters in which OM was diagnosed as a 'new' problem from BEACH data. BEACH defines a new problem as the first presentation of OM, including the first presentation of a recurrence of a previously resolved problem but excluding the presentation of a problem first assessed by another provider
- The proportion of GP encounters for OM-related diagnoses that involved a long surgery consultation from BEACH data (1.8%)
- The average number of GP visits per episode, derived from BEACH data (1.5 visits)
- The MBS fee 1 November 2007 for a long consultation (MBS item number 54)

AOM: Acute otitis media; BEACH: Bettering the Evaluation and Care of Health; GP: General practitioner; MBS: Medicare Benefits Schedule; OM: Otitis media.

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**Table 2. Cohort of children born 2008: number of children experiencing otitis media and number of episodes.**

| Year | Age of children (years) | Children with at least one episode of OM (%) | OM episodes per person per year | Children with OM (n) | Episodes of OM (n) |
|---|---|---|---|---|---|
| 2008 | 0 | 63 | 1.98 | 169,387 | 335,387 |
| 2009 | 1 | 59 | 1.80 | 158,302 | 284,944 |
| 2010 | 2 | 41 | 1.76 | 109,955 | 193,520 |
| 2011 | 3 | 43 | 1.86 | 115,295 | 214,449 |
| 2012 | 4 | 43 | 1.74 | 115,280 | 200,587 |

OM: Otitis media.
Data from [11] and population projections using Australian Bureau of Statistics information on fertility, mortality and life expectancy by single year of age.

### *Burden of disease*

Burden of disease was measured in disability-adjusted life-years (DALYs), with the same approach for OM as that used by Australian national burden of disease studies (Begg *et al.* [7] and Mathers *et al.*[16]). The disability weight for AOM is 0.09 with a duration of illness of 1 week [16]. The disability weight for chronic suppurative OM is 0.11 (representing mild-to-moderate hearing loss) [16] with a duration of 6.8 weeks [17]. The disability weight for permanent hearing loss from AOM is 0.233, with five in 100,000 cases of AOM in 0–14-year-olds resulting in permanent hearing loss [16].

## Results

Approximately 73% of children aged up to 12 months will have at least one episode of OM (~195,000 Australian infants) in 2008. Nearly all children will have experienced at least one episode of OM by the time they are 3 years old (Table 3). In the first 5 years of life, the cohort of children born in 2008 is likely to experience more than 1.2 million episodes of OM (Table 2).

In 2008, 659,495 Australians (~3% of the total population) will experience between 1 and 2.4 million episodes of OM. Rates of OM are much higher among young people (~8.9% of young people aged 0–14 years will experience a case of OM compared with 3.1% of the population overall). Indigenous children experience a disproportionate share of OM – 12.8% of episodes among children aged 0–14 years (Table 4). By comparison, in 2006, close to 5% of the total Australian population aged 0–14 years was indigenous [18].

Between April 2000 and March 2007, AOM accounted for approximately 7% of all problems managed by Australian GPs in children aged 0–14 years [19]. GPs prescribed antibiotics for diagnosed AOM in 82.3 out of every 100 OM problems they diagnosed and managed during the period 2000–2007. In particular, antibiotics prescribed by GPs for OM in children aged 0–14 years comprised 10% of all medications (antibiotics and other medicines) prescribed by GPs for children of that age. The most common medications (as a share of total medications for children with OM) were amoxycillin (40.8%), cefaclor monohydrate (15.6%), amoxycillin/potassium clavulanate (11.3%) and paracetamol (10.5%). GP clinical treatments were predominantly provision of advice and education (85%), followed by reassurance and support (5.4%) and observe/wait (2.9%). The RCH Melbourne data suggested that OM accounted for 2.9% of ED presentations.

Separation rates with a principal diagnosis of OM fell from 1.8 per 1000 Australians in 1998–1999 to 1.3 per 1000 in 2006–2007. Among children aged 0–14 years, separation rates fell over the same period from 7.7 to 5.4 per 1000 children in that age group. Myringotomy with tube insertion was the most frequent procedure for OM undertaken in Australian hospitals in 2006–2007 (Table 5).

Based on the AIHW method, the discounted net present value (NPV) of the cost for the 2008 birth cohort for the first 5 years of life is between AUS$182.8 and 448.7 million (Table 6). The cost of OM occurring in 2008 is AUS$391.6 million, of which OM among Indigenous Australians in 2008

**Table 3. Lifetime incidence of acute otitis media and middle-ear effusion.**

| Age (years) | Cumulative incidence of AOM from epidemiological studies (%) | | | Lifetime incidence of AOM* (%) | Cumulative incidence of MEE from epidemiological studies (%) | | | Lifetime incidence of MEE* (%) |
|---|---|---|---|---|---|---|---|---|
| | *Lower* | *Upper* | *Midpoint* | | *Lower* | *Upper* | *Midpoint* | |
| <1 | 42 | 62 | 52 | 52 | 49 | 96 | 73 | 73 |
| 1–2 | 50 | 84 | 67 | 9 | 61 | 98 | 80 | 13 |
| 2–3 | | | 66 | 5 | | | 99 | 13 |

*Lifetime incidence is the first episode of otitis media experienced in the child's lifetime. For example, 52% of children experience their first episode of otitis media aged less than 1 year, and a further 9% experience their first episode of otitis media between the age of 1 year and just under 2 years. The cumulative incidence of the presence of MEE is used as an indicator of the cumulative incidence of all otitis media.
AOM: Acute otitis media; MEE: Middle-ear effusion.
Data from [9,10].

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cost approximately AUS$38.7 million (Table 7). Notably, the cost estimates for Indigenous Australians are based on the same average cost per episode as for all Australians. While this was the only available approach for this project, using the Australian average unit cost is unlikely to represent the true cost of treating OM in remote indigenous communities, given the higher unit cost of health-service provision in remote areas, health-service access issues and differences in treatment for Indigenous Australian children.

Using the bottom-up approach, the discounted NPV of the cost of episodes of OM experienced for the first 5 years of life for the 2008 birth cohort was AUS$75.4 million (Table 8). The cost of episodes of OM occurring in 2008 (Table 9):

- For the lower-bound prevalence estimate, was AUS$85.6 million (~AUS$8.5 million on Indigenous Australians);
- For the upper bound prevalence estimate, was AUS$163.2 million (~AUS$16.2 million on Indigenous Australians).

Visits to GPs and medicines contributed most to the total cost of treating OM (Tables 8 & 9).

For the 2008 birth cohort, during the first 5 years of life, the burden of disease is estimated to be approximately 5438 DALYs. For all Australians experiencing OM in 2008, the burden of the lower-bound prevalence estimate is 3974 DALYs (887 DALYs among Indigenous Australians) and of the upper-bound prevalence estimate is 9758 DALYs (2178 DALYs among Indigenous Australians).

**Table 4. Approximate number of otitis media episodes among Indigenous Australians in 2008.**

| Age (years) | Low | High | Indigenous as a percentage of all episodes |
|---|---|---|---|
| 0–14 | 84,477 | 207,402 | 12.8 |
| All | 98,097 | 240,841 | 9.9 |

## Expert commentary

This is the first study of the costs of OM in Australia. International estimates have tended to only focus on children. Capra *et al.* estimated the direct and indirect (lost work time) costs of OM among children under 14 years of age in a Californian, managed-care population between August and November 1997 [20]. The total costs of treatment among a sample of 119,970 children were US$8.02 million, of which 78% were outpatient visits, 14% hospitalizations and 8% other treatment. Patients with a simple episode had average medical care costs of US$131 and parental lost-work-time costs of US$114, while patients with a complex case had medical costs of US$327 and parental lost-work-time costs of US$404. In a study that followed 25 children aged 1–3 years from the Medigan Army Medical Center in Washington [21], which utilized OM diaries filled in by parents, the mean direct costs per patient for the 3 months following diagnosis totalled US$137.94, of which US$124.25 was for physician visits and the remaining US$13.69 for medications [21]. The cost estimates appear to be in 1996 US dollars, although this is not clear from the article. Niemelä *et al.* (1999) estimated direct and indirect costs of OM among 736 day-care children between March 1991 and May 1992 in Finland [22]. The total cost of an OM attack was estimated as US$228 (including physician visits, parental absence from work, medication and travel). The cost of OM medication was estimated at US$34 per person years at risk (PYR), the cost of adenoidectomy and tympanostomy US$80 per PYR and the total costs of OM at US$415 per PYR. Projecting this sample group across the total population of Finland aged under 7 years, the annual costs associated with OM were estimated at approximately US$138 million. Finally, in Canada, Coyte *et al.* estimated the economic consequences of OM in 1994 [23]. Direct and indirect costs (work loss of caregivers and lost productivity due to disability) were estimated at CAN$470.7 and 140.3 million, respectively. Consistent with the findings here, physician costs,

**Table 5. Hospital separations with a principal or any diagnosis of otitis media during 2006–2007.**

| Procedure | Separations for OM only (n) | Total separations for OM and/or another diagnosis (n) | Total separations for OM (%) |
|---|---|---|---|
| *Principal diagnosis of H65 or H66* | | | |
| Myringotomy | 494 | 1594 | 31.0 |
| Myringotomy with tube insertion | 22,251 | 30,126 | 73.9 |
| Myringoplasty | 5324 | 34,392 | 15.5 |
| Tonsillectomy/adenoidectomy | 4365 | 45,294 | 9.6 |
| *Any diagnosis of H65 or H66* | | | |
| Myringotomy | 904 | 1594 | 56.7 |
| Myringotomy with tube insertion | 27,037 | 30,126 | 89.7 |
| Myringoplasty | 9674 | 34,392 | 28.1 |
| Tonsillectomy/adenoidectomy | 8770 | 45,294 | 19.4 |

H65/H66: International Classification of Diseases version 10 codes.
OM: Otitis media.
Data from Australian Institute of Health and Welfare special request.

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**Table 6. Australian Institute of Health and Welfare method: discounted cost of otitis media (present value in 2008) birth cohort to age 4 years.**

| Year | High cost estimate (AUS$m) | Low cost estimate (AUS$m) |
|---|---|---|
| 2008 | 129 | 52 |
| 2009 | 106 | 43 |
| 2010 | 70 | 29 |
| 2011 | 75 | 31 |
| 2012 | 68 | 28 |
| Net present value | 449 | 183 |

other miscellaneous expenditures and drug costs represented the largest components of the direct cost at 47.3, 22.7 and 18.3%, respectively. Total costs were CAN$611.0 million or approximately 0.08% of Canadian gross domestic product. Also consistent with our findings, there was considerable uncertainty in the economic cost of OM, ranging from a low of CAN$491.3 million to a high of CAN$728.8 million.

Deriving incidence and prevalence estimates for OM is difficult. Many cases of OM are either under- or over-diagnosed but to an unknown extent [10,24–26]. Young children are not able to articulate their symptoms (if any). Fever, ear ache and excessive crying are frequently present among those with AOM but are also prominent among children without AOM [27].

Furthermore, differences across epidemiological studies in definitions of disease, population characteristics (e.g., age and use of out-of-home child care or shared child care), diagnostic methods (e.g., observation vs pneumatic otoscopy) and the season in which the study was conducted mean that comparisons across studies need to be made with care (an otoscope is a handheld instrument fitted with lighting and magnifying-lens systems and used to facilitate visual examination of the auditory canal and eardrum. A pneumatic otoscope has a rubber bulb attached that allows the examiner to send a small puff of air into the ear. This changes the pressure inside, and the healthcare professional can watch how the eardrum responds to pressure changes). Since OME is asymptomatic, short observation intervals are important for better accuracy in determining the onset of each new episode and thus the frequency and duration of OME [28]. There is a wide range of international estimates found in studies of the cumulative incidence of middle-ear effusion [28].

There is also a paucity of recent robust epidemiological data for the broader Australian population. Estimates from the ABS National Health Survey and National Aboriginal and Torres Strait Islander Health Survey are based on self-reporting rather than clinical diagnosis. Aside from the unreliable nature of self-reporting versus clinical studies, OME is generally asymptomatic. The largest clinical study, the National Trachoma and Eye Health Program, was conducted in the late 1970s [17].

For these reasons, incidence and prevalence estimates are only available within a broad range, partly explaining the need to report cost estimates within a broad range. However, the range in the cost estimates also reflects the different data sources for estimating treatment costs.

The advantage of the AIHW approach is that cost estimates are derived across diseases using a consistent methodology, and the sum of the parts does not exceed the whole (i.e., total health expenditure in Australia). However, the most recent AIHW expenditure data available by disease for this study were for the year 2000–2001, and health service utilization patterns for OM may have changed since then. In particular, hospitalization rates have fallen [102] and antibiotic prescribing rates have probably fallen. The bottom-up method combines different data sets with different objectives and collection methodologies. The extent to which capital costs are included in the different cost data is not always clear. For example, the National Hospital Cost Database collects data from a survey of hospitals, and depreciation is not necessarily defined consistently across hospitals, and the user cost of capital is excluded. Furthermore, it is unclear whether the user cost of capital was included in initial derivations of Medicare Benefits Schedule fees. Another issue is that the bottom-up method used here does not account for research costs, which are, however, included by the AIHW in their expenditure estimates.

The lack of consistent definitions and terminology for the types of OM hampers links between epidemiological data and Australian databases for health-service utilization and costs. The ICD-10 and International Classification of Primary Care version 2 codes for OM are not necessarily closely aligned with definitions of OM used in epidemiological research; therefore, costs by type of OM were not estimated here.

**Table 7. Australian Institute of Health and Welfare method: prevalence cost of otitis media in 2008.**

| Age (years) | Males (AUS$m) | Females (AUS$m) | Persons (AUS$m) | Indigenous (AUS$m) |
|---|---|---|---|---|
| 0–14 | 144.0 | 108.5 | 252.6 | 32.43 |
| 15–24 | 6.5 | 12.5 | 19.0 | NA |
| 25–64 | 48.7 | 30.4 | 79.1 | NA |
| 65–74 | 14.2 | 10.0 | 24.0 | NA |
| 75+ | 6.3 | 12.6 | 18.9 | NA |
| Total | 217.8 | 173.8 | 391.6 | 38.72 |

NA: Not available.

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The burden of disease calculations may be conservative. The estimates here are based on the method used by Begg *et al.* [7]. The duration of illness used by Begg *et al.* [7] to calculate the burden of disease associated with AOM is shorter than the mean duration of AOM found by Mandel *et al.* [28]. The duration of illness used by Begg *et al.* [7] was 7 days compared with 22.4 days (±26.4) found by Mandel *et al.* [28]. If AOM is approximately 80% of all OM, using the duration found by Mandel *et al.* [28] would increase the estimates of the burden of disease here by a factor of 2.6.

**Table 8. Discounted bottom-up cost of otitis media for first 5 years of life of the 2008 birth cohort.**

| Service | Cost (AUS$1000s) of year: | | | | |
|---|---|---|---|---|---|
| | *2008* | *2009* | *2010* | *2011* | *2012* |
| General practitioner | 10,727 | 8848 | 5834 | 6277 | 5700 |
| Medicines | 5011 | 4133 | 2725 | 2932 | 2663 |
| Pathology and imaging | 128 | 105 | 69 | 75 | 68 |
| Referrals | 2187 | 1804 | 1189 | 1280 | 1162 |
| Hospitals | 1578 | 3455 | 2278 | 2451 | 2226 |
| Emergency department | 103 | 100 | 97 | 94 | 92 |
| Total | 19,733 | 18,446 | 12,194 | 13,109 | 11,910 |

Net present value: AUS$75,391,000.

Together with GP visits, medicines constitute a high proportion of the cost of treating OM. Antibiotic prescribing rates appear to remain high despite clear evidence for a limited benefit from antibiotics for most OM and concerns regarding bacterial resistance. In 1986, a survey of GPs found that 98.2% of Australian patients diagnosed with AOM were prescribed with antibiotics [24]. Steinmann and Babl reviewed medical records at the RCH Melbourne to audit antibiotic prescribing rates for AOM [29]. Antibiotic prescription rates were 74% for children less than 1 year of age, 65% for children aged 1–2 years and 66% for children older than 2 years. Differences in data sets and methods mean that these estimates cannot be directly compared with the estimates found in this study; therefore, it is not possible to determine whether Australian prescribing rates have changed over time. The contribution of antibiotics for OM to bacterial resistance is worthy of further research. Cost–effectiveness studies – ideally incorporating the impact of antibiotics prescribed for OM on the rates of both serious complications and antibiotic resistance – would be useful to inform health providers and government policy in general regarding interventions that reduce or prevent the burden of this common condition and its complications.

### Ethical conduct of research

*The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.*

**Table 9. Bottom-up prevalence cost of otitis media in 2008.**

| Service | Age (years) | | | | | Total |
|---|---|---|---|---|---|---|
| | 0–14 | 15–24 | 25–64 | 65–74 | 75+ | |
| *Lower-bound estimate (AUS$1000s)* | | | | | | |
| General practitioners | 21,045 | 3090 | 6680 | 545 | 374 | 31,734 |
| Medicines | 9832 | 1444 | 3120 | 255 | 175 | 14,825 |
| Pathology and imaging | 250 | 37 | 79 | 6 | 4 | 378 |
| Referrals | 4290 | 630 | 1362 | 111 | 76 | 6469 |
| Hospital admitted | 22,733 | 2286 | 3745 | 672 | 695 | 30,131 |
| Emergency department | 2015 | NA | NA | NA | NA | 2015 |
| Total | 60,165 | 7486 | 14,986 | 1590 | 1324 | 85,552 |
| *Upper-bound estimate (AUS$1000s)* | | | | | | |
| General practitioners | 51,669 | 7586 | 16,399 | 1339 | 918 | 77,912 |
| Medicines | 24,138 | 3544 | 7661 | 625 | 429 | 36,397 |
| Pathology and imaging | 615 | 90 | 195 | 16 | 11 | 927 |
| Referrals | 10,533 | 1547 | 3343 | 273 | 187 | 15,883 |
| Hospital admitted | 22,733 | 2286 | 3745 | 672 | 695 | 30,131 |
| Emergency department | 2015 | NA | NA | NA | NA | 2015 |
| Total | 111,703 | 15,053 | 31,344 | 2925 | 2240 | 163,264 |

NA: Not available.

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## Key issues

- Available evidence suggests that otitis media (OM) is very common among children; nearly all children have experienced at least one episode of OM by the time they are 3 years old. However, the lack of recent, large, robust Australian epidemiological studies and consistent definitions of types of OM and terminology internationally, plus the significant differences in epidemiological study methods and diagnostic uncertainty mean that the prevalence and incidence of OM in Australia can only be determined for costing purposes within a broad range.
- Between April 2000 and March 2007, acute OM accounted for approximately 7% of all problems managed by Australian general practitioners (GPs) in children aged 0–14 years.
- Together with GP visits, medicines constitute a high proportion of the cost of treating OM. Antibiotic prescribing rates appear to remain high, despite clear evidence for a limited benefit from antibiotics for most cases of OM and concerns regarding bacterial resistance. In Australia, GPs prescribed antibiotics for AOM for 82.3 out of every 100 problems managed in which the diagnosis was OM during the period 2000–2007. Antibiotics prescribed by GPs for OM in children aged 0–14 years comprised 10% of all medications (antibiotics and other medicines) prescribed by GPs to children in that age group. The contribution of antibiotics for OM to bacterial resistance is worthy of further research.
- Data from a tertiary pediatric hospital in Melbourne for the period 1 June 2007 to 31 May 2008 suggested that OM accounted for 2.9% of emergency-department presentations (including young people up to age 18 years) in 2007.
- Rates of hospital use by admitted patients for OM have fallen for the period during which hospital data are available – between 1998–1999 and 2006–2007.
- Treatment costs for OM in Australia in 2008 were between AUS$100 and 400 million in 2008. The wide range reflects the different sources of information on health expenditure by disease and treatment costs in Australia and the wide range of estimates of incidence and prevalence from epidemiological studies.

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# CHAPTER 3

# MICROBIOLOGIC, IMMUNOLOGIC AND GENETIC STUDIES IN OTITIS MEDIA

| Paper no* | Supporting Articles | Thesis page no |
|---|---|---|
| 9 [23] - | Chronic Suppurative otitis media without cholesteatoma | 94 |
| 10 [42] - | Biofilm, intracellular bacteria and neutrophil extracellular traps in otitis media. | 101 |
| 11 [25] - | The role of chronic infection in children with otitis media with effusion: evidence for intracellular persistence of bacteria. | 127 |
| 12 [50] - | Multi-species bacterial biofilm and intracellular infection in otitis media. | 131 |
| 13 [26] - | Neutrophil extracellular traps and bacterial biofilms in middle ear effusion of children with recurrent acute otitis media--a potential treatment target. | 141 |
| 14 [54] - | Predominance of nontypeable Haemophilus influenzae in children with otitis media following introduction of a 3+0 pneumococcal conjugate vaccine schedule | 151 |
| 15 [53] - | IgG responses to Pneumococcal and Haemophilus influenzae protein antigens are not impaired in children with a history of recurrent acute otitis media. | 159 |
| 16 [58] - | High pneumococcal serotype specific IgG, IgG1 and IgG2 levels in serum and the middle ear of children with recurrent acute otitis media receiving ventilation tubes | 168 |
| 17 [65] - | FBXO11, a regulator of the TGFbeta pathway, is associated with severe otitis media in Western Australian children | 175 |

| | | | |
|---|---|---|---|
| 18 | 63 | Genome-wide association study to identify the genetic determinants of otitis media susceptibility in childhood | 183 |
| 19 | 67 | Genetic and functional evidence for a role for SLC11A1 in susceptibility to otitis media in early childhood in a Western Australian population. | 195 |
| 20 | 68 | Genetic and functional evidence for a locus controlling otitis media at chromosome 10q26.3. | 203 |


*Superscript numbers correspond to the designated general bibliography numbers – when cited in the text.

This chapter will describe microbiologic and immunologic studies by the author and colleagues, which have contributed greatly to the understanding of the pathogenesis of OM, especially regarding bacterial persistence strategies in OM. Bacterial carriage and persistent infections are an important risk factor in the causal pathway for OM (Figure 5). The genetic contribution to the casual pathway of OM was also addressed by the author and his colleagues finding a specific gene (FBOX11) to be associated with high susceptibility to rAOM or chronic OME.

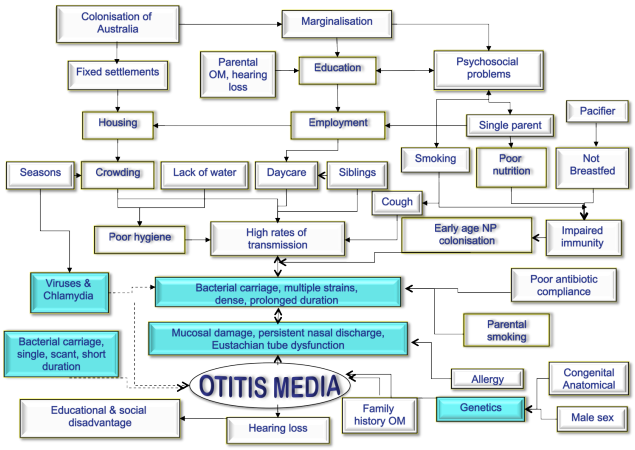


**Figure 5:** Causal pathways to OM addressed by the author and his colleagues through Microbiologic, Immunologic and genetic Studies.

## *3.1 Microbiologic Studies in Otitis Media*

In the causal pathways to OM, bacterial carriage and colonisation throughout the upper respiratory tract and the persistence of bacterial preservation, despite apparent appropriate medical treatment, have been contentious areas. The author became interested in this particular sphere of microbiology upon hearing a presentation of Professor Christopher Post on bacterial biofilm in the middle ear mucosa of chinchillas with middle ear infections. The author and his colleagues started a series of studies, including the investigation and first ever report of **bacterial biofilm** in the middle ear of an Aboriginal child with CSOM, and the

study of multiple modalities of bacterial persistence in the middle ear mucosa and fluid, such as **intracellular infection** and **Neutrophil Extracellular Traps (NETs)**. The discovery of these three bacterial preservation strategies have led to further innovative studies to eradicate or ameliorate the presence of biofilm and NETs in the middle ear, originating multiple papers, a PhD thesis co-supervised by the author, and a recent book chapter[42].

### 3.1.1 Bacterial Biofilm

Bacterial biofilm consists of clusters of bacteria embedded in a polymeric matrix with increased resistance to antibiotics and host defense mechanisms when compared to their "*planktonic*" (free floating) counterparts [43].

Since the 1970's when bacterial biofilm was discovered by Costerton and his group, bacteria biofilm have been noticed in cystic fibrosis, periodontitis, urinary tract infections and device related infections such as those of prosthetic heart valves, central venous catheters and contact lenses [44]. However, the biofilms on inert surfaces, such as devices, are physiologically separated from those on mucosal surfaces, where the host immune response contributes proteins and cells towards the composition of the biofilm matrix. Bacterial biofilm manifests as 98% of the bacteria in the human body, with only 2% being planktonic [45]. This means that only the minority of the bacteria present in the human body are susceptible to antibiotic treatment topically or systemically.

The bacterial biofilm may develop silently because of increased antibiotic resistance, decreased production of virulence factors and diminished neutrophil activity until such time as they are activated by viral infections, such as upper respiratory tract infections, or environmental or host defense changes [46]. At that

time, planktonic bacteria, that are more susceptible to antibiotics, can be dispersed. Effective antibiotic management of the planktonic bacteria and subsequent lack of ability to culture bacteria have led clinicians and microbiologists to assume the infection was cleared, however, through the use of PCR technology, bacteria have been demonstrated indicating that the underlying infection reservoir remains [47,48].

In 2001 at the Triological Meeting in California, the author heard Professor Christopher Post from the Center for Genomic Sciences (University of Pittsburgh) discussing a new concept of bacterial biofilm as a possible cause for the chronicity of not only systemic conditions such as cystic fibrosis (CF) and bacterial endocarditis, but also of middle ear disease, especially in OM, OME and rAOM in an animal model (chinchillas). The author and his team decided to investigate whether bacterial biofilm existed in the middle ear of human subjects, and in particular, in Aboriginal children with CSOM. Specimens from the middle ear mucosa of children with CSOM and OME were analysed utilising Scanning Electron Microscopy (SEM) and Confocal Laser Microscopy (CLM). We described the first human biofilm (*P. aeruginosa*) in the middle ear mucosa of an Aboriginal child with rAOM undergoing ventilation tube insertion [23]. Subsequently, our collaborators from the Center for Genomic Sciences in Pittsburgh, Professor Post and Dr. Garth Ehrlich, utilising the evidence shown, obtained a collaborative National Institute for Deafness and Other Communication Disorders (NIDCD) grant of over $4.3 million to further this research.

### 3.1.2 Intracellular Bacteria in the Middle Ear

An additional mechanism to the bacterial biofilm for persistent bacterial infection is the occurrence of intracellular bacterial infections. Intracellular bacteria have been recognised in multiple organ systems by multiple variations of bacteria, including *Escherichia coli* in the bladder mucosa of patients with recurrent urinary tract infections, *Helicobacter pylori* in chronic gastric ulcers, and *Mycobacterium tuberculosis* in lung infections. The bacteria, when located intracellularly, can evade both host immune responses and antibiotic therapies that are unable to penetrate the cell membrane [49].

The ability of bacteria to invade epithelial cells represents a useful strategy for colonisation of the respiratory tract and to avoid extracellular immune recognition. The author and his team believe that it is likely that a combination of intracellular infection and biofilm formation are responsible for the persistence and recurrence of ear disease in susceptible children.

Subsequently, the author and his colleagues described for the first time the occurrence of intracellular bacteria in the middle ear mucosa of children with rAOM and OME [25]. Through transmission electron microscopy, gram-positive bacteria were detected in the epithelial cells of middle ear mucosa in 4 of 11 children (36%). Bacteria were situated principally in the outside cytoplasmic portion of the surface epithelial cells and in particular, mucus secreting cells as well as the underlying connective tissue stroma. In addition, the author and colleagues postulated that this intracellular infection could be contributing to excessive mucus secretion seen in chronic OME as well as persistent inflammation. *S. pneumoniae*, NTHi, *Moraxella catarrhalis, Pseudomonas aeruginosa* and *S. aureus* are now well recognised pathogens associated with

recurrent and persistent OM and they are all capable of invading and persisting within cells[50].

The discovery of intracellular bacteria has great clinical impact, as certain classes of antibiotics, including the β-lactam class commonly used in the treatment of OM, do not penetrate the cellular membrane and are inefficient against intracellular bacteria. The introduction of antibiotics such as macrolides and fluoroquinolones, which concentrate intracellularly might be more effective in the management of intractable OM than the penicillin group.

### 3.1.3 Neutrophil Extracellular Traps (NETs) in OME

Added to the first report of bacterial biofilm and intracellular pathogens in the middle ear and middle ear mucosa of children with OM, OME and CSOM, the author and his research team also described for the first time the existence of Neutrophil Extracellular Traps (NETs) in the middle ear effusion of children with rAOM/OME, indicating bacterial preservation strategies throughout the entire middle ear (on the middle ear mucosa, in the middle ear mucosa and in the middle ear fluid) [26].

NETs have a central DNA structure with embedded enzymes and antibacterial peptides. Free DNA contributes to the formation of bacterial biofilm and is derived from middle ear fluid, necrotic neutrophils or produced by bacteria themselves. A high concentration of NETs leads to an increased viscosity of the middle ear effusion, which also leads to diminished resolution of the middle ear effusion by medical means, as opposed to surgical treatment with ventilation tube insertion (VTI).

The author and his team analysed nasopharyngeal cultures obtained from swabs of children with OME using culture and FISH (Fluorescence *In Situ* Hybridisation) in order to investigate the presence of biofilm and determine if this could represent infectious reservoirs similarly to those found on the mucosa. The viscous middle ear fluid of children with OME collected during surgery was also analysed (utilising Confocal Laser Scanning Microscopy (CLSM)). These tests revealed the presence of DNA strands associated with colonised bacteria entitled Neutrophil Extracellular Traps (NETs) where multiple live bacterial species and polymicrobial biofilms were identified within the middle ear fluid. Studies in the presence of NETs in the Middle Ear Effusion (MEE) of children with rAOM correlate with previous studies in animals [51,52] and accounts for the difficulty in resolution of MEE clinically, despite appropriate oral antibiotics management. The availability of MEE to study bacterial/NETs and free-floating bacteria is attractive, avoiding invasive middle ear mucosal biopsies.

Biofilm, mucosal disease and NETs are often seen in Cystic Fibrosis (CF), where a common modality of treatment is the use of Recombinant Human Deoxyribonuclease 1 (Dornase alfa - Pulmozyme®) which specifically lyses DNA reducing the viscosity of the airway mucus. Adaptation of this treatment to *in vitro* NETs in the middle ear fluid of MEE showed rapid and complete break down of NETs/DNA. The reduction in viscosity, in turn, may allow easier resolution of the MEE, unmasking of live bacteria and antibiotic susceptible planktonic form and degradation of bacterial biofilm in MEE and mucosa.

The author and his colleagues are currently conducting a double blind random controlled trial of Dornase alfa in the middle ears of children undergoing VTI for

rAOM to assess the effect that this has in reducing complications of OM and repeat VTIs (please refer to **Chapter 4, section 4.1.2** for more details on Dornase alfa studies conducted by the author and his team).

## *3.2 Immunologic Studies in OM*

Immunologic investigations concentrated on whether bacterial recurrent otitis media was related to an underlying humoral immunologic defect or whether other factors might be involved. We know from previous studies from the Menzies group [35] that bacterial carriage is higher in Aboriginal children with recurrent acute otitis media, and that it occurs at an earlier age than in non-Aboriginal children. Our **Genesis of Recurrent Otitis Media in Toddlers** study (GROMIT study) [53] evaluated infection, bacterial carriage and immunity in 186 children younger than 36 months with a history of recurrent OM undergoing ventilation tube insertion (healthy children, n=81) undergoing surgery for other clinical reasons were selected as controls for this study (details of this cohort was described in [54]). Examination of nasopharyngeal swabs, blood samples and middle ear fluid were conducted for bacteria, viruses and immune studies looking for antibodies to the seven bacterial proteins that are candidates for protein based vaccines against *S pneumoniae*. Our studies showed that the most common pathogens in the middle ear fluid and nasopharynx of children with recurrent AOM were NTHi and rhinovirus. There was no impaired immunological response as measured by levels of IgG to specific NTHi or pneumococcal proteins. This is in contrast to studies by Kaur and Hotami [55,56], which suggested reduced immune

response to these specific proteins. Increased otopathogens seen in Indigenous children are not associated with inadequate responses to Capsular polysaccharide synthesis or protein antigens. The Indigenous child may have increased intensity and frequency of infections secondary to an overwhelmed immune system where the mucosal immune response is down- regulated by persistent nasopharyngeal carriage.

In a subset of the children from the GROMIT study, serum IgG, IgG1, and IgG2 antibody levels against 11 pneumococcal polysaccharides from PCV7 immunised and un-immunised children under three year old with rAOM were measured. There were similar levels of these IgG subtypes in the children with rAOM and healthy controls. Also noted was a strong correlation between pneumococcal serotype specific IgG antibodies found in the serum and middle ear fluid with evidence that PCV7 serotype antibodies entered the middle ear fluid in vaccinated children. This implies that vaccination with pneumococcal vaccines with increased valencies such as PCV10 and PCV13 might lead to improved efficacy in preventing rAOM [53,54,57,58].

## 3.3 *Genetic Studies*

Several environmental risk factors have already been identified as playing an important role in OM susceptibility. More recently, heritability studies are also revealing that a significant component of OM risk is attributable to genetic factors [6].

The prevalence of OM is known to be greater in specific populations, such as Aboriginal, Indians and Eskimos. Although there are multiple theories of the causality and relationship of OM, the genetic predisposition to OM and rAOM in these populations remains unclear. The Bluestone team has demonstrated heritability of OM in twins and triplets studies and this was replicated by Rovers et al, in a longitudinal study of 1,373 twin pairs [59,60]. Heritability studies show that 45 to 74% of the risk of OM may be related to genetic factors [59-61]. Genetic mutations that result in OM have been identified in two mouse models in the United Kingdom [62].

In 2012 the author and colleagues published an article entitled: **"Genome-wide association study to identify the genetic determinants of otitis media susceptibility in childhood"** [63]. In this study, the genetic profile of patients with rAOM was analysed to determine if there was an association between specific genes (EV11 and FBOX11) and the susceptibility to rAOM or chronic MEE. Children were recruited from the longitudinal birth cohort Raine Study (Western Australian Pregnancy Cohort Study) which comprises 2,868 children whose mothers joined the study during early pregnancy, from 1989 to 1991 and had multiple clinical examinations from birth to the current 21st year follow-up [64]. For this study, clinical test results and questionnaires obtained in the first 3 years of life were used to define a phenotype for OM. In total, there were 644 cases and 2,221 controls.

For replication of the results, a second group, the Western Australian Family Study of Otitis Media (WAFSOM) was also included in the study. This family-based cohort was initiated in 2007 and currently has 434 families with 561

children who had three or more physician-diagnosed episodes of AOM or have had ventilation tube insertion for rAOM or OME (details of this cohort were described elsewhere [65]).

In total, 1,491 children from the Raine Study (428 children with severe OM and 1,101 controls) and 645 families (793 affected children) from the WAFSOM Study were included in the Genome-Wide Association Study. The GWAS is one method to identify novel genes, which confer susceptibility to diseases such as OM, and to date there has been no GWAS of OM susceptibility reported.

Analysis revealed that there was no association in either of the cohorts studied (Raine and WAFSOM) for any of the EV11 variants evaluated but there was a significant association between single nucleotide polymorphisms (SNPs) at FBOX11 and rAOM in both cohorts ($p<0.009$). The association at FBXO11 was independent of environmental risk factors for OM (day-care attendance, allergy). As both EVI1 and FBXO11 proteins appear to interact in the transforming growth factor beta (TGF-β) signalling pathway [66], our results confirm a genetic basis for susceptibility to AOM and indicate that this may be mediated by variants at FBXO11 that result in inflammatory dysfunction by altering regulation of the TGF-β pathway [65].

Further studies of the genetic heritability of disease, in particular of the OM and other severe diseases, such as diabetes, are being conducted on a thousand families in Western Australia, as well as in remote Aboriginal communities [67,68].

Chapter 60

# Chronic Suppurative Otitis Media without Cholesteatoma

Harvey Coates, MS, FRACS

The World Health Organization's (WHO) definition of chronic suppurative otitis media (CSOM) is a stage in ear disease in which there is a chronic infection of the middle ear cleft (eustachian tube, middle ear, and mastoid) in the presence of a persistent tympanic membrane (TM) perforation (World Health Organization, 1998). There may be intermittent or constant purulent otorrhea. Mastoiditis is a consistent part of the pathologic process. This definition of chronic otorrhea varies between various groups from 2 weeks to 3 months or longer. I believe that 2 months minimum is necessary to distinguish this process from acute otitis media (AOM) with perforation.

Synonyms, many of which are inaccurate and inappropriate, include chronic otitis media (OM) (active and inactive), suppurative OM, and chronic perforation. For the purposes of this chapter, CSOM is characterized by recurrent or persistent bacterial infection of the ear with conductive hearing loss, mucopurulent otorrhea, and a chronic perforation of the TM.

## Incidence

CSOM is a disease affecting especially indigenous people with poor health, hygiene, and nutrition throughout the world. In developing countries, in cases in which CSOM accounted for 60 to 80% of middle ear disease, suppurative intracranial complications occurred in about 10% (Davidson et al, 1989). The WHO reported that a prevalence of CSOM greater than 4% in a defined child population indicated a massive public health problem needing urgent attention in targeted populations (World Health Organization, 1998). In many underdeveloped countries throughout the world, the incidence of CSOM is significantly higher than this.

In Inuit Indians, the prevalence of CSOM varies up to 66%, whereas in Australia, the prevalence of CSOM in the Aboriginal population is similarly high. The majority of children with CSOM have associated conductive hearing loss as a result of a perforated TM, otorrhea, granulation, polyps, cholesteatoma, and ossicular chain problems. In Australia, screenings of conductive hearing loss in Aboriginal children in one series showed 41% to have a hearing loss of 25 dB or greater in at least one ear (Watson et al, 1992). In nonindigenous Australian children, hearing loss in a large series was in the range of 4 to 5% of children, with an average hearing loss of 20 dB (McPherson, 1990).

## Importance

### Mortality and Morbidity

In 1993, the WHO reported that OM (principally CSOM) was responsible for the deaths of 51,000 children under the age of 5 years each year in developing countries (WHO, 1994). Combining this loss of life from early death with loss from disability—a term known as disability-adjusted life-years—OM resulted in an impact similar to meningitis and trachoma. This burden of suffering was assessed cumulatively in Australia, and Aboriginal children would be expected to have 32 months with OM between the ages of 2 and 20 years (McGilchrist and Hills, 1986) and non-Aboriginal children only 3 months with OM for the same time period.

CSOM in children will often continue into adulthood if improperly treated in childhood. Nunez and Browning (1990) reported that the annual risk of developing an abscess was approximately 1 in 10,000 in an adult with active chronic OM, with males three times more likely to develop this. They commented that although this risk appeared to be low, the lifetime expectancy of an adult aged 30 years with active chronic OM developing an abscess was 1 in 200 (Nunez and Browning, 1990).

### Hearing Loss

Up to one-third of the population in developing countries has their quality of life affected by CSOM and its precursors, OM and otitis media with effusion (OME). CSOM is the most common cause of hearing impairment in children in developing countries. Between 30 and 80% of Aboriginal children suffer from hearing loss. The impact of OM on development of language in Aboriginal children with the early onset of OM shortly after birth, persistent otorrhea, and hearing loss is much greater than in non-Aboriginal children. Difficulties in acquisition of language, particularly when English may be a second language, together with auditory processing problems, create a milieu in which educational success is difficult to accomplish.

Difficulty hearing in class leads to poor scholastic performance, behavior problems, poor self-esteem and social isolation, truancy, and, subsequently, unemployment and interaction with the judicial system.

## Pathogenesis

CSOM is a sequela of acute or unresolved OM, particularly in children with poor socioeconomic conditions, and usually presents within the first 5 years of life. In the presence of a preexisting perforation or ventilation tube, infection may develop secondary to contamination from ear canal organisms or with an upper respiratory tract infection. Reflux of nasopharyngeal secretions and possibly reflux of gastric secretions may occur secondary to loss of the protective middle ear air cushion with a perforation. This is in addition to the fact that children have shorter eustachian tubes, making them more prone to reflux. Recent studies by Tasker and colleagues (2002) showed that in OME, up to 83% of children had high levels of pepsin/pepsinogen in their effusions, suggesting that the reflux of gastric juice into the middle ear could be more common than previously thought.

Active middle ear infection leads to increased vascularity of the middle ear mucosa and submucosa with infiltration by acute and chronic inflammatory cells, subsequent mucosal edema, and osteitis. This infection leads to mucosa/ulceration and chronic infection and metaplasia of the columnar epithelium, which changes to a cuboidal-type epithelium with capillary proliferation, development of granulation, polyps, osteitis, and new bone formation. Pathologic changes in the temporal bone occur with almost 100% ossicular involvement, osteitis of the temporal bone, and development of granulation tissue and polyps. In about 25% of cases, cholesterol granuloma or tympanosclerosis is present. Progression of CSOM may lead to complications both within the temporal bone and intracranially. Temporal bone complications include mastoiditis, ossicular chain destruction, facial nerve involvement, secondary cholesteatoma development, labyrinthitis, and sensorineural hearing loss. Intracranial complications may include meningitis, extradural and intracranial abscesses, and sigmoid sinus thrombosis.

## Bacteriology

The usual organisms cultured in CSOM are aerobic organisms, although anaerobic organisms and fungi have been implicated in the disease process. The most common organism is *Pseudomonas aeruginosa*, followed by *Staphylococcus aureus*, and these bacteria may derive from external ear canal contamination of the middle ear (Brook and Finegold, 1979). Brook (1989) believes that the role of anaerobes has been underplayed and that it is important to culture for these organisms from the middle ear aspirates. The importance of culturing from the middle ear rather than the ear canal is stressed. He found that 56% of ear aspirates in patients with CSOM isolated anaerobic organisms, and other studies have shown isolation of anaerobic organisms of between 8 and 59%. The most common anaerobic bacteria isolated are *Bacteroides fragilis* and *Bacteroides melaninogenicus* group 9. Fungal isolates are principally *Aspergillus* species and *Candida albicans* (Attallah, 2000). Tuberculosis has also been implicated as a cause of CSOM in developing countries.

No discussion of CSOM would be complete without consideration of the effects of biofilm formation by bacteria in middle ear disease.

Biofilm formation occurs when microorganisms present in middle ear fluid lead to a quorum with deposition of sessile bacteria with protective biofilm production on mucosal surfaces and foreign bodies. This biofilm acts to provide an almost impervious layer to many systemic and topical antibiotics and may increase the resistance to antibiotics (eg, to tobramycin by *P. aeruginosa* and *S. aureus*) from 20 to 100 times. Within the glycocalyx formed by the heaping of bacteria and supporting substances, some bacterial changes to an almost inert form may occur. The presence of a viral stimulation such as an upper respiratory tract infection may encourage these spore-like bacteria to become activated and undergo planktonic changes, reactivating otorrhea. Biofilm has been noted to attach to osteitic bone, epithelial pits, and foreign bodies in CSOM (Post, 2001). I have isolated bacterial biofilm from the middle ear mucosa of a child with CSOM (Figure 60-1).

Future management strategies for CSOM may need to take into account treatment for biofilm. These strategies could include using appropriate messages to signal the biofilm to "go planktonic," where it could be available to systemic and topical antibiotics, disrupting the biofilm mechanically, using other chemicals or detergents that may break down the biofilm layer or using

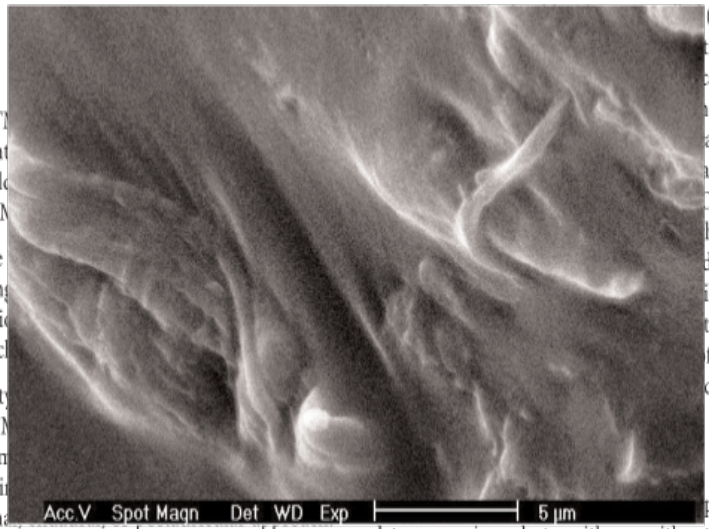


FIGURE 60-1 Scanning electron micrograph of middle ear mucosa from a child with chronic suppurative otitis media showing bacterial biofilm formation.

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indicated in most cases in which there is evidence of ossicular chain discontinuity or loss of the ossicles from chronic disease. Where there is chronic otorrhea unresponsive to conservative management, or if there are significant signs and symptoms such as profuse otorrhea, facial nerve involvement, vertigo, otalgia, and headaches, then a computed tomographic (CT) scan may indicate significant mastoid disease or cholesteatoma.

## Management: Treatment Options

Management of uncomplicated CSOM initially consists of conservative management with local treatment and topical medications and, occasionally, systemic antibiotic therapy. In complicated and persistent CSOM in which medical treatment has failed, consideration of surgical options using the principles of removal of disease and reconstruction and repair of the anatomy becomes an option. Treatment of CSOM is difficult because of structural and anatomic problems that prevent direct access to the diseased middle ear cleft. Also, problems with diffusion of systemic antibiotics into the temporal bone tissues to give sufficiently high mean inhibitory concentrations to eradicate the bacteria and problems of bacterial resistance and the new concept of the nonplanktonic growth modes of biofilm and other factors mitigate against easy control of the CSOM infection.

### Mastoidectomy

Mastoidectomy may be performed with the canal wall up (cortical mastoidectomy) or may be combined with a facial recess approach. Often, particularly in children, canal wall up procedures will need to have a second stage to ensure eradication of disease, and reconstruction of the ossicular chain will have to be considered if it has been involved in the disease process.

### Management of Complications

Management of complications of CSOM, including otogenic abscesses, generally follows the surgical principles of decompression of the abscess with drainage and meticulous attention to the mastoid cavity with closure of fistulae or defects between the temporal bone and intracranial cavities. A detailed description of surgical management of each complication of CSOM is beyond the scope of this chapter.

### Conservative Management

After a full history and clinical examination, consideration must be made to rule out underlying associated factors that may predispose to the infection. This demands obtaining an audiogram, performing a microscopic examination under the operating microscope, and taking a swab for culture and sensitivity of the middle ear discharge. The operating microscope, the surgical aural toilet, and the topical antibiotic therapy will result in the resolution of the otorrhea in the majority of cases. Aural toilet is the process of removing the debris and discharge from the middle ear. This is usually carried out by using a suction apparatus with a fine sucker or by mopping with cotton-tip applicators. Topical antibiotic therapy should be given initially as drops, three drops three times daily (three drops twice

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In cases in which the perforation is larger and occupying more than one-third of the TM, then I use a classic underlay myringoplasty with elevation of the tympanic membrane remnant and preparation of the ossicular chain as either tragal perichondrium or temporalis fascia graft. In this technique, the perforation is generally difficult to expose. Then the posterior approach may be necessary or a canal plasty may be performed. Postoperative antibiotics are given to reduce the incidence of postoperative infection, particularly in indigenous children.

### Ossicular Chain Reconstruction

Essentially, ossicular chain reconstruction replaces the damaged or malassembled ossicles with a new assembly to allow the tympanic membrane to vibrate with the sound waves and transmit the sound to the inner ear. Most commonly, the incus is removed because of erosion by cholesteatoma or chronic otitis media, leaving the stapes superstructure and the mobile footplate. The handle of the incus is then used to support a tragal cartilage graft that is placed between the TM and the stapes superstructure or footplate. Alternatively, a partial ossicular replacement prosthesis (PORP) or total ossicular replacement prosthesis (TORP) may be used. Occasionally, in the absence of the stapes superstructure, the head of the malleus is used to support the tragal cartilage graft. Always, the tragal cartilage graft that is placed between the TM and the ossicular chain or stapes superstructure or footplate is covered with temporalis fascia.

### My Preferences

After obtaining a history and performing an examination, particularly under the operating microscope, I recommend obtaining an audiogram, performing nasendoscopy, and taking a swab for culture and sensitivity of the middle ear discharge. I then start the patient on topical antibiotic drops, three drops twice daily (three drops twice

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### Elimination Surgery

In cases of canal wall down procedures, in which there has been no recurrence of otorrhea or chronic mastoiditis, consideration of reconstruction of the posterior canal wall and obliteration of the mastoid cavity can be made after a year of a dry mastoid cavity. The indications for this are to avoid the open cavity and reduce the chance of chronic otorrhea. In addition, vertigo secondary to air or water caloric effects, particularly when swimming, may be eliminated, and hearing aid fitting is easier than with a large cavity. Staged reconstruction may be preferred to allow optimal hearing and canal restoration. I prefer the method described by Black (1995), in which large low facial ridge cavities are managed with hydroxyapatite and high facial ridge small cavity sites are managed with an attic defect plate technique using tragal cartilage. Obliteration of the mastoid cavity and revascularization of the wall using a middle temporal artery flap lead to improved success rates.

## My Preferences in Specific Cases

### Under 5-Year-Old Child

Early myringoplasty is important for optimal language, hearing, and educational development, particularly in indigenous children. In those cases in which there is CSOM with bilateral perforations and greater than 30 dB hearing loss and recurrent otorrhea, I perform early myringoplasty using an inlay technique for small perforations and an underlay transcanal or postauricular approach for large perforations.

Postoperative antibiotics are given for 7 days to reduce the possibility of secondary infection and loss of the graft. Where there are bilateral perforations, I perform the transcanal or postauricular myringoplasty on the larger perforation on the ear with the worst hearing and may perform a circumcision of the TM edge and patching with Silastic sheeting on the contralateral side.

My experience parallels that of Denoyelle and colleagues (1999). In their series, the age of the patient and the size and location of the perforation did not affect the outcome, but poor prognostic factors were inflammatory changes in the middle ear mucosa, contralateral TM perforation, and contralateral cholesteatoma. They felt that the presence of one of these latter factors preoperatively should lead to a consideration of a more durable graft material, such as autologous cartilage.

### CSOM with Severe Granulation Tissue Formation

Merchant and colleagues (1997) noted that CSOM with granulation was more difficult to treat than CSOM with cholesteatoma. This is also my experience, and in these cases, canal wall down mastoidectomy, together with careful removal of granulation tissue from the middle ear and mastoid, is performed. Care must be taken with the obscuring of normal anatomic markers by the destructive disease, with the facial nerve, semicircular canals, and stapes superstructure being particularly at risk. A preoperative CT scan may help in preoperative planning and to avoid complications.

In those cases in which the surgeon is reluctant to proceed further owing to concerns regarding perilymph fistula formation or facial nerve injury, a planned staged procedure with intensive antibiotic eardrop therapy prior to the second procedure may be the best option. Often, after removal of the bulk of the infected granulation tissue and creation of an open mastoid bowl, the remaining granulation tissue may respond to topical therapy more easily.

### Children with Down Syndrome

In my experience, children with Down syndrome have long-standing eustachian tube dysfunction and anatomic differences requiring an aggressive approach with a canal wall down procedure with a meatoplasty to allow adequate exposure of the cavity, in view of the usual narrow external auditory canal.

## Justification for My Preferences

### Conservative Management

The majority of children with CSOM will respond to conservative management with intensive ear toilets using suction or irrigation followed by the use of antibiotic steroid eardrops, preferably one that is nonototoxic (fluoroquinolone). Topical therapy must be continued for an adequate period of time to allow eradication of bacterial disease. Rarely, persistent otorrhea, despite appropriate topical therapy, may require admission to the hospital or outpatient IV therapy with broad-spectrum culture-sensitive antibiotics.

My current first-line management for topical therapy in the presence of a wet perforation is fluoroquinolone antibiotic/steroid eardrops, such as ciprofloxacin hydrochloride, which are nonototoxic and have an excellent antibacterial spectrum.

Failure of this medical treatment despite patient compliance necessitates hospital or outpatient IV antibiotic therapy. If this is unsuccessful after 5 to 6 days with appropriate antibiotic therapy, a CT scan is performed to rule out significant mastoiditis or cholesteatoma. On occasion, an examination of the ear under anesthesia may be warranted for biopsy purposes and to rule out cholesteatoma and unusual conditions (eg, tuberculosis). This may be followed by a cortical mastoidectomy with or without myringoplasty.

### Myringoplasty

For simple myringoplasty with small perforations, the inlay tragal myringoplasty has a number of advantages:

1. In the absence of a significant hearing loss, this technique allows relatively straightforward and rapid closure of the TM perforation without tympanomeatal flap elevation.
2. The results appear similar to those of underlay myringoplasty but have the advantage of less discomfort. Also, the technique is a more straightforward procedure, particularly with anterosuperior perforations that might otherwise require a postauricular approach.
3. Even in the presence of postoperative infection, the graft may survive with the low oxygen and nutrition requirements of the cartilage and perichondrium
4. The procedure may be done as a same-day care procedure, and the child would be able to fly in the immediate postoperative period, which is not necessarily the case with other forms of myringoplasty.

Although there are concerns that this technique may mask an underlying cholesteatoma at the margins of the perforation, the advantages in most cases outweigh the disadvantages.

Underlay myringoplasty using temporalis fascia or tragal perichondrium for medium-size dry perforations remains my treatment of choice for medium- or large-size perforations, using either an endaural or a postauricular approach. These autograft tissues have graft-take rates of over 90% in many cases.

In the absence of cholesteatoma and in the presence of a large mastoid air cell system, a canal wall up technique is preferable to avoid a large mastoid cavity. Generally, however, if there is a small relatively sclerotic mastoid bone, then a canal wall down procedure will leave, in the presence of a wide meatoplasty, a small smooth cavity with a well-vascularized lining and an intact drum with objectives similar to those of elimination surgery (Black, 1995). However, canal wall down procedures in the presence of significant mastoid disease may be the only effective means of the eradication of the disease in children. Reconstruction of the canal wall can be considered in the future if the cavities remain dry and free of disease.

### Ossicular Chain Reconstruction

My preferred technique of ossicular chain reconstruction is to use spanner assemblies or oval top columellars because of the ease of use and reported reduction in extrusion through the TM.

## Alternative Approaches

My principles of otologic surgery dictate that if I perform two procedures on a patient and the procedure, whether it is myringoplasty, ossicular chain reconstruction, or mastoidectomy, is unsuccessful, then I will consider obtaining a second opinion from a colleague who specializes solely in otology. Generally, the success rate of myringoplasty on the first occasion is between 85 and 90%, but in special populations, such as indigenous children, the graft-take rate may be as low as 50%, particularly when factors such as early postoperative swimming in potentially contaminated water (eg, waterholes) and failure to comply with postoperative care are included.

With the inlay tragal cartilage myringoplasty, failure may be attributable to a small defect at the edge of the rim, and this can often be closed with a simple fat/subcutaneous tissue graft with good success. If the inlay technique fails, then an underlay temporalis fascia graft myringoplasty is performed, and at this procedure, careful examination of the status of the middle ear mucosa and the patency of the eustachian tube and consideration of a biopsy to exclude unusual disease are undertaken. If the middle ear mucosa is very thickened and polypoid, then clearance of this tissue in the middle ear cavity may result in successful revision myringoplasty.

The wet and open cavity remains a difficult problem for the otologist and may be related to exposed respiratory epithelium, failure of healing, or failure of the normal epithelial migration that is self-cleaning (Black, 1995). In the presence of a wet cavity, a number of surgical factors may have led to the presence of persistent otorrhea. These are stated in Table 60-1.

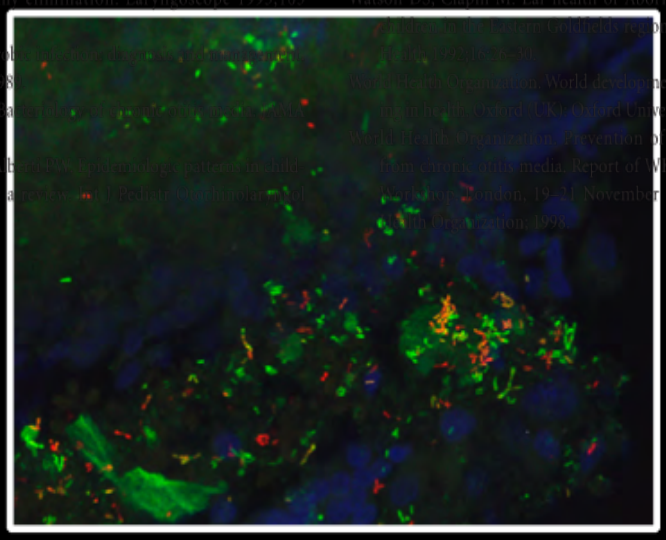
The factors in Table 60-1, together with postoperative infection, avascular changes, or problems with epithelial migration in the mastoid cavity, must be considered. These surgical factors and postoperative conditions may interact to create a poor prognosis for a dry mastoid cavity (Sadé et al, 1982). Prevention of a wet mastoid cavity requires appropriate surgical procedure selection and attention to those factors. Black (1995) noted a number of clinical features that will indicate a poor ossiculoplasty prognosis (Black, 1995) (Table 60-2).

A number of these conditions, including otorrhea, severe mucosal damage, myringitis, TM collapse, or a radical cavity, can be ameliorated by staging the surgery. Middle ear fluid or an absent malleus or stapes, together with severe audiologic losses, is more difficult to manage. Failure of canal wall reconstruction or complications of this surgery may be related to infection preoperatively, an excessively large mastoid cavity, the presence of a low facial ridge with its shorter healing distance, avascular atrophic cavity skin lining, or a lack of the pars tensa in radical mastoidectomy cavities (Black, 1995). Canal wall repair complications can be prevented by avoiding the

**TABLE 60-1. Surgical Factors Leading to Persistent Cavity Otorrhea**

| |
|---|
| Small meatus with inadequate cavity cleaning |
| Large cavity with exposed respiratory epithelium |
| Retained high facial ridge |
| Failure to close off the middle ear |
| Failure to adequately remove chronic infection and granulation |
| Failure to revascularize the mastoid cavity lining |

# Biofilm, intracellular bacteria and neutrophil extracellular traps in otitis media



*Ruth Thornton and Harvey Coates AO*

## Objectives

- To describe the multiple modalities of bacterial persistence in the middle ear.
- To utilize up-to-date knowledge of bacterial persistence to develop effective strategies to prevent and treat chronic infections of the head and neck.

# Biofilm, intracellular bacteria and neutrophil extracellular traps in otitis media

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## 1. Introduction

Historically biofilms were defined as a community of bacterial cells enclosed in an exopolymer matrix that are irreversibly associated with a surface (not removed by gentle rinsing).[1] In infections or when associated with a living organism, biofilms are more accurately defined as clusters of bacteria embedded in a polymeric matrix with increased resistance to antibiotics and host defense mechanisms when compared to their 'planktonic' or 'free-floating' counterparts.[2] These were described as far back as the 1600's by the Dutch microscopist Antonie van Leeuwenhoek who was the first person to observe bacteria and protozoa. Van Leeuwenhoek was also the first to describe one of the major resistance mechanisms adopted by bacteria in biofilms after observing penetration failure of acetic acid on the plaque removed from teeth.

This chapter aims to describe the multiple modalities of bacterial persistence in the host and how understanding these may lead to improvements in the development of improved treatment and prevention strategies facilitating the management of chronic head-and-neck infections.

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## 2. A role for biofilms in disease

Historically, otitis media (OM) became of lesser significance with the development of antibiotic treatments in the middle decades of the last century. However, the thought that this would eradicate the infections forever did not eventuate. Chronic infections such as long-standing otitis media with effusion (OME) and chronic suppurative otitis media (CSOM) are often recalcitrant to both systemic and topical antibiotic treatments. Despite the existence of excellent antibiotics for acute infections, challenges remain. Studies have shown that bacterial biofilm and the presence of intracellular bacteria may represent a bacterial strategy for preservation to avoid eradication by antibiotics, either systemically or topically, or by the host immune system.

Since the 1970's, biofilm research, both in the industrial and health settings, was brought into the public and research domains by the late William Costerton from the Centre for Biofilm Engineering in Montana, USA. Subsequently, biofilms have been implicated in more than 80% of all human microbial infections,[3] including those both natural and device-related. These include urinary tract infections, cystic fibrosis, periodontitis and device-related infections such as those of prosthetic heart valves, central venous catheters and contact lenses.[4] As human tissues are homeostatic and rich in simple nutrients, they represent attractive surfaces for biofilms.[5] It is increasingly apparent that biofilm characteristics resemble many chronic and/or recurrent infections yet to have their pathogenesis satisfactorily defined.[6] Biofilms on mucosal surfaces are physiologically distinct from those formed on inert surfaces and are modulated by host immune responses, with host cells and proteins contributing to the composition of the matrix.[7]

As bacteria and associated toxins are retained in the biofilm matrix, masking bacterial antigens and preventing a host inflammatory response, initial biofilm development may occur with no clinical signs of infection.[8-10] Increased antibiotic resistance, decreased production of virulence factors and diminished neutrophil activity facilitate bacterial growth for extended periods.[11] Bacteria forming biofilm are able to silently and persistently prolong infection, acting as a reservoir so that when environment permits and host defense mechanisms fail to contain them, planktonic bacteria can be dispersed.[8] It is these planktonic or aggregates of bacteria that cause acute clinical infections, and against which the antibiotics may be effective until such time as emboli or planktonic bacteria are dispersed again.[5,8,12-14] It is the eradication of the planktonic bacteria and subsequent lack of ability to culture bacteria which has led clinicians and microbiologists to assume the

infection is cleared, however, through the use of PCR technology, bacteria have been demonstrated indicating the underlying infection reservoir remains.[5,8] Acute clinical disease may recur weeks or months later making surgical removal of tissue or removal and replacement of medical device necessary, if possible,[5,12,13] or long-term antibiotic treatment, as seen in patients with cystic fibrosis (CF).[5]

## 3. Bacterial biofilms in otitis media

Historically it was thought that most OM was the result of sterile inflammatory processes, as there was no direct evidence of biofilms in human ear disease and many of the effusions were sterile on culture. The only evidence of biofilms in OM was in the chinchilla model and this was demonstrated using imaging methodologies.[15] Extrapolation of this evidence from the chinchilla model to other OM sequelae, including recurrent acute otitis media (rAOM) and OME, raised the hypothesis of bacterial biofilms causing chronic middle ear effusion.[16]

The use of non-specific imaging techniques, including scanning electron microscopy and LIVE/DEAD® staining combined with confocal laser scanning microscopy studies, have demonstrated bacterial biofilm in the ears of children. We first demonstrated biofilm on the middle ear biopsy of a child suffering from CSOM in 2005 (Fig. 1).[17] Then, these biofilms were demonstrated in a group of children with rAOM and chronic OME by Hall-Stoodley *et al.*,[18] *S. aureus* biofilm was shown on the biopsies of eight out of ten adults with CSOM[19] and six of ten middle ear biopsies from adults with CSOM, using LIVE/DEAD® staining and SEM.[20] Another more recent study by Saunders *et al.*, used scanning electron microscopy (SEM) to demonstrate that a low proportion of patients with cholesteatoma (three of five) and only one of seven patients with CSOM had any evidence of biofilm.[21] These studies were limited and bacterial species were only identified in a subset of the children. One study in Greenland found evidence of biofilm in smears of the middle ear effusion from children with CSOM, but no evidence in children with chronic OME.[19] To date, most reports (except Hall-Stoodley *et al.*[18]) have limited analysis to single organisms in OM biofilm, while multispecies biofilms are often observed in other disease processes and have significant clinical implications.

Biofilms have also been demonstrated on the adenoids of otitis prone children using SEM,[22] using LIVE/DEAD® viability staining[23,24] and using species-specific fluorescent *in-situ* hybridization (FISH).[25] Children with rAOM have more extensive biofilm coverage than those with OME (97.6%

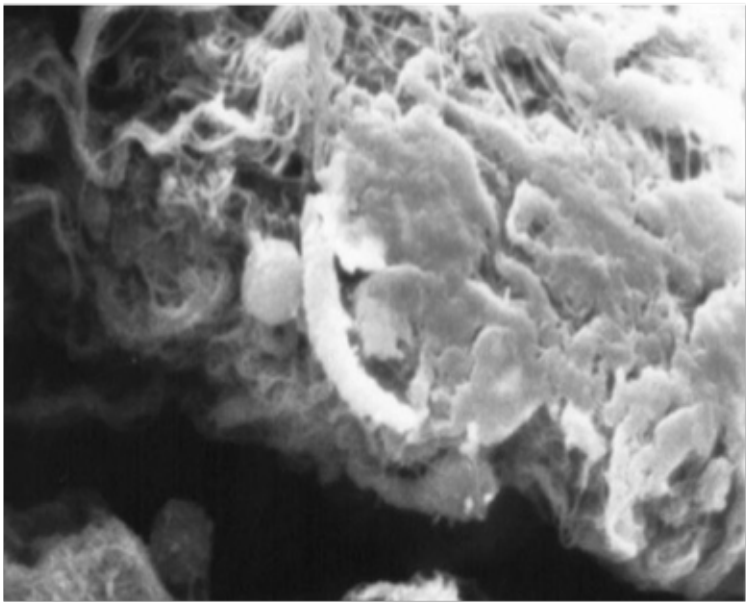

Fig. 1. Scanning electron micrographs of a middle ear mucosal biopsy taken from a child with CSOM, demonstrating a multispecies biofilm.

verses 27.7%).[22] FISH has shown that the biofilms present contain OM pathogens and are frequently polymicrobial.[25] Bacterial clusters were seen to be evident within the host epithelial cells.[25] It is thought that it is the removal of this reservoir that leads to the improved resolution and decrease in AOM recurrence following adenoidectomy.[25]

## 4. Intracellular infection – an additional mechanism of persistence

An additional mechanism for persistent and recurrent bacterial infection in children with chronic OM may be the persistence of intracellular infection by OM pathogens. When they become intracellular, microbes can evade both host immune responses and many antimicrobial therapies, which do not penetrate the cell membrane well. Bacteria are thought to be taken up by cells for three main reasons, including: (1) as an innate immune mechanism leading to bacterial killing; (2) initiated by bacteria as an initial step towards tissue invasion; and (3) internalization representing a bacterial mechanism to subvert host defenses, providing the bacteria with a protective niche.[26,27] It is this third idea that we believe may be important in pathogen's intracellular persistence in OM. Viruses, bacteria and parasites alike are all able to use intracellular infection to escape host immune responses and persist in the body. It is likely that a balance of intracellular infection and

biofilm formation are responsible for the persistence and recurrence of ear disease in a susceptible group of children. Whilst efforts are being exerted into the treatment of biofilm in OM, we believe that intracellular persistence should also be considered. Bacterial infection of human mucosal or immune cells can occur in several ways including through the exploitation of host immune mechanisms or pathways essential to the cell's survival and activity.

### 4.1. Intracellular infection in mucosal diseases

There are broad ranges of pathogens which exploit an intracellular environment for persistence in mucosal infections, including *Mycobacterium tuberculosis* in lung infections, *Streptococcus pyogenes* in tonsillitis, Helicobacter pylori in chronic gastric infection, *Escherichia coli* in recurrent urinary tract infections and *Salmonella* in gastrointestinal infections.[27-32] Bacteria may become intracellular through a variety of mechanisms including caveolae uptake and autophagy with the modification of compartments following ingestion to evade innate responses.[28,32] Little evidence of intracellular bacterial persistence exists in chronic and recurrent mucosal diseases such as OM. However, *in vivo* and *in vitro* data suggests the otopathogens are capable of invading and surviving within cells, though mechanisms for this are not well characterized. The ability of bacteria to invade epithelial cells may be a useful strategy for bacteria to colonize the respiratory tract and avoid extracellular immune recognition.[33]

### 4.2. Intracellular infection and the otopathogens

*Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are all well-recognized pathogens associated with recurrent and persistent OM and they are all capable of invading and persisting within cells. Intracellular persistence not only allows immune evasion from sIgA, bactericidal IgG and defensins,[34] but it also shields the bacteria from the action of antibiotics and provides a means by which clearance is avoided.[35,36] Once inside the epithelial cells, bacteria are also protected against certain classes of antibiotics including the β-lactam antibiotics[36,37] commonly used in the treatment of OM.

Intracellular persistence of NTHi has been demonstrated in many chronic conditions such as chronic obstructive pulmonary disease,[38] in adenoids in rAOM,[39-41] chronic bronchitis and cystic fibrosis.[42] NTHi is capable of invading and persisting within the macrophage-like cells of adenoids in children with rAOM[39-41] and epithelial cell lines and leukocytes *in vitro*.[36]

More recently, NTHi has been demonstrated to invade and form biofilm communities intracellularly in the middle ears of the chinchilla model of OM when subjected to nutrient limited conditions.[43] NTHi have been shown to gain entry through interaction of the phosphorocholine residue on the lipo-oligosaccharide with human platelet activating factor receptor (PAFR).[42] Entry to the cells in PAFR-linked pinocytic vacuoles is thought to limit the inflammatory response, which is likely essential for NTHi to be a successful commensal.[35] NTHi can also invade respiratory epithelial cells and trancytose to the sub-epithelial compartment leading to microcolony formation or end up in macrophage-like cells.[35]

*M. catarrhalis*, like non-typeable *H. influenzae* can colonize and exist either intracellularly or extracellularly in the human respiratory tract.[33] They have also been shown to be present in the crypts, intraepithelially, subepithelially and intracellularly in the adenoid, co-localizing with macrophages and B cells in lymphoid follicles.[34] While this has previously been considered an extracellular pathogen, *M. catarrhalis* has been shown to invade respiratory epithelial cells *in vitro* and appears to actively induce this process[34] by a macropinocytosis-like mechanism.[33]

*S. aureus* has been seen to internalize into non-professional phagocytes in a few patients with rhinosinusitis and in the tonsillar cells of children with recurrent tonsillitis.[44] Similarly, *P. aeruginosa* has been shown to enter epithelial cells *in vitro*, developing pod-like clusters with biofilm characteristics.[45] Intracellular bacteria were also shown to be insensitive to growth inhibition or killing by antibiotics capable of intraepithelial cell penetration.[45] This antibiotic resistance is thought to be due to the bacteria adopting the biofilm phenotype and this may have implications when considering antibiotic treatment, even when they are capable of accumulating within cells.[45] These findings suggest *P. aeruginosa* are able to persist in airway epithelial cells and this may contribute to the development of chronic infection.[45]

Variation in the ability of different *S. pneumoniae* strains to invade and persist intracellularly as well as their biofilm forming ability is apparent. *S. pneumoniae* strains isolated from the nasopharynx are seen to produce less capsule and are more prone to biofilm formation than isolates from the bloodstream.[46] *S. pneumoniae* in in-vitro systems can penetrate and persist in respiratory cell lines, although this ability is reduced in the presence of the polysaccharide capsule.[47] The polysaccharide capsule represents an important virulence factor in this bacterium, protecting against phagocytosis. It has been demonstrated in an *in-vitro* system that during contact with and invasion of epithelial cells the *S. pneumoniae* is

devoid of its capsular polysaccharide and using electron microscopy, a reduction in capsular material is observed.[48] It is thought that, while encapsulated, *S. pneumoniae* are poor at attaching and invading endothelial cells, inflammatory activation of endothelial cells induces expression of a choline binding platelet aggregating factor (PAF) receptor to which the bacteria bind and become internalized.[46]

### 4.3. Intracellular infection and otitis media

Despite the knowledge that otopathogenic bacteria are able to invade and survive intracellularly, these bacteria had not been located in the intracellular compartments of middle ear mucosal cells from patients with recurrent OM or chronic middle ear infections previously. Using transmission electron microscopy, our laboratory detected gram-positive bacteria in the epithelial cells of middle ear mucosa in 36% of 11 children (four); these were situated principally in the outside cytoplasmic portion of the surface epithelial cells and in particular, mucus secreting cells as well as the underlying connecting tissue stroma[49] (Fig. 2). In addition it was noted that coccal bacteria were presented in the mucus containing vacuoles and could be expelled into the middle ear fluid during mucus exocytosis. We postulate that this intracellular infection of the middle ear mucosal epithelial cells by gram-positive cocci in children with chronic OME may contribute to excessive mucus secretion seen in chronic OME as well as persistent inflammation.

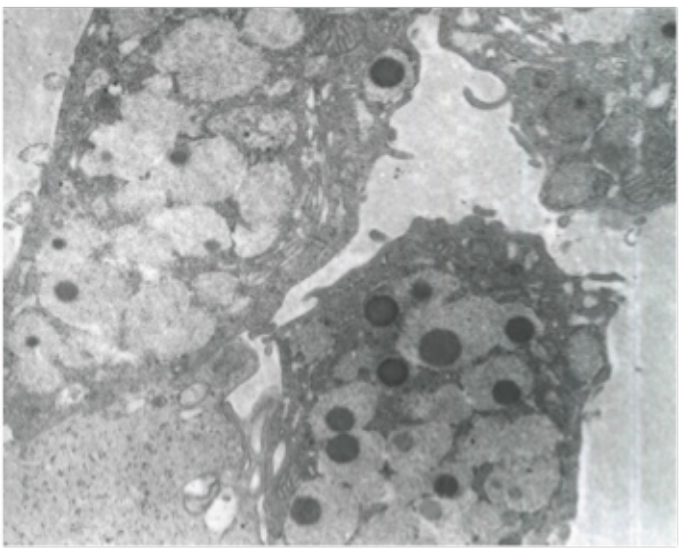

Fig. 2. Transmission electron micrograph of middle ear epithelial cells from a child with OME overwhelmed by cocci in mucus containing vacuoles.

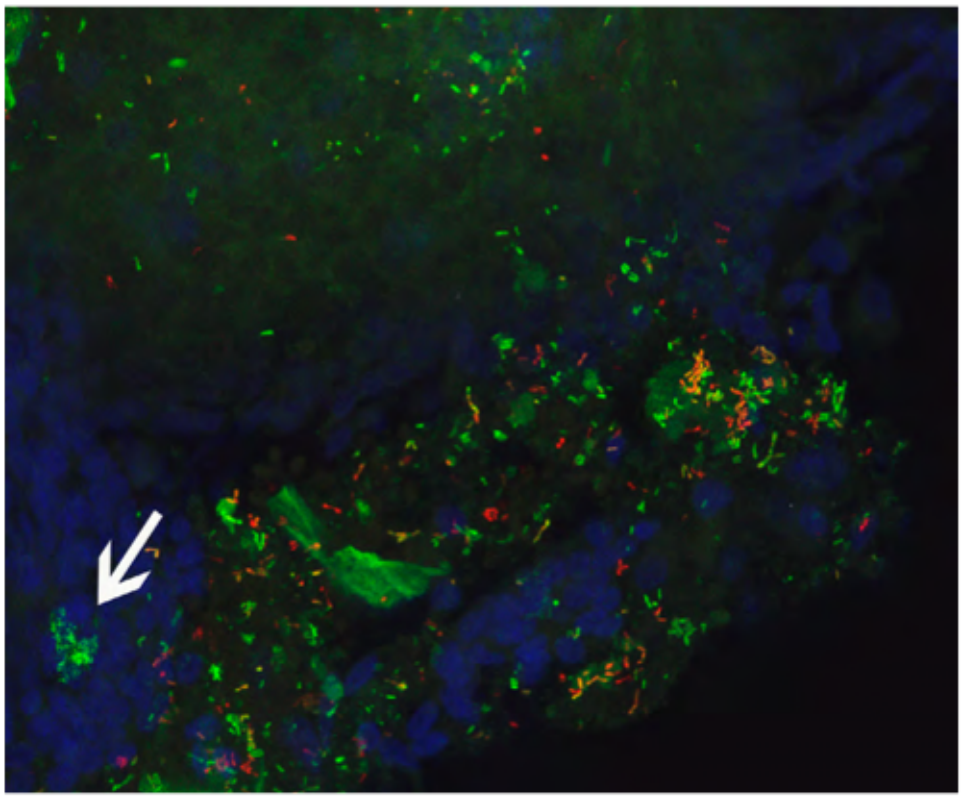

Fig. 3. Biofilm and intracellular infection in a mucosal biopsy from a child with chronic otitis media. The biopsy was labelled using Fluorescent in-situ hybridization for universal bacteria FISH - EUB (green), Streptococcus pneumoniae (red), nontypeable Haemophilus influenzae (grey) and Hoechst 33342 (Nuclei stain – blue) and imaged using confocal laser scanning microscopy. Extensive biofilm containing S. pneumoniae and other bacterial species can be observed over the middle ear mucosa as well as intracellular biofilm pods (arrow) of an unidentified bacterial species. Scale bar = 10 μm.

Following the early discovery of intracellular bacteria using non-specific electron microscopy techniques we established specialized imaging techniques in our laboratory which could be used to identify bacterial species in fully hydrated middle ear mucosal biopsies (Fig. 3). These allowed us to demonstrate that the intracellular biofilms are formed by known otopathogens and that when these bacteria are in this intracellular compartment, they consist of a single bacterial species.[50] This finding suggests that these bacteria invade and proliferate within the cells similar to what is observed by the uropathogenic *E. coli* rather than being taken up phagocytically from the multi-species biofilms. This discovery brings up the question of whether or not the intracellular bacteria precede the development of bacterial biofilm or whether the bacterial biofilm is a source of the bacteria which invade the middle ear mucosal lining.

## 5. Extracellular DNA and neutrophil involvement in mucosal disease NETs and/or necrotic neutrophils

Studies in CF indicate that bacterial persistence is also related to the presence of necrotic neutrophils. Animal and *in-vitro* studies have indicated that the presence of DNA, both host- and bacterially derived, may play a role in biofilm development and bacterial persistence in OM.[51-53] It was unclear if the host DNA is present due to active immune mechanisms or due to necrotic neutrophil presence.

Neutrophil extracellular traps (NETs) are formed by activated neutrophils and consist of a DNA backbone embedded with antimicrobial peptides and enzymes including histones and elastase.[51,54] These antimicrobial components interact with fungi and both Gram positive and negative bacteria, each showing different levels of resistance.[54] Mast cells have the ability to form similar structures and have been demonstrated in the OM inflammatory response.[51] NETs are thought to be important in confining infections to a local site,[54,55] however, some pathogens are able to resist NET killing.[54,56] The main differences used to distinguish between the NETs and necrotic neutrophils are that NETs lack membranes, membrane proteins and cytoplasmic markers (i.e., annexin I, actin and tubulin).[57,54]

Neutrophils enhance *P. aeruginosa* biofilm development in children with CF through formation of a polymer comprised of DNA and actin.[58] Biofilms in CF are not typical as they are not always surface associated, rather being small bacterial aggregates in highly viscous fluid; this is also true of many other clinical biofilm diseases.[59] In the CF lung, DNA is from necrotic cells where a loss of cellular integrity results in an uncoordinated release of nuclear and cytoplasmic contents, rather than active NET production or apoptosis.[59] F-actin (not DNA) enhanced biofilm development and was the site of *P. aeruginosa* attachment.[58] Extracellular DNA from *P. aeruginosa* biofilms play a critical role in triggering neutrophil pro-inflammatory responses and could exacerbate the viscosity of sputum by triggering NETs release.[60]

Pathogenic bacteria involved in OM, *S. pneumoniae*, non-typeable *H. influenzae* and *P. aeruginosa* are able to evade this mechanism of neutrophil killing.[61] Non-typeable *H. influenzae* are not all killed by phagocytosis and can survive extracellular neutrophil killing by means of lipo-oligosaccharide moieties promoting biofilm formation.[51] *S. pneumoniae* have also been shown to be resistant to neutrophil killing by capsule production,[46] alanylation of lipoteichoic acids in cell wall and by endonuclease production which may degrade DNA.[53] In the chinchilla model of OM, viable multicellular biofilm communities of both NTHi and *S. pneumoniae*

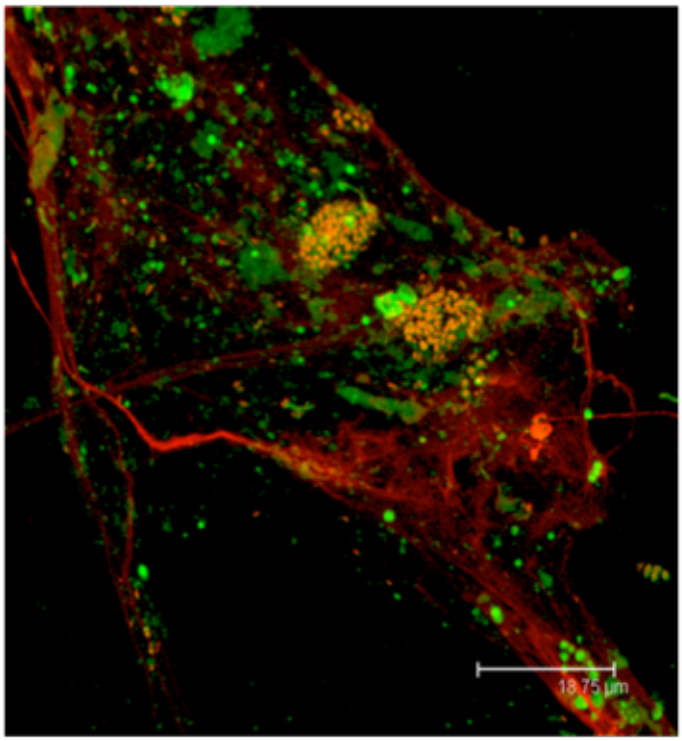


Fig. 4. Bacterial biofilm microcolonies contained within neutrophil extracellular traps from the middle ear from a child with rAOM. Host derived DNA strands are shown with associated microcolonies of bacteria.

were found within DNA lattices, it is believed that NETs play a primary role in facilitating the establishment of a stable biofilm community.[51,53]

We first described the presence of NETs in the middle ear effusions of children with a history of rAOM who were undergoing surgery for ventilation tube insertion[62] (Fig. 4). While the role of this novel finding is yet to be fully described we were able to demonstrate that the viscosity of the middle ear effusion is likely to be due largely to the presence of extensive DNA in the fluid. Furthermore, we were able to demonstrate that most of the DNA was neutrophil-derived and that it was released due to the active production of NETs rather than due to the presence of necrotic neutrophils.[62] The bacteria that we observed in these NETs consisted at least in part of the main described otopathogens and these were found within polymicrobial biofilms and were alive despite many of these children not having active infection and/or being on antibiotics at the time of the surgery.[62] *In vitro*, these NETs were seen to break down on application of the human recombinant Deoxyribonuclease I Dornase alfa (Pulmozyme®) and this now forms the basis of a clinical trial looking at the effectiveness of using this CF treatment in the middle ears at the time of ventilation

tube insertion to reduce associated complications and to reduce rates of reinsertion. We are also now exploring the use of this treatment as an adjunct to therapies for treating CSOM. We believe it may have the potential to dissolve persistent biofilms in osteal crevices in the mastoid and on the mucosa allowing for more effective treatment and subsequent disease resolution.

## 6. Clinical implications of our knowledge of biofilms and intracellular bacteria in otitis media

The application of what we now know regarding the involvement of both biofilm and intracellular infection in OM offers an explanation for the lack of effectiveness of many of the current treatment strategies, the recalcitrance of the infections and has major relevance clinically. Potential effects of persistent intracellular infection include the propagation of the middle ear inflammation, stimulation of mucus production into the middle ear whilst functioning as another modality to retain bacterial load for reinfection in addition to the biofilm covering the middle ear mucosa (Fig. 5). The question of which comes first, the intracellular bacteria or the bacterial biofilm, has not been elucidated, but the likelihood is that bacteria shed from the mucosal biofilm and invade the underlying mucosal cells, rather than excreted bacteria from the mucosa forming a mucosal biofilm.

Current treatment regimens for treating AOM with β-lactam antibiotics (including amoxicillin-clavulanate and cephalosporins) may be less effective in eradicating bacterial persistence and treating the underlying cause of these conditions, as they do not penetrate intracellularly. Antibiotics such as the macrolides, ketolides and fluoroquinolones, which penetrate and concentrate intracellularly may be more useful. A study conducted by Courter *et al.*, however, has indicated that more clinical failures are observed when children with AOM are treated with macrolides compared to those treated with amoxicillin and amoxicillin-clavulanate.[63] This difference may reflect the increased bacterial resistance to the macrolides when compared to the β-lactams.[63] This has also been observed in the Israeli studies where amoxicillin-clavulanate demonstrated a superior ability to eradicate bacterial pathogens from the middle ear fluid when compared to azithromycin.[64] These findings may reflect the differences in the target pathogens, the β-lactam antibiotics may still be more effective at treating the acute planktonic phase bacteria, while the macrolides may have a more important role in targeting underlying intracellular bacterial sequestration. This needs to be considered further though as some evidence from *in-vitro*

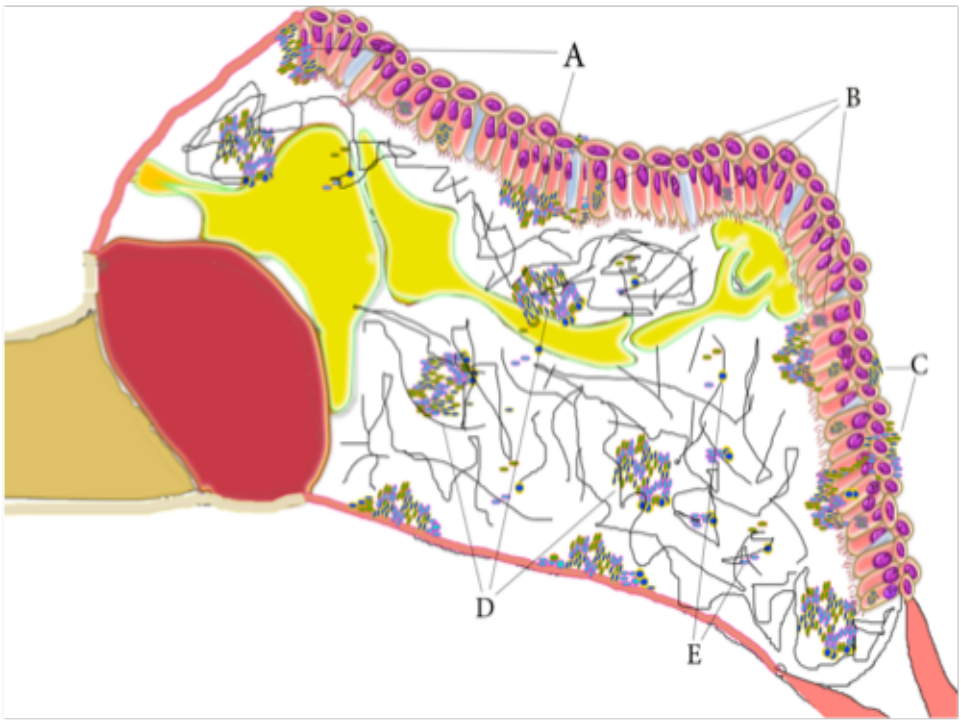


Fig. 5. Schematic diagram demonstrating the different types of bacterial persistence mechanisms within the middle ear. a: mucosally associated multi-species biofilm. b: single-species intracellular biofilm pods. c: intercellular biofilm formations extending into the basal layer of cells. d: multi-specie biofilms associated with neutrophil extracellular traps in viscous middle ear effusion. e: planktonic bacteria associated with neutrophil extracellular traps within the middle ear effusion.

studies into *P. aeruginosa* infection of an epithelial cell line suggests that bacteria within the cell adopt a biofilm phenotype and consequently are insensitive to antibiotic killing.[45] It may be important to consider using a combination of these antibiotics to improve treatment efficacy, though this would require clinical trials to determine.

The macrolide antibiotics may also affect nasopharyngeal carriage as was seen in a recent study by Morris *et al.*, where treatment of indigenous children with AOM with a single dose of azithromycin reduced nasal carriage of both *S. pneumoniae* and nontypeable *H. influenzae* when compared to children treated with amoxicillin for seven days.[65] It is known that in the nasopharynx of the Indigenous children that 36% of organisms exist in a non-cultureable state, detectable by PCR,[65] it is unclear if these are present in biofilm formation or intracellularly. It may be that these pathogens can persist in the nasopharynx intracellularly and that changing antibiotics may decrease pathogen carriage in the nasopharyngeal space and possibly those sequestered similarly in the middle ear. Given that pathogens are present both intracellularly and in biofilm it is likely effective treatment of

this condition will require combinations of antibiotics in conjunction with other treatments to target both mechanisms of persistence.

We believe that a combination of biofilm on the middle ear mucosa, intracellular invasion and those biofilms present in the extremely viscous NETs within the middle ear fluid are responsible for the recalcitrance and persistence of middle ear infections. This is likely to be particularly relevant in those children who suffer from rAOM superimposed on an underlying OME. The severity of this subtype of rAOM often necessitates surgical intervention, usually the insertion of ventilation tubes, while rAOM associated with complete resolution of middle ear effusion between episodes is less likely to require surgical intervention. While we are unsure of how this works the mode of action of ventilation tubes to counter biofilm and intracellular cocci may be through reduction of the bacterial load in the middle ear fluid, the increased oxygenation of the remaining biofilm, leading to decreased nutrients, collapse of the hydrated structure and thus a reduction in the fidelity of the biofilm. In a similar role, adenoidectomy reduces the bacterial load in the nasopharynx which has been conclusively shown to reduce the rate of subsequent grommet insertion for OME and rAOM by around 50%.[66,67]

Despite the widespread belief of the benefits of ventilation tubes in a number of children, recurrent or persistent otorrhoea may occur following the surgery. This has lead to various treatments prior to the final consideration of removing the grommet, including betadine ear toilets and topical fluoroquinolone therapy. Preventative measures have included the development of grommets with treated surfaces aimed in repelling or reducing the biofilm infection on the grommet's surface. Silver-impregnated tubes and Teflon tubes have been trialed with modest success.[68,69] In one to two percent of cases, conservative treatment of grommet-associated otorrhoea is unsuccessful and the otorrhoea persists for months or even years. Removal of the tube may be considered in those cases where localized causes have been excluded. For these patients an immunologic workup to rule out subclass immunoglobulin deficiency and mannose binding lectin deficiency are warranted.

Removal of middle ear fluid at grommet insertion is also likely to reduce the biofilm load in the middle ear of children with rAOM or OME. Examining this has led to the innovative use of Dornase alfa, a deoxyribonuclease, to reduce the residual biofilm associated with DNA in the middle ear and mastoid, specifically that sequestrated in bone crypts. The Dornase alfa works to digest the strands of neutrophil derived DNA remaining after suction of middle ear fluid via myringotomy incisions. It is impossible to

remove all the middle ear fluid, specifically that from the mastoid, during a myringotomy and grommet procedure. Whether the Dornase alfa will dissolve the biofilm in a mastoid region is uncertain and is under current investigation.

## 7. Ventilation tube insertion with or without adenoidectomy?

Most cases of OME resolve spontaneously and antibiotic therapy is seen to have limited efficacy.[70] When OME persists for four months or more ventilation tubes may be inserted depending on hearing status, a child's developmental risk, and/or structural damage to tympanic membrane or middle ear.[70] For children older than one year who have had previous ventilation tube insertion, adjunct adenoidectomy may be appropriate.[70] If rAOM is superimposed on non-resolving OME, the suggested management involves ventilation tube insertion as primary treatment.[71] While this is seen to be effective short term, almost one third of children will require reinsertion of ventilation tubes for the recurrence of OME and subsequent hearing loss or for rAOM.[72]

Debate exists as to whether adenoidectomy in addition to insertion of ventilation tubes should be used as a primary treatment in children with rAOM and/or chronic OME. It is unclear if adenoidectomy works due to removal of a mechanical obstruction of the Eustachian tube or the removal of a reservoir of pathogenic organisms.[67,73] Only one study has considered the change in nasopharyngeal carriage following adenoidectomy with ventilation tube insertion, this showed carriage of *H. influenzae* or *M. catarrhalis* was not affected, however, *S. pneumoniae* carriage was increased.[74] It is known that adenoids can harbor many of the known otopathogens and adenoidectomy often improves disease resolution and decreases AOM recurrence.[22] In the literature, one study saw no difference in time spent with effusion or OM, when comparing children with chronic OME who had undergone ventilation tube insertion alone with those who had undergone adenoidectomy and ventilation tube insertion during the [36] months of follow-up.[73] Other randomized controlled trials have indicated that adenoidectomy is more efficacious for chronic OME treatment in older children, while ventilation tube insertion is best advised for younger children.[73] A large population-based study was conducted in Western Australia, including 51,373 children under ten years of age undergoing ventilation tube insertion between 1981 and 2004.[67] Having an adenoidectomy or adenotonsillectomy at the time of first or subsequent ventilation

tube insertion was associated with a 57% and 28% (respectively) reduced risk of further ventilation tube insertion surgery compared to ventilation tube insertion alone.[67] As approximately one third of children will have more than one ventilation tube insertion procedure, the low complications rates from adenoidectomy and the short hospital stay may indicate this as an adjunct with ventilation tube insertion and could represent a potentially cost effective first-line management option for chronic OME.[67] Contradictory to the high rates of rAOM and OME in Indigenous children, there appears to be a reluctance to perform additional pharyngeal surgery at time of ventilation tube insertion, it is unclear if this is due to difference in disease pathogenesis or if this is a result of access issues.[67] In the population-based study in Western Australia, many children had their adenoids out in conjunction with the first or second insertion of ventilation tubes with chronic OME as the sole indication; this may reflect the growing awareness in targeting the underlying etiology.[67] Alternative management strategies for decreasing the need for reinsertion of ventilation tubes in children with rAOM and chronic OME are needed.

## 8. Treating chronic suppurative OM – why wash ears instead of using tissue spears?

CSOM in the Australian Aboriginal population remains a big problem with rates between 15-24%,[65,75] well above the WHO level of 4% indicating a massive public health problem.[76] Treatment of CSOM in Australia differs between states, particularly between Western Australia and the Northern Territory. Recommended treatment includes management with aural toileting, ototopical medications and possibly systemic antibiotics.[77] In Western Australian Aboriginal children experiencing CSOM, gentle syringing with a 0.5% povidone-iodine solution, followed by topical ciprofloxacin has proven effective at community level for achieving up to 64% cure rate for CSOM,[78] these rates are up to threefold that observed in children treated with previously recommended aminoglycoside treatments.[79] Rates are similar to those observed when treating CSOM with aural cleansing and irrigation in a Korean population.[80]

Comparisons of topical Ciprofloxacin and Sofradex (framycetin-gramicidin-dexamethasone) in indigenous children in the Northern Territory, showed low rates of improvement or cure of persistent CSOM regardless of choice of therapy.[81] Ear discharge failed to resolve in 70% of children and the severity of discharge failed to improve in more than 50% of children.[81]

Of the one third of children achieving dry ears by the end of intervention period, many relapsed before the end of the school year demonstrating the low cure rate in this population.[81] Rates are very different between states despite the use of the same topical Ciprofloxacin. The major difference between states is the ear cleaning prior to instilling the topical eardrops, in the Northern Territory dry mopping with tissue spears is practiced[81] as opposed to ear toileting in Western Australia. This may account for some differences in success rates between the children if the perforation is blocked and topical antibiotics are unable to reach the infection site.[82] Alternatively, it may be that as is observed in orthopedic biofilm infections, irrigation with any solution is better than no irrigation to reduce microbial numbers; however irrigation with a surfactant is superior to saline or antibiotics alone.[83] These findings are likely due to the effects of mechanical disruption of the biofilms when using aural toileting or irrigation. This likely reduces the biofilm load and fidelity allowing antibiotics and host immune responses to have enhanced efficacies.

Biofilm presence and intracellular infection with known otopathogens on the middle ear mucosa of indigenous children with CSOM may help to explain different treatment efficacies between Ciprofloxacin (a fluoroquinolone) and Framycetin-gramicidin-dexamethasone (Sofradex – an aminoglycoside). A study by Couzos et al. demonstrated ciprofloxacin to be more effective at achieving a community level cure for CSOM than the topical aminoglycoside.[78] These differences may be due to the ability of the fluoroquinolones to penetrate into the cells and target sequestered pathogens. The comparative ineffectiveness of the topical aminoglycoside may be related to the associated induction of biofilm formation in bacteria.[84] While the induction of biofilm formation by aminoglycosides has been demonstrated in *P. aeruginosa* this effect has not considered in other otopathogens. The ability of antibiotics to induce biofilm formation in bacterial targets should be considered when treating chronic or recurrent disease processes. While these differences between treatments with ciprofloxacin and Sofradex were not observed in a study conducted by Leach *et al.*[81] this may reflect the differences in ear cleaning prior to topical treatments. Studies in the chinchilla OM model have indicated that lavage of the middle ear space results in the removal of biofilm from the mucosal surface.[85] It is possible that the aural toileting of the children's ears prior to treatment in the Couzos study[78] reduced the biofilm load and allowed optimal penetration and action of the antibiotics. Topical aminoglycoside eardrops are no longer advised for use where there is a perforation of

the tympanic membrane due to ototoxicity issues.[86] Studies comparing aural toileting plus ciprofloxacin with dry mopping and ciprofloxacin are the subject of a national study in Australia at present.

## 9. Summary

OM is the most common disease in childhood. Almost all children will suffer at least one AOM episode, however a subset of children will suffer recurrent episodes of AOM and/or chronic middle ear effusion. Indigenous Australians are a particularly high-risk group experiencing a disproportionate rate of middle ear disease including CSOM and often suffer more serious complications. While OM is mainly attributed to bacterial infections, antimicrobials appear to have limited efficacy in treatment of chronic OME and preventing the recurrence of both AOM and OME. The mechanisms of bacterial persistence in the middle ear are not well documented though bacterial biofilm is thought to be involved. Limited studies exist demonstrating the presence of otopathogens in biofilm formation or in exploring alternative mechanisms of persistence in the middle ear and no data exists looking at the high-risk Indigenous Australian population.

Future treatments will need to involve combination treatments. These will likely include the use of antimicrobials capable of targeting both intracellular and extracellular pathogens, mechanisms to interfere with bacterial communication necessary for biofilm formation and maintenance[87,88] and novel therapies nontoxic to the mucosa that disrupt the biofilms directly.[87] Macrolides or quinolones may be useful as part of these combinations and warrant further investigations as they target not only intracellular pathogens, but are demonstrated to inhibit quorum sensing and reduce the host inflammatory response.[89] However, changing any treatments to target these persistence mechanisms will need to be closely monitored as less than complete biofilm eradication may stimulate the proliferation of the biofilm acting to increase mucosal inflammation and clinical disease severity.[87]

## Executive summary

1. Bacterial persistence in otitis media may take the form of bacterial biofilms or intracellular infection and exploit the release of neutrophil extracellular traps (NETs) in the middle ear fluid. In this chapter we have reviewed these persistence modalities and described how these mechanisms protect bacteria from the human's immune system and antibiotics

   Bacterial biofilm
   - masks bacterial antigens and toxins;
   - decreases production of virulence factors;
   - reduces host inflammatory response;
   - reduces neutrophil activity;
   - promotes antibiotic resistance.

   Intracellular infection
   - uses internalization of bacteria to provide a protective niche;
   - promotes evasion of host immune responses;
   - decreases antibiotic activity.

   Neutrophil extracellular traps
   - host derived DNA increases viscosity of middle ear fluid;
   - enhances establishment of biofilm;
   - enables persistence of middle ear fluid and the associated biofilms within the middle ear.

2. Bacterial persistence mechanism and the hijacking of the host response often leads to treatment failure. Thus, new and improved strategies to treat and prevent chronic infections are desperately needed. Here we described the innovative use of Dornase alfa to disrupt biofilm and NETs in middle ear fluid and using different antibiotics in treating these conditions to target intracellular bacteria including macrolides and fluoroquinolones.

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ORIGINAL RESEARCH—PEDIATRIC OTOLARYNGOLOGY

# The role of chronic infection in children with otitis media with effusion: Evidence for intracellular persistence of bacteria

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**OBJECTIVE:** Demonstrate mucosal bacterial infection in children with otitis media with effusion (OME).

**STUDY DESIGN AND SETTING:** Middle ear mucosal biopsies from 11 children with OME were examined for bacteria utilizing transmission electron microscopy. This was correlated with standard culture and polymerase chain reaction (PCR) of middle ear effusions.

**RESULTS:** Gram-positive coccal bacteria were demonstrated in middle ear mucosal epithelial cells of 4 of 11 (36%) children. Morphological appearance of bacteria and detection of pneumolysin DNA by PCR in middle ear fluid suggests a role for persistent intracellular infection with *Streptococcus pneumoniae* and other gram-positive cocci in some cases of OME.

**CONCLUSION:** Intracellular bacterial infection of middle ear mucosal epithelial cells in children with OME may be an important mechanism for bacterial persistence, and contribute to inflammation and mucus production in the pathogenesis of this condition.

**SIGNIFICANCE:** Persistent intracellular infection is a novel paradigm for OME pathogenesis in children and may influence antibiotic effectiveness in treatment of this condition.

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Otitis media (OM) in all its manifestations is a major health problem for children worldwide. It is the most common reason for children to see a doctor and be prescribed antibiotics.[1] Persistent otitis media with effusion (OME), which often occurs after acute OM (AOM), may also arise without preceding symptomatic infection. OME is the most common cause of hearing loss in children, and may contribute to language and learning difficulties if not treated. Hospitalization for insertion of ventilation tubes is the most common pediatric surgical procedure in many industrialized countries.[2]

In children the most common organisms found to cause AOM are *Streptococcus pneumoniae* (SP), nontypeable *Haemophilus influenzae* (NTHI), and *Moraxella catarrhalis*.[3] The pathophysiologic mechanisms resulting in the persistence of OME are not well understood. While dysfunction of the Eustachian tube is an important factor, there is increasing interest in the role of persistent bacterial infection and associated chronic inflammation as the cause of chronic OME. Commonly used antibiotics have had limited impact in treating this condition,[4] and when analyzed by bacterial culture, 40 percent to 60 percent of middle ear effusions were considered to be sterile in chronic OME.[5,6] However through the use of PCR, studies have identified the presence of metabolically active *Haemophilus influenzae* (Hi) bacteria in 31 percent of culture-negative effusions.[6] Although SP is a common cause of AOM, it is rarely cultured from children with chronic OME. In a recent Finnish study,[7] children recovering from acute pneumococcal OM had evidence of persistent infection with pneumolysin DNA in the middle ear fluid (MEF) remaining detectable by PCR months after the original infection despite being culture negative.

The presence of culture-negative but bacterial DNA–positive by PCR-positive middle ear effusion suggests that the bacteria are not in the free-swimming or planktonic state typically associated with acute bacterial infections.[8] Bacterial biofilm has been hypothesized as an explanation of this phenomenon. Biofilm formation is a universal strategy used by bacteria to aid survival in the environment and optimization of available nutrients in which colonies form 3-dimensional structures surrounded by an exopolysaccharide matrix.[8] Biofilms offer bacteria many advantages over planktonic bacteria, especially in their increased antimicrobial resistance and host immune response evasion.[9] The existence of bacterial biofilm on middle ear mucosa was demonstrated in animal models by using electron microscopy[10] and also from ventilation tubes (VTs) removed from children with recurrent AOM.[10] Recently, Hall-Stoodley et al[11] described pneumococcal biofilm in a high percentage of children with OME, while our group[12] has identified pseudomonal biofilm in an aboriginal child with chronic suppurative otitis media (CSOM) using scanning electron microscopy.

An additional explanation for persistent infection in

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OME may be the intracellular persistence of bacteria in the middle ear mucosa. This intracellular location of the bacteria has been described for Hi in tonsillar and adenoidal tissue[13] but not in association with OME. Although the pneumococcus is generally an extracellular pathogen, in vitro it can survive inside human respiratory epithelial cells.[14] We therefore performed a pilot study to look for evidence of bacterial biofilm and intracellular infection in middle ear mucosal biopsies from 11 children undergoing insertion of VTs for persistent OME.

## METHODS

### Study Population

Children undergoing VT insertion at Perth metropolitan hospitals for persistent OME (greater than 3 months duration) were recruited after informed written parental consent was obtained, and basic demographic information, recent antibiotic use, and history of ear disease were documented. This study was approved by the Ethics Committee of Princess Margaret Hospital for Children, Perth, Western Australia.

### Sample Collection and Preparation

When possible, middle ear effusions were collected from children at the time of tympanotomy by using a sterile syringe and drawing up a needle through incisions made in the tympanic membrane. Samples were placed into sterile jars and placed immediately on ice for transport. An aliquot of MEF was cultured by using appropriate selective media for pneumococcus and other OM pathogens. Any remaining MEF was processed for PCR as described below.

Middle ear biopsies of less than 2 mm in diameter were taken of the mucosa through the incision for the VTs with the use of microcup forceps and were immersed immediately in 2.5 percent glutaraldehyde in phosphate buffer (pH 7.4) until processing.

### Pneumolysin PCR

Pneumolysin is a protein unique to *S. pneumoniae* and pneumolysin PCR has been established for detection of pneumococcal infection in both invasive infection[15] and otitis media.[6,11] Real-time PCR to detect the pneumolysin gene of SP was performed on DNA extracted from the MEF as previously described by van Haeften et al.[15] In brief, nucleic acid was extracted from middle ear effusions with the use of Roche Total Nucleic Acid extraction kits (Roche Molecular Biochemical, Mannheim, Germany).

Pneumolysin PCR was conducted on all available specimens with the use of the primers described earlier on the LightCyler instrument using the LightCycler FastStart DNA Master$^{plus}$ Hybridization Probes Kit (Roche Diagnostics, Mannheim, Germany). All runs included a negative water control and a calibrator/positive control, and sample material was handled according to NCCLS guideline (NCCLS, 1995) to minimize potential for contamination. The calibrator/positive control was a $2 \times 10^3$ copies/μl dilution of the plasmid as prepared for the standard curve. Data were analyzed by using the F2/F1 mode with proportional baseline adjustment and fit points calculation method.

### Transmission Electron Microscopy (TEM)

Middle ear biopsies were immediately immersed in 2.5 percent glutaraldehyde fixative in phosphate buffer (pH 7.4) for a minimum of 24 hours, until processed. Biopsies underwent two 15-minute washes in phosphate buffer before being secondarily fixed for 1 hour in 1 percent aqueous osmium tetroxide. Further processing involved successive 1-hour intervals of dehydration in progressive concentrations of ethanol (25%, 50%, 70%, 95%, and 100%). Following ethanol dehydration biopsies underwent two 15-minute immersions in propylene oxide intermediate solvent before being embedded in araldite (Fluka Chemika AG, Zurich, Switzerland) and hardened in an oven at 70°C.

Blocks were sectioned by ultramicrotomy. Preliminary sections of 1-μm thickness were taken and stained for 1 minute with toluidine blue, and then examined for the presence of bacteria or epithelial cells. When an area of interest was reached, biopsies were further sectioned at 90-nm thickness and mounted on 200-mesh copper grids before double staining with uranyl acetate for 15 minutes and lead citrate for 3 minutes. A TEM (Philips CM10, Eindhoven, The Netherlands) was employed to examine the grids. Micrographs were recorded at the desired magnification on Kodak 4489 electron microscopy film (KODAK, Rochester, NY) and printed for review and reporting.

## RESULTS

A total of 17 middle ear biopsies were taken from 11 children (6 males, 5 females; aged 2-8.5 years). All children were well at the time of surgery and did not have signs of acute OM. Bacterial biofilm was not detected in any of the biopsies by TEM. The presence of bacteria was found in the middle ear mucosal epithelial cells in 4 of the 11 (36%) children but was found bilaterally in only 1 child. Bacteria appeared to be gram-positive coccal organisms, which were mostly present in the outer apical cytoplasmic portion of surface epithelial cells, particularly mucus-secreting cells, and were also found in the underlying connective tissue and stroma (Fig 1A). Coccal bacteria were generally present in mucus-containing vacuoles with evidence of expulsion into the MEF during mucus exocytosis (Fig 1B).

Middle ear effusions were collected from 10 of 17 ears and cultured for all relevant bacterial species. In only 1 sample a recognized pathogen (Hi) was cultured. The remainder showed no growth or grew skin contaminants (coagulase-negative *Staphylococci* or mixed skin flora). Pneumolysin PCR could be conducted on 8 samples; 2 were pneumolysin DNA–positive, indicating the presence of SP,

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and 1 of these had intracellular coccal bacteria present on EM (Fig 1A and B).

## DISCUSSION

We have demonstrated for the first time that intracellular infection of middle ear mucosa epithelial cells by gram-positive cocci occurs in children with chronic OME. While it is possible that this may represent a normal variant of upper respiratory tract bacterial carriage, the detection of a known OM pathogen, *S. pneumoniae*, makes this hypothesis less likely. Further studies are planned to examine the middle ear mucosa of children without OME undergoing cochlear implants to confirm the pathological importance of intracellular infection. The morphological appearance and detection of pneumolysin DNA in the middle ear fluid testing of 2 children suggest that SP can persist intracellularly in the middle ear mucosa of at least a subset of children with chronic OME. More specific bacterial identification techniques such as fluorescent in-situ hybridization will need to be used to confirm the identity of the bacteria within the cells. Although pneumococci are generally extracellular pathogens, in vitro they can survive inside human respiratory epithelial cells (type 2 pneumocytes; A549 cell line),[14] which suggests that at least in some cases of OME, persistent pneumococcal infection drives an inflammatory process and may also contribute to excessive mucus production found in chronic OME. The intracellular location of the bacteria may reduce the ability of mucosal (and systemic) antibody to kill the bacteria, and indicate a role for cell-mediated immunity in control of OME as is seen in other chronic intracellular infections such as tuberculosis. This location may also provide a reservoir of potentially viable bacteria that may contribute to subsequent acute episodes of OM in children with recurrent infections.

These findings potentially alter the paradigm for the treatment of this condition. Antibiotics that attain high intracellular concentrations such as macrolides, ketolides, and quinolones may have greater efficacy in the treatment of OME than other beta lactam antibiotics such as amoxicillin, which have previously been shown to have only modest effects in the treatment of OME.[16] In addition to pneumococcal infection, other bacteria may also be involved in chronic intracellular infection and may account for the negative pneumolysin PCR in the presence of gram-positive coccal bacteria on EM. *Alloiococcus otitidis* has also been implicated in OME through the use of PCR,[17] although the importance of this organism in OME remains to be determined and is being further investigated in follow-up studies. NTHI have also been shown to invade bronchial epithelial cells and subepithelial spaces in adults with chronic bronchitis, adenoidal epithelial cells, and subepithelial macrophages in children with OME.[13] SP have been shown to invade and survive within epithelial cells in tissue culture[18] and in animal models during development of invasive pneumococcal disease.[19]

Our inability to demonstrate intracellular infection in all biopsies may reflect the patchy nature of the infection, the limitations of electron microscopy to scan the entire sample, or sample disruption during biopsy, or that intracellular infection only occurs in a subset of children with chronic OME. While we were unable to demonstrate the coexistence of pneumococcal bacterial biofilm, as has been suggested by previous investigators, this may relate to the preparation techniques for electron microscopy resulting in dehydration of the polysaccharide matrix and disruption of the biofilm or it may be related to our biopsy techniques. These methods are being modified and we are utilizing confocal laser scanning microscopy to further identify bacterial biofilm

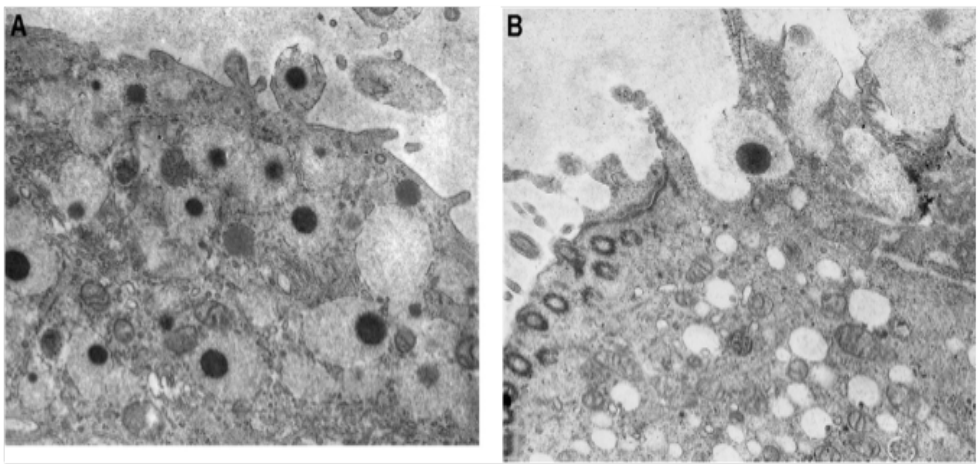


**Figure 1** TEM of middle ear mucosa from 25-month-old girl with chronic OME and rAOM, negative MEF culture, and PCR positive (PCR +ve) for pneumolysin. (**A**) Surface epithelia cell overwhelmed with coccal bacteria in mucus-containing vacuoles. (**B**) Cocci being expelled from ciliated epithelial cell.

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and intracellular bacteria. It is possible (indeed likely) that bacterial biofilm on the surface of the mucosa and intracellular infection may coexist and that both contribute to the development of OME as well as other factors such as Eustachian tube dysfunction in this multifactorial condition.

In summary this is the first study to demonstrate that chronic OME may be associated with intracellular infection of the middle ear mucosal epithelium, which we hypothesize contributes to middle ear inflammation and provides a bacterial reservoir, leading to recurrent infection.

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## FINANCIAL DISCLOSURE

Harvey Coates is affiliated with Alcon Laboratories. (attends scientific meetings, publishes and gives assistance with introduction of Ciprofloxin eardrops for indigenous people with CSOM.)

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# Multi-species bacterial biofilm and intracellular infection in otitis media

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## Abstract

**Background:** Bacteria which are metabolically active yet unable to be cultured and eradicated by antibiotic treatment are present in the middle ear effusion of children with chronic otitis media with effusion (COME) and recurrent acute otitis media (rAOM). These observations are suggestive of biofilm presence or intracellular sequestration of bacteria and may play a role in OM pathogenesis. The aim of this project is to provide evidence for the presence of otopathogenic bacteria intracellularly or within biofilm in the middle ear mucosa of children with COME or rAOM.

**Methods:** Middle ear mucosal biopsies from 20 children with COME or rAOM were examined for otopathogenic bacteria (either in biofilm or located intracellularly) using transmission electron microscopy (TEM) or species specific fluorescent *in situ* hybridisation (FISH) and confocal laser scanning microscopy (CLSM). One healthy control biopsy from a child undergoing cochlear implant surgery was also examined.

**Results:** No bacteria were observed in the healthy control sample. In 2 of the 3 biopsies imaged using TEM, bacteria were observed in mucus containing vacuoles within epithelial cells. Bacterial species within these could not be identified and biofilm was not observed. Using FISH with CLSM, bacteria were seen in 15 of the 17 otitis media mucosal specimens. In this group, 11 (65%) of the 17 middle ear mucosal biopsies showed evidence of bacterial biofilm and 12 demonstrated intracellular bacteria. 52% of biopsies were positive for both biofilm and intracellular bacteria. At least one otopathogen was identified in 13 of the 15 samples where bacteria were present. No differences were observed between biopsies from children with COME and those with rAOM.

**Conclusion:** Using FISH and CLSM, bacterial biofilm and intracellular infection with known otopathogens are demonstrated on/in the middle ear mucosa of children with COME and/or rAOM. While their role in disease pathogenesis remains to be determined, this previously undescribed infection pattern may help explain the ineffectiveness of current treatment strategies at preventing or resolving COME or rAOM.

**Keywords:** Bacterial biofilm, intracellular infection, otitis media, fluorescent in situ hybridisation, transmission electron microscopy

## Background

The important role of bacteria in otitis media (OM) pathogenesis has long been acknowledged, however the aetiology of recurrence and persistence of this condition is not well understood. Many characteristics suggest that chronic otitis media with effusion (COME) and recurrent acute otitis media (rAOM) are biofilm related [1-3]. Biofilms are defined as clusters of bacteria embedded in a polymeric matrix with increased resistance to antibiotics and host defence mechanisms when compared to their "planktonic" or "free floating" counterparts [4]. While biofilm has been demonstrated in OM animal models, [5,6] there is limited data available on biofilm formation in the middle ears of children with OM [7,8].

Our group has previously demonstrated intracellular infection of the middle ear mucosa in a small number of children with COME using transmission electron

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microscopy (TEM) [9]. However the bacterial species within these epithelial cells were not identified. Although it is known that some otopathogenic bacteria, including nontypeable *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*, are able to invade and survive within cells *in vitro* [10-15] and in adenoidal cells [16,17] it is unclear if this occurs *in vivo* in the middle ears of children with OM. This has important implications with regards to treatment, as the β-lactam antibiotics often used to treat OM episodes show poor penetration of cells and thus poor efficacy against bacteria sequestered intracellularly [12,18].

Despite polymicrobial biofilms being common [19], to date most reports (with the exception of Hall-Stoodley *et al* [8]) have not attempted to identify bacterial species present in the middle ear or have limited their analysis to a single species [20]. It is important to determine the presence of these otopathogenic species to determine the contribution of intracellular bacteria and biofilm formation to disease pathogenesis and for the development of new treatment strategies to combat this common childhood disease.

We hypothesise that multispecies bacterial biofilm and intracellular infection are both present in the middle ear mucosa of children with rAOM and COME. We believe this contributes to the chronic and recrudescent infections observed in these children. To investigate this hypothesis we used TEM and confocal laser scanning microscopy (CLSM) combined with bacterial-specific fluorescent *in situ* hybridisation (FISH) on middle ear biopsies taken from children undergoing ventilation tube insertion for rAOM and/or COME.

## Methods

### Patient population

Twenty children aged between 0 and 10 years were recruited at time of admission for ventilation tube insertion for a history of COME and/or rAOM through public hospitals in Perth, Australia. Due to public hospital waiting lists, several children were recruited between AOM or OME episodes.

COME was defined as the presence of a middle ear effusion without the symptoms or signs of suppurative infection for longer than 3 months [21]. rAOM was defined as having at least 3 acute OM presentations within a 6 month period or 4 or more episodes in a 12 month period, between which clinical symptoms resolved. Exclusion criteria included known immunodeficiency, chromosomal or craniofacial disorders.

One healthy mucosal control subject was recruited at time of cochlear implantation surgery. This child had no history of chronic or recurrent middle ear disease, nor any exclusion criteria for cases.

Clinical data including immunisation status was collected using parental questionnaires and medical records. Written informed consent was obtained from parents or guardians prior to enrolment in the study. Approval for this study was obtained from the Princess Margaret Hospital for Children, Armadale-Kelmscott and Osborne Park Hospital Ethics Committees.

### Specimen acquisition and tissue preparation

General laryngeal mask anaesthesia was used during the surgical procedure and utilizing the operating microscope, an anterior-inferior myringotomy incision was made. If middle ear effusion (MEE) was present, this was collected utilizing a sterile tympanostomy trap. MEE specimens were transported on ice to the PathWest clinical microbiology laboratory at Princess Margaret Hospital for Children for detection of bacteria using culture and PCR. Utilizing micro cup forceps via the myringotomy incision, middle ear mucosa biopsies of approximately 1 $mm^3$ were obtained from the promontory near the eustachian tube. One biopsy was obtained for assessment by microscopy for each child. Biopsies for TEM imaging were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) until processing. Biopsies for FISH and CLSM were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.2), washed three times with PBS and stored in 50% PBS/Ethanol at -20°C prior to hybridisation.

### Bacterial cultures

MEE samples were assayed using standard diagnostic culture techniques. Primary inoculations of MEE samples were made on blood agar, cysteine lactose electrolyte deficient agar, Filde's agar and colistin nalidixic acid (10 mg/L) blood agar plates. Plates were incubated at 35°C in 5% $CO_2$ and inspected for growth at 24 and 48 hours. In addition, inoculated anaerobic blood agar with vancomycin (2.5 mg/L) and nalidixic acid (10 mg/L) and colistin nalidixic acid blood agar were incubated anaerobically for 7 days with inspection at 48 hours and 7 days. All predominant bacteria were recorded including normal nasopharyngeal flora.

### Pneumolysin PCR

Pneumolysin (Ply) is a toxin present in *Streptococcus pneumoniae* and several closely related commensal bacteria including *Streptococcus mitis, Streptococcus oralis* and *Streptococcus pseudopneumoniae*. Genomic DNA was isolated from the MEE specimens using the Roche Total Nucleic Acid Extraction kits (Roche Diagnostics) following manufacturers instructions. Real time PCR to detect the *ply* gene was performed on the extracted DNA as previously described [22].

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### Microscopic examination

#### *Transmission electron microscopy*

Biopsies were processed as previously described [9]. In brief, 90nm sections were mounted on 200 mesh copper grids before double staining with uranyl acetate and lead citrate. Grids were then examined using a transmission electron microscope (Philips CM10, Eindhoven, The Netherlands). Micrographs were recorded on Kodak 4489 electron microscopy film (KODAK, NJ, USA) and printed for review and reporting.

#### *Confocal Laser Scanning Microscopy*

Microscopic examination was performed on biopsy specimens using CLSM imaging with a Nikon A1Si confocal microscope using 40x and 60x Plan Apo objectives.

### Evaluation of middle ear mucosal specimens with pathogen-specific probes

**16S rRNA FISH** - Biopsies were incubated for three min at room temperature in 50%, 80% and 100% ethanol, respectively. Each biopsy was incubated at 37°C in hybridisation chambers with 50 μl of 10 mg/ml of lysozyme in 0.1-M Tris and 0.05-M $Na_2$ EDTA and washed with sterile PBS.

Bacterial presence, biofilm structure and multi-species interactions were visualised using FISH and CLSM. Hybridisation probes were optimised for *S. pneumoniae*, nontypeable *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* as well as a universal eubacterial (EUB338) probe. Middle ear mucosal biopsies from all 18 children were examined for evidence of biofilm or intracellular infection using CLSM following FISH and generic nucleic acid labelling with Hoechst 33342. As mucosal biopsy size was a limiting factor, with samples being on average < 1 $mm^3$, only a single set of two bacterial species specific FISH probes could be performed on each specimen. In addition the universal EUB338 probe was used on all specimens for the specific detection of bacteria.

FISH was conducted as previously described by Hall-Stoodley *et al* [8] using 16S rRNA probes labelled with AlexaFluor 488, 546 or 633 dyes (Invitrogen Technologies) and incubated overnight in 1.2 ug/ml Hoechst 33342 (Invitrogen Technologies) to stain nuclei. Biopsies were mounted in low fade mounting media and imaged using four colour CLSM. Probes were selected for hybridisations based on bacterial culture and PCR results from MEE. If MEE was not present or if the sample was culture and/or PCR negative, probes for the most prevalent organisms were used (*S. pneumoniae*, *M. catarrhalis* or *H. influenzae*). The following probes were used: EUB338 for the domain bacteria (with a 5'-3' sequence, GCT GCC TCC CGT AGG AGT) [23], *S. pneumoniae* probe (GTG ATG CAA GTG CAC CTT) [24], nontypeable *H. influenzae* (CCG CAC TTT CAT CTT CCG) [25], *S. aureus* (GAA GCA AGC TTC TCG TCC G) [24] and *M. catarrhalis* (CCG CCA CUA AGU AUC AGA) [8]. Specificity of 16S rRNA probes was extensively tested and validated using ATCC and clinical isolates (data not shown).

### Image analysis and interpretation

As there is little data on characterisation and definitions of biofilms in middle ears of children, definitions by Hall-Stoodley et al [8] and from the chinchilla model of biofilm [5] were used as guides to assess and characterise bacterial presence in these specimens. Samples were scored as being positive for biofilm presence when characteristic biofilm morphology was apparent and included presence of aggregated bacteria (often in the form of microcolonies), usually adherent to a surface and surrounded by extracellular matrix [5,8]. When images were suggestive of biofilm these were further evaluated using high resolution electronic zoom with samples being recorded as positive when results demonstrated bacterial morphology, using size (approximately 0.5-2 μm) and shape (either cocci or cocco-bacilli), biofilm structure and fluorescence with appropriate signal. No attempt was made to quantify biofilm formation. Bacteria were marked as being intracellular when there was close association of bacteria (as demonstrated by size, morphology and appropriate fluorescent signal) with intact host cell nuclei, as described by Zautner *et al*, [26]. When intracellular location was suggested, images were assessed by examining individual focal planes to ensure true association of bacteria with host nuclei.

## Results

### Patient characteristics

General patient characteristics are described in Table 1 and 2. Twenty middle ear biopsies were obtained from 20 children undergoing ventilation tube insertion. Of these, 6 (30%) had a diagnosis of COME only, 6 (30%) had a diagnosis of rAOM only, and 8 (40%) had both diagnoses. Eleven (55%) were girls and the median age was 4.3 yr (range, 1.2-9.9). Control biopsies were obtained from a 1.6 year old female undergoing cochlear implantation due to sensorineural hearing loss. All

**Table 1 Gender, age, diagnosis and microscopic evidence of biofilm and intracellular bacteria in middle ear mucosal biopsies using Transmission Electron Microscopy.**

| Child No. | Sex (Age, yr) | Diagnosis | Biofilm | Intracellular bacteria |
|---|---|---|---|---|
| 1 | F (1.2) | RAOM and OME | - | - |
| 2 | M (2.1) | RAOM and OME | - | + |
| 3 | M (5.2) | RAOM | - | + |

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**Table 2 Gender, age, diagnosis, species presence and microscopic evidence of biofilm and intracellular bacteria using Fluorescence *In Situ* hybridisation (FISH) in middle ear mucosal biopsies and effusions assayed using culture, PCR and FISH methods for pathogen detection.**

| | | | | | PCR | | FISH | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Child No. | Sex (Age, yr) | Ear | Diagnosis | Current Abx | Culture | Spn | EUB | Spn | Hi | Mcat | Sau | Biofilm | Intracellular bacteria |
| **Children with recurrent acute otitis media** | | | | | | | | | | | | | |
| 4 | F (1.4) | R | RAOM | N | - | - | + | na | + | + | na | + | + |
| 5 | M (1.8) | L | RAOM | N | na | na | + | - | + | na | na | - | + |
| 6 | F (3.2) | L | RAOM | N | na | na | + | na | + | - | na | + | + |
| 7 | M (6.1) | L | RAOM | N | + mixed | + | + | + | na | - | na | - | + |
| 8 | F (9.3) | L | RAOM, Cholesteatoma | N | na | na | + | - | na | + | na | - | - |
| **Children with recurrent acute otitis media and otitis media with effusion** | | | | | | | | | | | | | |
| 9 | M (1.4) | R | RAOM, OME | Y | - | - | + | na | - | + | na | + | + |
| 10 | M (1.8) | L | RAOM, OME | N | + Spn, Mcat | + | + | + | na | + | na | + | + |
| 11 | F (1.8) | L | RAOM, OME | Y | - | + | + | - | + | na | na | - | + |
| 12 | F (3.5) | L | RAOM, OME | N | - | - | - | na | - | - | na | - | - |
| 13 | M (7.5) | L | RAOM, OME | N | - | - | + | na | + | + | na | + | + |
| 14 | F (8.9) | L | RAOM, OME | N | na | na | + | + | - | na | na | + | + |
| **Children with otitis media with effusion** | | | | | | | | | | | | | |
| 15 | M (1.5) | L | OME | N | na | na | + | - | - | na | na | + | - |
| 16 | F (5.4) | L | OME | N | + CNS | - | + | na | - | + | na | + | + |
| 17 | F (6.0) | L | OME | N | + Sau | - | + | na | na | - | + | + | + |
| 18 | F (6.3) | R | OME | N | na | na | - | - | na | - | na | - | - |
| 19 | F (8.0) | R | OME | N | na | na | + | - | - | na | na | + | - |
| 20 | M (9.9) | R | OME | N | na | na | + | + | na | + | na | + | + |
| **Control cochlear implant** | | | | | | | | | | | | | |
| 21 | F (1.6) | L | Sensorineural hearing loss | N | na | na | - | - | - | na | na | - | - |
| Positive from number tested (not including healthy control) | | | | 2/17 | 4/9 (44%) | 3/9 (33%) | 15/17 (88%) | 4/10 (40%) | 5/11 (45%) | 7/12 (58%) | 1/1 | 11/17 (64%) | 12/17 (71%) |

Biopsies are from children undergoing ventilation tube insertion for COME and recurrent AOM as well as a negative control from a child with no history of middle ear disease undergoing cochlear implantation surgery.

Abbreviations: EUB - eubacterial probe, Spn - *S. pneumoniae* (organism and probe), Hi - *H. influenzae* (organism and probe), Mcat - *M. catarrhalis* (organism and probe), Sau - *S. aureus* (organism and probe), CNS - Coagulase negative staphylococci (organism). na - Not available.

children in this group identified as Caucasian. Six of the 20 children with OM had been previously vaccinated with the 7-valent pneumococcal conjugate vaccine, as had the child undergoing cochlear implant surgery. The pneumococcal vaccination status for two children (No's 2 and 19) were unknown. Two children were receiving cephalosporin antibiotics at the time of surgery (Table 2) and one other child (Child 7) had received amoxycillin in the month prior to surgery.

### Microbiological findings in MEE

No MEEs were collected from those children for whom biopsies were examined using TEM (Table 1).

MEEs were collected from 9 of the 20 ears from which biopsies were taken. MEE was not present in all ears since several children were recruited between AOM or OME episodes. They were assayed via culture for respiratory bacterial pathogens and by PCR for the Ply gene for the presence of *S. pneumoniae* (Table 2). Of the 9 MEE cultured, one was positive for *S. pneumoniae* and *M. catarrhalis*, one was positive for *S. aureus*, one for Coagulase negative staphylococci and one was positive for unspecified mixed bacterial flora (not containing any middle ear pathogens). The Ply PCR was positive in three samples. Of these 3 Ply positive samples one was culture positive for *S. pneumoniae*, one was culture

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negative for any bacteria and the third sample was culture positive for mixed bacterial flora not including any of the middle ear pathogens. All remaining samples were Ply PCR negative.

**Evidence of biofilms and intracellular infection**

Two of the 3 samples examined using TEM were seen to have intracellular bacteria in mucus containing vacuoles within the epithelial cells (Table 1 Figure 1). No biofilm was observed using TEM.

Subsequently, 17 biopsy samples were examined using FISH and CLSM. Depending on biopsy size and the presence of bacteria, between 2 and 9 stacks of images were taken and examined for each specimen, representative maximum projections were presented. Universal bacterial EUB338 hybridisation was demonstrated in 15 of the 17 specimens (88%) (Table 2). Using 3 different probes for *H. influenzae, S. pneumoniae* and eubacterial 16S rRNA genes, no bacteria were observed in two biopsies taken from the same ear of a control cochlear implant case (Figure 2). Overall 11 (65%) of the 17 middle ear mucosal biopsies were positive for bacterial biofilm based on morphological and fluorescence criteria. Of these 11, 9 were also positive for intracellular bacteria demonstrating discrete aggregates of bacteria closely associated with host nuclei. Twelve (71%) of the 17 samples were positive for intracellular bacteria. Two samples showed scant bacteria which were scattered throughout the biopsies, without characteristics of biofilm or intracellular infection. Similar rates of bacterial biofilm and intracellular bacteria were seen in children with rAOM (4 out of 5 samples positive), COME (5 out of 6 samples positive) or both diagnoses (5 out of 6 samples positive; Table 2). Biofilm morphologies varied, ranging from microcolonies to large bacterial clusters, however no differences were observed between diagnostic groups (Figures 3, 4 and 5). Intracellular bacteria were frequently present throughout the positive specimens.

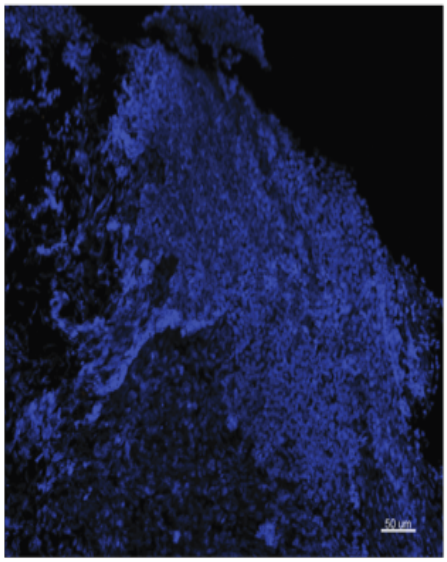


**Figure 2 Representative image from the middle ear mucosal biopsy of a child having a cochlear implant who has no history of middle ear disease**. Child 21. FISH -EUB338 (Yellow), *S. pneumoniae* (green), *H. influenzae* (pink) and Hoechst 33342 (nuclei stain - blue). This maximum intensity projection (Z = 20.5 µm) demonstrates the normal mucosal tissue with no evidence of bacteria. Scale bar = 50 µm.

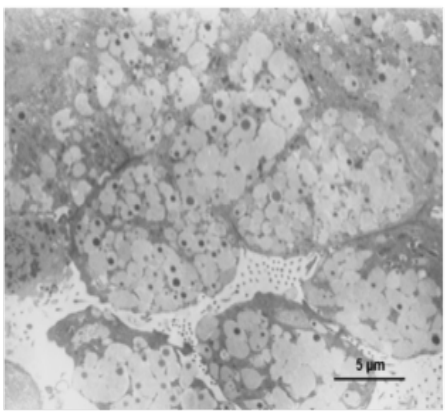


**Figure 1 Representative TEM of the middle ear mucosa from a child with a history of COME and recurrent AOM**. This biopsy was from a 25 month old male, MEE was not collected. Image shows mucus-secreting epithelial cells (12-15 µm) overwhelmed with coccal bacteria in mucus containing vacuoles.

When MEE was present and culture was positive, bacteria were identified by CLSM and FISH in all cases. For the MEE sample culture positive for *M. catarrhalis* and *S. pneumoniae* and PCR positive for *S. pneumoniae*, FISH was also positive for these bacteria. The MEE that was culture positive for *S. aureus* had a corresponding middle ear mucosal biopsy which was positive for *S. aureus* by FISH. This sample was negative for the additional *M. catarrhalis* probe but positive for other unidentified bacteria using the eubacterial probe.

Five of 11 biopsies (45%) assessed for *H. influenzae* by FISH were positive, including 3 of 3 rAOM and 2 of 5 rAOM/COME specimens (Table 2). None of the 3 COME only specimens tested were positive for *H. influenzae* by FISH. FISH identified *S. pneumoniae* in 4 of 10 biopsies (40%), including 1 of 3 rAOM, 2 of 3 rAOM/COME specimens and 1 of 4 children with COME only. *M. catarrhalis* was examined in 12 mucosal biopsies and of these 7 (58%) were positive. Two of 4 biopsies positive for *M. catarrhalis* were from children with

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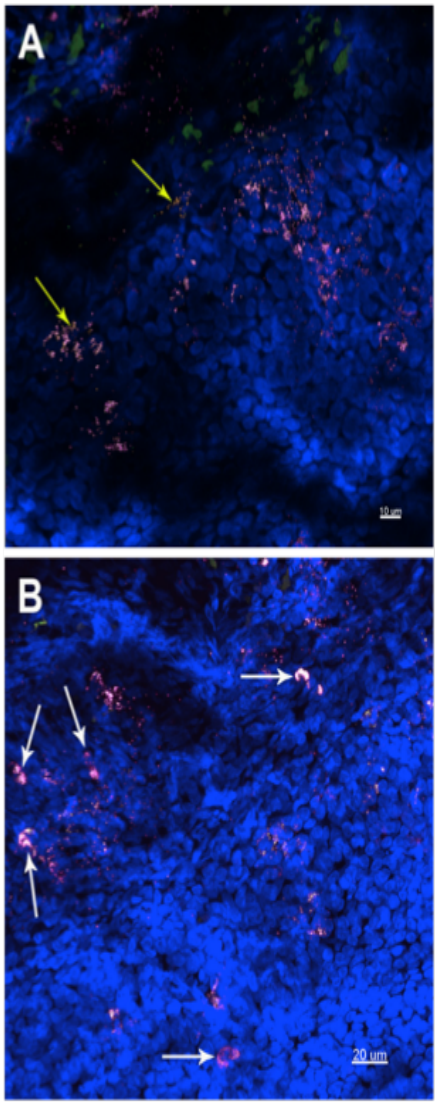


**Figure 3 Representative images of a mucosal biopsy from a child suffering with rAOM**. Middle ear fluid culture and Ply PCR negative, showing biofilm and intracellular infection. Child 4. FISH: EUB338 (yellow), *M. catarrhalis* (green), *H. influenzae* (pink) and Hoechst 33342 (nuclei stain - Blue). A) *H. influenzae* is the predominant pathogen, with other non-identified bacteria also evident (yellow arrows). Maximum projection image (Z = 29 µm), scale bar = 10 µm. B) Intracellular *H. influenzae* are apparent throughout the tissue as nuclei associated bacterial clusters (white arrows), as well as in biofilm. Maximum projection image (Z = 40 µm), scale bar = 20 µm.

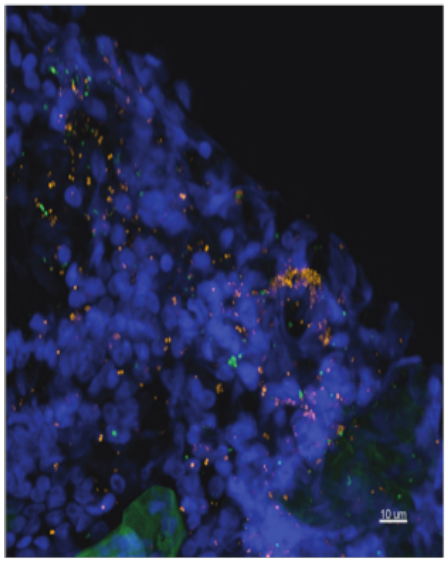


**Figure 4 Representative image of a mucosal biopsy from a child suffering from rAOM and COME**. MEE was not present. Child 14. FISH probes included EUB338 (yellow), *S. pneumoniae* (green), negative for *H. influenzae* (pink), Hoechst 33342 (nuclei stain - blue). This is a maximum intensity projection (Z = 39 µm) showing multispecies biofilm covering the mucosa. The biofilm is seen to consist of *S. pneumoniae* and other unidentified bacteria. These bacteria are also interspersed within the tissue. Scale bar = 10 µm.

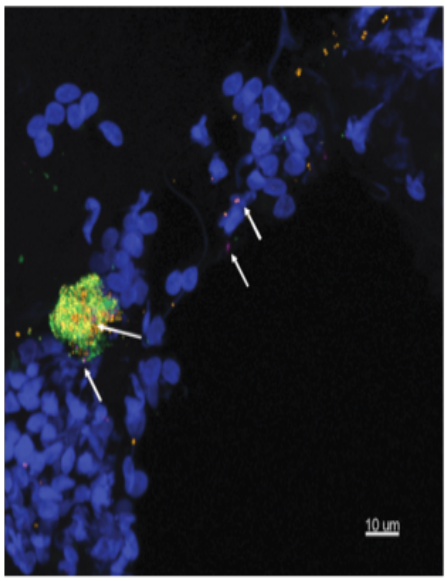


**Figure 5 Representative image of a mucosal biopsy from a child with a history of COME**. No middle ear fluid was taken. Child 20. FISH - EUB338 (Yellow), *M. catarrhalis* (green), *S. pneumoniae* (pink) and Hoechst 33342 (nuclei stain - blue). Maximum intensity projection (Z = 19.5 µm) showing microcolony formation, mainly *M. catarrhalis* and *S. pneumoniae* (arrows) scattered throughout the mucosa. Scale bar = 10 µm.

rAOM only and 3 of 4 positive specimens were from children with both rAOM and COME.

In 4 of the 5 *H. influenzae* positive samples, this pathogen was observed intracellularly only. In one of these 5 samples *H. influenzae* was found in biofilm and

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intracellularly concurrently (Figure 3). *S. pneumoniae* and *M. catarrhalis* were shown to reside both intracellularly and in biofilm structures.

## Discussion

Initial examination of middle ear mucosal biopsy samples using TEM indicated the presence of intracellular bacteria in mucus containing vacuoles, based on morphology and electron density. This mucus may be exocytosed onto the mucosal surface, contributing to the persistence of effusion as we have previously suggested in biopsies from children with COME [9]. Using TEM biofilm was not detectable in samples from children with rAOM, similarly to what was observed in children with COME [9] and, bacterial species were not able to be determined using this technique. It is apparent that while TEM may be a suitable technique to demonstrate intracellular bacteria within mucosal biopsies, it is less useful in examining biofilm presence or in determining bacterial species within a specimen. We therefore used FISH and CLSM. CLSM allowed us to examine hydrated specimens and the use of FISH allowed determination of bacterial structures and the species within these.

Using FISH and CLSM we have demonstrated that multiple bacterial species including pathogens associated with acute OM, are present intracellularly and/or in biofilm in the mucosa of 82% of children undergoing ventilation tube insertion for COME and rAOM. Separately, 64% of biopsies were positive for biofilm, while 71% were positive for intracellular bacteria. No difference was observed between children with either rAOM or COME, with biofilm and intracellular bacteria being demonstrated at similar rates in both. These findings are similar to those described by Hall-Stoodley *et al*, the only study using FISH to evaluate biopsies from a comparable cohort, showing bacterial clusters containing otopathogens, on the middle ear mucosa of children with both rAOM and COME [8]. Importantly, at least one of the known OM pathogens was demonstrated in 12 of the 14 samples positive for biofilm or intracellular infection, and no bacteria were seen on the healthy control middle ear mucosa. These findings support the hypothesis that the bacterial OM pathogens are present either intracellularly or in bacterial biofilms or both. The presence of otopathogens may play an important role in OM pathogenesis and could be a source of chronic inflammation and/or recurrent infection.

*H. influenzae* was found in 45% of biopsies, similar to levels detected in the MEEs of Australian children undergoing surgery for rAOM (47%) by PCR [27]. However, rates are lower than those detected by Hall-Stoodley et al who detected 70% of MEEs were positive for *H. influenzae* by PCR and 7 of 7 biopsies positive using FISH. This may reflect population differences or may reflect the differences in methodology. As the microbiology of our samples were unknown we chose to follow guidelines outlined by Hugenholtz *et al* for fixing specimens containing both Gram-positive and Gram-negative organisms [28]. All samples were fixed in paraformaldehyde. Furthermore we also pre-treated all biopsies with lysozyme which is recommended to permeabilise Gram-positive organisms. While this may affect the Gram-negative organisms and may be responsible for the differences observed between our study and that of Hall-Stoodley *et al* [8], during assay optimisation this was demonstrated to have no affect on hybridisation rates or on the bacterial morphology. We therefore believe this to be a true representation of *H. influenzae* presence in these biopsies.

Multiple bacterial species were present in all specimens suggesting that OM is a polymicrobial infection. For example, *S. pneumoniae* and *M. catarrhalis* were found in the same specimen in two of five cases (40%). *M. catarrhalis* and *H. influenzae* were found together in two out of six specimens (33%) tested. This may be important clinically as evidence in animal models suggests that in a polymicrobial biofilm bacterial species are able to confer protection from host defences and antimicrobials to each other and thus more resistant that single-specie biofilms [29]. Furthermore, in the nasopharynx of mouse OM models, bacterial composition and viral infection are shown to have significant effects on OM incidence and severity [30]. Although *S. pneumoniae* and *H. influenzae* have been described to reside together in the middle ear of children with OM [8,31], interestingly these pathogens were never observed together in the same specimen (0 of 5 specimens) in our study. While the number of samples tested for these species was small, our findings may reflect the competitive interactions observed between *S. pneumoniae* and *H. influenzae* [32-34]. In a significant proportion of biopsy samples, multiple unidentified bacterial species were observed. These bacteria were often not cultured even when shown to be present using FISH or PCR. Other fastidious organisms such as *Alloiococcus otitidis* may also play a role in this disease as they have been found using PCR and culture in children with both OME and rAOM [35,36].

Using TEM it is clear that intracellular bacteria were present within the epithelial cells of biopsies assessed in children with rAOM, similar to those seen in the middle ear mucosa of children with COME [9]. Biopsy samples using FISH were assessed as having intracellular bacteria when there was close association of bacteria with intact host cell nuclei, as described [26]. These results were consistent with TEM findings. In animal OM models, biofilm is often shown to be patchy [5] and may be missed during sampling or disruption of these structures

during processing [8,37]. This differs for intracellular bacteria which are more likely to be embedded within the biopsy and thus less likely to be dislodged or affected by processing techniques. We believe it is for these reasons we observed intracellular bacteria more often than bacterial biofilms and that they may represent continuums of the same disease process. In each of the cells, intracellular bacteria belonged to the same species. This may reflect the formation of intracellular "biofilm pods" similar to those formed within bladder epithelial cells by uropathogenic *Escherichia coli* [38-40]. These are important as in these intracellular compartments bacteria are able to proliferate and persist, subverting host immune responses and increasing resistance to antimicrobial treatments [10,12,41].

At the time of this study, PCRs for pathogens other than *S. pneumoniae* were not available in our laboratory. It has also since become apparent that not all strains of *S. pneumoniae* contain a functional Ply gene and as such these bacteria may not always be detected when using this PCR. Therefore the selections of probes to use on the biopsy specimens based on MEE PCR results were not optimal and represent limitations of this study. By using the eubacterial probe and combinations of probes for the most common otopathogens, we could successfully determine bacterial presence in either biofilm formation or intracellularly. While only one healthy mucosal specimen was available, on thorough examination no bacteria could be identified; this is in accordance with another study [8] which demonstrated that no bacteria were evident in healthy middle ear mucosal samples. Little is known regarding the progress of OME and rAOM. It is difficult to determine whether biofilm and intracellular infection are a reflection of the residual stage on the way to clearance of the infection or whether they represent the main mechanisms of persistence within the host. Whether the otopathogenic bacteria within these intracellular compartments demonstrate biofilm phenotypes is currently unknown and requires further investigation. Given the severity and chronic nature of infection in many of these children, it is clear however that these infections do not resolve readily on their own and that biofilm and intracellular sequestration represent ways in which bacteria can resist both host defences and current antimicrobial treatments.

Otopathogenic bacteria are known to form biofilms *in vitro* and in animal models [5], are able to invade and survive within cells *in vitro* [10-15] and in adenoidal cells [16,17]. While biofilm and intracellular infection with pathogenic species are known to play roles in the chronicity of many diseases [42-45] very few studies exist demonstrating the presence of biofilm and/or intracellular infection in the middle ear mucosa of children with chronic or recurrent OM. A more recent study examining MEE from children with COME detected no biofilm [20]. These differences may reflect the use of MEE smears rather than biopsies.

To our knowledge this is the first study that has concurrently identified both bacterial biofilm and intracellular bacterial sequestration of known otopathogens in the middle ear mucosa of children with COME and rAOM. Successful identification of otopathogenic species in biofilms or intracellularly may encourage the development of treatments that target both bacterial biofilm and intracellular infection, since both might contribute to persistence and recurrence of infection. Current treatment regimens with β-lactam antibiotics (including amoxycillin) which do not target intracellularly sequestered pathogens may be less effective than macrolide or fluoroquinolone class antibiotics which have been shown to penetrate and concentrate intracellularly depending on antibiotic resistance patterns. Studies examining the effectiveness of combination antibiotic treatment for rAOM and COME are urgently required to try and improve the outcome of non surgical interventions. Bacteria within biofilms and located within cells may also be less susceptible to killing by naturally acquired or vaccine-induced antibody which contributes to the recurrent nature of this disease. Investigational treatments specifically for biofilm diseases and those targeting intracellular bacteria may play an important role in OM management in the future.

## Conclusion

CLSM is a more robust imaging method to determine biofilm presence in middle ear biopsies when compared to TEM. Using FISH and CLSM, bacterial biofilm and/or intracellular infection with known otopathogens have been demonstrated on the middle ear mucosa of children with COME and rAOM. While the role of biofilm and intracellular infection in both of these disease presentations remains to be determined, these infection patterns may help explain the ineffectiveness of current treatments in preventing recurrent episodes. While larger studies on which quantitative analyses could be performed would be useful in the future this highlights the need for alternative strategies to target both biofilm and intracellular infection in the middle ear mucosa. Future studies looking at the mechanisms of bacterial persistence in high risk groups such as Indigenous Australians would be important in understanding these disease processes and to design more effective treatment and prevention.

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**Authors' contributions**
RT was involved in original study design, participant recruitment, sample processing, designed and conducted the fluorescent imaging experiments, and drafted the manuscript. PF, conducted the electron microscopy. PCR co-ordinated original study design and experimental design. SW, JL, HC & SV were involved with recruitment and sample collection. AK managed bacterial culture and PCR assays. PJR co-ordinated experimental design. All authors have revised and approved the final manuscript.

**Competing interests**
R.B. Thornton has received travel funding from GlaxoSmithKline.
S.P Wiertsema has received Institutional funding from GSK for investigator-led epidemiological studies in otitis media and has received travel support from GSK
H.L. Coates and S. Vijayasekaran consult for GlaxoSmithKline Biologicals.
P.C. Richmond has received Institutional funding from GSK for investigator-led epidemiological studies in otitis media and has received travel support from GSK, Wyeth and other vaccine companies to present scientific data and chair workshops. He has no shares, paid employment, or consultancies with any pharmaceutical companies.
Other authors have no competing interests to declare.

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# Neutrophil Extracellular Traps and Bacterial Biofilms in Middle Ear Effusion of Children with Recurrent Acute Otitis Media – A Potential Treatment Target

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## Abstract

***Background:*** Bacteria persist within biofilms on the middle ear mucosa of children with recurrent and chronic otitis media however the mechanisms by which these develop remain to be elucidated. Biopsies can be difficult to obtain from children and their small size limits analysis.

***Methods:*** In this study we aimed to investigate biofilm presence in middle ear effusion (MEE) from children with recurrent acute otitis media (rAOM) and to determine if these may represent infectious reservoirs similarly to those on the mucosa. We examined this through culture, viability staining and fluorescent *in situ* hybridisation (FISH) to determine bacterial species present. Most MEEs had live bacteria present using viability staining (32/36) and all effusions had bacteria present using the universal FISH probe (26/26). Of these, 70% contained 2 or more otopathogenic species. Extensive DNA stranding was also present. This DNA was largely host derived, representing neutrophil extracellular traps (NETs) within which live bacteria in biofilm formations were present. When treated with the recombinant human deoxyribonuclease 1, Dornase alfa, these strands were observed to fragment.

***Conclusions:*** Bacterial biofilms, composed of multiple live otopathogenic species can be demonstrated in the MEEs of children with rAOM and that these contain extensive DNA stranding from NETs. The NETs contribute to the viscosity of the effusion, potentially contributing to its failure to clear as well as biofilm development. Our data indicates that Dornase alfa can fragment these strands and may play a role in future chronic OM treatment.

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## Introduction

Recurrent acute otitis media (rAOM) affects between 10–20% of children [1,2] and is a major reason for children requiring surgery, predominantly for ventilation tube insertion (VTI). Biofilm and intracellular infection have been demonstrated on the middle ear mucosa of children with chronic suppurative otitis media (CSOM), rAOM and chronic otitis media with effusion (OME) and are mechanisms of bacterial persistence in the middle ear causing recalcitrance to treatment and disease recurrence [3–6].

Biofilms on mucosal surfaces are a result of interactions between bacteria and the host and these differ physiologically from biofilms formed on inert surfaces [7]. DNA, both host and bacterially derived, is important in biofilm formation, stabilisation and persistence of nontypeable *Haemophilus influenzae* (NTHi) and *Streptococcus pneumoniae in vitro* and in the chinchilla model of otitis media (OM) [8–12]. DNA may be derived from active immune mechanisms such as neutrophil extracellular traps (NETs) [13], necrotic neutrophil presence [14], or produced directly by the bacteria [8–11]. NETs consist of a DNA backbone embedded with antimicrobial peptides and enzymes [15] and are important in confining infections and killing bacteria [16]. Some pathogens, including *S. pneumoniae* and NTHi can resist NET killing [9,11,15–17] and NTHi has been shown to actively elicit NET formation, using the DNA as a protective niche [18]. High levels of NETs

may also lead to tissue damage as has been demonstrated in the liver and lung [19]. The presence of NETs and/or bacterial DNA in chronic OM may allow for novel treatments that breakdown DNA such as Dornase alfa used in the treatment of CF.

While middle ear mucosal biopsies have been used for demonstrating biofilm or intracellular presence of bacteria [3–6], biopsies are difficult to obtain and their small size limits analysis. The use of middle ear effusion (MEE) is an attractive alternative to examine the presence of biofilm-like formations in the middle ear and *Staphylococcus aureus* biofilm has been demonstrated in otorrhoea smears from children with CSOM [20]. Additional valuable data can be obtained from imaging the larger volume of MEE that cannot be acquired from single biopsies such as viability staining and fluorescent *in situ* hybridisation treatments, reducing bias in selecting probes and determining non-specific interactions between the probes and the sample. The presence of DNA in MEE from children with chronic and/or recurrent OM has not been previously demonstrated. In this study, we used FISH and viability staining to investigate whether bacterial biofilm, intracellular pathogens and extracellular DNA are present in MEE samples from children undergoing VTI for rAOM.

## Materials and Methods

### Patient population

MEE samples were obtained from children aged between 9 and 36 months with a history of at least 3 episodes of AOM and undergoing VTI as previously reported [21]. Children with diagnosed or suspected primary or secondary immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities and chromosomal or genetic syndromes were excluded. Subjects were recruited from metropolitan hospitals in Perth, Western Australia, following written informed consent from parents or guardians. Approval for this study was obtained from the Princess Margaret Hospital for Children Ethics Committee (1295/EP) and from institutional review boards of hospitals where recruitment took place.

### Specimen acquisition and preparation

Samples were collected as previously described [21]. In brief, MEEs were aspirated through a myringotomy incision using a sterile Leukotrap® (Pall Corporation, New York, USA) connected to the surgical suction system. The tubing system was rinsed with sterile saline to recover all the MEE present into the Leukotrap®. Nasopharyngeal samples were collected using a sterile flexible cotton-wool tip swab (Copan, Brescia, Italy) and stored in STGGB.

### Bacterial culture

MEE (100uL) and nasopharyngeal samples (10uL) were assayed with standard culture techniques [22] on horse blood agar, chocolate agar containing bacitracin (300 mg/L), vancomycin (5 mg/L) and clindamycin (0.96 mg/L), and colistin nalidixic acid blood agar plates (Oxoid, Australia). Plates were incubated at 37°C in 5% $CO_2$ for 48 h and all predominant bacteria recorded.

### Evaluation of bacterial viability and presence of DNA stranding in the MEE with BacLight staining kit

Viability of bacteria in the MEEs was assessed using the generic nucleic acid labelling of cells with the BacLight kit (Invitrogen Technologies). Briefly, 50ul of fresh MEE in saline was air dried for 30 min at room temperature on SuperFrost plus slides (Menzel-Glaser, Portsmouth, New Hampshire). Samples were incubated with 50 µl of the BacLight working solution (consisting of propidium iodide and SYTO9) (Invitrogen Technologies, Thornton, NSW) for 30 min at room temperature before three 5 min washes with PBS. Slides were mounted using an in-house low fade mounting media and stored at −20°C until CLSM imaging using the Leica TCS SP2 AOBS multiphoton confocal.

### Evaluation of MEE specimens using 16S rRNA FISH with pathogen-specific probes

FISH was conducted as described by Hall-Stoodley *et al* and Thornton *et al* [5,6] for mucosal biopsies using 16S rRNA probes labelled with AlexaFluor 488, 546 or 633 dyes (Invitrogen Technologies, Thornton, NSW). A panel of probes was used to assess 5 slides for each MEE. Due to the availability of 4 lasers a maximum of 3 probes and one nucleic acid stain could be used on each sample. The probes included EUB338, a universal probe that hybridises all bacteria (with a 5′-3′ sequence, GCT GCC TCC CGT AGG AGT) [23], Spn probe (GTG ATG CAA GTG CAC CTT) for *S. pneumoniae* [24], Hacinf (CCG CAC TTT CAT CTT CCG) for *H. influenzae* [25], Sau (GAA GCA AGC TTC TCG TCC G) for *S. aureus* [24], Mrc88 (CCG CCA CUA AGU AUC AGA) for *Moraxella catarrhalis* [5] and Psae (TCT CGG CCT TGA AAC CCC) for *Pseudomonas aeruginosa* [26] and NONEUB338 as the negative control for non-specific hybridisations [27]. Specificity of 16S rRNA probes was extensively tested and validated using ATCC and clinical isolates.

MEE samples stored in 50% PBS/Ethanol at −20°C were used for FISH. A 50 µl aliquot from each MEE was air dried as above, incubated for 30 min at room temperature in 80% and then 100% ethanol. Samples were transferred to hybridisation chambers and incubated with lysozyme (10 mg/ml lysozyme in 0.1 M Tris (hydroxymethyl) aminomethane hydrochloride and 0.05 M $Na_2$ EDTA) for 2 h at 37°C. Slides were then washed with PBS. Samples were treated with 40 µl of hybridisation buffer and 50 ng/ml of each probe at 46°C inside a hybridisation chamber for 2 h. Slides were then washed for 15 min at 48°C and incubated overnight with 1.2 µg/ml Hoechst 33342 (Invitrogen Technologies, Thornton, NSW) to stain the nuclei of the host cells. Slides were mounted in low fade mounting media and imaged using four colour CLSM using the Leica TCS SP2 AOBS multiphoton confocal. Images were analysed for presence of bacteria, specific bacterial species, biofilm structure and composition.

### Image analysis and interpretation

Samples were scored positive for biofilm presence when CLSM images showed bacterial aggregates resembling microcolonies or more extensive morphology, bacterial morphology based on size (0.5–2 µm) and shape (cocci or coccobacilli) was correct, biofilm ultrastructure was present and fluorescent signal was only present in the appropriate fluorescent channel. Host cells were identified through the use of the Hoechst 33342 nucleic acid stain. Bacteria were marked as being intracellular when discrete clusters of bacteria were in close proximity to intact host cell nuclei as per Thornton *et al* [6].

### Neutrophil elastase staining to determine whether the DNA in the samples was neutrophil derived

A subset of 10 MEEs was evaluated for neutrophil elastase by staining with anti-human neutrophil elastase antibodies (Abcam, UK). Fifty µl of MEE in saline was air dried for 30 min at room temperature on SuperFrost plus slides (Menzel-Glaser, Portsmouth, New Hampshire). MEE was blocked with Zuk's blocking buffer and incubated at room temperature for 30 min. Block

**Table 1.** Summary of demographic data and the pathogens identified in MEE from 24 children undergoing VTI for rAOM. Pathogens were identified using standard culture and FISH, and viability was determined using BacLight staining.

| | | | | | | FISH 16S RNA Probe | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Child No. | Sex (Age,m) | NP culture | Abx | Ear | MEE culture | EUB | Spn | Hi | Mcat | Sau | PA | BacLight (Live/ Dead/Both) | Biofilm present | Intracellular bacteria observed | NETs |
| 1 | M (16.6) | Spn, Hi | Y | L | – | + | + | – | + | – | – | na | + | – | na |
| | | Mcat | | R | – | + | + | – | + | – | – | na | + | – | na |
| 2 | M (28.3) | Spn, Hi | N | L | – | + | – | – | – | – | – | Both | – | – | + |
| 3 | M (16.8) | Hi | Y | L | Sau | + | + | + | – | – | – | Live | + | – | + |
| | | | | R | Sau | + | + | + | – | + | – | Live | + | + | + |
| 4 | M (20.0) | – | Y | R | – | + | – | – | – | – | – | Both | + | – | – |
| 5 | M (14.0) | – | Y | L | – | + | + | + | – | – | – | Both | + | + | – |
| | | | | R | – | + | + | – | – | – | – | Live | + | – | + |
| 6 | M (23.3) | Hi | Y | L | Hi | + | – | + | – | + | – | Both | + | + | + |
| | | | | R | Hi | + | + | + | – | – | – | Both | + | + | + |
| 7 | M (14.1) | Spn, Hi | N | R | Hi | + | + | + | – | + | – | Both | + | + | + |
| 8 | F (24.6) | Hi, | N | L | – | + | + | + | – | + | – | – | + | – | + |
| | | Mcat | | R | – | + | + | + | + | + | – | Both | + | + | + |
| 9 | F (18.0) | Spn | N | L | – | + | – | + | – | + | – | – | + | + | + |
| | | | | R | – | + | + | – | + | – | – | Both | – | – | + |
| 10 | M (10.7) | Spn, Hi | N | R | Spn | + | + | + | – | – | – | Live | + | + | + |
| 11 | M (13.2) | Hi | N | L | Hi | + | – | + | – | – | – | Both | + | – | + |
| | | | | R | Hi, Sau | + | – | + | – | – | – | Live | + | + | + |
| 12 | M (13.3) | Hi | N | R | – | + | + | – | + | – | – | Live | + | + | + |
| 13 | M (14.0) | Spn, Hi | Y | L | Spn, Hi | + | + | – | – | – | – | Live | + | – | + |
| | | | | R | Spn, Hi | + | + | – | – | – | + | Live | – | – | + |
| 14 | F (26.6) | Hi, | U | L | – | + | + | + | – | – | – | Live | + | – | + |
| | | Mcat | | R | – | + | + | + | + | + | – | Live | + | – | + |
| 15 | M (17.9) | – | Y | L | – | + | + | + | + | – | – | Live | + | + | + |
| | | | | R | AO | + | – | + | – | – | – | Live | + | + | + |
| 16 | M (18.5) | Sau | N | R | – | + | + | + | – | – | – | Live | + | + | – |
| 17 | F (16.9) | Spn, Hi | N | L | – | na | na | na | na | na | na | Live | + | + | + |
| | | Mcat | | R | – | na | na | na | na | na | na | Live | + | + | + |
| 18 | M (36.0) | – | Y | R | – | na | na | na | na | na | na | Both | + | – | + |
| 19 | M (9.7) | Mcat | N | R | – | na | na | na | na | na | na | Live | + | + | + |
| 20 | M (9.7) | Mcat, Hi | N | L | – | na | na | na | na | na | na | Live | + | – | + |
| | | | | R | – | na | na | na | na | na | na | Live | + | – | + |

**Table 1.** Cont.

| | | | | | | FISH 16S RNA Probe | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Child No. | Sex (Age,m) | NP culture | Abx | Ear | MEE culture | EUB | Spn | Hi | Mcat | Sau | PA | BacLight (Live/ Dead/Both) | Biofilm present | Intracellular bacteria observed | NETs |
| 21 | M (16.5) | – | Y | L | – | na | na | na | na | na | na | Both | + | – | + |
| 22 | M (32.0) | – | N | L | – | na | na | na | na | na | na | Both | + | + | + |
| | | | | R | – | na | na | na | na | na | na | Live | + | – | + |
| 23 | F (21.3) | Hi | U | L | – | na | na | na | na | na | na | Live | – | – | + |
| | | | | R | – | na | na | na | na | na | na | – | – | – | + |
| 24 | M (18.7) | – | Y | L | – | na | na | na | na | na | na | – | – | – | + |
| | | | | | 11/38 | 26/26 | 19/26 | 17/26 | 7/26 | 7/26 | 1/26 | 32/36 | 32/38 | 17/38 | 33/36 |
| Number positive | | | | | (29%) | (100%) | (73%) | (65%) | (27%) | (27%) | (4%) | (89%) | (84%) | (45%) | (92%) |

Biofilm and intracellular bacterial presence were also assessed.

Abbreviations: EUB – eubacterial probe, Spn – *S. pneumoniae* (organism and probe), Hi –*H. influenzae* (organism and probe), Mcat – *M. catarrhalis* (organism and probe), PA – *P. aeruginosa* (organism and probe), Sau – *S. aureus* (organism and probe), AO – *A. otitidis* (organism). na – Not available, Abx – taking antibiotics currently, U – Unknown, NP – nasopharyngeal culture.

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buffer was removed and MEE was incubated with a 1/100 dilution of rabbit anti-neutrophil elastase (Abcam, UK) in Zuk's buffer with 1% saponin (Sigma, St Louis, MO) for 60 min at room temperature. Antibody was aspirated and the MEE was washed 3×10 min in PBS. MEE was then incubated with 1/100 AlexaFluor 647 labelled goat anti-rabbit antibody (Invitrogen Technologies, Thornton, NSW) in Zuk's buffer with 1% saponin for 60 min at room temperature in the dark. Antibody was aspirated and the MEE was washed 3×10 min in PBS at room temperature before being counterstained with propidium iodide (Invitrogen Technologies, Thornton, NSW), mounted in low fade mounting media and stored at −20°C until imaging using the Leica TCS SP2 AOBS multiphoton confocal.

### Evaluation of actin presence using Phalloidin staining to determine whether neutrophil DNA was released via an active mechanism or necrosis

Seven MEEs were evaluated for actin presence by staining with Phalloidin. Briefly, 50ul of MEE in saline was air dried for 30 min at room temperature on SuperFrost plus slides. Samples were incubated with 50 µl of TRITC labeled Phalloidin (Invitrogen Technologies, Thornton, NSW) at room temperature for 60 min, washed 3 times with PBS, stained for 30 min at room temperature with 50 µl of SYTO9 and washed 3×5 min in PBS. Slides were mounted using a low fade mounting media and stored at −20°C until imaging using the Leica TCS SP2 AOBS multiphoton confocal.

### Lectin staining to elucidate whether bacterial formations were surround by a matrix

A subset of 10 MEEs was stained for lectins by staining with Concanavalin A (ConA) and Wheat Germ Agglutinin (WGA). Fifty µl of MEE in saline was air dried for 30 min at room temperature on SuperFrost plus slides. MEE were fixed in 4% formaldehyde for 15 min at 37°C. Samples were then washed 3×10 min with PBS at room temperature. Samples were incubated at room temperature in the dark with 50 µl of a 5 µg/ml of WGA conjugated with Oregon Green 488 (Molecular Probes, Eugene, Oregon) in PBS for 10 min. Samples were washed twice in PBS. MEEs were then stained with 50 µl of 50 µg/ml FITC labelled ConA (Sigma, St Louis, MO) for 30 min at room temperature. Samples were then washed 3×5 min in PBS before being counterstained with propidium iodide and mounted in low fade mounting media and stored at −20°C until imaging.

### Dornase alfa treatment of middle ear fluid

Seven MEEs were treated with two concentrations of a highly purified solution of recombinant human deoxyribonuclease I (Dornase alfa, Pulmozyme, Roche) which is an enzyme that selectively cleaves DNA. Briefly, 250 µl MEE was incubated with 250 µl of 5 ug/ml or 1 mg/ml Dornase alfa for 15 min at room temperature. Samples were centrifuged using a serofuge and rinsed three times using PBS and stained as previously described using the BacLight kit (Invitrogen Technologies) followed by CLSM imaging.

## Results

### Patient characteristics

Thirty eight MEEs obtained from 24 children undergoing VTI for rAOM were assessed for the presence of extracellular DNA and live bacteria using generic nucleic acid staining and CLSM. Five children were female (20%) and the median age was

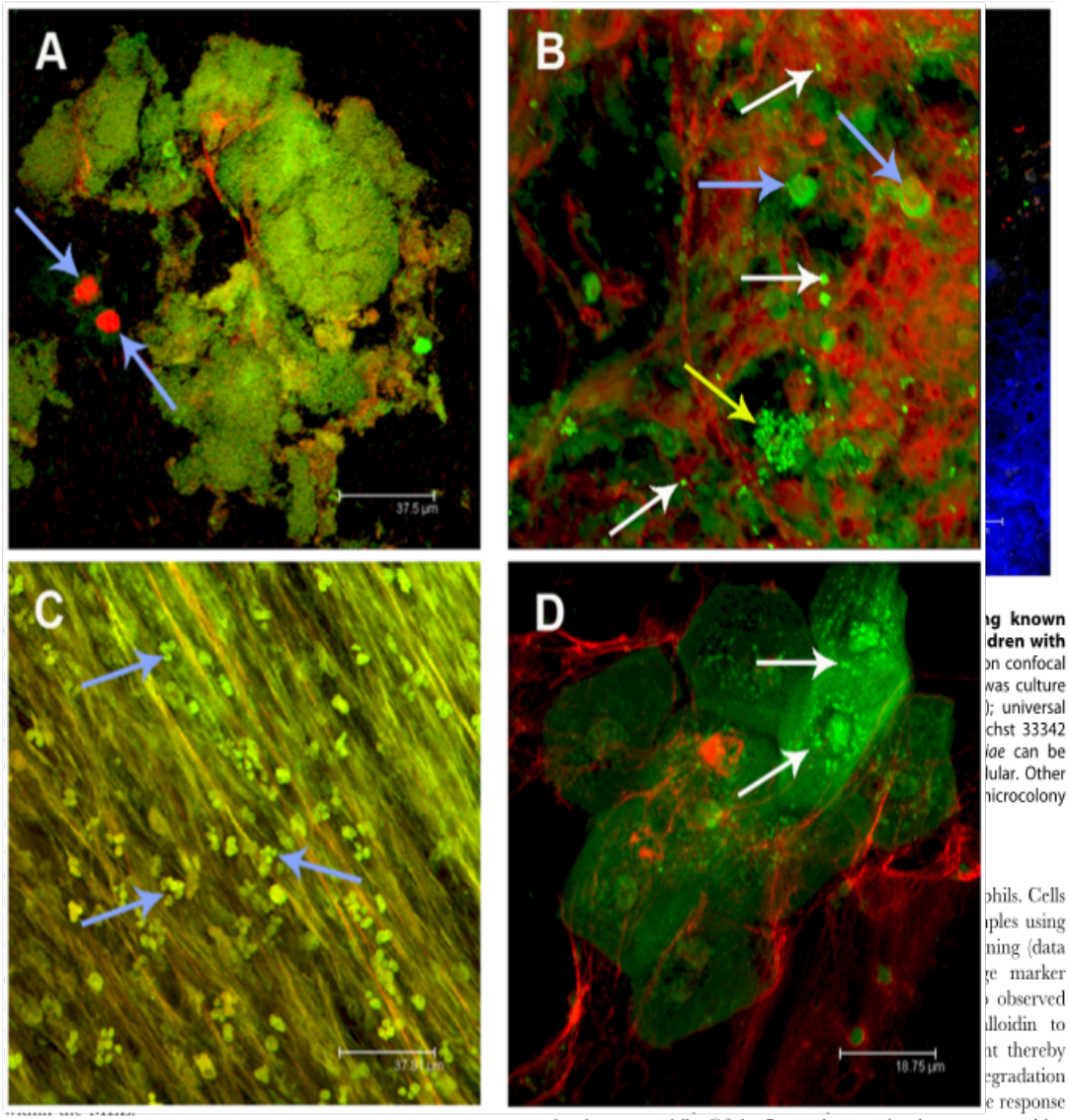


**Figure 1. Biofilms (containing live, dead and intracellular bacteria) and DNA stranding were observed in MEE from children with rAOM.** Representative images from MEEs stained with the BacLight kit. **A)** Large biofilm structures were observed. Both live (green) and dead bacteria (red) were present in MEE host cells (blue arrows). Bacteria with both dyes are evident (yellow). Host derived DNA strands were also present. **B)** Microcolonies of live bacteria (green, yellow arrows) within extensive nucleic acid matrices staining mainly with the propidium iodide component of the BacLight kit (red) were present throughout. Single bacteria (white arrows) and intact host nuclei were also observed (blue arrows). **C)** Extensive DNA stranding was present as were multi-lobed nuclei of intact neutrophils (blue arrow) apparent within the robotic DNA structure. **D)** Sloughed off epithelial cells containing live bacteria (arrows) were present in several MEE.
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## Polymicrobial biofilms containing known otopathogens

Polymicrobial biofilms containing known otopathogens were demonstrated in MEE samples using universal 16S rRNA and species specific PNA FISH probes (data not shown) with the universal EUB338 probe. Two or more otopathogenic species were observed within 70% of effusions (Table 1 and Figure 3) showing the presence of polymicrobial biofilms in the middle ear. Nineteen of 26 (73%) samples were positive for *S. pneumoniae*, 17 (65%) for *H. influenzae*, 7 (27%) for *M. catarrhalis*, 7 (27%) for *S. aureus* and 1 (4%) for *P. aeruginosa*. Using the NONEUB338 probe non-specific hybridisation was not observed for any of the MEE samples (data not shown). Bacteria were evident within epithelial cells (based on morphology – Figure 1D), though specific staining to identify these cells was not performed.

## Culture of nasopharyngeal swabs and MEE

Eighteen children had at least one pathogen isolated from the nasopharynx with 14 children carrying NTHi (58%), 6 *M. catarrhalis* (25%), 7 *S. pneumoniae* (29%) and 1 *S. aureus*. Nine children had 2 or more otopathogens isolated from the nasopharynx. Eleven MEEs (29%) were cultured positive for at least one of the otopathogens tested (Table 1).

## Extensive DNA stranding evident within the MEE is largely NET derived

Given the extensive DNA stranding present and high proportion of neutrophil nuclei evident by morphology (Figure 1C) we by the neutrophils. Of the 7 samples tested only one was positive

Treatment with Dornase alfa effectively breaks down the DNA structures and associated biofilms in the MEE

When MEEs were treated with Dornase alfa at a concentration used in CF therapy (1 mg/ml) and at 5µg/ml, complete fragmentation of the DNA matrix was observed in all samples within 15 minutes (Figure 6).

## Bacteria in MEE are viable and present in biofilm morphologies

To investigate whether bacteria within the MEE were viable despite low culture rates we used BacLight viability staining. Live bacteria were identified in 32 of the 36 MEE samples tested. In 20

## Discussion

Bacterial biofilm is increasingly recognised to play a role in the recurrence and persistence of infections such as OM. Demonstrating biofilm in very small tissue biopsies from the middle ear of children is technically difficult and may be biased by sampling and

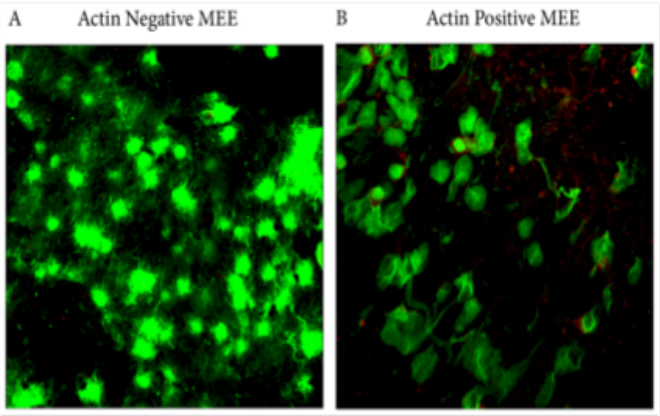


**Figure 4. Most DNA present in the MEE was negative for actin, indicating it was actively produced and not released due to necrosis.** MEE were stained with phalloidin to detect actin presence. MEEs were treated with the nucleic acid stain SYTO9 (green) and TRITC labelled Phalloidin (red). A) No red fluorescence was observed in most samples indicating actin was not present. This compared to B) where some actin is present (red). Even when the samples were positive for actin, this was not extensive, suggesting a combination of active DNA release and the presence of necrotic neutrophils is likely.
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probe selection [5,6]. This is the first study to identify multi-species biofilms consisting of live otopathogens in MEE samples from children with rAOM. Furthermore this is the first study to demonstrate the association of these biofilms with a DNA matrix produced by NETs which may represent a treatment target through the use of the therapeutic agent Dornase alfa which was able to fragment the DNA matrix.

In this study, bacterial biofilms were demonstrated in 84% of MEE from children undergoing VTI for rAOM. This is similar to rates of biofilm presence on the middle ear mucosa of children with chronic OME and rAOM (64–92%) [5,6] and in CSOM discharge samples (83%) [20]. MEE biofilms appear similar to those described in bronchoalveoloar lavage fluids from children with CF, where bacteria adhere indirectly to the mucosa [28] resulting in small bacterial aggregates in highly viscous fluid [29]. Biofilms in the MEE were not homogenous, suggesting discrete microcolonies, similar to patchy biofilm formations on the middle ear mucosa in the chinchilla model of OM [30].

Using FISH, biofilms within MEE were shown to be polymicrobial, consistent with those observed in the environment [31] and previously identified on the middle ear mucosa [5,6]. This is clinically relevant as in animal models, polymicrobial biofilms provide a protective advantage for bacteria compared to a single species biofilm [32]. Host inflammatory responses are

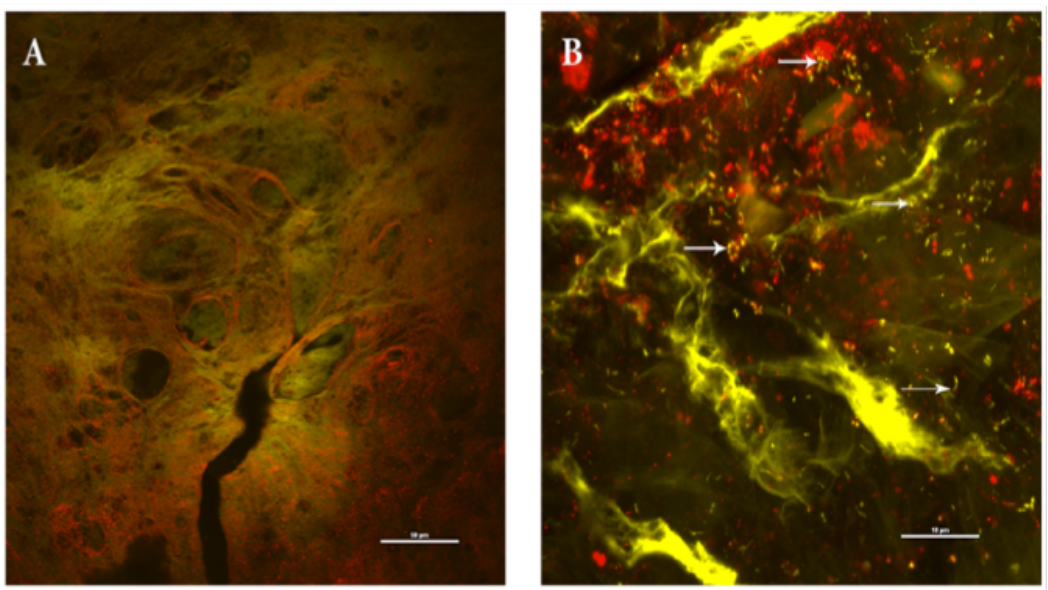


**Figure 5. Most DNA present in the MEE was neutrophil-derived.** MEE stained with neutrophil elastase AlexaFluor647 (Red) and propidium iodide (Yellow) and. A) Co-localisation of DNA and neutrophil elastase is present throughout the sample indicating the DNA that is present is neutrophil derived. Scale 50 µm. B) Co-localisation of DNA and neutrophil elastase is present throughout the sample as well as being bacterially associated throughout the sample (arrows). Scale 10 µm.
doi:10.1371/journal.pone.0053837.g005

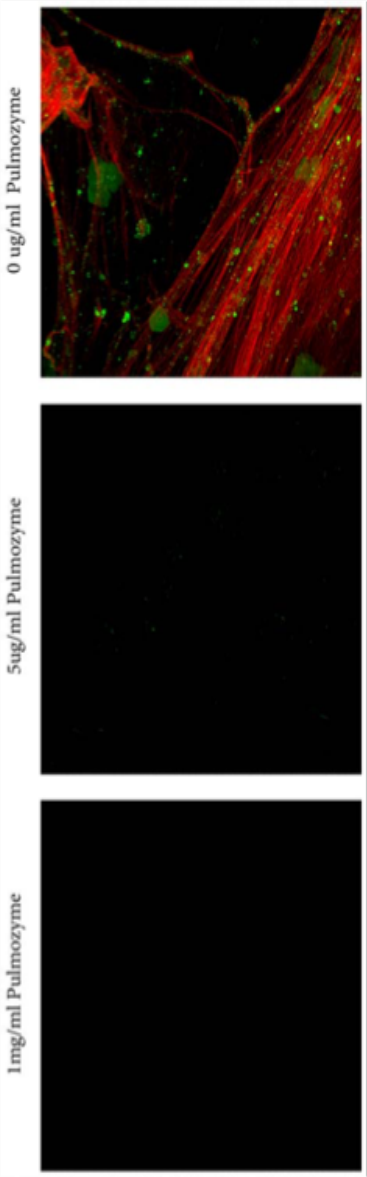


**Figure 6. Dornase alfa (Pulmozyme®) treatment of MEE results in disintegration of the NET structures.** DNA staining of MEEs from children with rAOM treated with Dornase alfa or left untreated. In the top panel, a control MEE untreated with Dornase alfa where extensive DNA stranding, epithelial cells and bacteria are evident. When treated with Dornase alfa even at concentrations as low as 5ug/ml complete fragmentation of the strands in the MEE are seen and nothing remains on the slide at 1 mg/ml.
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increased when multiple bacterial species are present regardless of whether they are able to be cultured [33,34]. While 92% of MEE's were positive for at least one of the known otopathogens, none bound the NONEUB338 (the nonsense or negative control) probe indicating the specificity of this technique. Large numbers of unidentified species were also present within the MEE (as identified by binding the EUB338 (universal) probe) and the high rates of bacterial detection using FISH suggest that this technique represents a sensitive and specific tool to assess bacteria present in MEE.

We demonstrated extensive extracellular DNA stranding in MEE, predominantly neutrophil derived through NET formation, and live bacteria within these. Both animal [10] and *in vitro* studies [8] suggest that the presence of such DNA, both host and bacterially derived, play a role in biofilm development and bacterial persistence in OM. NETs also play roles in interspecies competition for colonising the nasopharynx [35,36] and may also do so in the middle ear. Similarly to previous studies [21,37] we found *S. pneumoniae* and *H. influenzae* the most commonly identified pathogens in the MEE of children with rAOM. These species can resist NET killing despite high levels of antimicrobial proteins [9,16,18] due to production of DNases and through alteration of the bacterial surface [18]. The lipooligosaccharide modifications promoting NTHi survival in NETs are similar to those that promote biofilm formation [18]. In the chinchilla model of NTHi OM, a bacterial histone-like protein (IHF) is involved in structuring the extracellular DNA that plays a major role in stabilising biofilms [38]. The inability of NETs to clear otopathogens may contribute to establishing stable biofilm communities in the MEE [9,11,18]. This combined with the viscosity of the DNA may impede clearance of the MEE and the associated bacteria. Mast cells and eosinophils are also able to form extracellular traps [39] and their role in rAOM requires further investigation.

In the MEE, it is important to use both the nucleic acid staining and the specific FISH methodology to assess biofilms. Generic staining protocols such as the BacLight kit do not involve enzyme treatments or stringent wash steps, which are necessary to conduct FISH staining, which means that the biofilm morphology within the MEE is retained more reliably. Indeed biofilm presence was more often scored using this technique compared with using FISH. FISH however allows identification of specific species and their relationship within the sample. Intracellular pathogens were detected in 45% of effusions, and 36% of children. These rates are lower than those detected in biopsies from children in previous studies (71%) [6]. The lower detection rate likely reflects a higher proportion of cells containing pathogenic bacteria being sequestered within the tissue rather than shed into the MEE. While MEE may be useful to assess biofilm presence in the middle ear, it may not be as representative in determining intracellular sequestration.

The presence of non-cultureable but metabolically active bacteria in the MEE provides further evidence of biofilm involvement in OM. In this study 21% of children were culture positive for any of the main OM pathogens which is comparable with other published studies [5,40]. However, using viability staining, 89% of MEEs tested were positive for live bacteria, predominantly *H. influenzae* followed by *S. pneumoniae*. Discrepancies were present comparing FISH and culture, with bacteria observed more often using direct microscopic examination. These differences have been observed previously when comparing standard culture and a direct detection method such as FISH [41,42]. Detection of bacterial species in one ear of a single subject did not always correlate with the opposite ear using either culture or FISH. Findings of different pathogens in the ears of individual children are not isolated to our study [5] and may suggest the presence of different microbial reservoirs in each ear.

FISH represents a specific and sensitive tool for assessing bacteria in the MEEs and in addition provides spatial and morphological data. This could be important when considering larger scale studies, particularly monitoring clinical interventions to treat biofilm, where it is easier to collect and examine MEEs than biopsies. Using MEE to monitor intracellular persistence may not be as reliable as much lower rates of intracellular infection

were observed in the MEE when compared to mucosal biopsies. Direct comparisons of MEE and middle ear biopsies from the same child are required to confirm this. Data from animal models have also demonstrated a difference in the niches occupied by bacterial species in polymicrobial infections. Specifically, in the chinchilla model of *H. influenzae* and *M. catarrhalis* OM, where *M. catarrhalis* is seen to be exclusively associated with the epithelial surface while *H. influenzae* is detected in both the epithelium and the MEE [32].

Studies often use nasopharyngeal carriage of otopathogens as a surrogate for the infecting pathogens of the middle ear. This may be indicative with acute infection but less reliable in children with recurrent AOM. In this small study, we did not see a direct correlation between the pathogens in the middle ear with any of the detection methodologies with those cultured from the nasopharynx. This suggests that different bacteria may be found only in the middle ear space reflecting the presence of biofilm as a separate source of microbes causing the recurrent acute infections. Investigation of MEE and NPS samples using molecular detection techniques rather than relying solely on culture methodology is required as previous studies have demonstrated that non-culture-able organisms can be present in up to 36% of NPS using molecular methods [43].

Dornase alfa is the only licensed DNAse with efficacy in the treatment of CF [44] and has been demonstrated to improve lung function, reduce DNA levels, and to reduce the viscosity of airway secretions [45]. When we treated MEEs with Dornase alfa in the current study, complete and rapid degradation of the matrix was observed even at concentrations lower than those used clinically in CF. While the number of MEEs tested was small, this data suggests that Dornase alfa may be a useful adjunct treatment in recurrent or chronic OM. Directly degrading the DNA may improve bacterial clearance from the middle ear by reducing biofilm stability and causing bacteria to return to their planktonic form which are susceptible to locally (or systemically) administered antimicrobials and host immune mechanisms. In *in vitro* systems of neutrophil induced biofilms by *P. aeruginosa*, DNases had decreased activity in treating thicker, more mature biofilms or those containing mucoid strains of bacteria, possibly due to increased polysaccharide masking DNA cleavage sites [29]. This effect of DNase appears to be species specific, [8,12,38] and needs to be better characterized for otopathogens and for polymicrobial biofilms. Other biofilm components in the MEE also need to be better assessed as they may affect the efficacy of a DNase treatment.

This study includes a relatively small number of children with a specific clinical presentation of OM, being persistent effusion following rAOM episodes. Investigation of the role of biofilm in MEE and the middle ear mucosa in a population of children with a broader OM spectrum, including children with chronic OME and CSOM, and the potential of Dornase alfa are required.

These data provide an insight into the role of biofilm in the middle ear which is not only surface associated but present in the entire middle ear cavity. Live bacteria in microcolonies are present in extensive DNA stranding from NETs in the MEE of children with rAOM, contributing to effusion viscosity and may affect its failure to clear. The involvement of these biofilms with NET associated DNA indicates a potential for the novel use of Dornase alfa combined with currently used topical antibiotics at the time of surgery. We are currently conducting a clinical trial to assess the use of Dornase alfa as an adjunct therapy to VTI to prevent recurrent episodes of OM and possibly the need for repeat surgery. The potential use of Dornase alfa as a treatment for recurrent or chronic OM prior to surgery requires development of suitable methods of delivery to the middle ear and this warrants further investigation.

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## Author Contributions

Conceived and designed the experiments: RT SW LK PR SV HC PCR. Performed the experiments: RT SW. Analyzed the data: RT SW LK PR PCR. Contributed reagents/materials/analysis tools: RT SW LK PR SV HC PCR. Wrote the paper: RT SW LK PR SV HC PCR.

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# Predominance of nontypeable *Haemophilus influenzae* in children with otitis media following introduction of a 3+0 pneumococcal conjugate vaccine schedule

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ABSTRACT

In Australia the 7-valent pneumococcal conjugate vaccine (PCV7) is administered at 2, 4 and 6 months of age, with no booster dose. Information on bacterial carriage and the aetiology of recurrent acute otitis media (rAOM) after introduction of PCV7 using the 3+0 schedule is required to evaluate the potential impact of second generation pneumococcal vaccines. We found that 2–4 years after introduction of PCV7 in the National Immunisation Program, nontypeable *Haemophilus influenzae* (NTHi) was the predominant pathogen isolated from the nasopharynx and middle ear of children with a history of rAOM. Compared with healthy controls ($n=81$), NTHi and *Streptococcus pneumoniae* carriage rates were significantly higher in children with a history of rAOM ($n=186$) (19% vs. 56% $p<0.0001$ and 26% vs. 41%, $p=0.02$, respectively). Carriage of PCV7 pneumococcal serotypes was rare, whereas PCV7-related and non-PCV7 serotypes were isolated of 38% of cases and 24% of controls. Serotype 19A was the most common serotype isolated from the nasopharynx and middle ear and accounted for 36% (14/39) of total pneumococcal isolates with reduced susceptibility to cotrimoxazole. Of the 119 children carrying NTHi, 17% of isolates were β-lactamase positive.

The scarcity of PCV7 serotypes in children with and without a history of rAOM indicates that the 3+0 PCV7 schedule is preventing carriage and rAOM from PCV7 serotypes. Introduction of new vaccines in Australia with increased pneumococcal serotype and pathogen coverage, including 19A and NTHi, should decrease the circulation of antibiotic-resistant bacteria and reduce the burden of rAOM.

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## 1. Introduction

The global burden of recurrent acute otitis media (rAOM) on the population, health care systems and the economy remains significant, highlighting the need for effective preventative measures [1]. *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) together account for approximately 80% of cases of AOM, with *Moraxella catarrhalis* isolated in 3–20% of cases [2,3]. Colonisation of the nasopharynx with these otopathogens is directly related to the development of rAOM, with colonisation and first AOM episode at a young age being risk-factors for developing rAOM [4,5]. Despite high detection rates of these pathogens in the nasopharynx by bacteriological culture, middle ear effusions (MEE) are often culture-negative due to recent antibiotic treatment and bacterial survival in biofilms [6,7]. Polymerase chain reaction (PCR) provides a sensitive method for detection of non-cultureable bacteria in MEE [8,9].

Introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) at the beginning of this century significantly reduced invasive pneumococcal disease. Trials with this vaccine showed that the efficacy against PCV7-serotype OM was 57%. However, replacement with non-vaccine pneumococcal serotypes and other otopathogens

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such as NTHi has resulted in a limited reduction in overall AOM incidence of 6–9% [10,11]. PCV7 was introduced into the Australian National Immunisation Program for infants at 2, 4 and 6 months of age with no booster dose in 2005 as well as a catch-up program for all children up to 2 years of age at that time. It is important to investigate the bacterial aetiology of rAOM in PCV7-vaccinated children and whether the Australian 3+0 vaccine schedule, which is different from the booster schedules used in the UK (2+1) or the US (3+1), prevents nasopharyngeal carriage and OM from PCV7 serotypes.

Two pneumococcal conjugate vaccines with additional pneumococcal serotypes have recently been developed. PCV7 has been expanded to PCV13 by including 6 additional pneumococcal serotypes (1, 3, 5, 6A, 7F and 19A) [12]. This vaccine was shown to induce comparable immune responses to PCV7 and immunisation with PCV13 resulted in functional anti-polysaccharide antibodies against all 13 serotypes [13]. Pneumococcal nontypeable *H. influenzae* protein D conjugate vaccine (PHiD-CV) is a 10-valent PCV that in addition to the serotypes in PCV7 includes serotypes 1, 5 and 7F with the majority of the polysaccharides conjugated to NTHi Protein D [14]. A clinical trial of an 11-valent predecessor of PHiD-CV demonstrated protection against otitis media caused by *S. pneumoniae* and NTHi, leading to a reduction in NTHi carriage of 39% and NTHi AOM of 35% [15,16].

To estimate the impact of these or other new vaccines on nasopharyngeal carriage and AOM, especially in high-risk individuals, national data are important to ensure that the best vaccination strategy is chosen to give optimal serotype and pathogen coverage that is specific to a geographical area. Therefore, the aim of this study was to investigate nasopharyngeal carriage rates of common otopathogens (*S. pneumoniae*, NTHi, *M. catarrhalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and β-haemolytic streptococci) in children below 36 months of age and vaccinated with PCV7 at 2, 4 and 6 months, either with a history of rAOM requiring ventilation tube insertion or healthy children with no history of rAOM. MEE specimens from children with rAOM were cultured for the presence of the above otopathogens and PCR detection of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* was conducted to investigate the bacterial aetiology of rAOM in Australia.

## 2. Patients and methods

### 2.1. Recruitment of the study cohort

Between November 2007 and May 2009 children below 36 months of age were recruited to this study to investigate the microbiology, immunology and genetics of rAOM (the GROMIT Study). Of these children 98% had received three doses of PCV7 according to the Australian National Immunisation Program at 2, 4 and 6 months of age. Cases were defined as children with a history of at least 3 episodes of AOM requiring insertion of ventilation tubes. Children without rAOM undergoing general surgery (predominantly orthopaedics, strabismus, circumcision, cryptorchidism, hypospadias repair) were recruited as controls. Children with diagnosed immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities and chromosomal or genetic syndromes were excluded. Data on ear disease, vaccination status, antibiotic treatment and host- and environmental risk factors were collected by parental questionnaire and from medical records. The study was approved by the Ethics Committee of Princess Margaret Hospital for Children, Perth, Western Australia and by ethics committees and the institutional boards of hospitals in Perth where recruitment took place. Informed consent was obtained from parents of participating children before recruitment.

### 2.2. Nasopharyngeal swab collection and processing

Nasopharyngeal swabs were collected while the child was under general anaesthetic for the insertion of ventilation tubes (cases) or minor surgical procedures (controls). A small number of nasopharyngeal swabs (n = 10) from healthy controls were collected by a nurse during visits of healthy control children to the study centre's clinic. A sterile flexible cotton-wool tip swab (Copan, Brescia, Italy) was inserted trans-nasally, reaching the nasopharyngeal space. Swabs were immediately stored in sterile Skim-Milk–Tryptone–Glucose–Glycerol-Broth (STGGB; 3% tryptone soya broth [Sigma–Aldrich, Castle-Hill, Australia], 0.5% glucose, 2% skimmed milk powder and 10% glycerol in 100 mL distilled water), placed on ice and transported to the laboratory within 4 h. Samples were vortexed vigorously for 1 min and stored at −80 °C until analyses. A subset of samples was cultured immediately and after storage at −80 °C, showing no reduction in bacterial viability with storage.

### 2.3. Middle ear effusion (MEE) collection and processing

MEE samples were collected from anaesthetised children undergoing ventilation tube insertion for rAOM. An anterior-inferior myringotomy incision was made utilising the operating microscope and the MEE sample was collected with a sterile Leukotrap® [Pall corporation, New York, USA] connected to the surgical suction system. The tubing system was rinsed with approximately 1 mL of sterile saline to recover all of the MEE present. The MEE was immediately placed on ice and transported to the laboratory within 4 h where 200 μL of the collected sample was transferred into 1 mL sterile STGGB, vortexed vigorously for 1 min and stored at −80 °C until analyses.

### 2.4. Bacteriological culture and typing

All nasopharyngeal swabs and MEE specimens were examined for the presence of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *P. aeruginosa*, *S. aureus* and β-haemolytic streptococci using conventional culture methods as previously described [17]. In brief, 10 μL of the nasopharyngeal samples and 100 μL of each MEE sample were streaked onto horse blood agar, chocolate agar (containing 300 mg/L bacitracin, 5 mg/L vancomycin and 0.96 mg/L clindamycin), and colistin nalidixic acid blood agar plates [Oxoid, Australia]. The agar plates were incubated at 37 °C in a humid atmosphere containing 5% $CO_2$ and inspected at 24 h and 48 h for signs of bacterial growth. Four colonies of presumptive *S. pneumoniae*, two colonies each of NTHi and *M. catarrhalis*, and single colonies of *P. aeruginosa*, *S. aureus* and β-haemolytic streptococci were selected for sub-culture when present. When possible, morphologically distinct colonies of the same species were selected to increase the potential for detection of multiple subtypes. All sub-cultured *S. pneumoniae* isolates were serotyped at the Queensland Pneumococcal Reference Laboratory using type-specific capsular antiserum (Statens Serum Institute, Copenhagen, Denmark). All mucoid *H. influenzae* isolates ($n = 4$) and a 10% sample of non-mucoid isolates were tested with Phadebact *H.influenzae* type b reagent (Bactus AB, Huddinge, Sweden). All presumptive NTHi isolates were further tested by 16S rDNA colony PCR to distinguish true NTHi from non-haemolytic *Haemophilus haemolyticus* [18]. Primers and PCR cycle conditions are given in Table 1. Only true NTHi carriage rates are reported.

### 2.5. Antibiotic susceptibility testing

*S. pneumoniae* isolates were tested for susceptibility to oxacillin, chloramphenicol, cotrimoxazole, tetracycline, erythromycin and

**Table 1**
Primers used for the identification of *S. pneumoniae*, *H. influenzae*, *H. haemolyticus* and *M. catarrhalis*.

| Species | Target | Primer sequence (5′–3′) | PCR cycle conditions | PCR product size (bp) |
|---|---|---|---|---|
| *H. haemolyticus*[a] | *16S rDNA* | Fwd: TGACATCCAKRGRAYYYW<br>Rev: GCAGGTTCCCTACGGTTA | 30× 94 °C 30 s<br>55 °C 30 s<br>72 °C 60 s | 500 |
| *H. influenzae*[a] | *16S rDNA* | Fwd: TGACATCCTAAGAAGAGC<br>Rev: GCAGGTTCCCTACGGTTA | 30× 94 °C 30 s<br>55 °C 30 s<br>72 °C 60 s | 500 |
| *H. influenzae*[d] | *hpd* | Fwd: GCTAGCGGTTATTTACCAGAGC<br>Rev: GATGGTGGAACCAAGGTGC | 35× 94 °C 15 s<br>58 °C 15 s<br>72 °C 30 s | 424 |
| *S. pneumoniae*[b] | *ply* | Fwd: CTTGGCTACGATATTGGC<br>Rev: TACTTAGTCCAACCACGG | 35× 94 °C 30 s<br>58 °C 60 s<br>72 °C 150 s | 2400 |
| *S. pneumoniae*[c] | *lytA* | Fwd: CAACCGTACAGAATGAAGCGG<br>Rev: TTATTCGTGCAATACTCGTGCG | 30× 94 °C 15 s<br>60 °C 15 s<br>72 °C 30 s | 319 |
| *M. catarrhalis*[d] | *copB* | Fwd: GCTTTTGCAGCTGTTAGCCAG<br>Rev: CGATGATCGCCAGCAGTTAGC | 35× 94 °C 15 s<br>61 °C 15 s<br>72 °C 45 s | 677 |

bp, base pairs; *copB*, *M. catarrhalis* major outer membrane protein; Fwd, forward primer; *hpd*, *Haemophilus influenzae* protein D; *lytA*, autolysin A; *ply*, pneumolysin; Rev, reverse primer; rDNA, ribosomal DNA.

[a] [18].

[b] Jefferies JM, Johnston CH, Kirkham LA, Cowan GJ, Ross KS, Smith A, et al. Presence of nonhemolytic pneumolysin in serotypes of Streptococcus pneumoniae associated with disease outbreaks. J Infect Dis 2007;196:936–44.

[c] Nagai K, Shibasaki Y, Hasegawa K, Davies TA, Jacobs MR, Ubukata K, et al. Evaluation of PCR primers to screen for Streptococcus pneumoniae isolates and beta-lactam resistance, and to detect common macrolide resistance determinants. J Antimicrob Chemother 2001;48:915–8.

[d] This study.

moxifloxacin by agar disc diffusion [Oxoid] according to the 2009 Clinical Laboratory Standards Institute (CSLI) guidelines. For oxacillin resistant pneumococci, the penicillin and ceftriaxone minimum inhibitory concentrations (MIC) were then determined by E-test [bioMerieux, Australia], with the more conservative break-points for penicillin adopted (<0.06 mg/L susceptible, 0.12–1.0 mg/mL intermediate, and >2.0 mg/mL resistant). All NTHi isolates were tested for β-lactamase production using cefinase discs [Becton Dickinson, New Jersey, USA]. β-Lactamase positive NTHi were further tested by agar disc diffusion for susceptibility to ampicillin, amoxicillin, cefotaxime, ciprofloxacin, clarithromycin, cotrimoxazole, sulfamethoxazole/trimethoprim and tetracycline according to the CSLI guidelines.

### 2.6. PCR of MEE for detection of S. pneumoniae, H. influenzae and M. catarrhalis

MEE samples were defrosted, transferred to 1.5 mL microcentrifuge tubes and centrifuged at maximum speed on a benchtop centrifuge for 2 min. The supernatant was carefully removed and the pellet resuspended in 500 μL SDS-lysis buffer (20% 0.5 M EDTA, 1% 1 M Tris pH 8.0 and 0.5% SDS in nuclease free water). Samples were incubated at 37 °C for 1 h then at 50 °C for 3 h with 100 μg/mL Proteinase K to ensure complete lysis of all cells and to degrade DNAses. Samples were then processed using the Wizard SV genomic (g) DNA extraction kit [Promega, Alexandria, Australia] following the manufacturers e-note instructions for gDNA extraction from Gram-negative bacteria. The gDNA was eluted in 80 μL nuclease free water and stored in aliquots at −20 °C. PCR was conducted on gDNA prepared from all MEE for detection of *S. pneumoniae* (pneumolysin, *ply*, Genbank accession number: AAK75991.1; and autolysin A, *lytA*: NC_008533.1), *H. influenzae* (*Haemophilus* protein D, *hpd*: AAX87718.1) and *M. catarrhalis* (outer membrane protein, *copB*: L12346.1). Table 1 lists the PCR primers and cycle conditions. All PCR preparations were incubated at 94 °C for 5 min prior to cycling and 72 °C for 10 min for a post-cycle final extension. The sensitivity of each PCR was assessed on gDNA preparations from 2 colony forming units (cfu) of *S. pneumoniae* (D39, ATCC #7466), *M. catarrhalis* (ATCC #25238) and NTHi (86-028NP) reference strains.

### 2.7. Statistical analyses

Host and environmental risk-factors were compared between children with and without rAOM using *t*-tests for continuous variables (age) and Pearson Chi-square analyses (*p*-value asymptotic significant 2-sided) for categorical variables (day-care attendance, siblings, gender, PCV7-vaccination status and antibiotic use). Nasopharyngeal carriage of bacteria, specific combinations of co-occurring species, and antibiotic susceptibility patterns were compared between children with and without a history of rAOM using Pearson Chi-square analyses. In addition, carriage data were analysed in three age-groups (0–12 months, 13–24 months and 25–36 months) and binary logistic regression was performed correcting for age, gender, antibiotic use and day-care attendance. Difference in detection of bacteria in MEE using either culture or PCR was compared using McNemar's test. Data were analysed using the statistical analysis package SPSS for Windows, version 16.0.

## 3. Results

### 3.1. Study population

One hundred and eighty-six children with a history of at least three episodes of AOM (median of 7 AOM episodes) in the first 3 years of life and requiring the insertion of ventilation tubes were enrolled. Of the 186 cases, 43 children had no MEE present. Of the remaining 143 children, 97 had bilateral effusion and 46 had unilateral effusion present, resulting in a collection of 240 MEE samples. In the healthy control group 81 children were recruited. Hospital policy is to only proceed with surgery if the child is well and without current infection; therefore all children were asymptomatic at the time of sample collection. General characteristics of the study cohort are described in Table 2. The mean age of rAOM children was 20.8 (7.3–36.0) months and of controls 16.5 (3.4–35.0) months ($p = 0.001$), therefore carriage rates were also compared within three age-groups (0–12 months, 13–24 months and 25–36

**Table 2**
Host- and environmental risk factors in children with rAOM and healthy controls.

| | rAOM | Controls | *p*-Value |
|---|---|---|---|
| Number | 186 | 81 | n/a |
| Host factors | | | |
| Mean age in months (range) | 20.8 (7.3–36.0) | 16.5 (3.4–35.0) | 0.001 |
| Male (%) | 113 (61%) | 62 (77%) | 0.01 |
| AOM episodes total ($n = 174$) | | | n/a |
| 0–2 | – | 81 (100%) | |
| 3–4 | 57 (33%) | – | |
| 5–7 | 30 (17%) | – | |
| 8–9 | 63 (36%) | – | |
| ≥10 | 24 (14%) | – | |
| Median # AOM episodes | 7 | n/a | |
| Environmental factors[a] | | | |
| Antibiotic use | | | <0.001 |
| Currently | 44/172 (26%) | 13/79 (17%) | 0.1 |
| Last month | 61/172 (36%) | 6/79 (8%) | <0.001 |
| Not recently | 67/172 (39%) | 60/79 (76%) | <0.001 |
| Having siblings | 123/173 (71%) | 53/79 (67%) | 0.5 |
| Attending day-care ≥4 h/week | 105/171 (61%) | 20/79 (25%) | <0.001 |
| Fully vaccinated with PCV7 | 184/186 (99%) | 78/81 (96%) | 0.2 |
| Being breastfed | 160/173 (93%) | 74/79 (94%) | 0.7 |
| Exposed to cigarette smoke | 31/173 (18%) | 17/79 (22%) | 0.5 |
| Pacifier used | 106/173 (61%) | 50/79 (63%) | 0.8 |

[a] Environmental data were not available for all children included in this study.

**Table 3**
Bacterial carriage and co-colonisation in children with and without rAOM.

| Bacteria | rAOM 186 | Healthy control 81 | *p*-Value |
|---|---|---|---|
| Total children culture positive for at least one pathogen | 147 (79%) | 43 (53%) | 0.001 |
| NTHi (total) | 104 (56%) | 15 (19%) | <0.001 |
| *S. pneumoniae* (total) | 76 (41%) | 21 (26%) | 0.002 |
| VT | 6 (3%) | 2 (3%) | 0.8 |
| VRT | 38 (20%) | 14 (17%) | 0.2 |
| NVT | 33 (18%) | 6 (7%) | 0.2 |
| *M. catarrhalis* (total) | 80 (43%) | 32 (40%) | 0.6 |
| **Only one pathogen** | | | |
| *S. pneumoniae* | 18 (10%) | 6 (7%) | 0.6 |
| NTHi | 34 (18%) | 5 (6%) | 0.01 |
| *M. catarrhalis* | 10 (5%) | 12 (15%) | 0.01 |
| **Two pathogens together** | | | |
| *S. pneumoniae* + NTHi | 15 (8%) | 0 | 0.009 |
| *S. pneumoniae* + *M. catarrhalis* | 15 (8%) | 10 (12%) | 0.3 |
| NTHi + *M. catarrhalis* | 27 (15%) | 5 (6%) | 0.05 |
| **Three pathogens together** | | | |
| *S. pneumoniae* + NTHi + *M. catarrhalis* | 28 (15%) | 5 (6%) | 0.04 |

VT: PCV7 vaccine type; VRT: PCV7 vaccine related type; NVT: non-PCV7 vaccine type.
Number and percentage of children with either no, 1, 2 or 3 of the three main OM pathogens *S. pneumoniae, nontypeable H. influenzae* (NTHi), *M. catarrhalis* present in the nasopharynx of children with recurrent acute otitis media (rAOM) and healthy controls.

months). Antibiotic use was significantly higher in the cases compared with the controls with 61% of cases using antibiotics currently or within the last month, compared with 24% in the control group ($p < 0.001$). The control group consisted of more males than the group of children with rAOM ($p = 0.01$), which was due to the high frequency of male-specific surgical procedures in the control group (circumcision, cryptorchidism, hypospadias repair). Of the children with rAOM, 61% attended day-care ≥4 h a week, compared with 25% of the controls ($p < 0.001$) confirming that day-care attendance is a risk-factor for rAOM. No Aboriginal children were recruited in either of the two groups and the presence of siblings, PCV7 vaccine coverage, breast feeding, cigarette smoke exposure and pacifier use were similarly distributed between children with and without a history of rAOM.

### 3.2. Nasopharyngeal bacterial carriage in children with and without rAOM

In children with rAOM, 79% of the nasopharyngeal swabs were culture-positive for at least one major otopathogens (*S. pneumoniae*, NTHi and *M. catarrhalis*), compared with 53% of healthy children ($p < 0.001$; Table 3). NTHi was the most common pathogen isolated from the nasopharynx of children with a history of rAOM whereas this was the third most common pathogen isolated from the nasopharynx of healthy children (56% vs. 19%; $p < 0.001$), who were predominantly colonised with *M. catarrhalis* (40%). In addition, when adjusting for age, gender, antibiotic use and day-care attendance using binary logistic regression, carriage of NTHi was significantly associated with the development of rAOM (odds ratio 5.4; 95% CI 2.6–11.1). Children with a history of rAOM were also significantly more often colonised with *S. pneumoniae* compared with healthy controls (41% vs. 26%; $p = 0.02$). When adjusting for age, gender, antibiotic use and day-care attendance, the odds ratio for carriage of *S. pneumoniae* associated with development of rAOM was 2.2 (95% CI 1.1–4.3). There was no significant difference in nasopharyngeal carriage rates for *M. catarrhalis* (43% vs. 40%; $p = 0.59$), *S. aureus* (11% vs. 9%; $p = 0.52$) or β-haemolytic streptococci (3% vs. 0%; $p = 0.14$) between children with and without rAOM. *P. aeruginosa* was not isolated from the nasopharynx of any child in this study.

A similar percentage of children with rAOM and healthy controls was colonised with only one of the three major otopathogens at a time (33% vs. 28%; Table 3). In children with rAOM, single-species carriage was pre-dominantly observed with NTHi, which was significantly higher in cases compared with controls (18% vs. 6%; $p = 0.01$). However, in healthy children single-species colonisation with *M. catarrhalis* was most common and significantly higher in the controls compared with children with rAOM (14.8% vs. 5%; $p = 0.01$). *S. pneumoniae* and NTHi were found together in 15 of the 186 children with a history of rAOM (8%), but this was never observed in healthy controls ($p = 0.009$). Co-colonisation rates of *M. catarrhalis* either with NTHi or *S. pneumoniae* were not different between children with or without rAOM (Table 3).

Bacterial carriage was also analysed in three age-groups (Table 4). Carriage of NTHi was significantly higher in cases compared with controls in all three age-groups, being 59% vs. 19% ($p = 0.003$), 53% vs. 18% ($p = 0.001$) and 56% vs. 18% ($p = 0.009$) in the 0–12 month, 13–24 month and 25–36 month age-groups, respectively. For *S. pneumoniae*, carriage was significantly higher in cases compared with controls in the 13–24 month age-stratum only (46% vs. 23%, $p = 0.046$). There were no significant differences for carriage of the other bacteria when comparing age-groups.

### 3.3. Microbiological findings in middle ear effusion using culture and PCR methods

Of the 143 children with rAOM from which MEE was collected, 73% were culture-negative. NTHi was the most common bacterium, which was isolated from the MEE of 19 (13%) children, followed by *M. catarrhalis* from 9 (6%), *S. pneumoniae* from 8 (6%), *S. aureus* from 5 (4%), *P. aeruginosa* from 2 (1%) and β-haemolytic streptococci from 1 child (Table 5). Isolation of two different otopathogens from the MEE of the same child was rare. The pathogens isolated from the middle ear were also present in the nasopharynx, except *P. aeruginosa* which was isolated from the MEE of two children that were not colonised with this pathogen. The proportion of MEE samples positive for at least one of the three major otopathogens increased by PCR, with detection of NTHi increasing from 19 (13%) positive children by culture to 67 (47%) positive children by PCR ($p < 0.001$). *M. catarrhalis* detection

**Table 4**
Bacterial carriage according to age.

| Age | *N* | | NTHi | | Pnc | | Mc | |
|---|---|---|---|---|---|---|---|---|
| | rAOM | Control | rAOM | Control | rAOM | Control | rAOM | Control |
| 0–12 | 17 | 37 | 11 (59%)** | 7 (19%) | 5 (29%) | 10 (27%) | 7 (41%) | 14 (38%) |
| 13–24 | 114 | 22 | 62 (53%)** | 4 (18%) | 52 (46%)* | 5 (23%) | 53 (47%) | 10 (46%) |
| 25–36 | 55 | 22 | 31 (56%)** | 4 (18%) | 19 (35%) | 6 (27%) | 20 (36%) | 8 (36%) |

Number and percentage of children with recurrent acute otitis media (rAOM) and healthy controls carrying nontypeable *H. Influenza* (NTHi), *S. pneumoniae* (Pnc) and *M. catarrhalis* (Mc) according to three age-groups (months).
* $p < 0.05$ when carriage rate is compared with that in controls.
** $p < 0.01$ when carriage rate is compared with that in controls.

in the MEE increased from 9 (6%) to 19 (13%) children ($p = 0.013$), whereas *S. pneumoniae* detection was only increased by 1 child from 8 (6%) by culture to 9 (6%) using PCR analysis ($p = 1.00$) (Table 5). MEE samples from 6 children were culture-positive but PCR-negative. This occurred twice for *M. catarrhalis*, once for NTHi and 3 children were culture-positive *for S. pneumoniae* but negative for both pneumococcal PCR targets. Of these MEE samples, most had only 1 or 2 colonies isolated from 100 μL MEE. Direct PCR on cell lysate from the sub-cultured isolate gave a positive PCR result.

### 3.4. Pneumococcal serotypes in the nasopharynx and middle ear

Nasopharyngeal swabs of 76 children with a history of rAOM (41%) and 21 controls (26%) were positive for *S. pneumoniae*. PCV7 serotypes were infrequently found in both groups of children, with 5 children carrying serotype 19F and one serotype 4 in the rAOM group. Two healthy controls carried serotype 6B. PCV7-related serotypes were isolated from 38 children with rAOM, with 19A isolated from 24 cases (13%), 6A and 6C from 5 children each and 23B from 4 cases. In the healthy control group, 14 vaccine-related serotypes were isolated, which were serotype 19A and 6A from 5 healthy children, 23B from 2 children and 6C and 23A from one child each. Non-PCV7 serotypes were isolated from 33 cases, being 9 children with 11A (12%) and 7 with 15B (10%). Serotype 15C, 16F, 22F and 35B were isolated from 3 rAOM children each, 17F from 2 children, and 33F, 10A and 35F isolated once. Two healthy controls were colonised with serotype 15B and serotypes 15C, 35B, 10A and 35F where each found once. Two of the 76 children with rAOM carried two different pneumococcal serotypes concurrently (19A/6C and 19F/15B) and one pneumococcal isolate was nontypeable. One of the 21 pneumococcal positive healthy controls was concurrently colonised with serotypes 19A and 15C.

Of the 14 *S. pneumoniae* culture-positive MEE samples from 8 children, 3 were serotype 19A. The other serotypes (15C, 16F, 17F, 19F, 23B) were isolated from the middle ear of 1 child each (Table 5). In all 8 children, the pneumococcal serotype isolated from the middle ear was the same serotype identified the nasopharynx. Where a child had bilateral MEE and both samples were *S. pneumoniae* culture-positive, the pneumococcal serotype from each ear was identical. The one case of AOM from vaccine type 19F was in a 14-month-old child who was fully vaccinated according to the National Immunisation Program.

**Table 5**
Bacterial presence in middle ear effusion using culture and PCR.

| | Culture-positive | PCR-positive | *p*-Value |
|---|---|---|---|
| *H. influenzae* | 19 (13%) | 67 (47%) | <0.001 |
| *S. pneumoniae* | 8 (6%) | 9 (6%) | 1.00 |
| VT (19F) | 1 (1%) | nd | |
| VRT (19A, 23B) | 4 (3%) | nd | |
| NVT (15C, 16F, 17F) | 3 (2%) | nd | |
| *M. catarrhalis* | 9 (6%) | 19 (13%) | 0.013 |
| *S. aureus* | 5 (4%) | nd | |
| *P. aeruginosa* | 2 (1%) | nd | |
| β-Haemolytic streptococcus | 1 (1%) | nd | |

Number of otitis-prone children ($n = 143$) with middle ear effusion (MEE) that was culture or PCR positive for otopathogens. nd, not determined by PCR. VT, PCV7 vaccine type; VRT, PCV7 vaccine-related type; NVT, non-PCV7 vaccine type. *p*-Value McNemar's test to determine difference between culture and PCR methods.

### 3.5. Antibiotic usage, presence of bacteria and antibiotic susceptibility

Questionnaire data on antibiotic usage were available for 172 cases (93%) and 79 controls (98%). Cases had higher rates of antibiotic usage, either currently (26%) or in the last month (35%) compared with controls (16% and 8%, respectively; Table 2). The most commonly used antibiotics by children with and without rAOM were β-lactams (amoxicillin and/or penicillin) and cotrimoxazole. Despite the higher rates of antibiotic usage in children with rAOM, cases still had higher carriage rates for NTHi, *S. pneumoniae* or *M. catarrhalis* compared with controls (Table 3).

Of the 105 children using antibiotics, 53 children carried NTHi (51%) and that of the 67 not using antibiotics, 42 carried NTHi (63%), which is not significantly different ($p = 0.12$). Of the 105 children using antibiotics, 37 carried *S. pneumoniae* (35%) and of the 67 not using antibiotics, 36 carried *S. pneumoniae* (54%; $p = 0.003$). This indicates that recent antibiotic use decreases carriage rates of *S. pneumoniae* but not NTHi.

Similarly, current or recent antibiotic use did not influence the presence of NTHi in the middle ear as determined by PCR. There were 47 children with MEE present that were not using antibiotics. Of these, 23 had NTHi (49%) detected in the MEE. Of the 84 children that were currently or recently using antibiotics 37 (44%) had NTHi ($p = 0.6$).

Of the 119 children carrying NTHi (104 cases and 15 controls), 17% of isolates were β-lactamase positive (18/104 and 2/15). All of the β-lactamase positive NTHi were also resistant to ampicillin, and 7 of these strains had reduced susceptibility to sulfamethoxazole/trimethoprim, 5 to tetracycline, 4 to cefotaxime and 1 with reduced susceptibility to clarithromycin. Of the 19 children that had NTHi isolated from their ear, one was infected with a β-lactamase positive NTHi.

The percentage of pneumococci with reduced antibiotic susceptibility was similar between cases and controls, with resistance to cotrimoxazole (28/76 in rAOM group and 11/21 healthy children; $p = 0.2$) and penicillin (17/76 children with rAOM and 1/21 healthy children; $p = 0.07$) most common. Serotype 19A strains accounted for 39% (11/28) of isolates with reduced susceptibility to cotrimoxazole in the rAOM group and for 27% (3/11) in the healthy group. In the rAOM group 11 children carried pneumococci that had reduced susceptibility to multiple antibiotics. Of these 11, 6 were serotype 19A and 3 were serotype 19F. Of the 8 children that had a pneumococcus isolated from their ear, 1 child was infected with a 19A strain with reduced susceptibility to 5 antibiotics and one with a 19F strain with reduced susceptibility to 4 antibiotics.

## 4. Discussion

Australia introduced a national PCV7 immunisation schedule in 2005 where 3 primary doses of PCV7 are given at 2, 4 and 6 months of age, with no 12-month booster dose (3 + 0 schedule). A catch-up program was also implemented in the process of vaccine introduction for children under 2 years. We have found that 4 years after the implementation of a 3 + 0 PCV7 vaccination schedule, non-Aboriginal children below 36 months of age with a history of rAOM (but asymptomatic at the time of sample collection) predominantly have NTHi isolated from their nasopharynx and middle ear. Studies in several countries have identified NTHi as the leading cause of otitis media [8,19–22] both pre- and post-PCV7 vaccination, however there is evidence in the US that the proportion of NTHi AOM is increasing as a result of PCV7 vaccination [23,24]. We observed that following introduction of PCV7, carriage of PCV7 serotypes is rare (3%) in both children with and without rAOM, whereas carriage of PCV7-related and non-PCV7 serotypes is more frequent. This is in accordance with data from the US showing a reduction in carriage of vaccine serotypes from 36% in 2001, before widespread use of PCV7, to 3% in 2007 [19] and another study showing that carriage of PCV7 serotypes was as low as 1% 6 to 8 years after introduction of PCV7 using a 3 + 1 vaccine schedule [19,25]. Even though it was recently shown that children vaccinated at 2, 4 and 6 months of age with a 3 + 0 vaccine schedule had lower antibody levels at 13 and 19 months of age compared with children receiving a 2 or 3 dose primary series followed by a booster dose at 12 months [26], our data suggest that the Australian 3 + 0 vaccine schedule prevents carriage of PCV7 serotypes to a similar extent as a PCV7 vaccine schedule with a booster dose. Indeed Jones et al. observed no additional effect of a PCV7 booster dose on pneumococcal nasopharyngeal carriage in healthy US children [27]. In contrast, a study by Cohen et al. demonstrated that after an additional fourth dose, carriage of PCV7 serotypes in French children with AOM further decreased from 24% to 12% [28]. Recent data from Van Gils et al. support the proposal to explore the use of reduced dose PCV7 vaccine schedules, showing that even a 2-dose schedule, with or without a booster at 11 months, resulted in a significant reduction of PCV7 serotype carriage in the second year of life, compared with no pneumococcal vaccination [29]. This is of particular importance to developing countries where such a schedule is more feasible. Further studies are necessary to improve our understanding of the immunogenicity of different pneumococcal vaccine schedules and how this relates to nasopharyngeal bacterial carriage and disease.

We found that pneumococcal serotypes 19A, 6A, 15B and 11A were the most commonly carried serotypes in our study population, which is in accordance with studies from the US that described that by 2007 these were also the most commonly carried pneumococcal serotypes [19,25]. As the numbers of pneumococcal-positive MEE were low in our study, we cannot comment on the impact of PCV7 on pneumococcal AOM. However, serotype 19A was the most common pneumococcal serotype isolated from the MEE, which is in accordance with a recent report from the US where 19A was the most common cause of pneumococcal AOM [19]. Some studies using opsonophagocytic activity assays suggest that vaccines including serotypes 6B and 19F may induce functional antibodies against serotypes 6A and 19A, although the degree of this cross-protection appears greater for 6A than for 19A [30–33]. Surveillance data have shown that the widespread use of PCV7 including serotypes 6B and 19F prevented invasive pneumococcal disease from serotype 6A but not from 19A [34]. To reveal potential cross-protection induced by the 10-valent or 13-valent pneumococcal conjugate vaccine, close monitoring of pneumococcal carriage and OM after the widespread use of these vaccines is required.

A recent study by Prymula et al. demonstrated a reduction in nasopharyngeal carriage of NTHi as determined by culture and PCR by 37% in PHiD-CV vaccinated children when compared with a control group but this was not significant [16]. However, NTHi carriage was extremely low in the study population, with only 16 out of 177 (9%) children carrying NTHi in the vaccine group and 25 of 175 (14%) carrying NTHi in the control vaccine arm. Further investigations into the effects of PHiD-CV on NTHi carriage and disease in an environment with high NTHi carriage rates are warranted.

The MEE culture rates for NTHi, *S. pneumoniae* and *M. catarrhalis* were low in our study being 13%, 6% and 6%, respectively. A recent study by Casey et al. on MEE samples collected from US PCV7-vaccinated children with rAOM aged 6–36 months, reported considerably higher culture rates of 45%, 42% and 13% respectively [19]. This difference may be explained by the fact that Casey et al. collected MEE by tympanocentesis *during* an AOM episode, whereas we collected MEE from children *between* AOM episodes when they are undergoing ventilation tube insertion and are asymptomatic. The PCR detection rate for *S. pneumoniae* in our study (6%) is at the lower end of the range of previous reports from OME studies (4–8%) [8,20,35] and AOM studies (28–47%) [36–38], however, the PCR detection rate for NTHi in MEE for our study (40%) is within the range of 14–58% described in previous reports [8,20,21,35,36], such as a recent study by Kaur et al. found that 42% of MEE from tympanocentesis of US children with AOM were positive for NTHi (by culture and/or PCR) [36]. In studies where the prevalence of all three major otopathogens is compared between culture and PCR, NTHi is generally the pathogen with the greatest increase in detection by PCR [8,20,35], which we also observed. Intracellular persistence of NTHi, which has been implicated in conditions such as chronic obstructive pulmonary disease and rAOM [39–42], may explain the increased proportion of MEE that were NTHi PCR-positive but culture-negative when compared with other otopathogens. An OME study has also reported no increase in detection of pneumococci in MEE by PCR when compared with culture [20]. The reasons for this are unclear particularly as we used SDS-treatment of MEE, which has been shown to improve lysis of Gram-positive bacteria prior to gDNA isolation [43].

There is debate about the importance of PCV7 use in the development of antibiotic resistant pneumococcal strains and serotypes, with some papers describing an increase [24,28,44] and others a decrease [45,46] in penicillin susceptible pneumococci in OM after wide-spread use of PCV7. After introduction of routine PCV7 vaccination the prevalence of non-vaccine serotype 19A, often multi-drug resistant, has increased [45,46] however, care should be taken when assuming a causal relationship between PCV7 vaccination and an increase in 19A disease, since recent reports describe an increase in multi-drug resistant 19A in countries without routine PCV7 vaccination [47,48]. We also observe high carriage rates of pneumococcal serotype 19A and a high proportion of reduced susceptible pneumococcal isolates being 19A. However, due to the lack of pre-PCV7 data we cannot comment on the effect of PCV7 and the impact of a 3 + 0 vaccination schedule on current serotype distribution and antibiotic resistance patterns.

As described in Table 2 there are differences in the distribution of risk factors between the two groups of children either with or without OM in our study, which may influence the comparison between these groups, however we corrected for these factors in our statistical analyses.

In conclusion, local data will facilitate the decision of which second generation pneumococcal vaccine and vaccination schedule will suit a specific geographical region. NTHi was the predominant pathogen isolated from the nasopharynx and middle ear of PCV7-vaccinated non-Aboriginal Western Australian children with a history of rAOM. Introduction of second generation vaccines with

increased pneumococcal serotype and pathogen coverage, including NTHi and *S. pneumoniae* serotype 19A have the ability to reduce the burden but not solve the problem of rAOM in Australia, since new pneumococcal serotypes, like 11A and 15B, seem to become more common. Investigations into vaccines with increased coverage of pathogens, such as vaccines including conserved protein antigens, are warranted.

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# IgG Responses to Pneumococcal and Haemophilus Influenzae Protein Antigens Are Not Impaired in Children with a History of Recurrent Acute Otitis Media

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## Abstract

***Background:*** Vaccines including conserved antigens from *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) have the potential to reduce the burden of acute otitis media. Little is known about the antibody response to such antigens in young children with recurrent acute otitis media, however, it has been suggested antibody production may be impaired in these children.

***Methods:*** We measured serum IgG levels against 4 pneumococcal (PspA1, PspA 2, CbpA and Ply) and 3 NTHi (P4, P6 and PD) proteins in a cross-sectional study of 172 children under 3 years of age with a history of recurrent acute otitis media (median 7 episodes, requiring ventilation tube insertion) and 63 healthy age-matched controls, using a newly developed multiplex bead assay.

***Results:*** Children with a history of recurrent acute otitis media had significantly higher geometric mean serum IgG levels against NTHi proteins P4, P6 and PD compared with healthy controls, whereas there was no difference in antibody levels against pneumococcal protein antigens. In both children with and without a history of acute otitis media, antibody levels increased with age and were significantly higher in children colonised with *S. pneumoniae* or NTHi compared with children that were not colonised.

***Conclusions:*** Proteins from *S. pneumoniae* and NTHi induce serum IgG in children with a history of acute otitis media. The mechanisms in which proteins induce immunity and potential protection requires further investigation but the dogma of impaired antibody responses in children with recurrent acute otitis media should be reconsidered.

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**Competing Interests:** SW and LK have received institutional funding for studies investigating bacterial pathogens in children with rAOM and travel funding from GlaxoSmithKline. TM has received institutional funding from Wyeth Vaccines and GlaxoSmithKline. LK and TM are co-inventors on patents PCT/GB2007/002242, PCT/GB2005/001792 and PCT/GB2005/001774. HC and SV have served as consultants and have received travel funding from GlaxoSmithKline Biologicals. PR has received institutional funding for epidemiological research from GlaxoSmithKline and CSL and served on advisory boards for CSL and Wyeth Vaccines. All other authors have indicated they have no conflicts of interest.

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## Introduction

Acute otitis media (AOM) is the most common reason for physician visits and the prescription of antibiotics for children in industrialised countries, where 80% of children will have an episode of AOM at least once before age 3 and infants spend a mean of 42 days on antibiotics in the first year of life [1]. Data from the USA suggests that the costs associated with AOM are approximately US$3.5 billion per year and the World Health Organization estimates that in developing countries 51,000 deaths per year in children younger than 5 years of age are attributable to complications of AOM [1].

*Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) account for approximately 80% of AOM cases and exposure to these pathogens early in life is a risk-factor for the development of recurrent AOM (rAOM) [2]. Although *S. pneumoniae* is a predominant AOM pathogen, the 7-valent pneumococcal conjugate vaccine (PCV7) has had modest impact on overall otitis media rates, which is partly due to replacement of PCV7 serotypes with non-PCV7 serotypes and other pathogens after the widespread use of PCV7 [3,4,5]. Vaccines that confer species-wide protection against multiple bacterial pathogens, with the potential to prevent early bacterial colonisation and sub-

sequent rAOM, may be more successful and are urgently required. A trial with an 11-valent predecessor of the currently licensed 10-valent pneumococcal conjugate vaccine (PCV10) that included Protein D from NTHi as a carrier protein, showed that the incidence of NTHi AOM decreased by 35%, suggesting protein-based vaccines may be effective against AOM [6]. Indeed several protein antigens of *S. pneumoniae* and NTHi have been demonstrated to be protective against otitis media in animal models [7,8,9], however little is known about the antibody response to such antigens in young children with rAOM. The few studies investigating natural acquired humoral immunity against bacterial proteins in children with AOM are contradictory, with studies indicating that children with AOM produce antibodies against pneumococcal proteins [10,11,12,13,14], whereas others have shown impaired antibody production against pneumococcal and NTHi proteins in otitis-prone children, suggesting these children have a specific humoral immunodeficiency [15,16,17,18,19]. Group sizes in these studies are generally small, have a wide age-range and/or do not include healthy age-matched controls. In addition, antibody levels are often measured against a limited number of protein antigens due to the small blood sample volumes available from young children.

To address whether children with rAOM are able to mount antibody responses to surface exposed bacterial proteins that are potential vaccine candidates, we have measured serum IgG antibody levels to 4 pneumococcal and 3 NTHi proteins in a cross-sectional cohort of 172 children less than 3 years of age requiring the insertion of ventilation tubes for rAOM and 63 healthy age-matched controls. We developed a novel bead-based multiplex assay that permits the measurement of antibody levels to all 7 protein antigens in less than 10 µl of serum. Our study showed that humoral immunity against *S. pneumoniae* and NTHi is not impaired in children with rAOM, which are important data when developing protein-based vaccines that aim to reduce the burden of AOM.

## Methods

### Ethics Statement

The study was approved by the Ethics Committee of Princess Margaret Hospital for Children, Perth, Western Australia (1295/EP) and by the institutional boards of the private hospitals in Perth where recruitment took place. Written informed consent was obtained from parents of participating children before recruitment.

### Recruitment of the Study Cohort

From November 2007 to May 2009, children between 6 and 36 months of age were recruited from private hospitals in Perth, Western Australia, to this cross-sectional study to investigate the immunology and microbiology of rAOM. Cases were defined as children with a history of at least 3 episodes of AOM requiring insertion of ventilation tubes. The frequency of and the time-intervals between AOM episodes that the child had experienced before entry into the study at the day of surgery were based on the ENT surgeon's medical records and parental report. In a questionnaire, data on the number of GP visits for OM, the number of antibiotic courses, whether the child had been noticeably sick with otalgia, age at first AOM episode and number of AOM episodes in the past 12 months were collected to ascertain the recurrent AOM phenotype. When the child had not experienced at least 3 AOM episodes and was having ventilation tubes inserted for otitis media with effusion only, the child was not included in the study. Children with no history of any form of ear disease based on parental questionnaire data and undergoing general surgery (orthopaedics, strabismus, circumcision, cryptorchidism, hypospadias repair) were recruited as controls. None of the children had signs of acute infection at the time of surgery when the samples were collected. Children with diagnosed immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities, and chromosomal or genetic syndromes were excluded. Data on host- and environmental risk factors (age, gender, day-care attendance, siblings, and PCV7 vaccine status) were collected by parental questionnaires and from medical records. Infants in Australia received 3 doses of PCV7 with no booster in the second year of life (3+0 schedule) at the time of this study.

### Sample Collection

All samples were collected while the child was under general anaesthetic for surgery to insert ventilation tubes (rAOM group) or other minor surgical procedures (healthy controls). Blood samples were collected in a serum clot tube (Vacuette® Greiner Bio-one) and centrifuged for 10 min at 3200 g. The serum top-layer was collected and aliquoted. Nasopharyngeal swabs were collected by trans-nasal insertion of a sterile flexible cotton-wool swab (Copan, Brescia, Italy) reaching the nasopharyngeal space. Swabs were stored in sterile Skim-Milk-Tryptone-Glucose-Glycerol-Broth. An anterior-inferior myringotomy incision was made for the collection of middle ear effusion in 1 mL of saline with a sterile Leukotrap® (Pall Corporation, New York, USA). All samples were stored at −80°C until analyses [20].

### Protein Preparation

*S. pneumoniae* antigens pneumococcal surface protein A family 1 and 2 (PspA1 and PspA2) and choline binding protein A (CbpA) were constructed with C-terminal hexahistidine tags and without their repetitive choline binding sequences. Recombinant PspA1 was constituted by the 302 N-terminal amino acids of the mature PspA antigen of *S. pneumoniae* strain Rx1, and PspA2 by the 410 N-terminal amino acids of the mature PspA of *S. pneumoniae* Taiwan19F-14. Recombinant CbpA was comprised of amino acids 1 to 445 from *S. pneumoniae* strain L81905. Protein 4 (P4) and Protein 6 (P6) proteins were constructed from *H. influenzae* Eagen sequences and incorporating an N-terminal hexahistidine fusion tag [21]. Recombinant PspA1, PspA2, CbpA, P4, and P6 were expressed in *Escherichia coli* BL21 Star™(DE3)pLysS (Invitrogen, Victoria, Australia) and purified from the soluble lysate by Ni-NTA in the presence of 600 mM NaCl. Protein-containing fractions were dialysed in PBS and further purified by Bio-Rad Macro-prep High Q anion exchange chromatography (Bio-Rad, California, USA). These proteins were then purified by size exclusion over a HiPrep HR S200 26/60 column and endotoxin was removed using 0.2-µm Mustang E filters (Pall Life Sciences, Portsmouth, UK). Pneumolysin (Ply) from *S. pneumoniae* serotype 1 sequence type 306 was purified as previously described [22]. Protein purity was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Non-lipidated Protein D (PD) was kindly provided by GlaxoSmithKline (GlaxoSmithKline Biologicals, Rixensart, Belgium). All proteins were stored in aliquots at −80°C.

### Measurement of IgG Against Pneumococcal and NTHi Proteins using a Multiplex Bead Assay

Microspheres were incubated with bead activation buffer (1×PBS containing 5 mg/mL 1-ethyl-3-(-3dimethylaminopropyl)-carbodiimide hydrochloride and 5 mg/mL N-Hydroxysuccinimide) for 20 minutes. Recombinant purified proteins were then

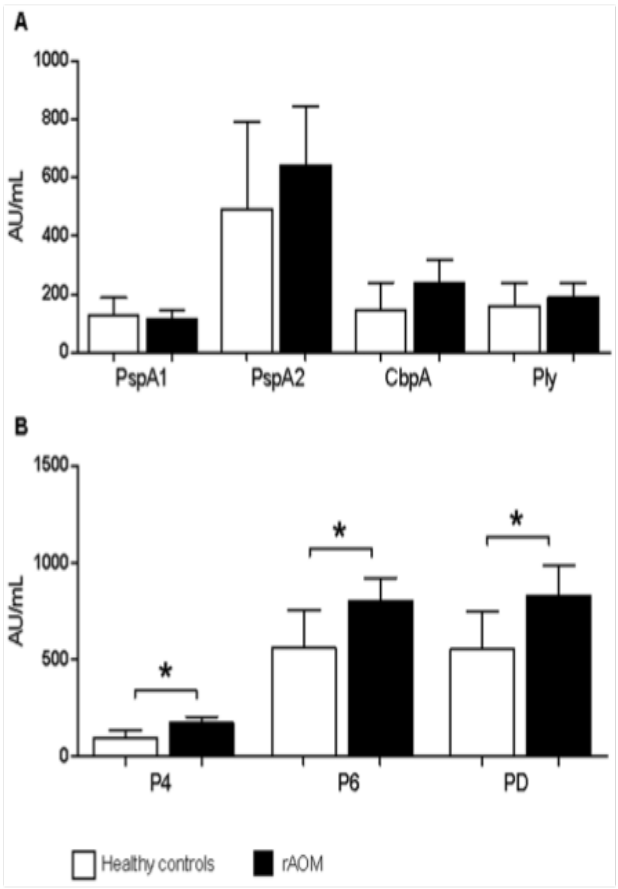


**Figure 1. Serum IgG levels.** Adjusted (age, gender, day-care attendance) geometric mean concentration of serum IgG antibody levels in Arbituary Units/mL (95% CI) against pneumococcal (top panel A) and NTHi (bottom panel B) proteins in healthy children (white bars) and children with a history of rAOM (black bars). * p≤0.01 when comparing IgG levels between children with and without a history of rAOM using linear regression correcting for age, gender and day-care attendance *PspA1/2, pneumococcal surface protein A families 1 and 2; CbpA, choline binding protein A; Ply, pneumolysin; P4, outer membrane protein 4; P6, outer membrane protein 6; PD, protein D.*
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covalently conjugated to these activated fluorescent carboxylated microspheres using a two-step carbodiimide reaction. To assess assay specificity, a selection of serum samples were pre-incubated with a single recombinant protein prior to performing the multiplex assay, which should only affect the antibody measurement against that specific protein. However, we found that addition of PspA1 interfered with measurement of PspA2, CbpA, and Ply antibody levels and that pre-incubation of serum with PD interfered with measurement of P4 antibody levels. To prevent this cross-reactivity and ensure optimal assay specificity, test samples were measured using a 2-plex assay including microspheres coated with PspA1 and PD and a 5-plex assay including microspheres coated with PspA2, CbpA, Ply, P4, and P6. In each assay, pooled sera from adult volunteers were used as a reference and quality control and IgG-deficient serum (Sigma-Aldrich) was used as a negative control.

With this optimised assay, test serum samples were diluted 1:250 in phosphate buffered saline containing 2% new born bovine serum and 0.05% Tween20 (All Sigma-Aldrich). Diluted samples (25 µL) were incubated with a mixture of 25 µL of the protein-coated microspheres for 30 minutes at room temperature. After washing (phosphate buffered saline, 0.05% Tween20), 100 µL of *R*-phycoerythrin conjugated anti-human IgG was added (Jackson ImmunoResearch Laboratories, Pennsylvania, USA), the mixture was incubated for 30 minutes at room temperature and washed again. The fluorescence of 100 beads within each specific bead region was measured on the BioPlex® 200 System (Biorad). Inter-assay variability was assessed by calculating the % CV of the mean fluorescence intensity of standard curve dilutions 4 and 7 over all assays which were in the range of 10–15%. Mean fluorescence intensity data was acquired electronically in real-time and analysed using Bio-plex Manager 5.0 software. Data in arbitrary units (AU/mL) were generated from a 5-PL standard curve of mean fluorescence intensity against the in-house reference human serum pool and plotted as the geometric mean concentration (GMC) with 95% confidence intervals (CI). Out of range values were repeated using appropriate higher or lower serum dilutions.

## Detection of *S. pneumoniae* and NTHi in Nasopharyngeal Swabs and Middle Ear Effusion

Nasopharyngeal swabs were examined for the presence of *S. pneumoniae* and NTHi using standard culture methods. PCR for detections of *S. pneumoniae* and *H. influenzae* was conducted on genomic DNA prepared from the middle ear effusion. Methods

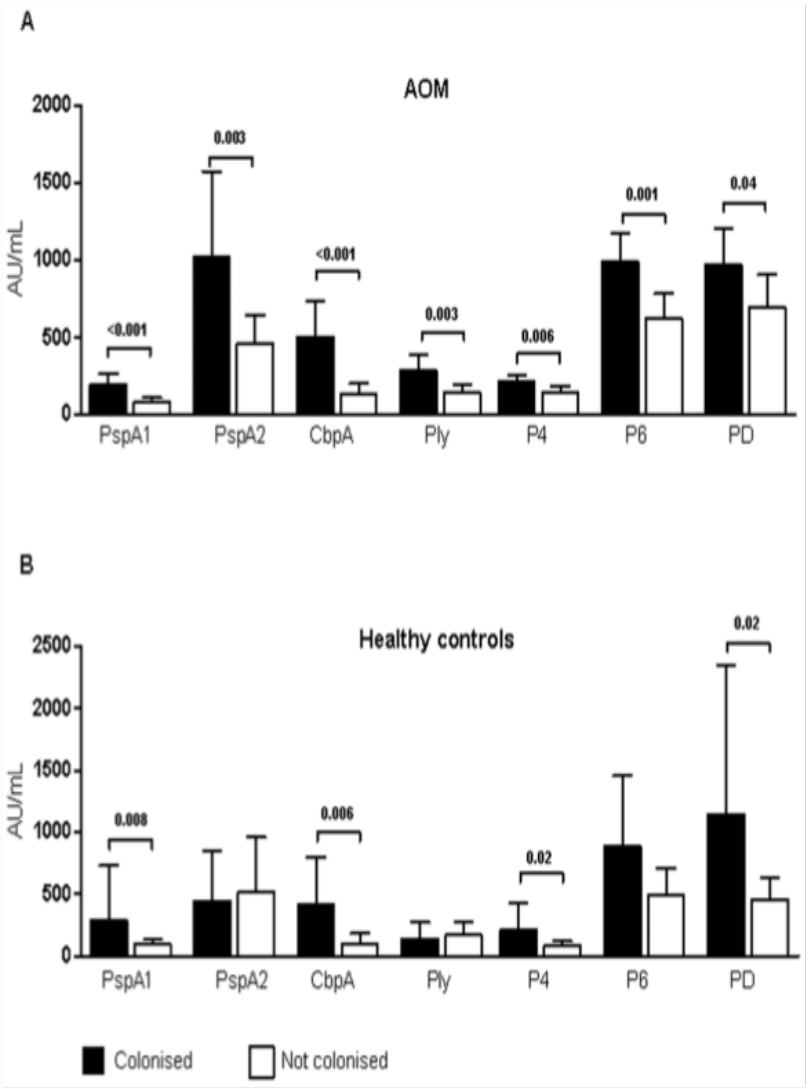


**Figure 2. Serum IgG levels according to AOM history and nasopharyngeal colonisation.** Adjusted (age, gender, day-care attendance) geometric mean concentration of serum IgG in Arbitrary Units/mL (95% CI) against pneumococcal and NTHi proteins in children positive (black bar) or negative (white bar) for nasopharyngeal colonisation with *S. pneumoniae* for IgG against PspA1, PspA2, CbpA, and Ply or nasopharyngeal colonisation with NTHi for IgG against P4, P6, and PD in children with a history of rAOM (top panel A) or healthy controls (bottom panel B). P-value determined using linear regression correcting for age, gender and day-care attendance… *PspA1/2, pneumococcal surface protein A families 1 and 2; CbpA, choline binding protein A; Ply, pneumolysin; P4, outer membrane protein 4; P6, outer membrane protein 6; PD, protein D.*
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and results of these analyses have been described in detail elsewhere [20].

## Statistical Analyses

Host and environmental risk factors were compared between children with or without rAOM using Student's *t* tests for continuous variables (age) and Pearson Chi-square analyses (p-value asymptotic significant 2-sided) for categorical variables (gender, day-care attendance, siblings, and PCV7 vaccine status). Antibody titers were expressed in Arbitrary units/mL (AU/mL) against a reference human serum pool and reported as adjusted antigen-specific geometric mean concentrations (GMCs) with 95% confidence interval (CI), correcting for confounding variables as appropriate. Adjusted GMCs were calculated using adjusted antibody concentrations that were computed in log values for the adjustment. The residual values that were derived in a linear regression model or general linear model with the corresponding antibody levels as dependent variable and the confounders as independent variables [23,24] were used for the calculation of the adjusted antibody levels. To analyse differences in anti-pneumococcal (PspA1, PspA2, CbpA, and Ply) and anti-NTHi (P4, P6, and PD) antibody levels between children with or without rAOM linear regression correcting for age, gender and day-care attendance was used. To analyse the difference in antibody levels between children who were either colonised or not colonised with *S. pneumoniae* or NTHi linear regression correcting for age, gender and day-care attendance was used. These analyses were conducted within the group of children with a history of rAOM and within

**Table 1.** Serum IgG according to detection of *S. pneumoniae* in the middle ear.

| | MEE Pnc positive (n = 8) | MEE Pnc negative (n = 126) | p-value |
|---|---|---|---|
| PspA1 | 189.6 (38.5–934.4) | 126·4 (93.6–170.9) | 0.5 |
| PspA2 | 355.5 (71.8–1760.2) | 784·3 (575.3–1069.4) | 0.2 |
| CbpA | 258.6 (31.0–2161.8) | 256·6 (180.8–364.1) | 1.0 |
| Ply | 165.5 (21.5–1273.5) | 199·9 (148.0–269.9) | 0.8 |

Geometric mean concentration of serum IgG in Arbitrary Units/mL (95% CI) against pneumococcal proteins in children with a history of rAOM who do or do not have *S. pneumoniae* (Pnc) detected in middle ear effusion (MEE) using PCR. P-value determined using Students *t* test.
*PspA1/2, pneumococcal surface protein A families 1 and 2; CbpA, choline binding protein A; Ply, pneumolysin.*
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**Table 2.** Serum IgG according to detection of NTHi in the middle ear.

| | MEE NTHi positive (n = 61) | MEE NTHi negative (n = 73) | p-value |
|---|---|---|---|
| P4 | 195.1 (149.9–254.1) | 167.0 (133.5–208.9) | 0.4 |
| P6 | 923.2 (731.1–1165.9) | 797.1 (641.8–990.1) | 0.4 |
| PD | 964.7 (748.4–1243.4) | 789.8 (587.0–1062.7) | 0.3 |

Geometric mean concentration of serum IgG in Arbitrary Units/mL (95% CI) against NTHi proteins in children with a history of rAOM who do or do not have NTHi detected in middle ear effusion (MEE) using PCR. P-value determined using Students *t* test.
*P4, outer membrane protein 4; P6, outer membrane protein 6; PD, protein D.*
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the group of healthy controls, separately. General linear model with Tukey honestly significant difference test was used to compare the difference in antibody levels in three different age groups (<12 months, 12–24 months, and 24–36 months of age) within the group of children with a history of rAOM and within the group of healthy children, after adjusting for gender and day-care attendance. General linear model was also employed to investigate the two-way interactions of rAOM with age (categorical variables), gender and day-care attendance. IBM SPSS Statistics 19 for Windows software package (IBM, New York, USA) was used for all statistical analyses and data were plotted using GraphPad Prism (Graphpad Software Inc, California, USA).

## Results

### Study Population

We enrolled 172 children with validated medical records meeting the study inclusion criteria of a history of at least 3 episodes of AOM in the first 3 years of life (median 7 AOM episodes) requiring the insertion of ventilation tubes. The healthy control group was comprised of 63 children without a history of ear disease. The mean age of the group of children with a history of rAOM was 21.1 months (range 7.3−36.0) and of controls 18.7 months (range 7.0–35.0; $p = 0.06$), with median ages of 20.6 months and 15.8 months, respectively. In the group of children with rAOM 61% were male and the control group consisted of 73% males ($p = 0.09$). None of the parents of children in the study self-identified as being Aboriginal or of Torres Strait Islander descent. In the rAOM group, 63% attended a day-care facility for more than 4 hrs/week, whereas this was 31% in the healthy control group ($p<0.001$). In both groups the percentage of children that had siblings was similar, being 70% in children with and 74% in children without a history of rAOM ($p = 0.6$). PCV7 vaccine coverage was also similar between children with (99% fully vaccinated) or without a history of rAOM (97% fully vaccinated; $p = 0.3$). *S. pneumoniae* and NTHi were more commonly isolated from the nasopharynx of children with a history of rAOM compared with healthy children (41% *vs* 27%; $p = 0.04$ for *S. pneumoniae* and 56% *vs* 21%; $p<0.001$ for NTHi).

### Serum IgG Levels to NTHi Proteins are Higher in Children with rAOM

In children with a history of rAOM the raw geometric mean serum IgG antibody concentration against pneumococcal proteins were 1.5−2.7 times higher than those in healthy children, however, these differences were not statistically significant using linear regression correcting for age, gender and day-care attendance (Figure 1). The group of children with rAOM had significantly higher (1.7−2.1-fold) GMCs against the NTHi proteins P4 ($p<0.001$), P6 ($p = 0.01$), and PD ($p = 0.01$) compared with the healthy control group (Figure 1) after adjusting for age, gender and day-care attendance.

### Serum IgG Levels According to AOM History and Nasopharyngeal Colonisation

We then investigated the effect of colonisation on antibody production in healthy children compared with children with a history of rAOM. Children with a history of rAOM and colonised with *S. pneumoniae* had 1.8−3.4 times higher antibody levels compared with cases that were not colonised (PspA1: $p<0.001$; PspA2: $p = 0.003$; CbpA: $p<0.001$; Ply: $p = 0.003$; Figure 2). Similarly, cases colonised with NTHi had significantly higher geometric mean IgG concentrations against P4 ($p = 0.006$), P6 ($p = 0.001$) and PD ($p = 0.04$) compared with cases that were non-carriers of NTHi (Figure 2). Healthy children that were colonised with *S. pneumoniae* had GMCs that were significantly higher against PspA1 (3.5 times higher, $p = 0.008$) and CbpA (4.4 times higher, $p = 0.006$), whereas the GMCs to PspA2 and Ply were not different in healthy controls carrying *S. pneumoniae* compared with those not colonised (Figure 2). The geometric mean antibody concentration against P4 ($p = 0.02$) and PD ($p = 0.02$) were significantly higher in healthy children colonised with NTHi compared with non-colonised healthy children (Figure 2).

### Serum IgG Levels do not Correlate with Detection of Pathogens in the Middle ear

Of the 134 children with rAOM that had middle ear effusion collected during surgery for the insertion of ventilation tubes, 8 were PCR positive for *S. pneumoniae* (6%) and 61 were PCR positive for NTHi (46%). There were no differences in geometric mean serum IgG levels against any of the pneumococcal and NTHi protein antigens between the children that did or did not have the respective pathogen in the middle ear (Table 1 and Table 2). The mean age, gender distribution and day-care attendance rates were not different between children that did or did not have *S. pneumoniae* and NTHi detected in the middle ear (data not shown). There were also no differences in geometric mean antibody concentrations against any of the protein antigens according to the number of AOM episodes experienced prior to entry in the study (data not shown).

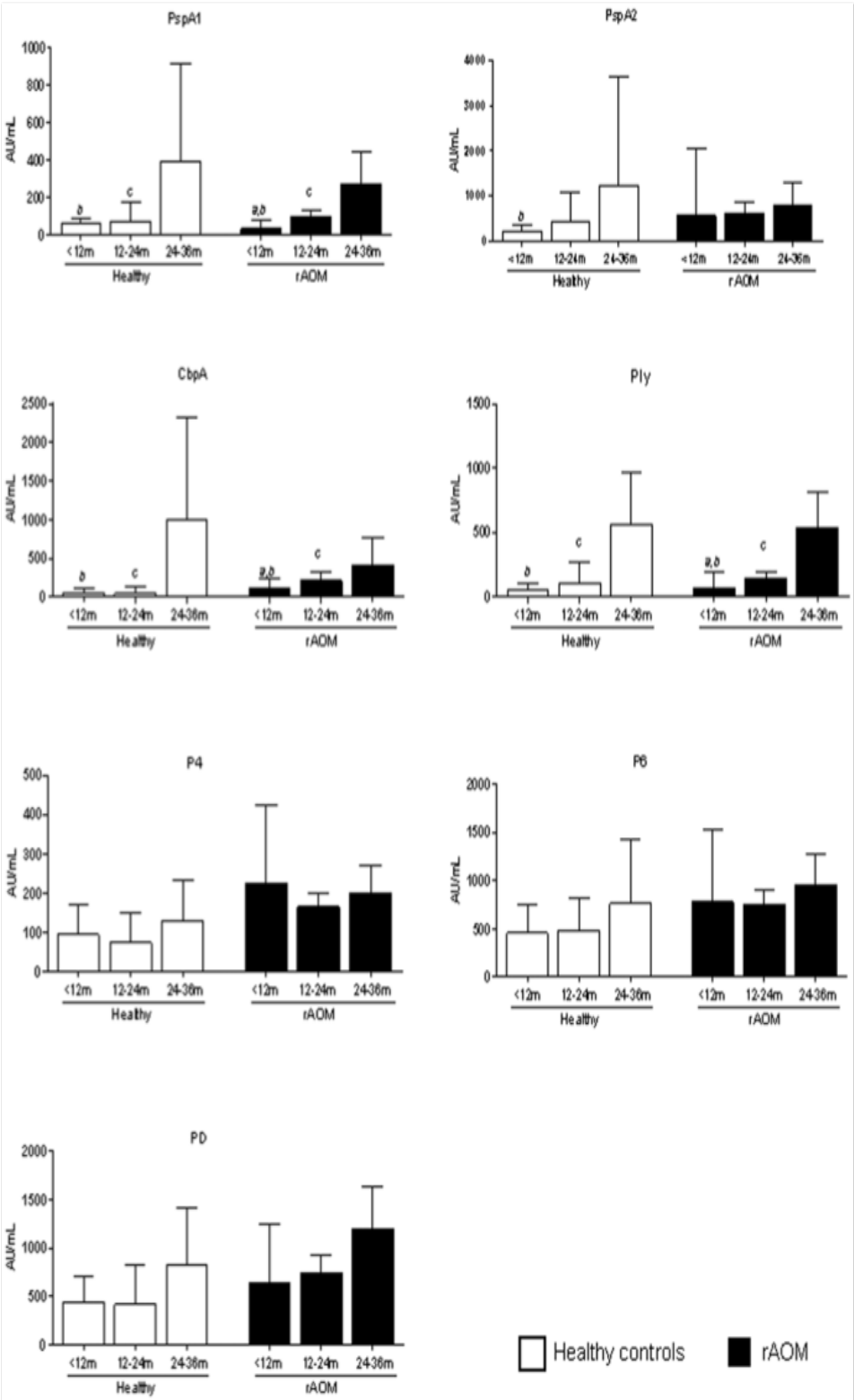


**Figure 3. Serum IgG levels according to AOM history and age.** Adjusted (gender, day-care attendance) geometric mean concentration according to age of serum IgG in Arbitrary Units/mL (95% CI) against pneumococcal and NTHi protein antigens in healthy children (white bars) and children with a history of rAOM (black bars).General linear model followed by Tukey honestly significant difference test was used to compare differences between age groups within the group of children with rAOM and within the healthy control group, where $p<0.05$ was considered

significant; *a* = p<0.05 when comparing <12 months with 12–14 months, *b* = p<0.05 when comparing <12 months with 24–36 months, *c* = p<0.05 when comparing 12–24 months with 24–36 months. *PspA1/2, pneumococcal surface protein A families 1 and 2; CbpA, choline binding protein A; Ply, pneumolysin; P4, outer membrane protein 4; P6, outer membrane protein 6; PD, protein D.*
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### Serum IgG Levels to Bacterial Proteins Increase with Age

To investigate the effect of age on antibody levels, data in both groups of children, either with or without a history of rAOM, were analysed in 3 age-brackets: <12 months old, 12–24 months old, and 24–36 months old (Figure 3) using a general linear model after adjusting for gender and day-care attendance. In both groups antibody levels increased with age. This rise was most apparent for IgG against pneumococcal proteins, with a significant increase in children with a history of rAOM for PspA1, CbpA, and Ply (Figure 3). In healthy controls, antibody titers against the pneumococcal antigens remained low until 24 months of age, but were significantly higher in the 24–36 months old age group. There was a trend for higher antibody levels against all NTHi protein antigens with increasing age in both groups of children but this did not reach statistical significance. When comparing antibody levels against NTHi proteins between children with and without a history of rAOM within a specific age-bracket, antibody levels in children with rAOM were generally higher.

### The Interactive Effects of rAOM with Age, Gender and Day-care Attendance on Serum IgG Levels

To clarify the effects of rAOM on serum IgG levels we further investigated the interactive effects of a history of rAOM with age, gender and day-care attendance. For the 7 antibody levels, we identified three significant interactions: rAOM*age on CbpA ($p = 0.003$), rAOM*day-care attendance on CbpA ($p = 0.04$) and rAOM*gender on P4 ($p = 0.05$). Consistent with the findings in paragraph 3.5, CbpA levels increased with age and the linear pattern was more apparent in children with rAOM (Figure 4a). However, after two years CbpA levels increased significantly in controls. Children with a history of rAOM who did not attend day-care had significantly higher levels of antibodies against CbpA ($p = 0.03$). In contrast, a history of rAOM did not increase the levels of CbpA in children who did attend day-care ($p = 0.79$) (Figure 4b). In boys, a history of rAOM had significantly increased the levels of P4 antibody levels ($p<0.001$), however, rAOM did not increase the levels of P4 IgG in girls ($p = 0.71$) (Figure 4c).

## Discussion

In this large cross-sectional study of young children with a history of rAOM undergoing ventilation tube insertion and healthy age-matched controls, we have shown that children with a history of rAOM have significantly higher naturally acquired serum IgG levels to the NTHi proteins P4, P6, and PD. In addition, serum IgG levels to protein antigens were higher in children colonised with *S. pneumoniae* or NTHi, suggesting that serum IgG is induced by carriage. Together this may imply that the higher antibody levels in children with a history of rAOM are a marker of previous exposure rather than a measure of susceptibility to recurrent disease. To address this, longitudinal studies are required. Our findings of higher antigen-specific antibody responses in children with rAOM or children colonised with *S. pneumoniae* or NTHi are in accordance with several studies in children with AOM [10,11,12,13,14] or colonised with *S. pneumoniae* or NTHi [11,14,25,26]. Our data contradict other studies, in which the observation of lower antigen-specific antibody levels in children with rAOM suggests that these antibodies are protective [15,16,17,18,19]. These conflicting findings may reflect differences in the timing of, or the response to colonisation in children with a history of rAOM compared with healthy children. One study showed that antibodies against CbpA and Ply were lower in children colonised with *S. pneumoniae*. Children in this study were undergoing surgery for adenoidal hypertrophy though and were not reported to suffer from rAOM, suggesting the aetiology of diseases is different [27]. Reduced antibody responses after colonisation may play a role in the progression of adenoidal hypertrophy, but may be less important in the development of rAOM.

It is important for these discrepancies to be resolved to improve our understanding of the aetiology of AOM, which could contribute to the development of diagnostics that would allow for the prediction of prognosis, early intervention and improved treatment options for children with AOM. Studies investigating pneumococcal and NTHi specific immune responses in children with rAOM should be compared cautiously though, especially when the aim is to draw general conclusions on the immune status of children with rAOM or the immunogenicity of vaccine candidate proteins in these children. Firstly, investigation of samples collected during or outside the acute phase of infection may lead to different results and conclusions. The high antibody levels in children with a history of rAOM between AOM episodes, as we show here, may reflect a booster-type of response induced by previous colonisation and/or disease, whereas during acute infection the antibody response in otitis-prone children may well lag behind. Two recent studies from the same investigators compared IgG levels to pneumococcal and NTHi proteins in serum all collected during an AOM episode from otitis-prone children, non otitis-prone children and children who failed to respond to antibiotic treatment [18,19]. The authors concluded that otitis-prone children are immunologically hypo-responsive to pneumococcal and NTHi proteins. Outside the acute infection however, the antibody titers in otitis-prone children deemed to be similar to those in controls, but direct statistical analyses were not described. These studies rather analysed the kinetics of antibody responses, which actually showed higher and earlier, but flatter antibody responses in otitis-prone children. Care should be taken in generalising results from these smaller and clinically less distinct cohorts, as these results may not reflect the immune status of the majority of children with rAOM.

In addition it is crucial to assess age-matched cohorts with a strict, limited age-range when investigating immunity in children with rAOM, since antibody titres against pneumococcal and NTHi protein antigens increase with age as shown here and by others [11,14,19,26,28]. Data from studies with a wide age-range [27] or where otitis-prone and non otitis-prone groups are not age-matched [17] should be interpreted cautiously. The conclusions drawn from such studies that state that otitis-proneness is related to several immunological derangements seem to be too strong.

Moreover, antibody levels against a wide range of antigens should be tested before conclusion on the general immune response in children with rAOM are made, since antibody responses against antigens are very different, partly due to variation in expression patterns and function of specific proteins in bacterial strains and species. The country and era that samples were collected in also needs to be taken into account, since pneumococcal and NTHi carriage patterns, and therefore

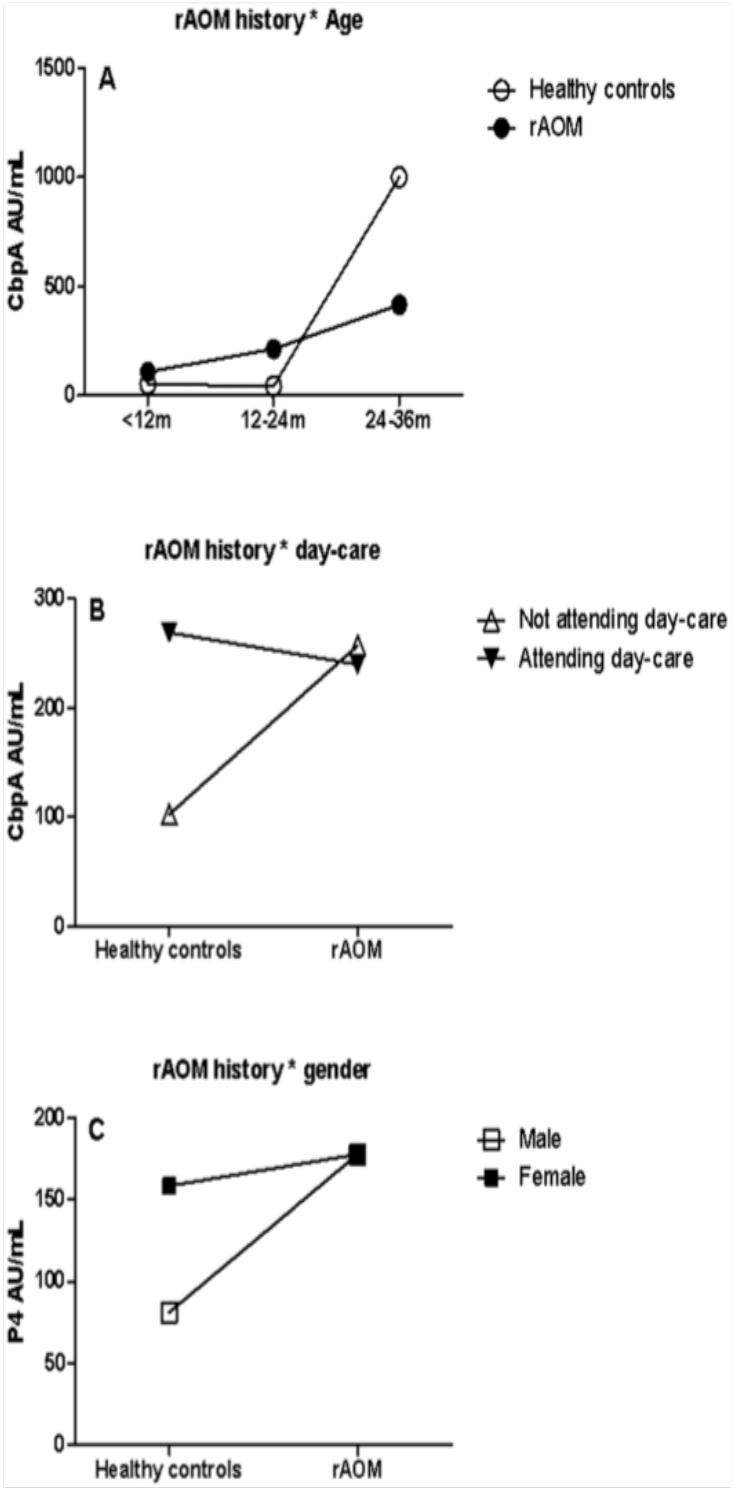


**Figure 4. Interactive effects.** Interactive effects calculated using general linear model of rAOM * age (panel A; p = 0.003) and rAOM * day-care attendance (panel B; p = 0.04) on geometric mean CbpA levels (AU/mL) and the effect of rAOM * gender on GMC of anti-P4 IgG levels (panel C; p = 0.05). *CbpA, choline binding protein A; P4, outer membrane protein 4.*
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immune responses, can vary, for instance due to the use of pneumococcal conjugate vaccines.

Finally, there were interactions between rAOM and gender, age and daycare attendance on antibody levels. The effects of rAOM on antibody levels varied between sub-groups of children with different combinations of confounders. This further complicates the investigation on the relationship between rAOM and antibody responses in children.

Considering the conflicting data, the complex interactions identified in this study and conclusions regarding antibody responses in children with rAOM in literature, partly due to the limitations described above, there is a clear requirement for larger, well age-matched studies in which standardised clinical definitions of the rAOM phenotype are applied and samples are collected during the same phase of the AOM episode. In addition it will be important to investigate the timing and load of bacterial colonisation and/or disease, antibody levels, antibody functionality, mucosal immunity and cell mediated immune responses to bacterial otopathogens. These factors are likely to be interacting and therefore large longitudinal studies following children from early on in life are necessary to better understand the aetiology and pathogenesis of rAOM. Data from our large cross-sectional study shows that several pneumococcal and NTHi proteins are immunogenic in young children with a history of rAOM. While a subset of children with humoral immune-deficiencies may suffer from rAOM, our findings indicate that previous statements suggesting that all children with rAOM have impaired antibody responses should be reconsidered. The fact that otitis-prone children can mount antibody responses to proteins from common otopathogens implies that a vaccine including a combination of protein antigens may offer protection from initial colonisation and otitis media when used early in life. The specific mechanisms in which bacterial proteins induce immunity and potential protection against rAOM when used in protein based vaccines, requires further investigation.

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## Author Contributions

Conceived and designed the experiments: SW KC PR WT TM SV HC LK. Performed the experiments: SW KC LK EM. Analyzed the data: SW GZ KC LK PR. Contributed reagents/materials/analysis tools: SV HC WT TM. Wrote the paper: SW GZ KC LK WT PR.

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# High pneumococcal serotype specific IgG, IgG1 and IgG2 levels in serum and the middle ear of children with recurrent acute otitis media receiving ventilation tubes

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ABSTRACT

Recurrent acute otitis media (AOM), frequently caused by *Streptococcus pneumoniae*, is a major paediatric health problem. A reduced antibody response against pneumococcal polysaccharides may contribute to an increased susceptibility to AOM. Using a multiplex bead-based assay we measured IgG, IgG1 and IgG2 levels against 11 pneumococcal polysaccharides in serum samples from 166 children below 3 years of age with a history of at least 3 episodes of acute otitis media receiving ventilation tubes, and 61 healthy controls. Pneumococcal serotype specific IgG was also determined in 144 middle ear effusion samples. Pneumococcal serotype specific IgG, IgG1 and IgG2 levels were similar in children with or without AOM, except for IgG and IgG1 levels against serotype 5, which were significantly higher in children with a history of frequent AOM (IgG: 137.5 μg/ml vs. 84.0 μg/ml; $p = 0.02$; IgG1: 24.5 μg/ml vs. 18.2 μg/ml; $p = 0.05$). The age-related development of pneumococcal serotype-specific IgG, IgG1 and IgG2 levels was similar in children with or without a history of AOM. Pneumococcal serotype specific IgG was present in middle ear effusion and these levels correlated significantly with serum titres. Children with a history of frequent AOM receiving ventilation tubes do not have a deficient IgG, IgG1 or IgG2 response against pneumococcal polysaccharides, either induced by vaccination or due to natural exposure. The strong correlation between IgG levels in serum and the middle ear suggests parenteral pneumococcal conjugate vaccination induces antibodies in the middle ear which may therefore contribute to reducing the burden of AOM.

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## 1. Introduction

Acute otitis media (AOM), frequently caused by *Streptococcus pneumoniae*, is one of the most common paediatric infectious diseases and a main reason for visits to a general practitioner, antibiotic use and surgery in children [1]. Pneumococcal infections are common in patients with primary antibody deficiency, which may indicate that children with recurrent AOM (rAOM) have an impaired immune response to *S. pneumoniae* [2,3]. Indeed, it has been shown that children with rAOM have lower levels of total IgG2 [4,5], the main IgG isotype induced by polysaccharides, and pneumococcal serotype specific IgG [6], IgG2 and IgA [7]. On the contrary though, others found normal levels of total IgG, IgA and its subclasses [8,9] or even higher IgG levels to pneumococcal polysaccharides in children with AOM [10,11]. In addition, studies have shown that the 7-valent pneumococcal conjugate vaccine (PCV7) elicits a pneumococcal serotype specific serum IgG and IgA response in otitis-prone children [12–15]. Data on pneumococcal polysaccharide antibodies in serum of children with rAOM is clearly conflicting. This is likely due to the fact that several studies have small group sizes, do not include healthy controls or investigated antibody responses against a limited number of pneumococcal serotypes. In addition, study populations vary since the type and frequency of each OM episode is ascertained in different ways. In clinical trials and prospective studies it is often feasible to have every acute OM episode confirmed, whereas in a cross-sectional observational study the phenotype is based on medical records and

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**Table 1**
Demographics and risk factors. Host and environmental risk-factors were compared between children with and without a history of rAOM using Student's t-tests for continuous variables and Pearson Chi-square analyses (*p*-value asymptotic significant 2-sided) for categorical variables.

| | rAOM $N=166$ | Healthy $N=61$ | *p*-Value |
|---|---|---|---|
| Mean age (months) | 21.1 | 18.5 | 0.04 |
| Gender (% male) | 60.8% | 68.9% | 0.3 |
| AOM episodes | | | |
| 3–4 | 32.5% | – | |
| 5–7 | 17.5% | – | |
| 8–9 | 35.7% | – | |
| 10+ | 14.3% | – | |
| Attending day care ≥4 h/week | 61.6% | 32.2% | <0.001 |
| Using antibiotic currently | 24.2% | 18.6% | 0.4 |
| Having siblings | 70.8% | 72.9% | 0.2 |

parental report. This hampers direct comparisons between studies, highlighting that study populations need to be clearly defined and reported [16,17].

In addition to serum antibodies, pneumococcal serotype specific antibodies in the middle ear may be essential to prevent recurrence of pneumococcal AOM. It has been shown that antibodies against pneumococcal polysaccharides are present in ~50% of middle ear effusion (MEE) samples [18,19], and that these antibodies are associated with clearance of the infection [20–22]. Whether such antibodies are produced locally or systemically is unclear, with one study showing that if antibodies are found in MEE they are always present in serum as well [19], whereas it has also been found that antibodies can be present solely in the middle ear [20] or that there can be no correlation between serum and MEE levels at all [23]. If pneumococcal serotype specific antibodies indeed transudate from serum to the middle ear, this would provide opportunities for systemic pneumococcal vaccination to contribute to protection against AOM. This is supported by one small study which showed that vaccination with a pneumococcal polysaccharide vaccine resulted in specific antibodies in the middle ear [24].

The aim of the current cross-sectional study was to investigate pneumococcal serotype specific antibody responses in children with a history of frequent AOM who required surgery for insertion of ventilation tubes. Using a multiplex bead-based assay we investigated whether serum IgG, IgG1 and IgG2 levels against 11 pneumococcal serotypes were different in children with history of frequent AOM compared with healthy age-matched controls. Secondly, we examined whether these IgG antibodies were also present in the middle ear of children with reoccurring AOM and whether these antibody levels correlated with levels found in serum. Together these data will further our understanding on whether children who suffer from recurrent AOM have a specific immunodeficiency and whether systemic vaccination with pneumococcal vaccines has the potential to contribute to the reduction of AOM.

## 2. Methods

### 2.1. Study design and participants

Details of this study have been described previously [25]. In brief, children between 6 and 36 months of age were recruited to this cross-sectional study to investigate the immunology and microbiology of rAOM. Cases were defined as children with a history of at least 3 episodes of AOM requiring insertion of ventilation tubes. AOM diagnosis was based on the ENT surgeon's medical records and parental report. Data on the number of GP visits for AOM, the number of antibiotic courses, age at first AOM episode and number of AOM episodes in the past 12 months were collected. Children with no history of any form of ear disease based on parental questionnaire data and undergoing general surgery (orthopaedics, strabismus, circumcision, cryptorchidism, hypospadias repair) were recruited as controls. All children had received three doses of the 7-valent pneumococcal conjugate vaccine according to the Australian National Immunisation Programme. The study was approved by the Ethics Committee of Princess Margaret Hospital for Children, Perth, Western Australia. Written informed consent was obtained from parents of participating children before recruitment.

### 2.2. Sample collection and processing

Details on sample collection and processing have been described previously [25]. In brief, blood samples were collected during surgery, the separated serum was aliquoted and stored at −80 °C. Middle ear effusion (MEE) samples were collected and stored at −80 °C until analyses. Total protein was determined in MEE using a Micro BCA Protein Assay Kit according to the manufacturers' instructions (Thermo Scientific, Rockford, IL, USA). Total IgG immunoglobulin was determined in MEE that had protein present using nephelometry. Only MEE samples with total IgG immunoglobulin present were analysed for pneumococcal serotype specific IgG.

### 2.3. Measurement of anti-pneumococcal polysaccharide specific IgG, IgG1 and IgG2

A microsphere-based flow cytometric assay was used to measure IgG, IgG1 and IgG2 antibody levels against 11 pneumococcal polysaccharides in serum and middle ear effusion samples (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F; American Type Culture Collection, Virginia, USA) modified from assays previously described [26,27]. In brief, pneumococcal polysaccharides were conjugated to poly-L-lysine (Sigma Aldrich, New South Wales, Australia) and then covalently bound to carboxylated microspheres (Bio-Rad Laboratories, Gladesville, Australia). Samples were diluted in adsorption buffer (phosphate buffered saline, 0.05% Tween 20 and 2% new born bovine serum; all from Sigma Aldrich), pneumococcal cell wall polysaccharide (Statens Serum Institute, Copenhagen, Denmark) and serotype 22F polysaccharide (American Type Culture Collection, Virginia, USA). Diluted samples were incubated with polysaccharide conjugated microspheres for 20 min at room temperature then washed (phosphate buffered saline, 0.05% Tween 20). For detection of IgG, *R*-phycoerythrin (R-PE) conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, Pennsylvania, USA) was added. For the detection of subclass antibodies, mouse anti-human IgG1 or IgG2 (Invitrogen, Victoria, Australia) was added, incubated for 20 min, washed and incubated with *R*-phycoerythrin conjugated goat anti-mouse IgG (Invitrogen, Victoria, Australia). The fluorescence in each specific bead region was measured on the BioPlex® 200 System (Bio-Rad Laboratories, Gladesville, Australia). Data in μg/ml was generated based on the standard reference serum 89-SF (Food and Drug Administration, Maryland, USA) [28–30]. A weight-based assignment for 19A IgG1 and IgG2 antibody levels has not been determined and these levels are therefore expressed in arbitrary units/ml. A limitation of this assay is the potential cross-reactivity between antibodies against serotypes 19A and 19F, which cannot be ruled out. To control for day-to-day assay variability, a quality control (QC) was included on each assay plate. Assay reproducibility was determined by calculating the %CV of these QCs over all assays, which fell between 4.1% and 13.9% for IgG, 4.9% and 13.7% for IgG1 and between 3.3% and 10.2% for IgG2.

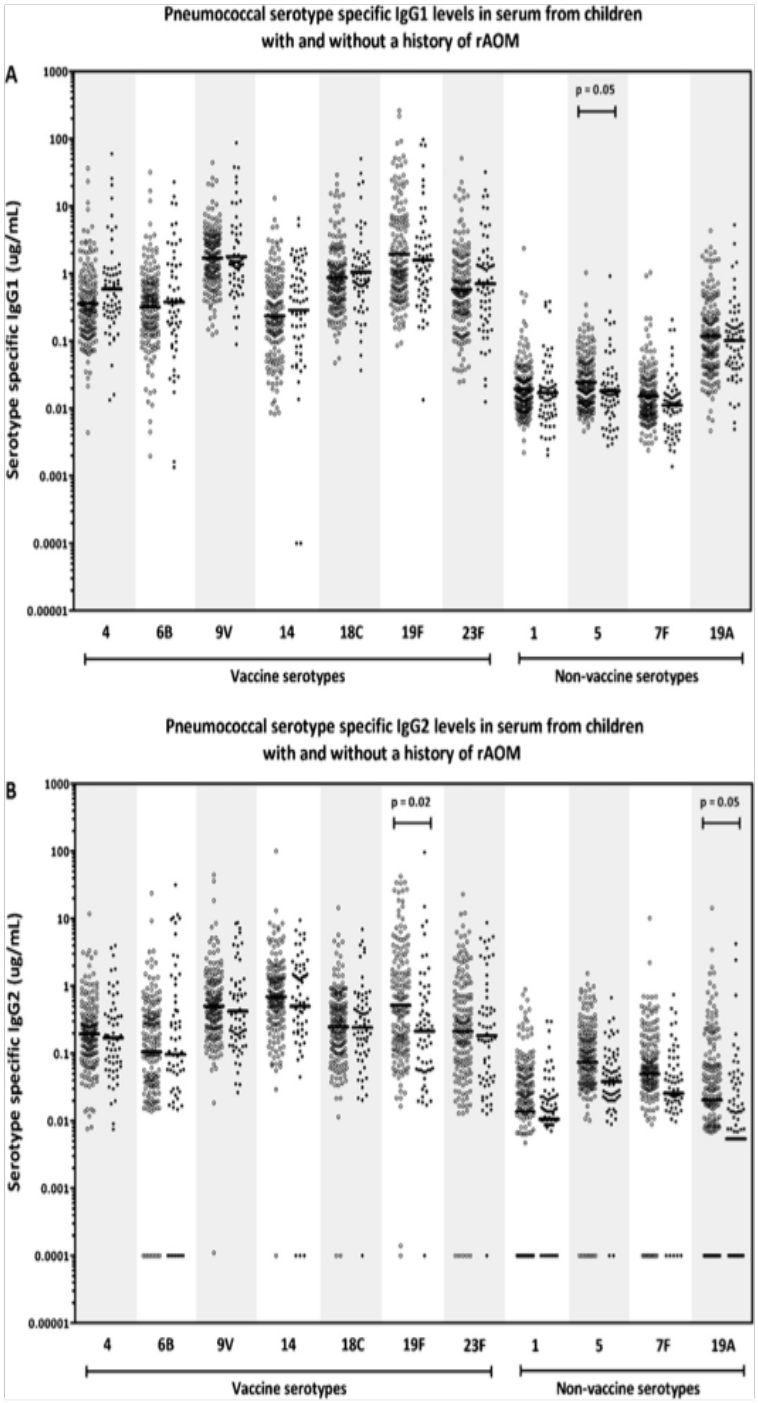


**Fig. 1.** Pneumococcal serotype specific IgG1 (panel a) and IgG2 (panel b) levels in μg/ml in serum samples collected from children with a history of recurrent acute otitis media (open circles) and healthy age matched controls (closed circles). The horizontal bars depict the geometric means and *p*-values were determined using linear regression correcting for age, gender and day-care attendance. *Note*: 19A IgG1 and IgG2 antibody levels are expressed in arbitrary units/ml.

### 2.4. Statistical analyses

Host and environmental risk-factors were compared using Student's *t*-tests for continuous variables (age) and Pearson Chi-square analyses for categorical variables (day-care attendance, siblings, gender and antibiotic use). Serum IgG, IgG1 and IgG2 levels were reported as antigen-specific geometric mean concentrations (GMCs). Antibody levels in serum were compared between the two groups using linear regression correcting for age, gender and day-care attendance. In children with a history of rAOM, serotype specific IgG, IgG1 and IgG2 levels were compared between children who had experienced 3–4, 5–7, 8–9 or 10+ episodes using linear regression correcting for age, gender and day-care attendance. IgG levels in MEE were reported as a ratio (arbitrary units) of

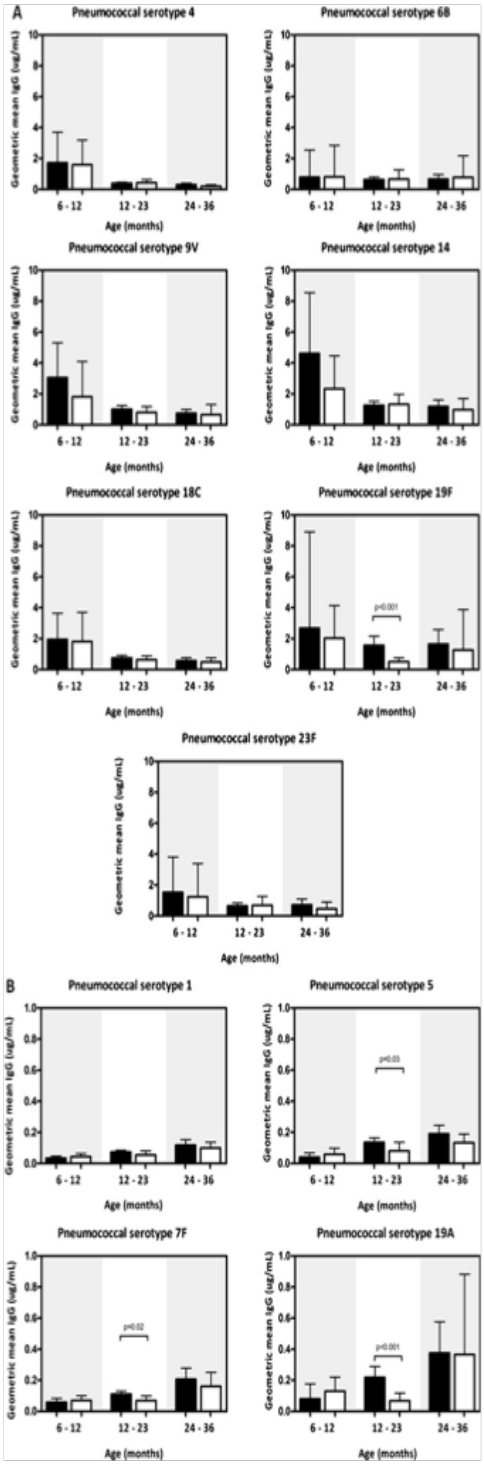


**Fig. 2.** Geometric mean and 95% confidence intervals of pneumococcal serotype specific IgG levels in μg/ml against pneumococcal serotypes included in PCV7 (panel a) and non-PCV7 serotypes (panel b) in serum of children with a history of rAOM (black bars) and healthy controls (white bars) according to three age categories.

pneumococcal serotype specific IgG over total IgG to control for variability in sample dilution. If two MEE samples were collected from one child, the ratio values of the two MEE samples were averaged to allow for correlation with serum IgG levels, which was determined using Pearson's correlation coefficient.

## 3. Results

### 3.1. General characteristics

In this study 166 young children below 36 months of age with a history of at least 3 episodes of AOM having surgery for the insertion of ventilation tubes and 61 healthy controls younger than 36 months without a history of ear disease were included. All children were undergoing surgery at the time of sample collection and were free of acute infection, as hospital policy is to only perform elective surgery on children who are free of infection. Table 1 describes the general characteristics of the study cohort. The mean age of children with a history or rAOM was 21.1 months and of children without ear disease 18.5 months ($p = 0.04$). The percentage of males was similar in both groups, being 60.8% in the rAOM group and 68.9% in healthy controls ($p = 0.3$). Children with rAOM significantly more often attended a day-care facility (61.6%) than healthy controls (32.2%; $p < 0.001$). The group of children with rAOM were at the severe end of the AOM spectrum, with half of them having experienced 8 or more AOM episodes based on medical records and parental report.

### 3.2. Pneumococcal serotype specific IgG, IgG1 and IgG2 antibody levels are not deficient in children with a history of rAOM

Geometric mean serum IgG, IgG1 and IgG2 levels against pneumococcal polysaccharides were compared between children with a history of rAOM and healthy controls (supplementary table 1). Pneumococcal serotype specific IgG and IgG1 levels were similar in children with or without rAOM, except for IgG and IgG1 levels against serotype 5, which were significantly higher in children with a history of frequent AOM (IgG: 137.5 μg/ml vs. 84.0 μg/ml; $p = 0.02$; IgG1: 24.5 μg/ml vs. 18.2 μg/ml; $p = 0.05$; Fig. 1A). Geometric mean IgG2 levels against 19F and 19A were significantly higher in children with rAOM ($p = 0.02$ and $p = 0.05$, respectively), whereas there was no difference in IgG2 levels for any of the other serotypes (Fig. 1B).

In children with a history of rAOM, 15 (9%) had an IgG2 level below the lower limit of detection for one or more PCV7 serotypes, compared to 7 (11%) of the healthy controls ($p = 0.6$). For the non-PCV7 serotypes 51 (31%) cases and 23 (38%) of the healthy controls had an IgG2 level below the lower limit of detection against at least one serotype ($p = 0.3$). Interestingly, a significantly higher percentage of children in the healthy control group had IgG2 levels against serotype 19A below the limit of detection (31%) compared with the children with a history of rAOM (15%; $p = 0.007$; Fig. 1B). In children with a history of rAOM, serotype specific IgG, IgG1 and IgG2 levels were compared between children who had experienced 3–4, 5–7, 8–9 or 10+ episodes using linear regression correcting for age, gender and day-care attendance. There was a statistically significant difference for IgG2 levels against serotype 19A between the groups, being 9.5 AU/ml in children with 3–4 episodes, 13.2 AU/ml in children with 5–7 episodes, 26.2 AU/ml in children with 8–9 episodes and 57.9 AU/ml in children with 10+ episodes ($p = 0.04$).

### 3.3. The age-related development of pneumococcal serotype-specific IgG, IgG1 and IgG2 responses is similar in children with or without rAOM

We compared pneumococcal serotype specific IgG, IgG1 and IgG2 antibody levels in 3 age brackets (6–11 months, 12–23 months and 24–36 months) between children with and without a history of rAOM. In the 12–23 months age-bracket, IgG (Fig. 2) and IgG1 levels against serotype 5, 7F, 19A and 19F were significantly higher in children with rAOM, whereas there were no differences in the other age groups. In both groups of children, IgG, IgG1 and IgG2 antibody levels against PCV7 serotypes were highest in the youngest

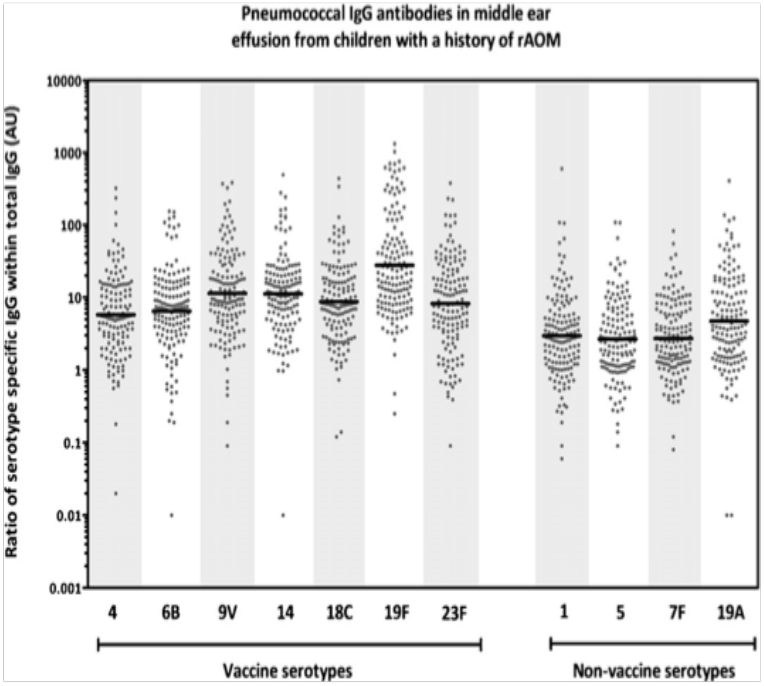


**Fig. 3.** Pneumococcal serotype specific IgG levels in middle ear effusion, expressed as a ratio over total IgG (AU, arbitrary units). 1 unit reflects 0.01% of total IgG is serotype specific.

age groups, with levels declining over time. On the contrary, IgG, IgG1 and IgG2 levels against non-PCV7 serotypes were lowest in the group of children between 6 and 12 months and levels increased with age.

### 3.4. *Pneumococcal serotype specific IgG is present in the middle ear of children with a history of rAOM and correlates with serum IgG levels*

We analysed 144 MEE samples, collected from 94 children with a history of rAOM, for the presence of pneumococcal serotype specific IgG (Fig. 3). Pneumococcal serotype specific IgG antibodies, as expressed as a ratio over total IgG, were detected in all MEE samples. IgG levels against PCV7 serotypes were higher compared with IgG levels against non-PCV7 serotypes. IgG levels against pneumococcal serotype 19F and 19A were higher than IgG levels against the other investigated PCV7 and non-PCV7 serotypes, respectively.

In the children with bilateral MEE, the correlation between MEE collected from the left and the right ear were strong (data not shown). Therefore, the average of serotype specific IgG in the left and right MEE sample was used to determine the correlation with serotype specific IgG in serum. There was a significant correlation between IgG in MEE and serum for all serotypes, except serotype 1. The correlations were stronger for IgG levels against PCV7 serotypes compared with non-PCV7 serotypes (Fig. 4).

## 4. Discussion

This study describes serum IgG, IgG1 and IgG2 antibody levels against a wide range of pneumococcal polysaccharides, both PCV7 and non-PCV7 serotypes, in a large cohort of young children below 36 months of age with or without a history of frequent AOM. In addition we measured IgG levels in middle ear effusion (MEE), demonstrating a strong correlation between IgG levels detected in serum and MEE.

We found that compared to healthy age matched controls, children with a history of frequent AOM had similar pneumococcal serotype specific IgG, IgG1 or IgG2 levels, either induced by vaccination or naturally acquired, which confirms previous data [9,10,12,13,15]. In contrast, one study showed lower pneumococcal serotype specific IgG levels in children with rAOM, however, in this study only 13 children with rAOM were included and IgG levels against only 3 pneumococcal serotypes were assessed [6]. Dhooge et al. found lower pneumococcal serotype specific IgG2 levels in otitis-prone children compared to healthy controls, but the IgG1 levels were not lower in otitis-prone children in this study, arguing against a more general antibody deficiency in otitis-prone children [7].

It is important to note that the case-definition in different study populations varies, which complicates comparisons between studies. Some studies only include children that comply with the classical "otitis-prone" condition as defined by Howie et al. [31], whereas other studies have to rely on medical records or parental report. Because of the retrospective nature of our study the classical "otitis-prone" condition could not be established and the term "otitis-prone" was therefore not used.

The higher serum IgG antibody levels against non-PCV7 serotypes that were observed in children with a history of reoccurring AOM compared with healthy controls may be due to the higher pneumococcal nasopharyngeal carriage rates in these children [25], which could be related to the higher rate of day care attendance in the group of children with rAOM. There is currently no generally accepted antibody level that is required to prevent nasopharyngeal colonisation and further studies, preferably large longitudinal cohort studies, are required.

The children with a history of rAOM did not have a delay in acquisition of serotype-specific antibodies or a more rapid decline in vaccine-induced antibody levels compared with healthy controls. In both children with and without a history of rAOM, antibody levels against PCV7 serotypes decreased with age, reflecting the waning antibody response after vaccination. In both children with

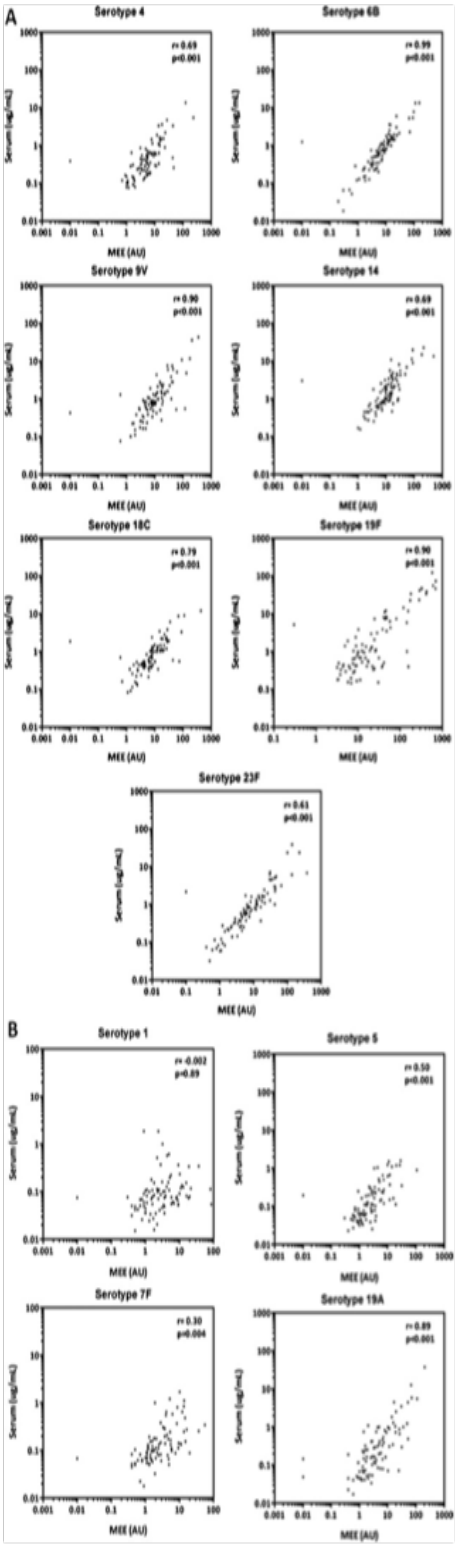


**Fig. 4.** Correlation between pneumococcal serotype specific IgG against serotypes included in PCV7 (panel a) and non-PCV7 serotypes (panel b) in serum (μg/ml) and middle ear effusion (arbitrary units) from children with a history of rAOM. *R* and *p*-value are determined using Pearson's correlation coefficient.

and without a history of rAOM antibody levels against non-PCV7 serotypes increased over time, which is likely due to an increase in pneumococcal exposure in combination with maturation of the immune response and an increased capacity to respond the polysaccharide antigens [32].

Children with rAOM may have a more subtle immune impairment, such as low IgG1 or IgG2 subclass levels to pneumococcal antigens, however, we did not find evidence for such a subclass immune-deficiency in children with a history of rAOM. It has been suggested that the IgG1 response mainly reflects the antibody response to proteins and the pneumococcal conjugate vaccine and that IgG2 is predominantly a measure of the natural antibody response to pneumococcal polysaccharides [33,34]. This hypothesis is supported by our observation that IgG1 levels against PCV7 serotypes are markedly higher than naturally induced IgG1 levels to non-PCV7 serotypes. It has also been shown though that children predominantly mount an IgG1 response, whereas adults mainly respond with IgG2 antibodies [35], which may therefore also explain the higher IgG1 levels in our cohort.

Debate on the potential cross-reactivity between antibodies against PCV7 pneumococcal serotype 19F and non-PCV7 serotype 19A is ongoing. After the wide-spread use of PCV7, carriage of and disease caused by 19A has increased, arguing against cross-protection of vaccine induced 19F antibodies [36]. This is in accordance with data showing that vaccination with PCV7 does not induce functional antibodies against 19A as measured by opsonophagocytic assays [36,37]. The high IgG1 levels against 19A compared to IgG1 levels against other non-PCV7 serotypes we found in this study suggests that vaccination with PCV7 including 19F induces cross-reactive antibodies against 19A. However, since children predominantly raise an IgG1 response, the high carriage rate of serotype 19A in our cohort may also contribute to the high anti-19A IgG1 antibody levels [25].

We frequently detected pneumococcal serotype specific IgG antibodies in MEE, however, only a small number of MEE samples were positive for *S. pneumoniae* (6%) [25], which may suggest that these antibodies contributed to the elimination of the pneumococcus from the middle ear. Indeed, it has been shown that the presence of antibody in the middle ear is associated with clearing of the clinical infection [20–22], however, further research is warranted.

We found a strong correlation between pneumococcal serotype specific IgG antibodies detected in serum and the middle ear, which suggests that at least part of the pneumococcal serotype specific IgG antibodies in serum transudate into the middle ear space. This correlation is stronger for PCV7 serotypes compared with non-PCV7 serotypes, which may imply that antibodies induced by systemic vaccination reach the middle ear, which confirms previous findings [24]. Investigation of pneumococcal serotype specific IgA in serum and MEE would be an important next step in understanding the contribution of systemic and local antibody production in the protection against rAOM.

To conclude, we did not find evidence that children with frequent AOM have an impaired pneumococcal serotype specific antibody response. While a subset of children with humoral immune-deficiencies may suffer from rAOM, the suggestion that children with rAOM *generally* have impaired antibody responses should be reconsidered. Pneumococcal serotype specific IgG is also present in the middle ear and strongly correlated with serum IgG levels. Together these data support the suggestion that vaccination with pneumococcal vaccines with broader valencies (PCV10 and PCV13) could further contribute to reducing the burden of rAOM, however, an approach that also targets social and health-behavioural risk factors for rAOM, such as crowding, breastfeeding, day-care and smoke exposure, is required.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.12.078.

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ORIGINAL ARTICLE

# *FBXO11, a regulator of the TGFβ pathway, is associated with severe otitis media in Western Australian children*

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*Otitis media (OM) is a common childhood disease characterised by middle ear inflammation following infection. Susceptibility to recurrent acute OM (rAOM) and chronic OM with effusion (COME) is highly heritable. Two murine mutants, Junbo and Jeff, spontaneously develop severe OM with similar phenotypes to human disease. Fine-mapping of these mutants identified two genes (Evi1 and Fbxo11) that interact with the transforming growth factor β (TGFβ) signalling pathway. We investigated these genes, as well as four Sma- and Mad-related (SMAD) genes of the TGFβ pathway, as candidate rAOM/COME susceptibility genes in two predominantly Caucasian populations. Single-nucleotide polymorphisms (SNPs) within FBXO11 (family-based association testing Z-Score = 2.61; $P_{best}$ = 0.009) were associated with severe OM in family-based analysis of 434 families (561 affected individuals) from the Western Australian Family Study of OM. The FBXO11 association was replicated by directed analysis of Illumina 660W-Quad Beadchip data available for 253 cases and 866 controls (OR = 1.55 (95% CI 1.28–1.89); $P_{best} = 6.9 \times 10^{-6}$) available within the Western Australian Pregnancy Cohort (Raine) Study. Combined primary and replication results show $P_{combined} = 2.98 \times 10^{-6}$. Neither cohort showed an association with EVI1 variants. Family-based associations at SMAD2 (P = 0.038) and SMAD4 (P = 0.048) were not replicated. Together, these data provide strong evidence for FBXO11 as a susceptibility gene for severe OM.*

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**Keywords:** *acute otitis media; otitis media with effusion; genetic polymorphisms; mouse-to-man; association; Raine Study*

## Introduction

Otitis media (OM), one of the most common diseases of early childhood, is characterised by inflammation of the middle ear, often with effusion. The inflammation is triggered by infection and leads to a variety of related clinical phenotypes. Acute otitis media (AOM) is an acute, usually bacterial infection of the middle ear, characterised by fever and painful inflammation of the tympanic membrane. The majority of children have at least one episode of AOM by school age, with 40% suffering from recurrent AOM[1] (rAOM: ⩾3 episodes in 6 months or ⩾4 episodes in 12 months). OM with effusion, a common sequelae of rAOM, is a non-purulent middle ear effusion, which may become chronic (COME). Both rAOM and COME may lead to perforation of the tympanic membrane, conductive hearing loss, delayed speech development and poor educational outcomes.[2] Treatment for rAOM and COME may include insertion of tympanostomy tubes. In Australia, the cost of OM treatment was estimated at AUD$100–$400 million in 2008 (see ref. 3).

Both genetic and environmental factors predisposing children to rAOM and OM with effusion have been described. The genetic risk has been quantified by heritability data from twins and triplets. Self-reported data from Norwegian twins gave a heritability estimate of 0.74 for females and 0.45 for males.[4] In the United States, analysis of twins and triplets with physician-diagnosed OM in the first few years of life gave a heritability of 0.73 for the total time with middle ear effusion, but with no sex differences.[5] A longitudinal study reported that the heritability of symptom scores, composed of ear infection and respiratory symptoms, increased with age, from 0.49 at age 2 to 0.71 at age 4 (see ref. 6). These studies confirm a substantial heritable

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component to the broad clinical spectrum of OM. Environmental risk factors,[7] including attendance at day care, exposure to tobacco smoke and short duration of breastfeeding, also have an important role and may be important covariates of heritable risk.

To identify genes involved in OM susceptibility, mouse mutagenesis studies have investigated murine models with a conductive deafness phenotype and identified two mouse models, *Junbo* (*Jbo*) and *Jeff* (*Jf*), with pathological signs comparable to OM in humans. Homozygous *Jbo*/*Jbo* and *Jf*/*Jf* mice are lethal *in utero* or early life, with a range of developmental abnormalities. However, heterozygous *Jbo*/+ mice develop bilateral chronic suppurative OM 29 days after birth, which is not associated with major developmental malformations or immune deficiencies.[8] The *Jbo* mutation maps to a zinc-finger domain at the *Evi1* gene. *Jf* homozygotes die shortly after birth and display a number of developmental abnormalities, including cleft palate and eyes open at birth. Heterozygous *Jf*/+ mice develop COME 28 days after birth, again in the absence of severe craniofacial abnormalities.[9] The *Jf* mutation maps to the *Fbxo11* gene. Interestingly, both mutant mouse strains develop chronic OM in specific pathogen-free conditions. Allelic association analysis of single-nucleotide polymorphism (SNP) variants in the human *FBXO11* gene carried out in the predominantly Caucasian Minnesota COME/ROM Family Study cohort identified nominal evidence for association at rs2134056 ($P = 0.02$) (see ref. 10).

Given the phenotypic similarities between $Jbo^{+/-}$ and $Jf^{+/-}$ mice and human OM, the human orthologues were selected as candidate genes for association analysis with rAOM and COME susceptibility. Preliminary functional[11] and pathway analyses revealed that both EVI1 and FBXO11 proteins interact in the transforming growth factor β (TGFβ) signalling pathway. On the basis of this, four Sma- and Mad-related (*SMAD*) mediators of the TGFβ pathway (*SMAD2*, *SMAD3*, *SMAD4* and *SMAD7*) were also investigated as susceptibility determinants of OM. To establish whether these genes have a role in determining susceptibility to rAOM and COME, family-based allelic association analysis with replication in an independent case–control cohort was carried out.

## Results

*Allelic association analysis of FBXO11 and EVI1 polymorphisms*

Family-based association testing results are shown in Table 1. Significant association under a recessive model was observed at two of five polymorphisms in *FBXO11*, rs12712997 (Z-score +2.61; $P = 0.009$) and rs330787 (Z-score +1.94; $P = 0.053$). In both cases the major allele was disease associated (A for rs12712997; C for rs330787). No other polymorphisms in *FBXO11* showed significant association with disease, including *FBXO11*/rs2134056, which was previously shown to be associated with OM.[10] There was no significant association with any *EVI1* polymorphism.

Analysis using univariate logistic regression analysis of data available from the Western Australian Pregnancy Cohort (Raine) Study ('the Raine Study') replicated the significant results observed in the family-based association testing analysis for *FBXO11* polymorphisms (see Table 2 and Supplementary Table 3b). The major alleles of *FBXO11*/rs330787 (Odds ratio (OR) = 1.55; $P = 6.9 \times 10^{-6}$) and *FBXO11*/rs12712997 (OR = 1.45; $P = 2.0 \times 10^{-4}$) were disease associated. These significant associations are robust to correction for the number of independent tests carried out in the Raine Study data (that is, 92 haplotype blocks across all genes analysed; $P_{corrected} = 0.05/92 = 5x10^{-4}$). Significant association was also observed at a further six polymorphisms in *FBXO11*. No significant association was observed for any *EVI1* variants in the replication analysis (data not shown). Multivariate analysis, taking account of epidemiological risk factors shown to be independently associated with OM in the Raine Study population (that is, day care attendance (OR = 2.31; $P = 2.1 \times 10^{-20}$) and allergy (OR = 1.47; $P = 5 \times 10^{-4}$)), did not significantly alter the univariate association analyses for *FBXO11* or *EVI1* (data not shown). A combination of results from the primary and replication cohort was carried out using Fisher's trend test carried out in MetaP[12] and gives combined *P*-values at *FBXO11*/rs12712997 of $P_{combined} = 1.41 \times 10^{-5}$ and at *FBXO11*/rs330787 of $P_{combined} = 2.98 \times 10^{-6}$. Overall, these results confirm that *FBXO11* is significantly associated with OM susceptibility in two separate cohorts of Western Australian children and exert an effect independently of known environmental determinants.

*Linkage disequilibrium and haplotype analysis at FBXO11*

Pairwise linkage disequilibrium (LD) between the two associated *FBXO11* polymorphisms (rs330787 and rs12712997) in the primary cohort is high ($r^2 = 0.75$; $D' = 0.89$), indicating that these polymorphisms likely exist on the same haplotype. Forward stepwise case–pseudocontrol conditional logistic regression analysis supported this, as neither polymorphism contributed significant additional main effects to a model containing the other (that is, adding rs330787 to rs12712997: $P = 0.858$; and adding rs12712997 to rs330787: $P = 0.518$). Haplotypic analysis using haplotype-based association tests (HBAT) identified one haplotype, carrying both major alleles, as significantly associated with OM under a recessive model (rs330787C: rs12712997A; Z-score +2.31; $P = 0.021$).

LD analysis in the replication cohort showed a similar pattern of pairwise LD between rs330787 and rs12712997 in the replication cohort ($r^2 = 0.82$; $D' = 0.91$). Further analysis of pairwise LD between all associated *FBXO11* SNPs in the Raine Study shows that although D′ is generally high (that is, $D' = 0.50$–$1.00$) between all markers (Figure 1a), the $r^2$ values (Figure 1b) identify two overlapping LD blocks (designated as block 1 and block 2; see Figures 1c and d) that show high pairwise $r^2$ within each group (that is, $r^2 \geqslant 0.68$) but not between each group (that is, $r^2 \leqslant 0.14$). All markers that fall in LD block 1 show significant disease associations at the major allele, whereas those in LD block 2 show significant disease associations at the minor allele (Table 2). *FBXO11*/rs330787 and *FBXO11*/rs12712997, which are associated in both the primary and replication cohorts, fall in LD block 1. *FBXO11*/rs2134056, which was associated with COME/ROM in the Minnesota Family Study[10] but was not associated in our study, does not belong to either LD block ($r^2 \leqslant 0.12$).

**Table 1** FBAT analysis under additive and recessive inheritance models of *FBXO11* and *EVI1* polymorphisms and rAOM/COME

| *SNPs* | *Allele* | *Allele freq* | *Additive inheritance model* | | | *Recessive inheritance model* | | |
|---|---|---|---|---|---|---|---|---|
| | | | *N° Families* | *Z-score* | *P-value* | N° Families | *Z-score* | *P-value* |
| ***EVI1*** | | | | | | | | |
| rs6795291 | A | 0.662 | 112 | −0.506 | 0.613 | 240 | −0.387 | 0.699 |
| | T | 0.338 | 240 | 0.387 | 0.699 | 112 | 0.506 | 0.613 |
| rs7615092 | T | 0.726 | 82 | 0.006 | 0.995 | 245 | 0.410 | 0.682 |
| | C | 0.274 | 245 | −0.410 | 0.682 | 82 | −0.006 | 0.995 |
| rs6778131 | A | 0.636 | 127 | 0.668 | 0.504 | 230 | −1.307 | 0.191 |
| | T | 0.364 | 230 | 1.307 | 0.191 | 127 | −0.668 | 0.504 |
| rs10936575 | T | 0.634 | 135 | 1.016 | 0.309 | 240 | −0.534 | 0.593 |
| | C | 0.366 | 240 | 0.534 | 0.593 | 135 | −1.016 | 0.309 |
| ***FBXO11*** | | | | | | | | |
| rs330787 | C | 0.640 | 285 | 1.480 | 0.139 | 234 | 1.937 | **0.053** |
| | A | 0.360 | 285 | −1.480 | 0.139 | 139 | −0.041 | 0.967 |
| rs2134056 | C | 0.828 | 187 | −1.005 | 0.315 | 178 | −0.967 | 0.333 |
| | T | 0.172 | 187 | 1.005 | 0.315 | 37 | 0.390 | 0.697 |
| rs12712997 | A | 0.627 | 284 | 1.692 | 0.091 | 232 | 2.610 | **0.009** |
| | C | 0.373 | 284 | −1.692 | 0.091 | 145 | 0.439 | 0.660 |
| rs874869 | C | 0.545 | 296 | 0.402 | 0.688 | 209 | 0.097 | 0.922 |
| | G | 0.455 | 296 | −0.402 | 0.688 | 183 | −0.525 | 0.599 |
| rs13035558 | T | 0.737 | 252 | −0.896 | 0.370 | 225 | −0.364 | 0.716 |
| | C | 0.263 | 252 | 0.896 | 0.370 | 89 | 1.261 | 0.207 |

Abbreviations: AOM, acute otitis media; FBAT, family-based association testing; SNPs, single-nucleotide polymorphisms.
Significant *P*-values are shown in bold.

**Table 2** Univariate logistic regression analysis of *FBXO11* polymorphisms and OM in the Raine study

| *SNPs* | *Allele* | *SNP position* | *Allele Freq.* | *Genotyped status* | *P-value* | *Odds ratio* | *95% Confidence interval* |
|---|---|---|---|---|---|---|---|
| rs2710163 | T | Intronic | 0.57 | Imputed | **0.0003** | 1.43 | 1.18–1.79 |
| rs6713506 | A | Intronic | 0.15 | Genotyped | **0.0074** | 1.38 | 1.09–1.75 |
| rs330787 | C | Intronic | 0.61 | Genotyped | **0.0000069** | 1.55 | 1.28–1.89 |
| rs12712997 | A | Intronic | 0.60 | Imputed | **0.0002** | 1.45 | 1.19–1.76 |
| rs10182633 | T | Intronic | 0.20 | Imputed | **0.0009** | 1.44 | 1.16–1.77 |
| rs6728843 | C | Intronic | 0.16 | Genotyped | **0.0061** | 1.39 | 1.10–1.76 |
| rs13430439 | C | Intronic | 0.16 | Imputed | **0.0061** | 1.39 | 1.10–1.76 |
| rs12620679 | G | Intronic | 0.61 | Imputed | **0.0001** | 1.50 | 1.22–1.83 |

Abbreviations: OM, otitis media; SNPs, single-nucleotide polymorphisms.
Significant *P*-values are shown in bold.

To determine whether the association observed with members of LD block 2 in the replication study is indicative of an independent disease effect at *FBXO11* in this population, we undertook both haplotype analysis in Haploview and a forward stepwise logistic regression analysis in STATA v10. The haplotype analysis confirmed that significantly overtransmitted haplotypes for LD block 1 markers always comprised major alleles (Table 3a), whereas the significantly overtransmitted haplotypes for LD block 2 markers always comprised minor alleles (Table 3b). Haplotype analysis across all eight associated SNPs also shows that the minor alleles at LD block 2 SNPs are only significantly overtransmitted when on a haplotype with the major alleles of LD block 1 SNPs (Supplementary Table 4), suggesting that the markers in LD block 2 do not represent an independent disease effect.

To further confirm this, stepwise logistic regression analysis was used to determine whether independent effects existed at the significantly associated markers within each LD block and between each LD block. Within LD block 1, only rs330787 and rs12620679 contributed significant independent effects when compared with other LD block 1 markers, whereas in LD block 2 only rs10182633 contributed significant independent effects when compared with other LD block 2 markers (Supplementary Table 5). This suggests that rs330787 and rs12620679 account for the association observed with LD block 1 markers, whereas rs10182633 accounts for the association observed with LD block 2 markers. When comparing between the two LD blocks, rs330787 (used as the most significantly associated SNP in LD block 1) adds independent main effects ($P = 0.001$) to rs10182633, but the converse does not hold true ($P = 0.06$), again suggesting that the markers in LD block 2 do not represent an independent disease effect. In summary, the haplotype and stepwise logistic regression analyses are most consistent with a single disease association in this

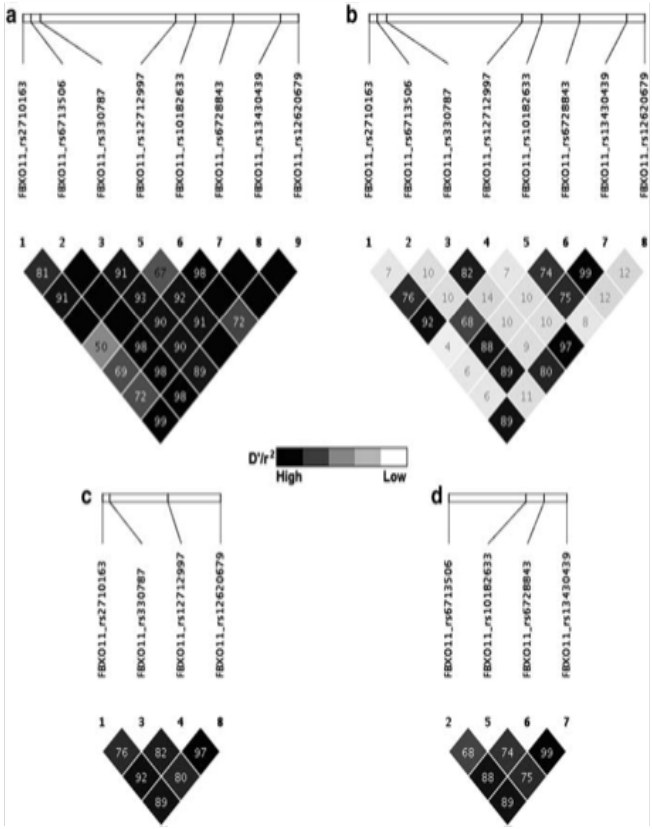


**Figure 1** Pairwise linkage disequilibrium between *FBXO11* SNPs in the Raine Study cohort showing (**a**) D′ across all SNPs, (**b**) $r^2$ across all SNPs, (**c**) $r^2$ across markers 1, 3, 4 and 8 (designated as LD block 1) and (**d**) $r^2$ across markers 2, 5, 6 and 7 (designated as LD block 2). For both D′ and $r^2$, LD measures are indicated at the matrix intercept between two markers. For D′ measures, in which no value is indicated at the intercept, D′ = 1. For D′ measures, black and darker shades of grey indicate a higher degree of confidence (that is, LOD ⩾2.0).

replication cohort, which is attributable to an aetiological variant in strong LD with rs330787.

*Allelic association analysis of TGFβ signalling pathway members*
Analysis of TGFβ signalling pathway members in the primary OM cohort (Supplementary Table 2) identified significant association at the major alleles of rs1792658 in *SMAD2* (Z-score +2.08; $P = 0.038$) and at rs10502913 in *SMAD4* (Z-score +1.98; $P = 0.048$). No significant associations were identified at *SMAD3* or *SMAD7* ($P > 0.05$). Replication in the Raine Study cohort using univariate and multivariate analysis of all *SMAD2*, *SMAD3*, *SMAD4* and *SMAD7* polymorphisms from the primary cohort, plus an additional 306 polymorphisms, did not reveal any significant associations robust to multiple testing correction ($P_{\text{uncorrected}} \geqslant 0.03$). Univariate and multivariate analyses of polymorphisms at an additional 13 TGFβ signalling pathway members (Supplementary Table 3A) in the Raine Study data did not reveal any significant associations robust to multiple testing correction ($P_{\text{uncorrected}} \geqslant 0.001$).

## Discussion

This study investigated whether variants at the human orthologues, *FBXO11* and *EVI1*, for the well-characterised mouse mutants *Jeff* (*Fbxo11*) and *Junbo* (*Evi1*) are associated with OM in humans. In both our primary sample of families from the Western Australian Family Study of OM and in a replication cohort derived from the Raine Study we found significant association with variants at *FBXO11* (best combined $P$-value $= 2.98 \times 10^{-6}$) but not at *EVI1*. Together, these two independent data sets provide strong evidence of a role of the *FBXO11* gene in susceptibility to severe/recurrent OM in this predominantly Caucasian population, which seems to exert an effect independently of known environmental determinants.

In our study, the *FBXO11* association was observed across the spectrum (rAOM/COME) of clinically severe OM. This is broadly concordant with a previously reported association between *FBXO11* and recurrent OM/COME in The Minnesota COME/ROM Family Study,[10] although we did not replicate association with the same SNP (rs2134056), which falls outside the haplotype blocks containing our disease-associated SNPs. Conversely, the previous study did not observe an association with rs330787, the most significantly associated SNP observed here, despite the fact that all three cohorts comprise predominantly Caucasian children. Further work is required to determine whether different haplotypes occur in the United States of America compared with Australian children used in

**Table 3** Haplotype analysis performed in Haploview for SNPs genotyped across *FBXO11* for (A) markers 1, 3, 4 and 8 in LD block 1 (see Figure 1) and (B) markers 2, 5, 6 and 7 in LD block 2 (see Figure 1)

(A)

| Over transmitted haplotypes | | Frequency | rs2710163 | rs330787 | rs12712997 | rs12620679 |
|---|---|---|---|---|---|---|
| | | | 1 | 3 | 4 | 8 |
| Global single point analysis | | | Major (T) $\chi^2 = 7.39$; $P = \mathbf{0.0066}$ | Major (C) $\chi^2 = 11.584$; $P = \mathbf{0.0007}$ | Major (A) $\chi^2 = 7.793$; $P = \mathbf{0.0052}$ | Major (G) $\chi^2 = 7.503$; $P = \mathbf{0.0062}$ |
| 2 | T.C | 0.58 | $\chi^2 = 6.825$; $P = \mathbf{0.0090}$ | | | |
| | C.A | 0.60 | | $\chi^2 = 7.865$; $P = \mathbf{0.0050}$ | | |
| | A.G | 0.62 | | | $\chi^2 = 9.006$; $P = \mathbf{0.0027}$ | |
| 3 | T.C.A | 0.58 | $\chi^2 = 7.418$; $P = \mathbf{0.0065}$ | | | |
| | C.A.G | 0.60 | | $\chi^2 = 7.77$; $P = \mathbf{0.0053}$ | | |
| 4 | T.C.A.G | 0.58 | $\chi^2 = 8.445$; $P = \mathbf{0.0037}$ | | | |
| **Under transmitted haplotypes** | | **Frequency** | **rs2710163** | **rs330787** | **rs12712997** | **rs12620679** |
| | | | **1** | **3** | **4** | **8** |
| 2 | C.A | 0.36 | $\chi^2 = 6.825$; $P = \mathbf{0.0009}$ | | | |
| | A.C | 0.36 | | $\chi^2 = 12.184$; $P = \mathbf{0.0005}$ | | |
| | C.C | 0.38 | | | $\chi^2 = 8.973$; $P = \mathbf{0.0027}$ | |
| 3 | C.A.C | 0.36 | $\chi^2 = 12.608$; $P = \mathbf{0.0004}$ | | | |
| | A.C.C | 0.35 | | $\chi^2 = 12.947$; $P = \mathbf{0.0003}$ | | |
| 4 | C.A.C.C | 0.35 | $\chi^2 = 13.840$; $P = \mathbf{0.0002}$ | | | |

(B)

| Over transmitted haplotypes | | Frequency | rs6713506 | rs10182633 | rs6728843 | rs13430439 |
|---|---|---|---|---|---|---|
| | | | 2 | 5 | 6 | 7 |
| Global single point analysis | | | Minor (A) $\chi^2 = 3.192$; $P = \mathit{0.074}$ | Minor (T) $\chi^2 = 5.278$; $P = \mathbf{0.0216}$ | Minor (C) $\chi^2 = 3.700$; $P = \mathbf{0.0544}$ | Minor (C) $\chi^2 = 3.223$; $P = \mathit{0.0726}$ |
| 2 | A.T | 0.15 | $\chi^2 = 3.187$; $P = \mathit{0.0742}$ | | | |
| | T.C | 0.17 | | $\chi^2 = 3.331$; $P = \mathit{0.068}$ | | |
| | C.C | 0.16 | | | $\chi^2 = 3.586$; $P = \mathbf{0.0583}$ | |
| 3 | A.T.C | 0.15 | $\chi^2 = 3.377$; $P = \mathit{0.0661}$ | | | |
| | T.C.C | 0.17 | | $\chi^2 = 3.326$; $P = \mathit{0.0682}$ | | |
| 4 | A.T.C.C | 0.15 | $\chi^2 = 3.486$; $P = \mathit{0.0619}$ | | | |
| **Under transmitted haplotypes** | | **Frequency** | **rs6713506** | **rs10182633** | **rs6728843** | **rs13430439** |
| | | | **2** | **5** | **6** | **7** |
| 2 | G.A | 0.79 | $\chi^2 = 5.035$; $P = \mathbf{0.0248}$ | | | |
| | A.T | 0.79 | | $\chi^2 = 4.808$; $P = \mathbf{0.0283}$ | | |
| | T.T | 0.83 | | | $\chi^2 = 3.741$; $P = \mathbf{0.053}$ | |
| 3 | G.A.T | 0.79 | $\chi^2 = 4.995$; $P = \mathbf{0.0254}$ | | | |
| | A.T.T | 0.79 | | $\chi^2 = 4.653$; $P = \mathbf{0.0310}$ | | |
| 4 | G.A.T.T | 0.79 | $\chi^2 = 5.160$; $P = \mathbf{0.0231}$ | | | |

Abbreviations: LD, linkage disequilibrium; SNPs, single-nucleotide polymorphisms.
Significant *P*-values are shown in bold, those approaching significance are shown in italics.

these studies, and whether this could be capitalised on to map the precise aetiological variants for disease in both geographical locations.

The FBXO11 protein is a member of the F-box family, a group of SCF E3 ligases containing a protein domain with a role in ubiquitination and degradation of phosphorylated proteins.[13] There are limited data on the role of FBXO11 in humans; however, functional studies in mice have identified a role in the regulation of the TGFβ signalling pathway.[11] During embryonic development, mice homozygous for the *Jf* mutation show increased expression and accumulated nuclear

localisation of phospho-Smad2 (pSmad2) in epithelial cells of the eyelid, palate and lung.[11] Furthermore, it has been shown that FBXO11 neddylates p53 suggesting a role for FBXO11 in the stabilisation of p53, (see ref. 14) a known cofactor of pSmad2. This proposed role is supported by the observation that *Jf/Jf* mice show reduced levels of p53 (see ref. 11) As such, FBXO11 may have a putative role that relates to the diverse functions of the TGFβ signalling pathway, including embryonic development and immunological regulation, both of which may be important determinants of OM susceptibility and are discussed briefly here.

On a C3H/HeH background,[9] *Jf* homozygous mutant mice have severe developmental defects, including cleft palate, which is a developmental condition known to increase the prevalence of OM in humans,[15] whereas heterozygous *Jf/+* mice demonstrate mild craniofacial abnormalities, such as a shortened snout and smaller eustachian tubes. Whilst these craniofacial abnormalities may contribute to the OM susceptibility observed in these mice, it has been shown that on a C57BL/6 background *Jf/+* mice have no apparent craniofacial defects although they do still develop OM (SD Brown, unpublished data[16]). This suggests that craniofacial abnormalities are not the major mechanism of FBXO11 function in OM susceptibility, although subtle differences in craniofacial or Eustachian tube development cannot be completely discounted.

Instead, a role in inflammatory dysfunction may be the key mechanism underlying the increased susceptibility to OM. In support of this is the observation that *Jf/+* mice develop COME 28 days after birth under specific pathogen-free conditions. This is possibly because of an inability to control chronic inflammation in the middle ear, due to either a constitutive inflammatory process or in response to commensal bacteria, consistent with a defect in the known anti-inflammatory activity of the TGFβ pathway.[17]

Analysis of other key genes in the TGFβ pathway, specifically *SMAD2*, *SMAD3*, *SMAD4* and *SMAD7*, revealed only borderline genetic associations at *SMAD2* and *SMAD4*. These were not replicated in the Raine Study cohort. We cannot rule out that the lack of replication at *SMAD2* and *SMAD4* maybe due to subtle differences in phenotype or exposures to environmental determinants between the primary and replication cohorts; thus, further replication in additional cohorts should be undertaken. No additional genetic associations were observed with other members of the TGFβ pathway in the Raine Study cohort.

Our study was not sufficiently powered (or designed) to determine whether *FBXO11* was more strongly associated with rAOM or COME. Future studies using larger sample sizes of children with a specific rAOM or COME phenotype are needed to firmly establish the relationship between *FBXO11* variants and different OM phenotypes. In our study the tag-SNPs selected to cover the *FBXO11* gene in the Western Australian Family Study of OM, plus those analysed for the Raine Study, were all intronic and unlikely to represent functional variants, although enhancers are known to fall within intronic regions. Ongoing fine-mapping aims to identify the aetiological variants at *FBXO11* associated with OM. For the moment, associations with polymorphisms at *FBXO11* in three separate cohorts confirm a role for this gene in susceptibility to childhood OM. These results provide some progress to improved understanding of the pathogenesis of this disease in humans.

## Study population and methods

*Sample collection and phenotype definition*

Recruitment to the Western Australian Family Study of OM commenced in 2007. The study was approved by the human research ethics committee at Princess Margaret Hospital for Children (#1428/EP). Probands with a history of tympanostomy tube insertion for rAOM or COME were identified from medical records of collaborating ear, nose and throat (ENT) specialists. Parents and affected siblings (defined as ⩾3 physician-diagnosed episodes of AOM or tympanostomy tube insertion for rAOM/COME) were also recruited to the family-based study. No exclusion was made on the basis of ethnicity.

Following informed consent, study participants provided either a non-invasive saliva sample or, for a subset of probands, a blood sample at the time of tympanostomy tube insertion for DNA extraction, and parents completed a study-specific questionnaire. Information on exposure to known environmental risk factors such as attendance at day care, duration of breastfeeding and exposure to tobacco smoke was also obtained, together with data on episodes of OM and their management. Children with predisposing medical conditions (that is, immune deficiencies, craniofacial abnormalities and specific genetic syndromes) were excluded from the study. Family members with no history of rAOM were classified as unaffected, whereas all others were classified as unknown. In total, 561 affected individuals from 434 families, containing up to four affected siblings, were available for inclusion in the study. In all, 92% of families self-identified themselves as Caucasian.

For replication, we used the Western Australian Pregnancy Cohort (Raine) Study ('the Raine Study'), a longitudinal cohort of children whose mothers were recruited during early pregnancy and who are now in the 20th year of follow-up.[18] Genome-wide Illumina 660W-Quad Beadchip data are available on 1198 Caucasian Raine Study participants. Clinical data collected during the first 3 years of life indicating retracted or scarred tympanic membranes, middle ear effusion or tympanostomy tube insertions or parental reporting of ⩾3 episodes of AOM by age 3 years were used to identify 253 children with a history consistent with OM (cases). As for the family-based study, these children represent the broad spectrum (rAOM/COME) of severe OM. Of the remaining children, 866 had no OM history and were used as controls. Data on relevant epidemiological risk factors, including tobacco smoke exposure, attendance at day care and duration of breastfeeding, are available for Raine Study participants.

*DNA extraction*

Genomic DNA was extracted from 2 ml of saliva for the majority of participants using the Oragene technology (DNA Genotek, Ontario, Canada) as per the manufacturer's instructions. For a subset of children, blood samples were collected at the time of tympanostomy tube insertion and DNA was extracted from neutrophil pellets using a salting-out method.[19] Extracted DNA was

resuspended in TE buffer, quantified using spectrophotometry and stored at −20 °C.

*Selection of genes and SNPs—Western Australian family study of otitis media*

The *FBXO11* and *EVI1* genes were initially selected as candidates for human OM on the basis of data from murine models. Functional data from mouse models,[8,9] combined with Ingenuity pathway analysis (Ingenuity Systems, http://www.ingenuity.com), indicated that both gene products interact with the TGFβ pathway and thus other key genes in this pathway—*SMAD2*, *SMAD3*, *SMAD4* and *SMAD7*—were also investigated. For genotyping in the family cohort, a minimum of two haplotype tagging (tag) SNPs were selected for each gene using data from the Caucasian population in the International HapMap Project.[20] Where possible, tag-SNPs with a minor allele frequency (MAF) $\geqslant 0.2$ were selected. The previously OM-associated *FBXO11* rs2134056 polymorphism[10] was also included in the family-based analysis (Supplementary Table 1). All SNPs were intronic, apart from *EVI1*/rs10936575, which lies in the 3′-untranslated region.

*Selection of genes and SNPs—the Raine study*

For replication, we used directed analysis of cleaned,[21] imputed[22] genome-wide Illumina 660W-Quad Beadchip data available on 1198 Caucasian Raine Study participants. This included replication analysis of all SNPs genotyped in the primary OM cohort, plus analysis of additional SNP markers within these genes/regions available in the genome-wide data set. Access to genome-wide data also afforded the opportunity to expand the analysis in the Raine Study cohort to include additional members of the TGFβ pathway. In total, data for 1504 polymorphisms across 19 TGFβ pathway members were available for analysis (see Supplementary Table 3A).

*SNP genotyping and quality control*

TaqMan (Life Technologies, Carlsbad, CA, USA) allelic discrimination was carried out according to the manufacturer's instructions. Allele calling was carried out by two independent researchers using an Applied Biosystems 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA, USA). Mendelian inconsistencies were identified in family data using PedCheck software[23] and removed before statistical analysis. Hardy–Weinberg equilibrium analysis in the primary and replication data was carried out in STATA v10 (see ref. 24) using unaffected, unrelated individuals. No SNPs were found to significantly deviate from Hardy–Weinberg equilibrium in either cohort.

*Analysis*

Transmission disequilibrium test power approximations determined that 434 families have 96% power to detect allelic association, with an effect size or OR of 2 at $P = 0.001$ for SNPs with a MAF $\geqslant 0.2$. This underestimates the true power, as multiple affected siblings were available in some families (total 561 affected individuals). Comparison of 253 cases vs 866 controls available within the Raine Study has 98% power to detect OR = 2 at $P = 1 \times 10^{-4}$ for SNPs with a MAF $\geqslant 0.2$. Family-based allelic association tests, based on transmission disequilibrium test under a generalised programme that carries out an analysis of additive, dominant and recessive inheritance models, were conducted within the program FBAT under the null hypothesis of 'no linkage and no association'.[25] Family-based association testing analysis is robust to the presence of multiple affected individuals in families. Family-based haplotype analysis was carried out in HBAT[25] for genes with multiple significantly associated SNPs. Unconditional logistic regression was used to analyse replication data from the Raine Study. Forward stepwise logistic [regression analysis, using either a case–pseudocontrol (family cohort) or case–control (Raine Study) data set, was used to determine the presence of independent effects between multiple associated polymorphisms within a gene/region.[26] LD and case–control haplotype association analyses were performed in Haploview 3.32 (see ref. 27)

## Conflict of interest

The authors declare no conflict of interest.

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# Genome-Wide Association Study to Identify the Genetic Determinants of Otitis Media Susceptibility in Childhood

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## Abstract

***Background:*** Otitis media (OM) is a common childhood disease characterised by middle ear inflammation and effusion. Susceptibility to recurrent acute OM (rAOM; ≥3 episodes of AOM in 6 months) and chronic OM with effusion (COME; MEE ≥3 months) is 40–70% heritable. Few underlying genes have been identified to date, and no genome-wide association study (GWAS) of OM has been reported.

***Methods and Findings:*** Data for 2,524,817 single nucleotide polymorphisms (SNPs; 535,544 quality-controlled SNPs genotyped by Illumina 660W-Quad; 1,989,273 by imputation) were analysed for association with OM in 416 cases and 1,075 controls from the Western Australian Pregnancy Cohort (Raine) Study. Logistic regression analyses under an additive model undertaken in GenABEL/ProbABEL adjusting for population substructure using principal components identified SNPs at *CAPN14* (rs6755194: OR = 1.90; 95%CI 1.47–2.45; $P_{adj\text{-}PCA} = 8.3\times10^{-7}$) on chromosome 2p23.1 as the top hit, with independent effects (rs1862981: OR = 1.60; 95%CI 1.29–1.99; $P_{adj\text{-}PCA} = 2.2\times10^{-5}$) observed at the adjacent *GALNT14* gene. In a gene-based analysis in VEGAS, *BPIFA3* ($P_{Gene} = 2\times10^{-5}$) and *BPIFA1* ($P_{Gene} = 1.07\times10^{-4}$) in the *BPIFA* gene cluster on chromosome 20q11.21 were the top hits. In all, 32 genomic regions show evidence of association ($P_{adj\text{-}PCA} < 10^{-5}$) in this GWAS, with pathway analysis showing a connection between top candidates and the TGFβ pathway. However, top and tag-SNP analysis for seven selected candidate genes in this pathway did not replicate in 645 families (793 affected individuals) from the Western Australian Family Study of Otitis Media (WAFSOM). Lack of replication may be explained by sample size, difference in OM disease severity between primary and replication cohorts or due to type I error in the primary GWAS.

***Conclusions:*** This first discovery GWAS for an OM phenotype has identified *CAPN14* and *GALNT14* on chromosome 2p23.1 and the *BPIFA* gene cluster on chromosome 20q11.21 as novel candidate genes which warrant further analysis in cohorts matched more precisely for clinical phenotypes.

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## Introduction

Otitis media (OM) is a common disease of early childhood characterised by inflammation of the middle ear cavity. There are two related clinical phenotypes of OM; acute otitis media (AOM) and otitis media with effusion (OME). Acute OM is defined as the presence of a purulent middle ear effusion (MEE) due to acute infection and is accompanied by fever and painful inflammation of the tympanic membrane (TM). Whilst the majority of children experience at least one episode of AOM by school age, up to 40% suffer from recurrent AOM (rAOM; ≥3 episodes in 6 months or ≥4 episodes in 12 months) [1,2]. In contrast, OME presents with a serous, non-purulent MEE which can become chronic (COME; MEE ≥3 months with no fluid free intervening period). Severe OM (rAOM or COME) can cause mild to moderate conductive hearing loss and perforation of the TM leading to possible delays in speech development and educational difficulties in school [3]. In view of this, severe OM is frequently managed via surgical insertion of tympanostomy tubes.

Whilst epidemiological analyses have identified several environmental risk factors [4] that play an apparent role in OM susceptibility, heritability studies also reveal that a significant component of OM risk is attributable to genetic factors. Three heritability studies performed using twins and triplets have been published to date with results suggesting that genetic factors contribute between 45–74% of the risk for OM [5,6,7]. Genome-

wide linkage studies have implicated several chromosomal regions, including 10q26, 19q13.43, 3p25.3, 17q12 and 10q22.3, as harbouring susceptibility loci, but the genes underlying these linkage peaks have yet to be identified [8,9,10]. In addition, candidate gene studies have identified a handful of genes contributing to OM susceptibility including several immune genes, such as *IL10* and *TNF* (reviewed in [11]), as well as *FBXO11*, which has been significantly associated with OM in three independent cohorts [12,13].

One method to identify novel genes that confer susceptibility to complex diseases such as OM is via a genome-wide association study (GWAS). In recent years, GWAS have increased in popularity with numerous loci robustly associated with various common, complex conditions [14]. However, there has been no GWAS of OM susceptibility reported to date. Here we present results from the first GWAS of OM carried out using cases and controls derived from The Western Australian Pregnancy Cohort (Raine) Study ('the Raine Study') [15], a longitudinal birth cohort based in Western Australia, with replication performed in the Western Australian Family Study of OM (WAFSOM) [13].

## Methods

### Study participants and phenotype definition

The Raine Study was used for a 'discovery' GWAS. The Raine Study is a longitudinal birth cohort of 2,868 children whose mothers were recruited from antenatal clinics in Western Australia during early pregnancy in 1989–1991 [15]. Children were initially assessed at birth and subsequently through comprehensive follow-up assessments at specific ages to the current 21st year follow-up. For the purposes of this study, data collected from clinical examinations and parental questionnaires at the (average) ages of one, two and three years was used to define a phenotype for OM. In the full cohort, there were 644 cases and 2,221 controls. Three children were excluded from all analyses due to diagnosis of cleft palate, a known medical risk factor for OM susceptibility [16]. A total of 1532 Raine Study participants (428 cases and 1,101 controls) had genome-wide data available from an Illumina 660W Quad Beadchip (cf. below) and so were included in this discovery GWAS. Children (N = 279; 65%) were defined as a case if clinical examination in the first three years of life indicated presence of inflamed, retracted or scarred TM, MEE or tympanostomy tubes *in situ*. Participants (N = 245; 57%) were also classified as a case where parents reported ≥3 episodes of AOM by the age of 3 yrs; 35% qualifying on the basis questionnaire criteria alone. Children with no clinical or parental reported history of OM by the age of 3 yrs were classified as controls. Based on questionnaire data, 94% self-identified their ethnicity as Caucasian. Recruitment to the Raine Study and all follow-ups were approved by the Human Ethics Committee at King Edward Memorial Hospital and/or Princess Margaret Hospital for Children (PMH), with specific adult re-consent for DNA for those individuals participating in this study.

For replication of selected top hits, the WAFSOM was used. This is a family-based cohort that recruited probands on the basis of tympanostomy tube insertion for rAOM or COME prior to 3 years of age. Their parents and any affected siblings (defined as having ≥3 episodes of AOM by age 3 yrs and/or COME with tympanostomy tubes inserted or recommended) were also recruited. Details of this study have been previously described [13]. In total, 645 families consisting of 793 affected children (190 rAOM only; 546 rAOM and OME/COME; 57 COME only) were available for replication genotyping of which 93.2% self-reported their ethnicity as Caucasian. Recruitment to WAFSOM was approved by the Human Ethics Committee at PMH. Written, informed consents were obtained both for participation in the study and for DNA consent from all adults or from the parents of participants less than 18 years of age.

### Epidemiological analysis of potential covariates

Retrospective data collated from parental completed questionnaires at the year 1, 2 and 3 follow-up for the Raine Study provided information on potential covariates. This includes information on environmental covariates, such as day care attendance, breast feeding duration, exposure to furry pets, exposure to post-natal tobacco smoke and socio-economic status as well as relevant clinical diagnoses (i.e. reflux requiring medication, allergy and asthma) in childhood. For analysis purposes, each variable was converted into a two-tier categorical variable based on the data available in the questionnaire and analysed for association with OM using logistic regression models in STATA v10 [17].

### Sample preparation

For the majority of Raine Study participants genomic DNA (gDNA) was extracted from whole blood, collected via venapuncture utilising 4 mL $K_2$EDTA vacuum tubes, from 1532 participants who attended the 14- or 17-year follow-up. Extraction of DNA from whole blood was performed utilising Qiagen PureGene chemistry. For a small subset of individuals (~5%), gDNA was extracted from saliva using the Oragene technology (DNA Genotek, Ontario, Canada) as per manufacturer's instructions. Samples were quantified using spectrophotometry, diluted to a normalised concentration with reduced EDTA TE buffer and stored at −80°C. For the majority of WAFSOM participants, gDNA was extracted from saliva using the Oragene technology (DNA Genotek, Ontario, Canada). For a subset of WAFSOM cases (~8%), blood samples were collected at the time of tympanostomy tube insertion and DNA was extracted from neutrophil pellets using a salting-out method [18]. WAFSOM samples were quantified using spectrophotometry, diluted in TE buffer and stored at −20°C.

### Discovery GWAS and quality control

Genotyping and quality control for the Raine Study DNAs has been described elsewhere [19]. Briefly, genome-wide data were generated at the Centre for Applied Genomics (Toronto, Ontario, Canada) using an Illumina 660W Quad Beadchip. Quality control (QC) checks were performed for individuals and single nucleotide polymorphisms (SNPs) in the discovery GWAS using PLINK [20]. Samples were genotyped in two separate batches; no batch effects were detected. Replicate samples with the lower genotyping success rate and plate controls were removed. Individuals were then excluded if they had a gender mismatch between reported gender and that determined on the basis of X chromosome data (N = 7), had a genotyping success rate <97% (N = 16), were related to other participants at the level of half-siblings or first cousins by IBD sharing (i.e. $\pi > 0.1875$; N = 68) or if they had a low level of heterozygosity (i.e. $h < 0.3$; N = 4). In total, 1,491 individuals passed QC and were available for analysis (416 cases and 1,075 controls). SNPs were excluded on the basis of deviation from Hardy-Weinberg Equilibrium (HWE $P < 5.7 \times 10^{-7}$; N = 919 SNPs), having a genotype call rate <95% (N = 97,718 SNPs) or having a minor allele frequency <1% (N = 119,246 SNPs). A total of 535,632 SNPs passed QC checks and were available for analysis.

### Discovery GWAS analysis

After QC, principal components (PCs) analysis (PCA) was carried out in EIGENSTRAT [21] using a pruned subset of 42,888 SNPs in linkage equilibrium (pairwise $r^2 \leq 0.1$). To account for population substructure, the first two PCs have been used as covariates in association analyses. Imputation of un-typed or missing genotypes was also performed using MACH v1.0.16 for the 22 autosomes with the CEU samples from HapMap Phase2 (Build 36, release 22) used as a reference panel and $r^2 > 0.3$ as the default threshold in MACH for accepting imputed genotypes. After imputation, 2,509,905 SNPs (i.e. 535,632 genotyped; 1,974,273 imputed) were available for analysis. Power approximations estimated for a disease prevalence of 0.3 show that the Raine Study (416 cases and 1,075 controls) has 54.6% power to detect associations at an alpha level of $P = 1 \times 10^{-5}$ with an effect size or odds ratio (OR) of 1.5 for SNPs with a MAF = 0.1; 76% power at $P = 1 \times 10^{-4}$, and 92% power at P = 0.001. Association analysis under an additive model was performed using logistic regression within GenABEL (for genotyped SNPs) or ProbABEL (imputed data) available at http://www.genabel.org/ [22,23]. Analysis was initially performed adjusting for 2 PCs, and then repeated adjusting for 2 PCs and independently associated covariates as indicated. Nominal P-values are reported throughout. A conservative threshold of $P_{adj\text{-}PCA} < 10^{-5}$ was used to identify SNPs/regions of interest for follow-up. Logistic regression modelling for independent effects between pairs of SNPs was undertaken in R version 2.15.0 (URL http://www.R-project.org/), and results presented as $P_{LRT}$ for the likelihood ratio comparison. Gene based tests of association were performed using Versatile Gene-based Association Study (VEGAS) [24]. For gene based analysis the GenABEL single-point $P_{adj\text{-}PCA}$-values for all SNPs were assigned to one or more genes (defined by a boundary of +/−20 kb from the 5′ and 3′ untranslated regions). Linkage disequilibrium (LD) and the number of SNPs per gene were taken into account. A total of 17,206 autosomal genes were analysed. Hence, the P-value needed to achieve statistical significance taking multiple testing into account for this gene-based analysis is $P_{Gene} = 2.9 \times 10^{-6}$ (= 0.05/17,206) [24]. Regional plots of association were created using LocusZoom [25] in which $-\log_{10}(P_{adj\text{-}PCA}$ values) were graphed against their chromosomal location. Pairwise LD patterns between all regional SNPs and the top SNP were calculated specifically for this Raine Study data.

### Replication analysis - Selection of Genes and SNP genotyping

Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) was used to identify potential functional pathways based on genes in which associated SNPs from the discovery GWAS ($P < 10^{-5}$) were clustered. Replication of top hits from the discovery GWAS plus genes identified via pathway analysis was undertaken in samples from the WAFSOM cohort. Genes selected for replication were genotyped using their respective top SNPs from the discovery GWAS with at least two further haplotype tagging (tag-) SNPs ($r^2 > 0.8$ in the CEU HapMap population) genotyped for each gene [26]. A total of 20 SNPs were selected for genotyping, which was carried out at KBiosciences (Hertfordshire, UK) using the KASPar chemistry. Analysis of HWE in the replication data was performed in STATA v10 using unaffected, unrelated individuals.

### Analysis of the Replication cohort

Power approximations using the method of Knapp [27] estimate that the 645 families within the WAFSOM cohort have 99% power to detect association with an effect size (OR) of 2 at P = 0.001 for SNPs with MAF ≥0.15. This may be an underestimation as some families contain multiple affected children (793 in total). Prior to association analysis Mendelian inconsistencies in family data were identified and removed using the PedCheck software [28]. Family-based association analysis of replication data was performed using conditional logistic regression under an additive model. For this analysis case/pseudo-control (CPC) datasets are generated in which each affected offspring is matched with one to three pseudo-controls derived from the remaining potential genotypes of the parental mating [29]. OR, 95% confidence intervals and P-values are calculated using a robust sandwich estimator of the variance and a Wald $\chi^2$ test statistic to control for clustering of affected siblings within families for 1df (allele-wise) tests. CPC was implemented in STATA v10.0. Patterns of LD were analysed in Haploview 4.2 [30]. As confirmation, association analyses were also undertaken using a conventional transmission disequilibrium test in the family-based association test (FBAT) [31].

## Results

### Discovery GWAS

Following quality control checks (see methods), Illumina 660W Quad Beadchip genome-wide data was available for 535,544 genotyped SNPs (2,524,817 after imputation) in 416 OM cases and 1,075 controls. Although the Raine Cohort was recruited as a predominantly Caucasian cohort, there was evidence for some level of population substructure from PCA (Figure S1A). A quantile-quantile plot of the observed versus expected $-\log_{10}(P_{adj\text{-}PCA}$-value) shows no inflation of association test scores (inflation factor $\lambda = 0.989$; Figure S1B) when the first two PCs are included as covariates in the association analysis for OM. Our genome-wide analysis for OM susceptibility genes tested the additive effects of each SNP, adjusted throughout for the first 2 PCs, using GenABEL [22] for genotyped data or ProbABEL for imputed data [23]. Manhattan plots showing the genome-wide results for the genotyped and full imputed datasets are presented in Figure 1.

In the Raine GWAS analyses (Figure 1), no single variant achieved genome-wide significance, commonly accepted as $P < 5 \times 10^{-8}$ [33]. Although we cannot be certain that associations observed were not due to type I errors, we highlight here top hits which are supported by strong biological candidacy. Genome-wide analysis adjusted for 2 PCs (Figure 1A) identified 4 genotyped SNPs showing association at $P_{adj\text{-}PCA} < 10^{-6}$ and 42 genotyped SNPs showing association at $P_{adj\text{-}PCA} < 10^{-5}$ (20 expected by chance at $P < 10^{-5}$) in 32 genomic regions. The top 25 genotyped SNPs from the PC adjusted analysis are summarised in Table 1. The top hit was at two polymorphisms (rs13386850 and rs13408922; OR = 1.86; $P_{adj\text{-}PCA} = 1.32 \times 10^{-6}$) which were in complete LD ($r^2 = 1$) and located ~4.9 kb upstream of the canonical calcium-activated neutral proteinase or calpain 14 (*CAPN14*) gene on chromosome 2p23.1. PC adjusted analysis of the full imputed dataset (Figure 1B) confirmed the *CAPN14* region as the most significantly associated region with improved evidence for association (rs6755194: OR = 1.90; 95%CI 1.47–2.45; $P_{adj\text{-}PCA} = 8.3 \times 10^{-7}$). Other genes of known function highlighted by the top 25 SNPs (Table 1) include *CCD27*, *LIN28*, *FBXO11*, *GRID2*, *SORBS2*, *CDK14*, *ROR2*, *SORCS3* and the psuedogene *BPIFA4* immediately upstream of functional *BPIFA3*.

To provide in-depth gene-wide evidence for specific genes associated with OM we undertook a gene-based analysis in VEGAS using $P_{adj\text{-}PCA}$ results from the GWAS as input data. This revealed a number of additional associated genes (Table 2), none

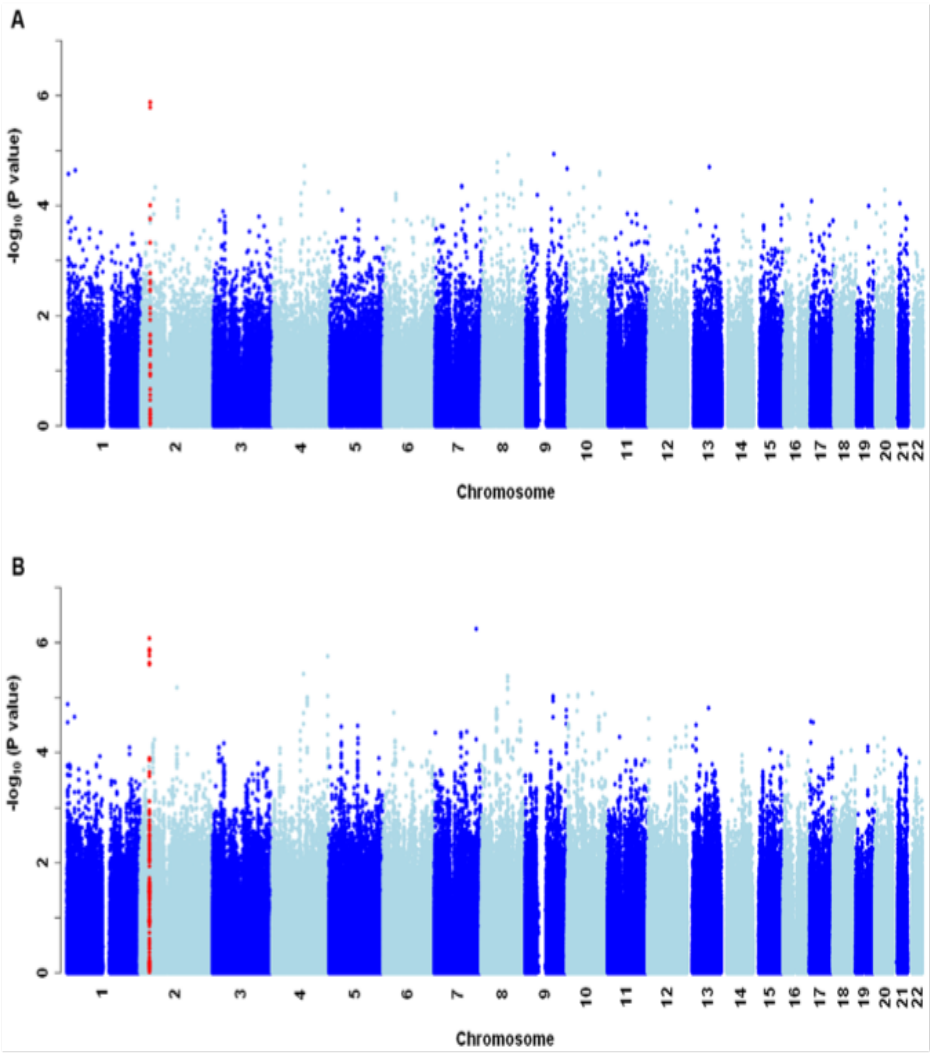


**Figure 1. Manhattan plots for the discovery GWAS carried out in the Raine Study.** Association analysis was performed on (A) genotyped SNPs, and (B) the full imputed dataset, corrected for the first two principal components to account for population stratification. Plots show $-\log_{10}$(P values) of association ordered by chromosomal location. Red dots highlight the region of the top hits CAPN14 and GALNT14 on chromosome 2p23.1. doi:10.1371/journal.pone.0048215.g001

of which withstand strict correction for the 17,206 genes interrogated ($P_{Gene} = 2.9 \times 10^{-6}$). However, *CAPN14* ($P_{Gene} = 5.3 \times 10^{-5}$) was amongst the top hits, with additional genes belonging to the BPI/PLUNC structural superfamily of palate, lung and nasal epithelium proteins also amongst the top hits; *BPIFA3* ($P_{Gene} = 2.00 \times 10^{-5}$) and *BPIFA1* ($P_{Gene} = 1.07 \times 10^{-4}$). The top SNP at *BPIFA3* is rs17396317 (Table1), lying within the pseudogene *BPIFA4* 15.258 kb upstream of *BPIFA3*. The VEGAS analysis lends support to the region of chromosome 20q11.21 carrying the cluster of BPI superfamily genes as of particular interest as an OM susceptibility locus. Figure 2A plots the fully imputed dataset across this region, highlighting the peak of association at rs17396317. Adjusting for this top SNP (Figure 2B) reduces significance ($P_{adj\text{-}PCA} \geq 0.01$) across the region. Two residual signals at rs17304572 in *BPIFA2* and at rs2275082 in *BPIFB1* each provided weak evidence ($P_{LRT} = 0.002$ and $P_{LRT} = 0.01$, respectively) for independent effects when added to a model with the top SNP rs17396317. Conversely, rs17396317 provided strong evidence for independent effects when added to a model with either rs17304572 ($P_{LRT} = 3.2 \times 10^{-4}$) or rs2275082 ($P_{LRT} = 3.02 \times 10^{-5}$). The weight of evidence from the VEGAS analysis (Table 2) suggests that the etiological gene in this region is *BPIFA3* ($P_{Gene} = 2.00 \times 10^{-5}$), although *BPIFA1* ($P_{Gene} = 1.07 \times 10^{-4}$) cannot be discounted.

## Analysis of environmental covariates in the Raine Study cohort

One advantage of using a longitudinal birth cohort such as the Raine Study is the availability of extensive data at multiple time points on the disease phenotype and potential covariates. As outlined in the methods, Raine Study participants were defined as having OM if clinical examination at follow-up in the first three years of life indicated presence of inflamed, retracted or scarred TM, MEE or tympanostomy tubes *in situ*, or if parents reported ≥3 episodes of AOM by the age of 3 yrs. Participants with no clinical or parental reported history of OM by the age of 3 yrs

**Table 1.** Top 25 genotyped polymorphisms from the discovery GWAS carried out in the Raine Study ordered by chromosome and physical location with analysis adjusted for the first two PCs using GenABEL.

| SNP | Chr | Physical Location | Major/Minor | MAF | Risk Allele | Gene/Region[a] | $P_{adj\text{-}PCA}$ | $OR_{adj\text{-}PCA}$[b] (95% CI) |
|---|---|---|---|---|---|---|---|---|
| rs1175549 | 1 | 3681587 | A/C | 0.237 | A | CCDC27 | $2.65\times10^{-5}$ | 1.56 (1.27–1.91) |
| rs12728900 | 1 | 26619394 | G/A | 0.249 | A | LIN28 | $2.28\times10^{-5}$ | 1.47 (1.23–1.75) |
| rs13408922 | 2 | 31298330 | C/A | 0.095 | A | CAPN14 | $1.32\times10^{-6}$ | 1.86 (1.44–2.38) |
| rs13386745 | 2 | 31299119 | A/G | 0.096 | G | CAPN14 | $1.63\times10^{-6}$ | 1.84 (1.44–2.37) |
| rs13386850 | 2 | 31299195 | A/C | 0.095 | C | CAPN14 | $1.32\times10^{-6}$ | 1.86 (1.44–2.38) |
| rs330787 | 2 | 47894881 | G/A | 0.358 | G | FBXO11 | $4.64\times10^{-5}$ | 1.43 (1.21–1.70) |
| rs11097383 | 4 | 94802863 | A/G | 0.158 | G | GRID2 | $5.97\times10^{-5}$ | 1.55 (1.25–1.92) |
| rs10008015 | 4 | 106224696 | A/G | 0.112 | G | Intergenic | $3.89\times10^{-5}$ | 1.66 (1.30–2.11) |
| rs1859161 | 4 | 106262141 | A/G | 0.087 | G | Intergenic | $1.90\times10^{-5}$ | 1.78 (1.37–2.32) |
| rs11940126 | 4 | 187027903 | G/A | 0.045 | A | SORBS2 | $5.70\times10^{-5}$ | 2.09 (1.46–3.00) |
| rs10242197 | 7 | 90097848 | G/A | 0.194 | G | CDK14 | $4.38\times10^{-5}$ | 1.59 (1.27–1.98) |
| rs10488001 | 7 | 90493976 | C/A | 0.089 | A | CDK14 | $4.53\times10^{-5}$ | 1.72 (1.33–2.24) |
| rs1496306 | 8 | 50703261 | G/A | 0.095 | A | Intergenic | $1.63\times10^{-5}$ | 1.76 (1.36–2.28) |
| rs2132528 | 8 | 50768208 | A/G | 0.093 | G | Intergenic | $2.41\times10^{-5}$ | 1.75 (1.35–2.26) |
| rs13438948 | 8 | 87927894 | G/A | 0.087 | A | Intergenic | $1.19\times10^{-5}$ | 1.82 (1.39–2.39) |
| rs7846284 | 8 | 130439417 | G/A | 0.431 | A | Intergenic | $4.06\times10^{-5}$ | 1.40 (1.19–1.65) |
| rs7846684 | 8 | 130439596 | A/G | 0.432 | G | Intergenic | $3.60\times10^{-5}$ | 1.41 (1.20–1.65) |
| rs11790808 | 9 | 93628319 | A/G | 0.054 | G | ROR2 | $1.15\times10^{-5}$ | 2.09 (1.50–2.91) |
| rs10776851 | 9 | 137857942 | G/A | 0.134 | A | CAMSAP1 | $2.13\times10^{-5}$ | 1.63 (1.30–2.05) |
| rs4575213 | 10 | 53095334 | C/A | 0.396 | C | PRKG1 | $4.65\times10^{-5}$ | 1.41 (1.20–1.67) |
| rs10884043 | 10 | 106521693 | A/G | 0.261 | A | SORCS3 | $2.74\times10^{-5}$ | 1.53 (1.25–1.86) |
| rs4556466 | 10 | 106548015 | A/G | 0.259 | A | SORCS3 | $2.47\times10^{-5}$ | 1.53 (1.26–1.87) |
| rs9564897 | 13 | 71810339 | C/A | 0.340 | A | Intergenic | $1.98\times10^{-5}$ | 1.43 (1.22–1.69) |
| rs17396317 | 20 | 31254038 | G/A | 0.118 | A | BPIFA4P[c] | $5.15\times10^{-5}$ | 1.62 (1.28–2.05) |
| rs1894516 | X | 123961302 | G/A | 0.320 | A | Intergenic | $6.64\times10^{-6}$ | 1.37 (1.19–1.57) |

Where MAF = minor allele frequency, OR = odds ratio, 95% CI = 95% confidence intervals. [a] - Gene/Region is based on annotation from Ensembl build 54; intergenic SNPs fall >20 kb from an annotated gene. [b] - Odds ratios are shown for the risk allele; [c] - rs17396317 lies within the pseudogene *BPIFA4*, 15.258 kb upstream of *BPIFA3*.
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were classified as controls. Previous studies have identified several environmental and clinical variables as potentially influencing OM risk, including duration of exclusive breast feeding, day care attendance, allergy, pacifier use and exposure to tobacco smoke, as reviewed by Daly *et al.* [4]. Therefore, we assessed several covariates that could influence OM with results presented in Table 3. Attendance at day care was the most significant environmental covariate associated with OM risk in this cohort (day care in 1st year OR = 2.23; $P = 4.86\times10^{-8}$; day care ever in yrs1–3 OR = 1.76; $P = 1.03\times10^{-5}$) followed by diagnosis of allergy (in 3rd year OR = 1.94; $P = 3.14\times10^{-6}$; allergy diagnosis ever in yrs1–3 OR = 1.47; P = 0.0005) and non-exclusive breastfeeding (i.e. being introduced to other milks or supplements) before the age of 6 months (OR = 1.23; P = 0.03).

## Influence of environmental and clinical covariates on genetic associations

To determine whether environmental or clinical covariates influenced genetic associations we examined data for a subset of 831 (256 cases; 575 controls) genotyped Raine Study participants for whom full covariate data were available. Genome-wide analysis adjusting for PCs in this subset of participants highlighted several other genes/regions (Table 4) containing multiple significantly associated SNPs ($P_{adj\text{-}PCA}<10^{-5}$). This includes the *GALNT14* (located ~34 kb distal of *CAPN14* on 2p23.1; best $P_{adj\text{-}PCA} = 2.2\times10^{-5}$), *ASPH* (8q12.3; best $P_{adj\text{-}PCA} = 3.85\times10^{-6}$), *ALDH1A2* (15q21.3; best $P_{adj\text{-}PCA} = 3.72\times10^{-5}$), and *RPTOR* (17q25.3; best $P_{adj\text{-}PCA} = 4.92\times10^{-5}$) genes. Estimates of the $\beta$ coefficient for each SNP at these genes, and in the top genes of interest (*CALPN14*, *BPIFA1*, *BPIFA3*, *BPIFA4P*) derived from the full discovery GWAS and VEGAS analyses, did not change appreciably after adjustment for covariates (Table S1) in this dataset, indicating that genetic associations were independent of these covariates.

This analysis further highlighted the region of chromosome 2p23.1 as of particular interest in containing both *GALNT14* encoding N-acetylgalactosaminyltransferase 14 and *CAPN14*. Figure 3A plots the imputed dataset across this region from the full discovery GWAS analysis, highlighting the peak of association at *CAPN14*. The imputed analysis identified 7 additional strongly associated SNPs ($8.3\times10^{-7}<P_{adj\text{-}PCA}<2.5\times10^{-6}$) located 3.25–8.32 kb upstream of *CAPN14* (Figure 3A) at chromosome 2p23.1. Figure 3B plots the imputed dataset across this region in the subset of Raine participants for whom full covariate data were available, highlighting the peak of association at *GALNT14*. This imputed analysis supports association at *GALNT14* with a total of 31 SNPs

**Table 2.** Top 15 genes from VEGAS gene-based analysis based on GenABEL P-values from the Raine Study discovery GWAS after adjustment for PCs.

| Gene | Chr | Start - End | No. SNPs | $P_{adj\text{-}PCA}$ | Top SNP |
|---|---|---|---|---|---|
| LRRC47 | 1 | 3686643–3702928 | 13 | $1.19\times10^{-4}$ | rs1175549 |
| LIN28 | 1 | 26609855–26628806 | 14 | $3.63\times10^{-4}$ | rs12728900 |
| PALMD | 1 | 99884185–99932847 | 27 | $6.55\times10^{-4}$ | rs1338571 |
| CAPN14 | 2 | 31249428–31310228 | 48 | $5.30\times10^{-5}$ | rs13408922 |
| EHD3 | 2 | 31310706–31344764 | 32 | $4.03\times10^{-4}$ | rs13408922 |
| MSH6 | 2 | 47863724–47887596 | 14 | $5.89\times10^{-4}$ | rs330787 |
| CNNM4 | 2 | 96790365–96841355 | 6 | $8.87\times10^{-4}$ | rs968470 |
| CTDSPL | 3 | 37878672–38000964 | 27 | $1.70\times10^{-4}$ | rs6776745 |
| VILL | 3 | 38010081–38023680 | 9 | $2.27\times10^{-4}$ | rs11711286 |
| RBPJ | 4 | 25930429–26042376 | 18 | $8.94\times10^{-4}$ | rs968470 |
| PALM2 | 9 | 111442892–111753577 | 208 | $5.60\times10^{-4}$ | rs16914575 |
| AQR | 15 | 32935843–33049287 | 30 | $6.81\times10^{-4}$ | rs963425 |
| ZNF770 | 15 | 33057835–33067746 | 10 | $4.68\times10^{-4}$ | rs2060810 |
| BPIFA3 | 20 | 31268795–31279220 | 8 | $2.00\times10^{-5}$ | rs17396317 [a] |
| BPIFA1 | 20 | 31287462–31294776 | 8 | $1.07\times10^{-4}$ | rs17305657 |

[a]- rs17396317 lies within the pseudogene *BPIFA4*, 15.258 kb upstream of *BPIFA3*.

Ordered by chromosome and physical location.

doi:10.1371/journal.pone.0048215.t002

($2.65^{-5}<P_{adj\text{-}PCA}<9.86\times10^{-5}$) associated at $P_{adj\text{-}PCA}<10^{-4}$ (Figure 3B). In both the full discovery sample analysis (Figure 3C) and the Raine dataset with full covariates (Figure 3D), adjusting for the top *CAPN14* SNP rs6755194 reduced the signal at *CAPN14* but maintained (Figure 3C) or improved (Figure 3D) the signal at *GALNT14*. Conversely, adjusting for the top *GALNT14* SNP rs13029054 reduced the signal at *GALNT14* but maintained (Figure 3E) or improved (Figure 3F) the signal at *CAPN14*. Logistic regression modelling confirmed that addition of SNP rs13029054 compared to a model in which rs6755104 was considered alone added significant independent effects ($P_{LRT}=0.0027$ for the full discovery GWAS dataset; $P_{LRT}=4.25\times10^{-6}$ for the covariate dataset). Conversely, addition of rs6755104 compared to a model in which rs13029054 was considered alone added significant independent effects ($P_{LRT}=5.67\times10^{-7}$ for the full discovery GWAS dataset; $P_{LRT}=1.98\times10\text{-}4$ for the covariate dataset). Independent association signals at *CAPN14* and *GALNT14* is consistent with analysis showing that there is no long range LD between these two genes (pairwise $D'\leq0.20$; $r^2=0$). The presence of independent association signals *CAPN14* ($P_{Gene}=5.3\times10^{-5}$) and *GALNT14* ($P_{Gene}=2.4\times10^{-3}$) is also supported by the gene-based analysis in VEGAS in the full discovery GWAS sample. Adjusting for both SNPs (Figures 3G and 3H) reduced the signal across *GALNT14*, but retained a weaker signal (Figure 3G; $P_{adj\text{-}PCA}=7.34\times10^{-4}$ at rs1443711), suggesting that there may be more than one association signal at *CAPN14*. This second signal was also retained in the full discovery cohort following adjustment for the top SNPs at *CAPN14* SNP rs 6755194 (Figure 3C).

## Interrogating the GWAS data for replication of published associations for OM

A number of genes have previously been implicated in the pathogenesis of OM, through both murine models and human candidate gene studies. To determine whether any of these previously implicated genes show evidence of association in the Raine Study, we performed a directed interrogation of the discovery GWAS data for all SNPs across each of the previously associated genes (see Table S2). Human candidate gene studies have previously identified 20 genes as showing significant association with OM susceptibility (Table S2A) [11]. Evidence for association (nominal $P\leq0.05$, i.e. without correction for multiple testing) is observed at several of these genes, including *IL10* ($P_{adj\text{-}PCA}=0.010$), *TNF* ($P_{adj\text{-}PCA}=0.035$), *SFTPD* ($P_{adj\text{-}PCA}=0.033$) and *MUC2* ($P_{adj\text{-}PCA}=0.003$), none of which achieved the required threshold ($P=0.002$; 0.05/20) to take account of multiple testing of 20 genes included in this candidate gene hypothesis. Murine models of OM have also identified several OM genes [32]. In addition to our previously published data on *FBXO11* [13], investigation of the human homologues of 25 of these genes (Table S2B) provided evidence of association (nominal $P\leq0.05$) at four additional genes, including TP73 ($P_{adj\text{-}PCA}=0.011$), *DNAH5* ($P_{adj\text{-}PCA}=0.015$), *DNAH11* ($P_{adj\text{-}PCA}=0.016$) and *FAS* ($P_{adj\text{-}PCA}=0.021$), with only *FBXO11* ($P_{adj\text{-}PCA}=4.64\times10^{-5}$) achieving a P-value that would meet the threshold required to allow for multiple testing of 25 genes ($P=0.002$; 0.05/25) to test this mouse-to-man hypothesis.

## Interrogating the GWAS data for replication of published linkages for OM

A number of regions of linkage (chromosomes 3p25.3, 10q22.3, 10q26.3, 17q12, 19q13.4) have also been reported following genome-wide analysis of microsatellite panels in family studies of otitis media [8,9,10]. We looked across these regions of linkage (Figure S2) for evidence provided by top SNPs (Table S2C). VEGAS support for gene-based associations were observed at *SRGAP3* ($P_{Gene}=0.044$) but not *ATP2B2* ($P_{Gene}=0.069$) at 3p25.3 (Figure S2A), at *TCERG1L* ($P_{Gene}=0.012$) and *PP2R2D* ($P_{Gene}=0.007$) but not *GLRX3* ($P_{Gene}=0.5$) at 10q26.3 (Figure S2B), and at *CCT6B* ($P_{Gene}=0.026$) and *ZNF830* ($P_{Gene}=0.022$) but not *SLFN5* ($P_{Gene}=0.3$) at 17q12 (Figures S2C and S2D). There was no VEGAS gene-based support for association at *LILRA2* ($P_{Gene}=0.1$) which contained the best SNP-hit across the region (Table S2C), or any other genes in the region of linkage at 19q13.4 (data not shown).

## Replication analysis of GWAS candidates

As no single variant achieved genome-wide significance in the Raine GWAS analyses, and many regions were observed with SNPs at $P_{adj\text{-}PCA}<10^{-5}$ (32 genomic regions), we carried out Ingenuity Pathway Analysis (Ingenuity® Systems) seeded with top genes from the discovery GWAS to identify potentially interesting candidate genes for replication in the WAFSOM. Given our published data showing replication at *FBXO11* across the Raine Study and WAFSOM cohorts [13], and the evidence of others for activation of the transforming growth factor beta (TGFβ) pathway in OM [34], we focused initially on a number of genes that interact within this pathway (Figure S3), including the *GALNT14* gene. In total seven candidate genes were selected for replication analysis, including the top hits from the PC and fully adjusted analyses plus 5 additional genes identified via pathway analysis. Specifically we genotyped 20 tag-SNPs (Table S3) within the *CAPN14*, *GALNT14*, *GALNT13*, *BMP5*, *NELL1*, *TGFB3* and *BPIFA1* genes in 645 families, containing 793 affected individuals (55% rAOM; 45% COME), from the WAFSOM. No SNPs were found to significantly deviate from HWE.

Results from family based robust case/pseudo-control conditional logistic regression analysis under an additive model showed that none of the 20 tag-SNPs included in the replication analysis

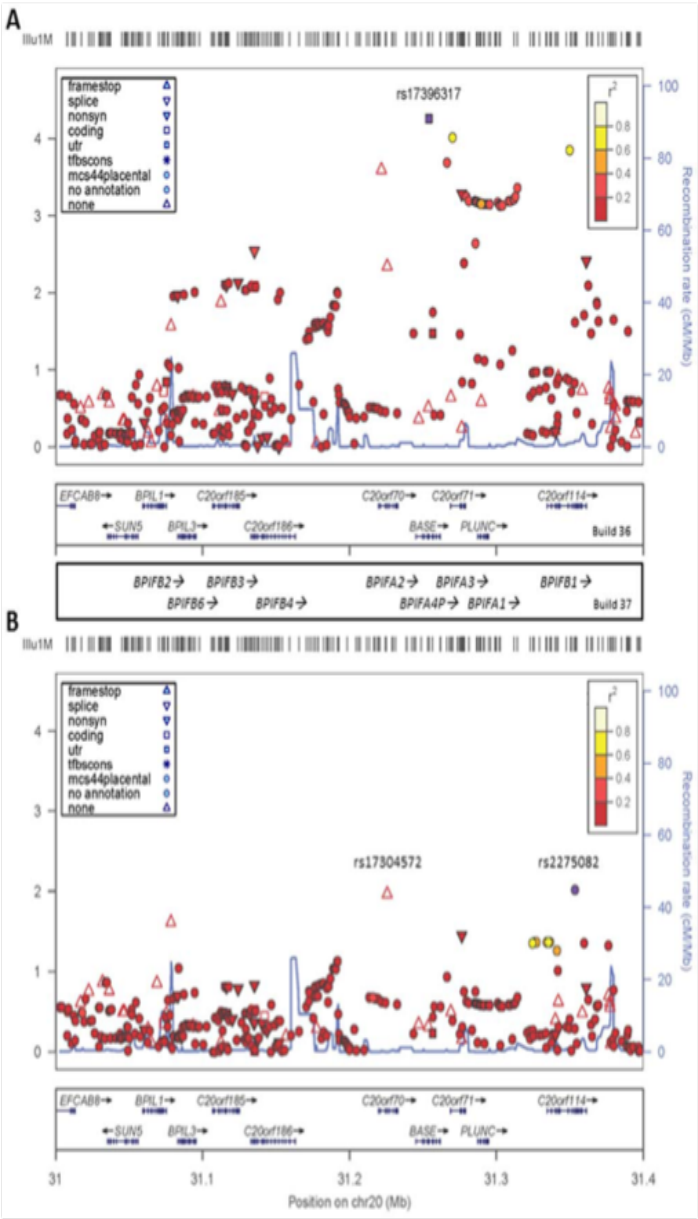


**Figure 2. Regional LocusZoom plots of association across the region of chromosome 20 containing the *BPIFA* genes.** (A) LocusZoom plots of association in the imputed dataset adjusted for PCs across the *BPIFA4P-BPIFA3-BPIFA1* gene region in the full discovery set of 1491 Raine Study participants. (B) The same region after adjusting for the top SNP rs17396317. The top associated SNP (rs17396317) is represented by purple circle in (A), the colour of all other SNPs is representative of the pairwise $r^2$ value with the relevant top SNP using patterns of LD from the Raine Study cohort. Recombination rates are shown by the solid blue line. Physical positions and gene designations are based on NCBI Build 36 of the human genome. Gene designations for NCBI Build 37 are indicated in the panel between (A) and (B).
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was associated (nominal $P_{robust}<0.05$) with OM in the WAFSOM cohort (Table S4). This was confirmed using FBAT (data not shown). Stratified analysis (data not shown) using only families that self-reported as Caucasian (601 families), or restriction of analysis to just those children diagnosed with rAOM only (N = 190) or COME only (N = 57), did not reveal any other significant associations that withstood correction for multiple testing, although the latter were underpowered. Analysis of LD patterns between replication SNPs in the Raine Study and WAFSOM cohorts show that patterns of LD are comparable between the two cohorts (Figure S4).

## Discussion

Here we report the results from the first GWAS of OM carried out using data from 1,491 participants in the Raine Study. Although we cannot be certain that associations observed were not due to type I errors, a number of SNPs located at or close to novel

**Table 3.** Odds ratios (OR) and P values for demographic, environmental and clinical covariates potentially influencing OM susceptibility in childhood in the Raine Study cohort.

| Covariate | % Controls | % Cases | OR | P value | CI |
|---|---|---|---|---|---|
| Gender (% Male) | 49.8 | 53.9 | 1.17 | 0.07 | 0.99–1.40 |
| Breast feeding duration (% <6 months) | 47.8 | 49.2 | 1.06 | 0.55 | 0.88–1.27 |
| Other milk introduced (% <6 months) | 60.4 | 65.2 | 1.23 | **0.03** | 1.01–1.49 |
| Other milk introduced (% <4 months) | 47.7 | 50.7 | 1.13 | 0.20 | 0.94–1.36 |
| Gestation <37 weeks | 11.1 | 10.7 | 0.96 | 0.77 | 0.72–1.27 |
| Low Birth Weight (<2500 g) | 9.2 | 7.5 | 0.80 | 0.16 | 0.57–1.10 |
| Pet exposure ever (years 1–3) | 80.6 | 81.1 | 1.03 | 0.80 | 0.81–1.31 |
| Reflux (i.e. vomit feeds) | 30.0 | 32.6 | 1.12 | 0.23 | 0.93–1.38 |
| Serious Reflux (i.e. medicated, GP visit) | 9.9 | 10.8 | 1.10 | 0.53 | 0.81–1.49 |
| Attended day care ever in year 1–3 | 67.2 | 78.2 | 1.74 | **$1.03\times10^{-5}$** | 1.36–2.26 |
| Attended day care in year 1 | 11.3 | 22.1 | 2.23 | **$4.86\times10^{-8}$** | 1.68–2.96 |
| Attended day care in year 2 | 18.8 | 31.7 | 1.99 | **$9.61\times10^{-8}$** | 1.56–2.57 |
| Attended day care in year 3 | 51.2 | 63.9 | 1.68 | **$8.77\times10^{-7}$** | 1.36–2.08 |
| Asthma diagnosed ever | 45.6 | 48.1 | 1.10 | 0.34 | 0.91–1.35 |
| Smoke exposure ever (years 1–3) | 44.7 | 41.5 | 0.88 | 0.20 | 0.72–1.07 |
| Allergy ever (years 1–3) | 29.0 | 37.5 | 1.47 | **0.0005** | 1.18–1.81 |
| Allergy in year 1 | 9.6 | 14.2 | 1.55 | **0.002** | 1.18–2.05 |
| Allergy in year 2 | 11.6 | 14.8 | 1.33 | 0.06 | 0.99–1.78 |
| Allergy in year 3 | 9.3 | 16.6 | 1.94 | **$3.14\times10^{-6}$** | 1.48–2.55 |
| Income yr 1 (Below av*; <$24,000AUD) | 41.5 | 38.7 | 0.89 | 0.24 | 0.74–1.08 |
| Income yr 2 (Below av*; <$27,000AUD) | 46.8 | 42.7 | 0.85 | 0.11 | 0.69–1.04 |
| Income yr 3 (Below av*; <$27,000AUD) | 43.1 | 40.1 | 0.89 | 0.22 | 0.73–1.07 |
| Maternal education ≤ year 12 | 26.3 | 26.4 | 1.00 | 0.95 | 0.80–1.26 |
| Maternal education > year 12 | 19.3 | 23.2 | 1.27 | 0.06 | 0.99–1.61 |

Covariate data collected as part of the Raine Study follow-up in years 1–3. P values and 95% confidence intervals (CI) determined via logistic regression. * - below average income for years 1 to 3 determined via the Australian Bureau of Statistics (http://www.abs.gov.au/) for the year in question. Significant P values are highlighted in bold.

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candidate genes were identified that may have a putative role in OM susceptibility. This includes the *CAPN14* and *GALNT14* genes at 2p23.1, and the *BPIFA3* and *BPIFA1* at 20q11.21, none of which has previously been implicated in OM pathogenesis. Other than *FBXO11* [12,13], no SNPs within regions identified in previous candidate gene or genome-wide linkage studies showed association at $P<10^{-5}$. Pathway analysis was used to identify additional genes that cluster within relevant functional pathways related to the data from the discovery GWAS presented here. This revealed that several genes from the discovery GWAS are either members of, or interact with, the TGFβ pathway.

To replicate the results from this discovery GWAS we undertook additional genotyping of variants in seven genes, including the *CAPN14* and *GALNT14* genes plus five additional genes from the TGFβ pathway (*BMP5*, *GALNT13*, *NELL1*, *TGFB3* and *BPIFA1*), in families that were available as part of the larger WAFSOM cohort. The results of this replication analysis did not provide evidence for association with OM at any of the seven genes.

The principle factors that may have contributed to the lack of replication between cohorts used in this study are sample size and phenotypic heterogeneity. For example, the sample of 416 Raine Study cases and 1,075 controls was only 54.6% powered to detect associations at an alpha level of $P=1\times10^{-5}$ with effect size (OR = 1.5) for SNPs with MAF>0.1. For most common, complex diseases where small genetic effect sizes (i.e. OR<1.5) are expected, a sample size in excess of 1000 cases and 1000 controls is recommended [35]. In addition, there was phenotypic heterogeneity as there was incomplete overlap in phenotypes across the OM spectrum in the Raine discovery and WAFSOM replication cohorts indicating that the WAFSOM cohort may not represent a true replication cohort. The phenotype in the Raine Study is based on a once yearly clinical examination or parental reporting of ≥3 AOM episodes during the first three years of life, with 65% of individuals documented with AOM, MEE, a scarred tympanum, or tympanostomy tubes at the time of a clinical examination with/without parent report of >3 episodes of OM per year, and 35% based on the parent-reported phenotype alone. This phenotype is therefore biased towards the milder end of the OM spectrum compared to the WAFSOM study, where all probands and their affected siblings were referred to an ENT specialist who had either undertaken or recommended tympanostomy tube insertion for the treatment of rAOM (i.e. ≥3 episodes in 6 months or ≥4 episodes in 12 months; 55% of cases) or COME (i.e. MEE ≥3 months; 45% of cases). Thus, the WAFSOM cohort includes only the severe end of the OM spectrum. Our results might also reflect "winner's curse", which predicts that genetic effect sizes reported from discovery GWAS are generally inflated, leading to under estimation of replication sample sizes required to replicate observed associations [36]. Whilst the 645 WAFSOM

**Table 4.** Top 25 genotyped polymorphisms, adjusted for the first two PCs, ordered by chromosome and physical location, from the GWAS analysis for the subset of Raine Study participants for whom full covariate data (day care attendance, allergy and non-exclusive breast feeding) were available.

| SNP | Chr | Physical Location | Major/Minor | MAF | Risk Allele | Gene/Region[a] | $P_{adj\text{-}PCA}$ | $OR_{adj\text{-}PCA}$ (95% CI) |
|---|---|---|---|---|---|---|---|---|
| rs2098787 | 2 | 31150148 | G/A | 0.470 | G | GALNT14 | $3.17\times10^{-5}$ | 1.58 (1.28–1.97) |
| rs1862981 | 2 | 31151028 | C/A | 0.463 | C | GALNT14 | $2.20\times10^{-5}$ | 1.60 (1.29–1.99) |
| rs2377445 | 2 | 106027133 | G/A | 0.303 | G | INTERGENIC | $5.20\times10^{-5}$ | 1.57 (1.26–1.96) |
| rs17624623 | 3 | 61595506 | A/G | 0.410 | A | PTPRG | $2.02\times10^{-5}$ | 1.60 (1.29–1.98) |
| rs10008015 | 4 | 106224696 | A/G | 0.113 | A | RP11-556I14.1 | $9.32\times10^{-6}$ | 2.08 (1.50–2.87) |
| rs1859161 | 4 | 106262141 | A/G | 0.090 | A | RP11-556I14.1 | $2.57\times10^{-6}$ | 2.32 (1.63–3.29) |
| rs6826919 | 4 | 113023321 | G/A | 0.428 | A | RP11-269F21.1 | $4.05\times10^{-5}$ | 0.63 (0.51–0.79) |
| rs1457955 | 6 | 77468785 | C/A | 0.133 | C | INTERGENIC | $4.98\times10^{-5}$ | 1.85 (1.37–2.49) |
| rs4709819 | 6 | 164383345 | G/A | 0.395 | A | INTERGENIC | $2.67\times10^{-6}$ | 0.63 (0.50–0.78) |
| rs10242197 | 7 | 90097848 | G/A | 0.193 | A | CDK14 | $4.84\times10^{-5}$ | 0.54 (0.41–0.73) |
| rs2882460 | 8 | 62688450 | C/A | 0.208 | C | ASPH | $4.96\times10^{-5}$ | 1.66 (1.30–2.12) |
| rs6471969 | 8 | 62729386 | C/A | 0.184 | C | ASPH | $3.85\times10^{-6}$ | 1.83 (1.42–2.37) |
| rs11990408 | 8 | 62747549 | A/G | 0.192 | A | ASPH | $1.10\times10^{-5}$ | 1.76 (1.27–2.27) |
| rs11787089 | 8 | 62783393 | G/A | 0.150 | G | ASPH | $6.83\times10^{-6}$ | 1.88 (1.43–2.47) |
| rs16919668 | 10 | 20319164 | A/G | 0.163 | G | PLXDC2b | $3.28\times10^{-5}$ | 0.50 (0.36–0.70) |
| rs1336708 | 13 | 101763004 | A/G | 0.225 | G | FGF14 | $5.17\times10^{-5}$ | 0.57 (0.44–0.75) |
| rs4512966 | 13 | 109880059 | G/A | 0.500 | G | COL4A2 | $5.01\times10^{-5}$ | 1.55 (1.25–1.91) |
| rs6493973 | 15 | 56079928 | G/A | 0.029 | G | ALDH1A2 | $4.40\times10^{-5}$ | 3.43 (1.90–6.20) |
| rs2218261 | 15 | 56081314 | G/A | 0.029 | G | ALDH1A2 | $4.40\times10^{-5}$ | 3.43 (1.90–6.20) |
| rs2704219 | 15 | 56115740 | A/G | 0.031 | A | ALDH1A2 | $3.72\times10^{-5}$ | 3.39 (1.90–6.05) |
| rs11658127 | 17 | 64225877 | A/G | 0.127 | A | INTERGENIC | $2.29\times10^{-5}$ | 1.93 (1.42–2.61) |
| rs11658297 | 17 | 64240578 | G/A | 0.126 | G | INTERGENIC | $1.89\times10^{-5}$ | 1.94 (1.43–2.63) |
| rs4627412 | 17 | 76138839 | A/G | 0.307 | A | RPTOR | $4.92\times10^{-5}$ | 1.58 (1.27–1.96) |
| rs9911978 | 17 | 76139002 | A/G | 0.307 | A | RPTOR | $4.92\times10^{-5}$ | 1.58 (1.27–1.96) |
| rs2839520 | 21 | 42742732 | A/G | 0.464 | G | UBASH3A | $1.63\times10^{-5}$ | 0.63 (0.51–0.77) |

Where MAF = minor allele frequency, 95% CI = 95% confidence intervals. [a] - Gene/Region is based on annotation from Ensembl build 54; intergenic SNPs fall >20 kb from an annotated gene.
[b]- Odds ratios are shown for the major allele.
Data for GWAS analyses adjusted for these clinical and environmental covariates are presented in Table S1.
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families provided >80% power at an alpha level of 0.0025 (i.e. 0.05/20 SNPs) to detect the genetic effect sizes (i.e. OR≥1.58) observed from the discovery GWAS at both *CAPN14* and *GALNT14* for variants with MAF≤0.10, this may be an under estimation of the replication sample size required. If the true genetic effect sizes were indeed smaller (i.e. OR≤1.4; MAF = 0.10), a replication cohort of at least 1700 families would be required to provide >80% power to detect these genetic effects. In summary, the lack of replication for the genes identified in the Raine discovery GWAS may be explained by factors of sample size and phenotypic heterogeneity, and may replicate in further well-powered cohorts in which these factors are more rigorously controlled.

Despite the lack of replication in WAFSOM and the plausible likelihood that some associations arose due to type I errors, the discovery GWAS analysis in the Raine Study has highlighted genetic risk factors that could play a role in OM, and are of interest in relation to their biological candidacy. For example, CAPN14 is a member of the calpain family of cytosolic calcium-activated cysteine proteases [37], other members of which mediate host cell invasion by known otopathogens [38] and are up-regulated in cholesteatoma associated with OM and its sequelae, including OME, COME and chronic suppurative OM [39]. CAPN14 is up-regulated in human conjunctival epithelial cells by interleukin-4 [40], a key T helper 2 cytokine involved in infection control and allergic responses. GALNT14 belongs to a large subfamily of glycosyltransferases that catalyse the transfer of N-acetyl-D-galactosamine (GalNAc) in O-glycosylation of mucin substrates such as MUC2, MUC5AC, MUC7, and MUC13 [41]. GalNAc containing sugar residues are essential for initial adherence of respiratory bacteria epithelial cell surfaces [42]. As is the case for FBXO11 [43], which regulates anti-inflammatory TGFβ via SMAD2 [44], this could include a role in response to commensal bacteria in determining OM pathogenesis. *BPIFA3* (previously known as *SPLUNC3*) and *BPIFA1* (previously known as *PLUNC* or *SPLUNC1*) are members of the bacterial permeability-increasing (BPI) protein family [45]. Proteins in this family are one of four groups of proteins involved in the early recognition of bacterial pathogens as part of the host defence at the nasopharyngeal, oral and lung entrances [46]. BPIFA1 decreases levels of *Mycoplasma pneumoniae* and epithelial IL-8 in the airways [47].

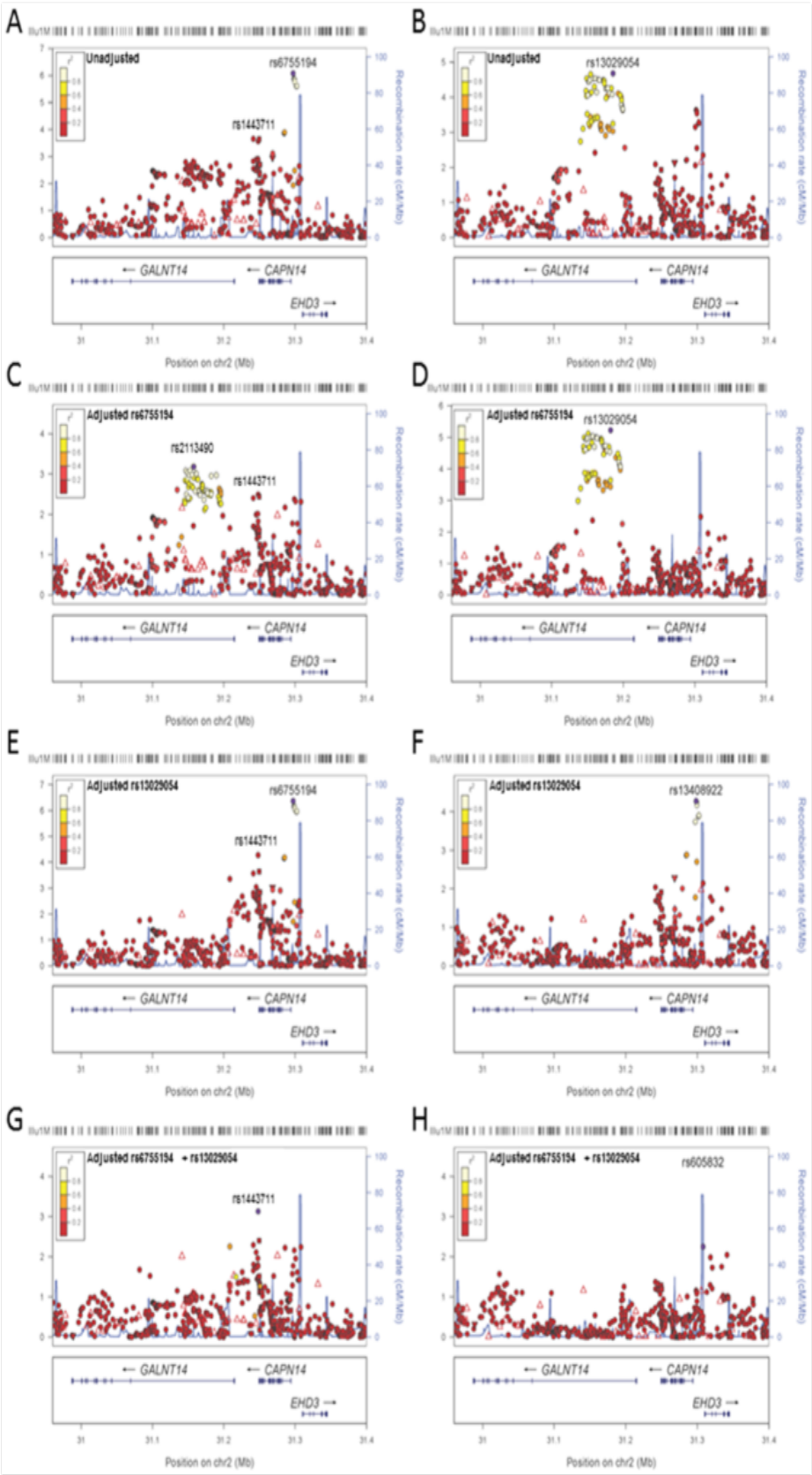
A
Unadjusted
rs6755194
rs1443711
B
Unadjusted
rs13029054
C
Adjusted rs6755194
rs2113490
rs1443711
D
Adjusted rs6755194
rs13029054
E
Adjusted rs13029054
rs6755194
rs1443711
F
Adjusted rs13029054
rs13408922
G
Adjusted rs6755194 + rs13029054
rs1443711
H
Adjusted rs6755194 + rs13029054
rs605832
GALNT14
CAPN14
EHD3
Position on chr2 (Mb)
Recombination rate (cM/Mb)

**Figure 3. Regional LocusZoom plots of association for the *CAPN14* and *GALNT14* genes.** LocusZoom plots of association in the imputed dataset adjusted for PCs across the *CAPN14-GALNT14* gene region in (A) the full discovery set of 1491 Raine Study participants and (B) the subset of 831 Raine Study participants for whom full covariate data were available. (C) and (D) present results in these two study groups, respectively, after conditioning on the top SNP rs6755194 at *CAPN14*; (E) and (F) after conditioning on the top SNP rs13029054 at *GALNT14*; and (G) and (H) after conditioning on both of these SNPs. Top associated SNPs in each plot are represented by purple circles, the colour of all other SNPs is representative of the pairwise $r^2$ value with the relevant top SNP using patterns of LD from the Raine Study cohort. Recombination rates are shown by the solid blue line. Physical positions are based on NCBI Build 36 of the human genome.
doi:10.1371/journal.pone.0048215.g003

In conclusion, we present the results from the first GWAS performed to identify the genetic determinants of OM in childhood. Several genes/pathways of plausible biological relevance are identified and replication of these observations in additional OM cohorts is recommended. This discovery GWAS also highlights factors that should be addressed in future GWAS studies of OM, namely that of phenotypic heterogeneity and sample size. The recent formation of the OTIGEN Consortium (www.otigen.org), an international collaboration between several research groups possessing well-powered, well-phenotyped OM cohorts, should address both of these issues allowing the genetic determinants of OM to be confidently elucidated.

## Supporting Information

**Figure S1 (A) Ethnic spread of Raine Study participants compared to the four HapMap populations.** The Raine Study population (RAINE) is in red, Caucasian population (CEU) in blue, Japanese and Chinese population (JPT+CHB) in green and African population (YRI) in purple. (**B**) Quantile-quantile plot of the observed versus expected $\log_{10}$(P-value) following PC adjustment.
(PDF)

**Figure S2 Regional LocusZoom plots of association in the full imputed dataset across three genomic regions previously linked to OM susceptibility: (A) the 3p25.3 region [8] adjusted for PCs; (B) the 10q26.3 region (linkage peak microsatellite D10S212, [8]) adjusted for PCs; (C) the 17q12 region [9] adjusted for PCs, and (D) a smaller region of the 17q12 plot to highlight the gene position for the top SNPs.** The top associated SNPs are represented by purple circles with the colour of all other SNPs representative of the pairwise $r^2$ value relative to the top SNP using patterns of LD from the CEU HapMap populations. Recombination rates are shown by the solid blue line. Physical positions and gene designations are based on NCBI Build 36 of the human genome.
(PDF)

**Figure S3 Pathway analysis using Ingenuity Pathway Analysis reveals many genes harbouring associated SNPs from the discovery GWAS (highlighted in gray) are part of, or interact with, the TGFβ pathway.**
(PDF)

**Figure S4 Comparison of LD patterns in replicated genes between the Raine Study and WAFSOM cohorts.**
(PDF)

**Table S1 Analysis in GenABEL showing the influence of adjustment for clinical and environmental covariates on associations with OM in 831 Raine Study participants for whom full covariate data were available.** Data are shown for PCA adjustment only ($P_{adj\text{-}PCA}$), and with additional adjustment for covariates including daycare attendance <3 yrs ($P_{adj\text{-}daycare}$), allergy at < 3 yrs ($P_{adj\text{-}allergy}$), non-exclusive breast feeding at <6 mths ($P_{adj\text{-}supp}$), and all covariates ($P_{adj\text{-}allCov}$).
(PDF)

**Table S2 Directed interrogation of the Raine Study Discovery GWAS results across genes/regions previously implicated in childhood OM susceptibility in the literature: (A) candidate genes previously reported to be associated with OM susceptibility; (B) the human homologues of genes identified from murine models of OM; and (C) regions of the human genome previously implicated in genome-wide linkage studies.** For all genes/regions and SNPs the physical location is based on annotation from Ensembl release 54 with gene boundaries defined as +/- 20 kb from the 5′ and 3′ UTR. Results are presented from the discovery GWAS adjusted for PCs alone. This analysis was undertaken to find any evidence of association at these genes, not as a direct replication of specific SNPs reported in other studies. Nominal P<0.05 are highlighted in bold. No associations were robust to correction for the number of genes interrogated under each hypothesis (see main text).
(PDF)

**Table S3 SNPs/Genes selected for replication analysis in the WAFSOM cohort.**
(PDF)

**Table S4 Replication of selected genes in the WAFSOM cohort using case/pseudo-control conditional logistic regression under an additive model.** Where possible, tag-SNPs were selected to match SNPs genotyped in the Raine cohort. For some genes we selected additional tag-SNPs to cover areas of the gene not well covered on the SNP-chip used to analyse Raine. $P_{adj\text{-}PCA}$ values from the Raine cohort are provided for ease of comparison.
(PDF)

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## Author Contributions

Conceived and designed the experiments: SEJ JMB CEP. Performed the experiments: MSR. Analyzed the data: MSR NMW SEJ DA. Wrote the paper: MSR SEJ JMB. Raine management, recruitment and sample preparation: CEP. Clinical care and characterization for WAFSOM: SV HLC. WAFSOM recruitment: ESHS SEJ MSR. WAFSOM sample preparation: MSR ESHS SEJ. Supervised the work: SEJ JMB CEP.

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# Genetic and functional evidence for a role for *SLC11A1* in susceptibility to otitis media in early childhood in a Western Australian population

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ABSTRACT

Otitis media (OM) is a common disease in early childhood characterised by inflammation of the middle ear. Susceptibility to recurrent acute OM (rAOM; ⩾3 episodes AOM in 6 months) and chronic OM with effusion (COME; middle ear effusion ⩾3 months) is 40–70% heritable. Three bacterial pathogens commonly associated with OM, *Streptococcus pneumoniae* (Sp), non-typeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* (Mc), have been observed within adenoids and as facultative intracellular pathogens that invade and survive in mononuclear cells. Case/pseudo-control conditional logistic regression analysis of variants in the *SLC11A1* gene, initially identified for its role in resistance to intra-macrophage pathogens in mice, revealed association with OM at four polymorphisms ($P_{best}$ = 0.025) in 531 families (660 affected children) from the Western Australian Family Study of Otitis Media. This included association at the functional promoter GTn polymorphism (rs34448891) with alleles that regulate high (allele 3; odds ratio = 1.2, 95% CI 1.00–1.44, $P$ = 0.04) versus low (allele 2; odds ratio = 0.83, 95% CI 0.69–0.99, $P$ = 0.04) SLC11A1 expression. Haplotype and stepwise conditional logistic regression analyses support a single genetic effect in the proximal region of *SLC11A1*, with the haplotype 3_C_C_G across rs34448891_rs2276631_rs3731865_rs2695343 significantly ($P$ = 0.008) over-transmitted to affected offspring. Stratified analysis showed no association with OM in children who had undergone adenoidectomy (296 children), whereas children with adenoids intact (364 children) showed improved significance at the GTn polymorphism (allele 3: odds ratio = 1.38, 95% CI = 1.10–1.75, $P$ = 0.006). Quantitative RT/PCR demonstrated high expression of SLC11A1 in mononuclear cells isolated from adenoid tissue, with a trend for decreased expression with increasing copies of GTn allele 2. Expression of SLC11A1 was enhanced at 12 ($P = 1.2 \times 10^{-3}$) and 24 h ($P < 1.0 \times 10^{-4}$) after infection of Mono-Mac-6 cells with NTHi. This study identifies *SLC11A1* as a novel candidate for OM susceptibility, particularly in children with adenoids intact. Further analysis in other cohorts is required to validate these observations.

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## 1. Introduction

Otitis media (OM) is a common disease in early childhood characterised by inflammation of the middle ear. Acute OM (AOM) is an acute infection presenting with fever and a purulent effusion whilst OM with effusion (OME) presents with a non-purulent effusion that can become chronic (COME) (defined as ⩾3 months of middle ear effusion). This disease has significant morbidity with 80% of children having an episode in their first three years of life and up to 40% of children experiencing recurrent episodes of AOM prior to starting school (rAOM; more than 3 episodes in 6 months or more than 4 episodes in 12 months) (Teele et al., 1989). Episodes of OM often occur subsequent to viral upper respiratory tract infections, with three common bacterial pathogens isolated from middle ear fluid (Kilpi et al., 2001): *Streptococcus pneumoniae* (Sp), non-typeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* (Mc).

It is now apparent that the three common otopathogens can exist as facultative intracellular pathogens that are able to invade and survive within macrophages, monocytes and epithelial cells, acting as a potential reservoir contributing to recurrent and chronic disease. For example, analysis of middle ear biopsies from children undergoing insertion of tympanostomy tubes (TT) for COME/rAOM

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has revealed the presence of intracellular bacteria in middle ear mucosa epithelial cells (Coates et al., 2008). All three main otopathogens have subsequently been identified intracellularly in the middle ear mucosa (Thornton et al., 2011). Furthermore, viable, replicating NTHi have been isolated *ex vivo* from mononuclear cells within the subepithelial layers of human adenoid tissue (Forsgren et al., 1994; Nistico et al., 2011), with many bacteria observed in macrophages staining strongly for CD14 (Forsgren et al., 1996). The subepithelial, intraepithelial and intracellular regions of adenoids have also been shown to contain reservoirs of intracellular Mc that colocalise with macrophages (Heiniger et al., 2007). These results confirm the presence of intracellular bacterial reservoirs, both in the middle ear and adenoid, which may contribute to recurrent and chronic OM. The observation of intracellular otopathogen reservoirs within adenoidal tissue also suggests that the reduced incidence of recurrent OM when adenoidectomy or adenotonsillectomy (but not tonsillectomy alone) is performed in conjunction with TT insertion (Kadhim et al., 2007) stems, at least in part, from the removal of this bacterial reservoir.

The solute carrier family member 11a member 1 (*SLC11A1*) gene plays a role in resistance to intra-macrophage infections (Blackwell et al., 2001). *SLC11A1* encodes a divalent cation ($Fe^{2+}$, $Zn^{2+}$ and $Mn^{2+}$) transporter (Goswami et al., 2001) that is localised to membranes of late endosomes and lysosomes of cells of the myeloid lineage (Gruenheid et al., 1997; Searle et al., 1998; Stober et al., 2007). SLC11A1 plays a role in the regulation of iron homeostasis (Mulero et al., 2002) and has numerous pleiotropic effects on macrophage activation (Blackwell et al., 2003). The direction of cation transport across the late endosome and lysosome membrane remains contentious (Jabado et al., 2000; Lafuse et al., 2002; Techau et al., 2007) but is thought to influence the survival of intracellular pathogens either by starving them of essential nutrients (Nairz et al., 2009) or via the Fenton reaction that uses $Fe^{2+}$ to generate hydroxyl radicals with antimicrobial activity (Zwilling et al., 1999). Thus, polymorphisms in *SLC11A1* can influence the efficiency of ion transport in and out of intracellular vesicles, which may lead to differences in intracellular survival of bacterial pathogens in the host. A GTn repeat polymorphism identified in the promoter region (Blackwell et al., 1995) was shown to be functional in regulating transcription (Bayele et al., 2007; Searle and Blackwell, 1999). Allele 3 of the GTn drives higher expression of SLC11A1 and is associated with susceptibility to autoimmune diseases and pro-inflammatory infections including meningococcal meningitis, whilst allele 2 drives lower SLC11A1 expression and has been associated with susceptibility to pulmonary tuberculosis in humans (Blackwell et al., 2003).

Given the role of SLC11A1 in regulating survival of intra-macrophage pathogens (Blackwell et al., 2001, 2003), and the observation that bacterial reservoirs of otopathogens survive within mononuclear cells in adenoid tissue (Forsgren et al., 1994, 1996), we hypothesised that variants in the *SLC11A1* gene could influence susceptibility to recurrent and chronic OM through modulation of intracellular survival of bacterial reservoirs. In the event, we here show that OM is associated with *SLC11A1* polymorphisms that enhance the innate pro-inflammatory macrophage activation response to otopathogens in adenoids.

## 2. Materials and methods

### 2.1. Study population and phenotype definition

All aspects of this study were approved by the human research ethics committee at Princess Margaret Hospital for Children, Subiaco, WA. Study participants with a history of rAOM or COME requiring TT insertion were recruited to the Western Australian Family Study of Otitis Media (WAFSOM) as previously detailed (Rye et al., 2011). Upon recruitment to the study, participants provided either a non-invasive saliva sample or a blood sample at the time of TT insertion (for 133 affected children). Questionnaire data pertaining to ear health and history of ENT surgical procedures was collected. Children with medical conditions known to predispose to OM, for example immune deficiencies, specific genetic syndromes or cleft palate, were excluded. A total of 660 affected individuals from 531 families were included in this analysis. 93.6% of families self-described their ethnicity as Caucasian.

### 2.2. Extraction of DNA

Genomic DNA was extracted from Oragene saliva kits (DNA Genotek, Ontario, Canada) as per manufacturer's instructions, or from neutrophil pellets isolated from whole blood using a salting-out method (Miller et al., 1988). Extracted DNA was resuspended in TE buffer, quantified using spectrophotometry and stored at −20 °C.

### 2.3. Marker selection and genotyping

Twelve *SLC11A1* polymorphisms, including the functional promoter microsatellite (GTn), two in/dels (CAAA and TGTG) and nine SNPs, were selected for genotyping. These represent the most commonly typed *SLC11A1* markers in previously published studies (Table 1) including both the only known functional variants at *SLC11A1* and tagging SNPs/variants for major linkage disequilibrium blocks across the gene (Mehrotra et al., 2011). Two *SLC11A1* polymorphisms have been shown to influence expression of SLC11A1: the promoter GTn polymorphism (Bayele et al., 2007; Searle and Blackwell, 1999) and the −237[C/T] SNP (Zaahl et al., 2004). The latter is rare (minor allele frequency or MAF < 0.05) and was not studied in our population. All reagents were from Life Technologies (Carlsbad, CA) unless otherwise specified. The CAAA and TGTG in/dels were genotyped using 15 ng genomic DNA, 1X PCR buffer, 2.6 mM $MgCl_2$, 0.45 mM dNTPs, 0.04 M betaine, 1.1 μM forward and reverse primers, and 0.25 U of AmpliTaq Polymerase. Cycling was performed using a Touchdown protocol (Korbie and Mattick, 2008), at 94 °C for 5 min, 14 cycles of 94 °C for 20 s, 63–56 °C (−0.5 °C/cycle) for 60 s and 72 °C for 60 s, followed by an additional 14 cycles of 94 °C for 20 s, 56 °C for 60 s and 72 °C for 60 s and final extension of 72 °C for 5 min. Genotyping of the GTn polymorphism was performed using 12.5 ng DNA, 1X Gold PCR buffer, 3.0 mM $MgCl_2$, 0.25 mM dNTPs, 0.44 M betaine, 0.5 μM primer and 0.25 units of AmpliTaq Gold Polymerase. Cycling conditions were 94 °C for 12 min, 9 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s, followed by 19 cycles of 89 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s then 72 °C for 10 min. Amplification products for both in/dels and the GTn were pooled, fragments resolved using an Applied Biosystems 3130xl Genetic Analyzer and alleles assigned via GeneMapper v4.1. All SNPs were genotyped using TaqMan allelic discrimination assays carried out according to manufacturer's instructions. Alleles were called using an Applied Biosystems 7900HT Sequence Detection System. Mendelian inconsistencies for microsatellites, in/dels and SNPs were identified using the PedCheck software (O'Connell and Weeks, 1998) and corrected or removed prior to statistical analysis. No markers significantly deviated from Hardy–Weinberg equilibrium in unaffected, unrelated founders.

### 2.4. Allelic association analysis

Power calculations performed using the method of Knapp (1999) provides estimated power of ⩾88% (effect size or odds ratio

**Table 1**
Microsatellite, in/dels and single nucleotide polymorphisms genotyped across *SLC11A1* using in the Western Australian Family Study of Otitis Media. MAF indicates minor allele frequency for the WAFSOM cohort.

| Polymorphism | Common name | Location | Position | Major/Minor allele | MAF |
|---|---|---|---|---|---|
| rs34448891 | GTn repeat | Promoter | 219246650 | 3/2[a] | 0.26 |
| rs7576974 | 263[C/T] | Exon | 219247790 | C/T | <0.02 |
| rs2276631 | 274[C/T] | Exon | 219249013 | C/T | 0.26 |
| rs3731865 | 469 + 14[C/G] | Intron | 219250003 | C/G | 0.27 |
| rs3731863 | – | Intron | 219252208 | G/A | 0.06 |
| rs17221959 | 823[C/T] | Exon | 219252630 | C/T | 0.04 |
| rs2695343 | – | Intron | 219255423 | G/A | 0.40 |
| rs2279015 | 1465–85[A/G] | Intron | 219259270 | C/T | 0.38 |
| rs17235409 | 1703[G/A]; D543 N | Exon | 219259732 | G/A | <0.02 |
| rs17235416 | 1729 + 55del4; TGTG ins/del | 3′UTR | 219259814 | INS/DEL | 0.02 |
| rs17229009 | 1729 + 263del4; CAAA ins/del | 3′UTR | 219260022 | INS/DEL | 0.36 |
| rs2279014 | – | 3′UTR | 219261176 | G/A | 0.35 |

[a] Relative to the two common (MAF > 0.2) alleles analysed in this study; allele 1 was present at frequency 0.004.

(OR) = 1.5; $P = 0.01$; MAF $\geqslant$ 0.2) for the 531 families included from the WAFSOM cohort. This is likely to be an underestimate of power as some families contain multiple affected children. For markers with MAF $\leqslant$ 0.05, power is estimated to be $\leqslant$31% for an effect size equivalent to an odds ratio of 1.5, i.e. our sample does not have sufficient power to look for associations with markers where the minor allele is at a frequency <0.05. These criteria were used to filter out markers not suitable for association analysis in our sample. Polymorphisms were analysed using case/pseudo-control (CPC) conditional logistic regression in STATA v10. For this family-based analysis, CPC datasets were generated to match each affected offspring with one to three pseudo-controls derived from the remaining genotype combinations possible from the parental genotypes (Cordell et al., 2004). Odds ratios, 95% confidence intervals and *P*-values are calculated using a robust sandwich estimator of the variance and a Wald $\chi^2$ test statistic to control for clustering of affected siblings within families for 1df (allele-wise) and 2df (genotype-wise) tests. A likelihood ratio test comparing 1df and 2df tests was used to determine whether there was evidence for one model over the other, i.e. provide evidence for a dominant model. Intra-locus conditional logistic regression analysis was performed in STATA v10 for combinations of significant polymorphisms to determine the presence of single or multiple genetic effects. Haplotype analyses were performed using TRANSMIT (Clayton and Jones, 1999). Patterns of LD were analysed in Haploview 4.2 (Barrett et al., 2005).

### 2.5. Analysis of adenoidectomy sub-groups

Information on adenoidectomy and TT insertion was extracted from the questionnaire data to generate descriptive statistics on age of adenoidectomy and whether it coincided with TT insertion. Data were not available on associated adenoidal pathologies, such as adenoid hypertrophy, obstructive sleep disorder etc. Statistics were generated using the Mann–Whitney test. Questionnaire data was also used to form two stratified groups of children, one of children whose parents ticked a box to say that children had undergone adenoidectomy for rAOM/COME (296 children) and one of children whose parents did not report adenoidectomy (364 children). Stratified analysis on the two groups of children was performed using CPC in STATA v10. Power for the 296 families/trios for which adenoidectomy was performed was estimated to be $\geqslant$61% (OR = 1.5; $P = 0.01$; MAF $\geqslant$ 0.2), and $\geqslant$71% (OR = 1.5; $P = 0.01$; MAF $\geqslant$ 0.2) for the 364 families/trios with cases with adenoids intact.

### 2.6. Extraction of RNA from adenoid and tonsil tissue

Twelve adenoid samples collected with parent/guardian consent during adenoidectomy performed for OM, obstructive sleep disorder or recurrent acute tonsillitis were available for analysis from a separate study of biofilms approved by the human research ethics committee at Princess Margaret Hospital for Children, Subiaco, and at St John of God Hospital, Subiaco, WA (authors RT, HLC and SV). Adenoids were collected into RPMI medium containing penicillin, streptomycin and fungizone. Adenoid tissue in RPMI was homogenised over a stainless steel mesh and the cell suspension passed through a non-absorbent cotton wool column and layered over a density gradient to isolate mononuclear cells. RNA was extracted from these mononuclear cells using the TRI Reagent (Sigma–Aldrich, St Louis, MO) protocol (Chomczynski, 1993).

### 2.7. Bacterial infection of cell lines and RNA extraction

Three macrophage cell lines were also available for expression analyses. The acute monocytic leukemia (THP-1) and leukemic monocyte lymphoma (U937) macrophage cell lines were grown in RPMI 1640, 10% FCS, 2 mM L-glutamine and 100U/ml penicillin/100 μg/ml streptomycin. The Mono-Mac-6 (MM6) macrophage cell line additionally required 1X non-essential amino acids and 9 μg/ml OPI bovine insulin (Sigma–Aldrich). Cell lines were stimulated *in vitro* in 6-well plates with either NTHi or Sp at a multiplicity of infection of 10:1. Bacterial cultures were kindly provided by Dr. Lea-Ann Kirkham (School of Paediatrics and Child Health, University of Western Australia, Perth). Bacteria were streaked and incubated overnight at 37 °C/5% $CO_2$ on an agar plate (blood for Sp and chocolate for NTHi) for growth of live bacteria. A viable count of bacteria was performed prior to infection using a Helber bacterial counting chamber with Thoma ruling (ProScitech) and diluted to an appropriate concentration. At the 0hr time-point, 1 ml of bacteria ($1 \times 10^8$ cfu/ml), or 1 ml of media for control samples, was added to 1 ml of cells ($1 \times 10^7$ cells/ml) to initiate bacterial infection. After co-culture for an hour, media containing non-adherent bacteria was removed and adherent extracellular bacteria were subsequently killed using fresh media containing penicillin and streptomycin. Cells were harvested for RNA extraction at six time-points (0, 1, 3, 6, 12 and 24 h) in TRI Reagent.

### 2.8. Gene expression analysis

Extracted total RNA ($\geqslant$1 μg) was reverse-transcribed into cDNA using the Applied Biosystems High Capacity cDNA RT Kit as per manufacturer's instructions. Quantitative analysis of *SLC11A1* gene expression was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using a TaqMan gene expression assay and normalised against the 18S rRNA endogenous control. Raw expression data were analysed in RQ Manager v1.2 (Life Technologies). Results are presented as Delta $C_T$ ($dC_T$) with statistical analysis performed in PRISM v5 (GraphPad Software, http://www.graphpad.com) using two-tailed unpaired t-tests.

## 3. Results

### 3.1. Allelic association analysis of *SLC11A1*

Details including MAF for the 12 polymorphic markers genotyped in 660 affected individuals from 531 families are provided in Table 1. Of these, 7 polymorphisms had MAF ⩾ 0.2 providing sufficient power to be taken forward in association analyses. Markers with MAF ⩽ 0.06 were excluded from association analyses. At rs34448891, the functional GTn repeat, 3 alleles were observed in the WAFSOM cohort. However, only alleles 2 and 3 observed at a frequency >0.2 were compared in the association analysis. Results of CPC logistic regression analysis for the 4 variants at MAF > 0.2 that demonstrate associations with nominal $P < 0.05$ for allele-wise or genotype-wise tests are shown in Table 2. Allelic models (1df) reveal association at rs34448891[GTn] (allele 3: OR = 1.20, 95%CI 1.00–1.44, $P = 0.04$) and at rs2695343 (allele G: OR = 1.18, 95%CI 1.00–1.39, $P = 0.038$). Associations at rs2776631/274[C/T] (genotype CC: OR = 1.76, 95%CI 1.07–2.88, $P = 0.025$) and rs3731865/469+14[G/C] (genotype GG: OR = 1.70, 95%CI 1.05–2.76, $P = 0.032$) were only observed under a genotypic model (2df). However, since the likelihood-ratio test statistics (Table 2) comparing 1df and 2df tests were not significant at any marker, there was no formal evidence to invoke dominant/recessive models over an additive model. Stepwise logistic regression analysis (Supplementary Table 1) showed that none of the 4 markers added independent effects to each other, suggesting that they are all tagging a single association signal in this 5′/proximal end of *SLC11A1*. Analysis in Haploview (Supplementary Fig. 1) provided evidence for two stronger haplotype blocks (as defined by $r^2$) for the 3 most proximal markers ($D' > 0.84$; $r^2 > 0.68$), rs34448891, rs2276631 and rs3731865, and for the 3 most distal markers, rs2279015, rs17229009 and rs2279014 ($D' > 0.88$; $r^2 > 0.70$). SNP rs2695343 showed moderate LD ($r^2 > 0.57$) with the distal 3 markers, and with the 2 nearest markers ($r^2 > 0.54$) with which it created the strongest LD block across *SLC11A1* as defined by $D' = 0.99$. The LD data are consistent with analysis in TRANSMIT (Supplementary Table 2) showing over-transmission of the common 3.C.C.G (frequency 0.58; $P = 0.008$) haplotype for the 4 proximal SNPs, with slightly stronger evidence for the haplotype comprising the 3 most proximal rs34448891, rs2276631 and rs3731865 ($P = 0.006$) that excludes rs2695343. There was no evidence for association (data not shown) for the 3 distal variants at MAF > 0.2 (Table 1) located in the 3′/distal region of *SLC11A1* (Supplementary Fig. 1). No association reported here is robust to strict Bonferroni correction for the number of polymorphisms tested in the association analysis (i.e. 0.05/7; $P < 0.007$). However, this correction is over-conservative as the markers are not all independent. A less stringent correction that takes account of the non-independence of the markers used in the association analysis provides a cut-off of $P < 0.025$ (i.e. 0.05/2 LD blocks; Supplementary Fig. 1). Association at the single major haplotype is robust to application of this cut-off.

### 3.2. Analysis of adenoidectomy sub-groups

Using population level data, adenoidectomy or adenotonsillectomy (but not tonsillectomy alone) at the time of TT insertion has been shown to reduce the need for further TT insertions in

**Table 2**
Results of logistic regression analyses for association between *SLC11A1* polymorphisms and OM under allelic (1 df) and genotypic (2 df) models in (A) the full WAFSOM cohort ($N = 531$ families; 660 affected individuals) and (B) the subset of WAFSOM families/children with adenoids intact ($N = 364$ affected individuals). LRT = likelihood ratio test comparing 1 df and 2 df tests. Bold indicates nominal $P \leq 0.05$.

| | Allelic model (1 df) | | | | Genotypic model (2 df) | | | | LHR test (*P*-value) |
|---|---|---|---|---|---|---|---|---|---|
| | Alleles | *P*-value | OR | 95% CI | Alleles | *P*-value | OR | 95% CI | |
| *(A) Polymorphism* | | | | | | | | | |
| *SLC11A1*_rs34448891 [GTn] | 3 v 2 | **0.040** | 1.20 | 1.00–1.44 | 33 v 22 | 0.056 | 1.55 | 0.99–2.43 | 0.616 |
| | | | | | 32 v 22 | 0.195 | 1.33 | 0.87–2.03 | |
| | 2 v 3 | **0.040** | 0.83 | 0.69–0.99 | 23 v 33 | 0.155 | 0.86 | 0.69–1.06 | |
| | | | | | 22 v 33 | 0.056 | 0.64 | 0.41–1.01 | |
| *SLC11A1*_rs2276631 274[C/T] | C v T | 0.100 | 1.16 | 0.97–1.39 | CC v TT | **0.025** | 1.76 | 1.07–2.88 | 0.092 |
| | | | | | CT v TT | **0.034** | 1.66 | 1.04–2.65 | |
| | T v C | 0.100 | 0.86 | 0.72–1.03 | TC v CC | 0.607 | 0.95 | 0.76–1.17 | |
| | | | | | TT v CC | **0.025** | 0.57 | 0.35–0.93 | |
| *SLC11A1*_rs3731865 469 + 14[G/C] | G v C | 0.062 | 0.84 | 0.70–1.01 | GG v CC | **0.032** | 0.59 | 0.36–0.96 | 0.190 |
| | | | | | GC v CC | 0.384 | 0.91 | 0.73–1.13 | |
| | C v G | 0.062 | 1.19 | 0.99–1.42 | CG v GG | 0.063 | 1.55 | 0.98–2.45 | |
| | | | | | CC v GG | **0.032** | 1.70 | 1.05–2.76 | |
| *SLC11A1*_rs2695343 | A v G | **0.038** | 0.85 | 0.73–0.99 | AA v GG | **0.028** | 0.69 | 0.50–0.96 | 0.413 |
| | | | | | AG v GG | 0.369 | 0.90 | 0.73–1.13 | |
| | G v A | **0.038** | 1.18 | 1.00–1.39 | GA v AA | 0.072 | 1.30 | 0.98–1.74 | |
| | | | | | GG v AA | **0.028** | 1.44 | 1.04–2.00 | |
| *(b) Polymorphism* | | | | | | | | | |
| *SLC11A1*_rs34448891 [GTn] | 3 v 2 | **0.006** | **1.38** | **1.10–1.75** | 33 v 22 | **0.007** | **2.41** | **1.28–4.54** | 0.301 |
| | | | | | 32 v 22 | **0.046** | **1.87** | **1.01–3.48** | |
| | 2 v 3 | **0.009** | **0.73** | **0.58–0.92** | 23 v 33 | 0.104 | 0.79 | 0.59–1.05 | |
| | | | | | 22 v 33 | **0.008** | **0.43** | **0.23–0.80** | |
| *SLC11A1*_rs2276631 274[C/T] | C v T | 0.082 | 1.24 | 0.97–1.58 | CC v TT | 0.059 | 1.97 | 0.97–3.98 | 0.303 |
| | | | | | CT v TT | 0.125 | 1.71 | 0.86–3.40 | |
| | T v C | 0.082 | 0.81 | 0.63–1.03 | TC v CC | 0.335 | 0.87 | 0.65–1.16 | |
| | | | | | TT v CC | 0.059 | 0.51 | 0.25–1.03 | |
| *SLC11A1*_rs3731865 469 + 14[G/C] | G v C | **0.047** | **0.78** | **0.62–0.99** | GG v CC | **0.043** | **0.49** | **0.24–0.98** | 0.334 |
| | | | | | GC v CC | 0.231 | 0.84 | 0.63–1.12 | |
| | C v G | **0.047** | **1.28** | **1.00–1.62** | CG v GG | 0.119 | 1.73 | 0.87–3.43 | |
| | | | | | CC v GG | **0.043** | **2.06** | **1.02–4.15** | |
| *SLC11A1*_rs2695343 | A v G | 0.236 | 0.88 | 0.72–1.09 | AA v GG | 0.162 | 0.73 | 0.46–1.14 | 0.345 |
| | | | | | AG v GG | 0.840 | 0.97 | 0.72–1.30 | |
| | G v A | 0.236 | 1.13 | 0.92–1.39 | GA v AA | 0.158 | 1.34 | 0.89–1.99 | |
| | | | | | GG v AA | 0.162 | 1.38 | 0.88–2.16 | |

Western Australian children (Kadhim et al., 2007). In view of this, relevant data on TT insertion and adenoidectomy was extracted from questionnaire data for generation of descriptive statistics (Supplementary Table 3). In the WAFSOM cohort, children that underwent adenoidectomy had a higher number of TT insertions overall (1.91 vs 1.15; $P < 1.0 \times 10^{-4}$) and had their first set of TTs at an older age (28.8 vs 23.4 months; $P = 1.8 \times 10^{-3}$) compared to children with adenoids intact. Nearly 45% of children who underwent adenoidectomy did so at the time of their first TT insertion and following adenoidectomy more than 70% of children did not need further TT insertions.

In our study SLC11A1 could be acting at the level of response to infection in the ear itself, or by influencing response to infection in the adenoids. To determine whether the presence of adenoids influence the association observed at *SLC11A1*, we performed a stratified analysis of two sub-groups of children, those whose parents reported that they had their adenoids removed (296 children) and those children with adenoids intact at the time of sample collection (364 children). No significance was seen at any *SLC11A1* polymorphism in the sub-group of children who underwent adenoidectomy (data not shown). In contrast, significance of the *SLC11A1* association at rs34448891/GTn was improved (allele 3: OR = 1.38, 95% CI = 1.10–1.75, $P = 0.006$; Table 2B) in the sub-group of children with adenoids intact compared to the total WAFSOM sample (allele 3: OR = 1.20, 95%CI 1.00–1.44, $P = 0.04$; Table 2A). In this sub-group the genotype-wise test (Table 2B) also suggested that allele 3 may be (incompletely) dominant over allele 2 (cf. section 3.3 below), although there was no formal statistical support from the likelihood ratio test for a dominant model (2df test) over the additive model tested in the allele-wise 1df test. Association with the C allele at rs3731865 (OR = 1.28, 95%CI 1.00–1.62, $P = 0.047$) was now significant (nominal $P < 0.05$) under an additive model (Table 2B). It should be noted, however, that associations at nominal $P < 0.05$ were not observed at rs2276631 or rs2695343 (Table 2B), possibly due to reduced power of the sample. Nevertheless, the results do suggest that association with the known functional GTn promoter polymorphism is only observed in children with adenoids intact, supporting the notion that SLC11A1 may be contributing to regulation of the innate immune response in relation to the bacterial reservoir in adenoid macrophages.

### 3.3. Expression of SLC11A1 in adenoids

Given our preliminary evidence that association between the GTn polymorphism at *SLC11A1* and susceptibility to recurrent/chronic OM is only observed in children with adenoids intact, along with published data (Heiniger et al., 2007) showing the facultative intracellular capability of NTHi and Mc in mononuclear cells of adenoids, the expression of SLC11A1 was measured in mononuclear cells isolated from adenoid tissues of donors genotyped for the GTn repeat. SLC11A1 was highly expressed in adenoid mononuclear cells from all donors, independent of GTn genotype (mean ± SD $dC_T = 20.00 \pm 0.35$, $N = 12$; Fig. 1). Previous studies (Searle and Blackwell, 1999) have shown that allele 3 of the GTn polymorphism drives higher expression of *SLC11A1* relative to allele 2. Consistent with these findings, our results (Fig. 1) suggest a trend towards lower expression (higher $dC_T$) of SLC11A1 relative to the number of copies of allele 2 being carried. However, pairwise comparison of 3,3 and 2,3 genotypes was not statistically significant, which may be explained in part by (incomplete) dominance of allele 3 over allele2 in the association analysis (Table 2B). Statistical comparisons of 3,3 or 2,3 genotypes against the 2,2 genotype were not possible due to availability of only one adenoid sample carrying this rarer genotype for the GTn.

Given the role of SLC11A1 in regulating innate immunity to intra-macrophage infections we also analysed expression in three

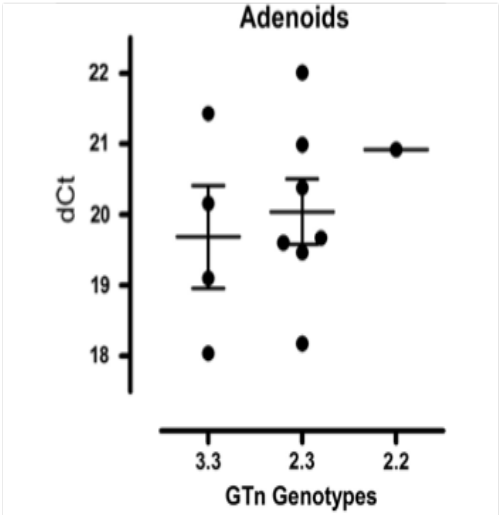


**Fig. 1.** Real time PCR expression of SLC11A1 in adenoids. Delta Ct ($dC_T$) values, where a lower value indicates greater expression, are graphed against GTn genotype for each sample.

macrophage cell lines, U937, THP1 and MM6, across different time points before and after infection with NTHi and Sp to determine whether bacterial infection influences the level of SLC11A1 expression in macrophages (Fig. 2; Supplementary Fig. 2). Baseline $dC_T$ values were lowest (i.e. expression highest) in THP1 cells (25.64 ± 0.06, $N = 3$) compared to MM6 (29.3 ± 0.26, $N = 3$) and U937 (31.18 ± 0.34, $N = 3$) cell lines. Whilst significant changes in expression levels occurred with time in culture for all 3 uninfected cell lines (Fig. 2 MM6; Supplementary Fig. 2 U937 and THP1), marked effects of infection were only observed for MM6 (Fig. 2). For this cell line, Sp showed transiently enhanced expression (lower $dC_T$) in infected over uninfected cells at 3 h post infection, which returned to below baseline levels at 12 and 24 h post infection (Fig. 2C). In contrast, NTHi stimulated sustained expression over the relevant uninfected baseline through 12 and 24 h post infection (Fig. 2B). These data suggest that SLC11A1 may have its biggest impact in relation to the reservoir of NTHi bacteria (although Mc was not tested in our experiments).

## 4. Discussion

Here we introduce *SLC11A1* as a novel candidate gene for OM susceptibility. This gene was first identified for its role in regulating intra-macrophage pathogens in mice, including bacterial (*Salmonella typhimurium*, *Mycobacterium spp.*) and protozoan (*Leishmania donovani*) infections (Blackwell et al., 2001, 2003). The murine Slc11a1 protein is a divalent cation transporter (Goswami et al., 2001) that localises to late endosomal/lysosomal membranes of cells of the myeloid lineage (Gruenheid et al., 1997; Searle et al., 1998; Stober et al., 2007), where it can directly influence pathogen survival (Nairz et al., 2009; Zwilling et al., 1999). However, SLC11A1 is also known to have many pleiotropic effects on macrophage activation, which have highlighted its role in pro-inflammatory autoimmune diseases (Blackwell et al., 2004) as well as in resistance to infectious disease (Blackwell et al., 2001, 2003). Here we show that four variants in the human *SLC11A1* gene are associated with susceptibility to OM in this predominantly Caucasian WAFSOM cohort, with evidence for a single genetic effect associated with a haplotype that tags the rs34448891 functional GTn promoter polymorphism along with rs2276631/274[C/T], rs3731865/469+14[G/C] and rs2695343. In

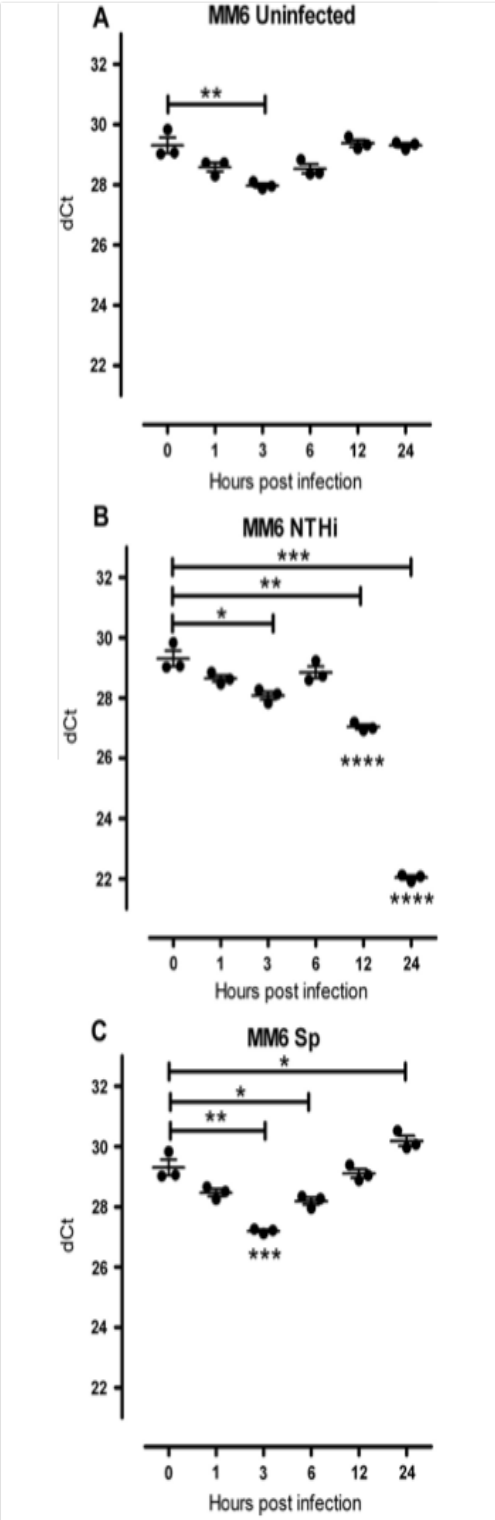


**Fig. 2.** Expression of SLC11A1 in the MM6 macrophage cell line either uninfected (A) or infected with non-typeable *Haemophilus influenzae* (NTHi: B) or *Streptococcus pnuemoniae* (SP: C). Delta Ct ($dC_T$) values are graphed against time-points for each sample (analysed in triplicate). Significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$) differences for time points within each treatment are indicated by the bars above the $dC_T$ values, while asterisks below the $dC_T$ values indicate significant differences between either NTHi compared to uninfected controls, or Sp compared to uninfected controls, at the relevant time points.

reporter gene studies, allele 3 at the GTn polymorphism was shown to drive high levels of expression relative to allele 2 (Searle and Blackwell, 1999). This led to the hypothesis that this high expressing allele would drive pro-inflammatory activated macrophage responses, accounting for the association of GTn allele 3 with multiple autoimmune diseases (Blackwell et al., 2004), while allele 2 was observed to be associated with anti-inflammatory interleukin-10-producing macrophage responses (Awomoyi et al., 2002) and susceptibility to pulmonary tuberculosis in a number of independent studies (Awomoyi et al., 2002; Bellamy et al., 1998; Gao et al., 2000; Shaw et al., 1997). Over time, association of the pro-inflammatory allele 3 has been demonstrated for a number of infectious diseases, including visceral leishmaniasis (Mohamed et al., 2004), meningococcal meningitis (Blackwell et al., 2003), and HIV (Marquet et al., 1999), suggesting that in some situations a strong pro-inflammatory innate macrophage activation response to infection driven by SLC11A1 can be deleterious (Blackwell et al., 2003). Here we find that susceptibility to OM is again associated with GTn allele 3, particularly in those children where adenoids are present. This suggests that a pro-inflammatory activated macrophage response driven by SLC11A1 in the adenoids in response to bacterial pathogens may be detrimental.

Adenoids sit at the first line of defence against bacteria and viruses, providing protection against respiratory tract infections. Nevertheless, children who harbour bacteria in adenoid tissue are thought to be at increased risk of OM. It has been assumed, therefore, that the protective effect of adenoidectomy is due to the removal of this reservoir of bacterial infection, although the removal of an obstruction to the Eustachian tube has also been proposed. Of interest, in the group of children with adenoids present, we observed increased significance at two *SLC11A1* markers, including the association with allele 3 at the functional GTn promoter region polymorphism. Failure to demonstrate the association in children who had adenoidectomy in early childhood implies that SLC11A1 has its influence in response to infection in the adenoids. This association with allele 3 implies that higher expression of SLC11A1 in the adenoids driven by this functional variant, as suggested by our quantitative analysis of expression using RNA from mononuclear cells isolated from adenoid tissue, is a risk factor for recurrent/chronic OM. This is perhaps counterintuitive, since we might expect that increased macrophage activation would lead to enhanced killing of otopathogens and hence protection against disease. Instead, our results suggest that a heightened macrophage activation response stimulated by otopathogens might contribute to the pathology of disease. This would also be consistent with the potent and sustained stimulation of SLC11A1 expression we observed in the MM6 macrophage cell line infected with NTHi. This MM6 line is considered to be representative of a mature tissue macrophage compared to the more monocyte-like THP1 and U937 cell lines (Lee et al., 2003). A vigorous pro-inflammatory response driven by SLC11A1 in resident macrophages in response to otopathogens in the adenoids could act as a potent trigger for polymorphonuclear neutrophil and monocyte infiltration to the site of infection. Our results also suggest that, of the two otopathogens studied, NTHi may be the more potent trigger of this innate SLC11A1-regulated macrophage response in adenoid tissue.

Our observation that the functional GTn promoter polymorphism is associated with OM susceptibility is also of interest in relation to the recent research highlighting the role of hypoxia and hypoxia inducible factor (HIF)-mediated pathways in OM pathogenesis (Cheeseman et al., 2011). In a recent study (Bayele et al., 2007) we showed that HIF-1 regulates allelic variation in *SLC11A1* expression by binding directly to the GTn microsatellite during macrophage activation by infection or inflammation. More

specifically, luciferase expression was enhanced approximately 3.4- to 11.3-fold by HIF-1α, with allele 3 driving the highest HIF-1α-induced luciferase expression. Targeted *Hif-1* ablation in murine macrophages attenuated *Slc11a11* expression and responsiveness to *S. typhimurium* infection (Bayele et al., 2007). Conversely, RNA interference-mediated down-regulation of SLC11A1 reduced HIF-1α induction following PMA-induced differentiation of U937 and THP1 cells, due to the influence of SLC11A1 on the labile iron pool (Knowles et al., 2006). This suggests that there is a strong interplay between these two genes which would come into play under hypoxic conditions. Interest in the role of hypoxia and HIF-1α has so far focused on the inflamed middle ear, the purulent bulla fluids, foamy macrophages within the bulla, the epithelium and in the connective tissues of the thickened, inflamed middle ear mucosa (Cheeseman et al., 2011). However, hypoxia is a common feature of inflamed microenvironments. Our data suggest that further genetic and functional analysis of the roles of HIF-1 and SLC11A1, and the inflammatory pathways with which they interact (including TNF and IL-1B), could contribute to our understanding of the role played by adenoids in pathogenesis of OM and therefore the protective role of adenoidectomy. In particular, it would be of interest to determine whether a heightened pro-inflammatory response to otopathogens under hypoxic conditions of inflamed adenoid tissue, rather than regulation of bacterial numbers *per se*, contributes to disease pathogenesis.

In summary, we present genetic and functional evidence for a role for *SLC11A1* in OM that provide novel insight into pathogenic processes occurring in adenoids, particularly in relation to the otopathogen NTHi. Further work is required to determine the specificity of this host gene/otopathogen interaction. In particular a larger longitudinal cohort study with greater statistical power is required to determine precisely when polymorphism at *SLC11A1* is having its effect, and whether the carriage of allele 3 at the GTn polymorphism could be used as a genetic biomarker for those children who would most benefit from early adenoidectomy in the treatment of OM. Nevertheless, our data provides an important step towards understanding the pathogenesis of OM and the influence disease-associated polymorphisms can have in the interplay between bacterial otopathogens and disease progression.

## Authors' contributions

Conceived and designed the experiments: JMB SEJ MSR. Clinical care and characterization for WAFSOM: SV HLC. WAFSOM recruitment and preparation: ESHS SEJ MSR SPW (Gromit Study included within WAFSOM). Development of in-house software tools for entry and storage of epidemiological and genetic data for Gromit and WAFSOM: RFW. Adenoid collection and preparation: RT SV HLC. WAFSOM genotyping, cell culture, RNA preparation and RT-PCR expression: MSR. analysed the data: MSR SEJ JMB. Supervised the work: SEJ JMB. Wrote the paper: MSR SEJ JMB. Reviewed the manuscript: all authors.

## Conflicts of interest

All authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.03.023.

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RESEARCH ARTICLE Open Access

# Genetic and functional evidence for a locus controlling otitis media at chromosome 10q26.3

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## Abstract

**Background:** Otitis media (OM) is a common childhood disease characterised by middle ear effusion and inflammation. Susceptibility to recurrent acute OM and chronic OM with effusion is 40-70% heritable. Linkage studies provide evidence for multiple putative OM susceptibility loci. This study attempts to replicate these linkages in a Western Australian (WA) population, and to identify the etiological gene(s) in a replicated region.

**Methods:** Microsatellites were genotyped in 468 individuals from 101 multicase families (208 OM cases) from the WA Family Study of OM (WAFSOM) and non-parametric linkage analysis carried out in ALLEGRO. Association mapping utilized dense single nucleotide polymorphism (SNP) data extracted from Illumina 660 W-Quad analysis of 256 OM cases and 575 controls from the WA Pregnancy Cohort (Raine) Study. Logistic regression analysis was undertaken in ProbABEL. RT-PCR was used to compare gene expression in paired adenoid and tonsil samples, and in epithelial and macrophage cell lines. Comparative genomics methods were used to identify putative regulatory elements and transcription factor binding sites potentially affected by associated SNPs.

**Results:** Evidence for linkage was observed at 10q26.3 ($Z_{lr}$ = 2.69; P = 0.0036; D10S1770) with borderline evidence for linkage at 10q22.3 ($Z_{lr}$ = 1.64; P = 0.05; D10S206). No evidence for linkage was seen at 3p25.3, 17q12, or 19q13.43. Peak association at 10q26.3 was in the intergenic region between *TCERG1L* and *PPP2R2D* (rs7922424; P = $9.47 \times 10^{-6}$), immediately under the peak of linkage. Independent associations were observed at *DOCK1* (rs9418832; P = $7.48 \times 10^{-5}$) and *ADAM12* (rs7902734; P = $8.04 \times 10^{-4}$). RT-PCR analysis confirmed expression of all 4 genes in adenoid samples. *ADAM12*, *DOCK1* and *PPP2R2D*, but not *TCERG1L*, were expressed in respiratory epithelial and macrophage cell lines. A significantly associated polymorphism (rs7087384) in strong LD with the top SNP (rs7922424; $r^2$ = 0.97) alters a transcription factor binding site (CREB/CREBP) in the intergenic region between *TCERG1L* and *PPP2R2D*.

**Conclusions:** OM linkage was replicated at 10q26.3. Whilst multiple genes could contribute to this linkage, the weight of evidence supports *PPP2R2D*, a TGF-β/Activin/Nodal pathway modulator, as the more likely functional candidate lying immediately under the linkage peak for OM susceptibility at chromosome 10q26.3.

**Keywords:** Acute otitis media, Otitis media with effusion, Genetic polymorphisms, Linkage, Association, Raine study, WAFSOM, Australia

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## Background

Otitis media (OM) is a common childhood disease characterised by the presence of infection (acute otitis media; AOM) or fluid (otitis media with effusion; OME) in the middle ear cavity. Most children experience at least one episode of AOM by 1 year of age with up to 40% experiencing recurrent episodes of AOM (rAOM; ≥3 episodes of AOM in 6 months) or chronic episodes of OME (COME; middle ear effusion (MEE) ≥3 months) in childhood [1]. Recurrent disease can result in perforation of the tympanic membrane and/or conductive hearing loss, leading to deficits in language development and poor educational outcomes. Treatment for recurrent disease may include insertion of tympanostomy tubes. High prevalence rates of OM result in substantial health care related costs and significant childhood morbidity in many countries [2].

The causal mechanisms that lead to recurrent disease are poorly understood but familial clustering and high heritability estimates point to a genetic component. Using self-reported AOM history from 2,570 Norwegian twin pairs, Kvaerner *et al.* [3] determined heritability estimates of 0.45 in males and 0.74 in females. Two prospective twin and triplet studies have confirmed these estimates. Casselbrant *et al.* [4] reported heritability estimates of 0.73 for time with MEE in the first two years of life. Likewise, Rovers *et al.* [5] reported heritability estimates of 0.49 at 2 years increasing to 0.71 at 4 years with a concomitant decrease in shared environment estimates from 0.41 at 2 years to 0.16 by 4 years of age. Overall these epidemiological studies confirm that susceptibility to OM has a substantial genetic component that increases with age.

To date, there have been few studies undertaken to pinpoint the genes involved. Two genome-wide linkage scans using multi-case families of Caucasian origin have identified specific regions of the genome harbouring putative susceptibility genes. The first identified two regions of linkage on chromosome 10q26.3 and 19q13.43, and a further region on 3p25.3 after conditioning on the linked regions, suggesting epistatic interactions [6]. The second identified two different regions of linkage on 17q12 and 10q22.3 [7]. Whilst the region of 19q13.43 has since been refined [8], there has been no replication of any of these regions in independent studies and the causal genes underlying them have yet to be identified.

To determine whether these genomic regions are important in recurrent/severe OM in a Western Australian population we have carried out linkage analysis using families who contain at least two individuals diagnosed with rAOM or chronic OME (COME) recruited to the Western Australian Family Study of Otitis Media (WAFSOM) [9]. To identify the putative disease susceptibility locus in the only region that replicated in this population, association using SNPs was undertaken in the Western Australian Pregnancy Cohort (Raine) Study [10]. Functional and bioinformatic studies were used to further clarify the putative etiological gene.

## Methods

### Sample collection and phenotype definition

Linkage studies utilised samples from the WAFSOM where probands with a history of tympanostomy tube insertion due to rAOM or COME were identified from the records of collaborating ear, nose and throat (ENT) specialists (HC and SV) as previously described [9]. Parents and full siblings with a history of recurrent disease, defined as ≥3 physician diagnosed episodes of AOM or tympanostomy tube insertion for rAOM or COME, were also invited to participate. No exclusion was made on the basis of ethnicity or gender; 93.2% of the families in the WAFSOM self-identified as Caucasian. Recruitment to the WAFSOM was approved by the Human Ethics Committee at Princess Margaret Hospital for Children (PMH). Written, informed consents were obtained both for participation in the study and for DNA collection from all adults or from the parents of participants less than 18 years of age. For linkage analysis family members with no history of recurrent disease were classified as unaffected whilst all others were classified as unknown. A total of 101 multicase families (107 nuclear families; 208 affected individuals; 468 total individuals) with 2 to 4 affected individuals were included in the linkage analysis.

For association mapping, we utilised data from the Western Australian Pregnancy Cohort (Raine) Study ('the Raine Study'), a longitudinal cohort of children whose mothers were recruited during early pregnancy [10]. For the purposes of this study, data collected from clinical examinations and parental questionnaires completed each year at the (average) ages of one, two and three years was used to define a phenotype for OM. Children were defined as a case if clinical examination in the first three years of life indicated presence of inflamed, retracted or scarred TM, MEE or tympanostomy tubes *in situ*. Participants were also classified as a case where parents' yearly reports indicated ≥3 episodes of AOM had occurred up to the age of 3 yrs; 35% of cases qualified on the basis of yearly questionnaire criteria alone. Children with no clinical or parental reported history of OM by the age of 3 yrs were classified as controls. Based on questionnaire data, 94% of the Raine Study participants self-identified their ethnicity as Caucasian. We used a subset of 831 Raine Study participants (256 cases and 575 controls) for whom both genome-wide data from an Illumina 660 W Quad Beadchip and complete epidemiological data for covariates were available [11]. Covariates included day care attendance at <3 years of age, allergy diagnosed at <3 years of age, and non-exclusive

breastfeeding from <6 months of age, all of which were significant risk factors for OM in the Raine Study cohort [11]. Recruitment to the Raine Study and all follow-ups were approved by the Human Ethics Committee at King Edward Memorial Hospital and/or PMH, with specific adult re-consent for DNA for those individuals participating in this study.

### DNA extraction

High molecular weight genomic DNA was extracted from a 2 mL saliva sample using the Oragene technology (DNA Genotek) as per manufacturer's instructions for the WAFSOM study. Extracted DNA was re-suspended in TE buffer, quantified by spectrophotometry, and stored at 50 ng/μl at -20C.

For the majority of Raine Study participants genomic DNA (gDNA) was extracted from whole blood collected at the 14- or 17-year follow-up via venipuncture utilising 4 mL $K_2$EDTA vacuum tubes. Extraction of DNA from whole blood was performed utilising Qiagen PureGene chemistry. For a small subset of individuals (~5%), gDNA was extracted from saliva using the Oragene technology (DNA Genotek) as per manufacturer's instructions. Samples were quantified using spectrophotometry, diluted to a normalised concentration with reduced EDTA TE buffer and stored at −80°C.

### Marker selection

For linkage analysis a minimum of four microsatellite markers [12] spanning each reported [6,7] region of linkage were identified. Marker names and primer sequences are provided in Additional file 1: Table S1. For 10q26.3, 19q13.43 and 3p25.3 the reported marker at the peak of linkage [6] was chosen in addition to four flanking markers. Association mapping across the 10q26.3 region was undertaken in the Raine Study cohort by extracting data for 10,185 SNPs (2,270 genotyped, 7,915 by imputation [13]) from cleaned [14], imputed [13] Illumina 660 W-Quad Beadchip data for 831 individuals (256 cases and 575 controls).

### Genotyping

Microsatellite PCRs were performed on WAFSOM DNAs using 15 ng genomic DNA, 1X PCR buffer, 2.6 mM $MgCl_2$, 0.45 mM dNTPs, 0.04 M betaine, 0.87 μM - 1.1 μM forward and reverse primers, and 0.25U of AmpliTaq Polymerase. Cycling was performed using a touchdown protocol [15]. Amplification products were pooled, fragments resolved using an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies, California) and alleles assigned via GeneMapper v4.1. All microsatellites were in Hardy-Weinberg equilibrium in genetically unrelated founders. Mendelian inconsistencies were identified using the Pedcheck software [16] and corrected or removed prior to analysis.

Genotype data for the Raine Study was generated at the Centre for Applied Genomics (Toronto, Canada) using an Illumina 660 W Quad Beadchip (Illumina, San Diego, California). Quality control (QC) and imputation for the Raine Study has previously been described [17]. Briefly, QC checks were performed for individuals and SNPs using PLINK [18]. Individuals were excluded based on gender mismatch, low genotyping rate (<97%), related to other participants or low level of heterozygosity. SNPs were excluded on the basis of deviation from Hardy-Weinberg Equilibrium (HWE $P < 5.7 \times 10^{-7}$), a genotype call rate <95%, or minor allele frequency <1%. For the chromosome 10q26.3 region, a total of 2,270 genotyped SNPs passed QC checks. Imputation was performed using MACH v1.0.16 with CEU samples from the HapMap Phase2 (Build 36, release 22) used as a reference panel and a default threshold of $r^2 > 0.3$. After imputation, 10,185 SNPs (2,270 genotyped, 7,915 imputed) were available for analysis.

### Linkage and association analysis

For microsatellite markers single-point and multi-point non-parametric linkage (NPL) analysis was carried out using all 101 multicase families with the $S_{all}$ scoring function in the program ALLEGRO [19] with genetic map distances obtained from the Rutgers Map v2 [12]. Conditional linkage analysis was also performed in ALLEGRO using only families showing evidence for linkage ($Z_{lr} > 0$) at a specified region.

Association mapping of the 10q26 region was performed using ProbABEL [20] for 10,185 SNPs in the region 127 Mb to qter. Power approximations [21] estimated for a disease prevalence of 0.4 show that the Raine Study has 86% power to detect associations at an alpha level of $P = 1 \times 10^{-5}$ with genotype relative risk (GRR) of 1.5 for SNPs with a MAF = 0.2 and 95% power at $P = 1 \times 10^{-4}$. Analysis was initially performed adjusting for population substructure (i.e. including the first two Principal Components; PCs), and then repeated, where appropriate, adjusting for PCs and independently associated covariates. If a strict Bonferroni correction was applied then the P-value required to correct for the number of SNPs analysed in the Raine Study data is $P = 4.9 \times 10^{-6}$ ($P = 0.05/10{,}185$). However, this is likely to be highly conservative due to the known presence of linkage disequilibrium (LD) in this region (i.e. 629 LD blocks across this 10q26 region, data not shown). Therefore, as this is a replicated linkage region [6,7], a relaxed threshold of $P < 10^{-3}$ was used to identify SNPs/genes of interest for follow-up. Logistic regression modelling for independent effects between pairs of SNPs was undertaken in R version 2.15.0 [22], and results presented as $P_{LRT}$ for the likelihood ratio test

comparison. Regional plots of association were created using LocusZoom [23] in which -$\log_{10}$(P values) were graphed against their chromosomal location. Pairwise LD patterns between all regional SNPs and the respective top SNP were calculated using the HapMap CEU population.

### Qualitative PCR expression

For qualitative RT-PCR, RNA was extracted from mononuclear cells isolated from 12 paired adenoid and tonsil samples collected with parent/guardian consent during adenoidectomy and/or tonsillectomy using TRI Reagent (Sigma-Aldrich) according to manufacturer's instructions [24]. Ethical approval for this part of the study was obtained from the Ethics Committee of PMH. RT-PCR was also performed on two respiratory epithelial and three macrophage cell lines with/without infection with otopathogens. All reagents were from Life Technologies, Australia unless otherwise stated. Adenocarcinomic alveolar basal epithelial cells (A549) were cultured in DMEM, 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100U/ml penicillin/100 μg/ml streptomycin. A pharyngeal carcinoma epithelial cell line (Detroit 562) was grown in MEM with Earle's salts, 10% FBS, 2 mM L-glutamine, 1X non-essential amino acids, 1 mM sodium pyruvate and 100U/ml penicillin/100 μg/ml streptomycin. Both the promonocytic U937 and monocytic THP-1 cell lines were grown in RPMI 1640, 10% FBS, 2 mM L-glutamine and 100U/ml penicillin/100 μg/ml streptomycin whilst the MonoMac 6 (MM6) mature monocytic cell line additionally required 1X non-essential amino acids and 9 μg/ml OPI bovine insulin (Sigma-Aldrich).

Cell lines were stimulated *in vitro* in 6-well plates with either non-typeable *Haemophilus influenzae* (NTHi) or *Streptoccocus pneumoniae* (SP) at a multiplicity of infection of 10:1. Bacterial cultures were kindly provided by Dr Lea-Ann Kirkham (School of Paediatrics and Child Health, University of Western Australia, Perth). Bacteria were streaked and incubated overnight at 37°C/5% CO2 on agar plates (blood for SP and chocolate for NTHi). A viable count of bacteria was performed prior to infection using a Helber bacterial counting chamber with Thoma ruling (ProScitech). At the 0 hr time-point, 1 ml of bacteria ($1 \times 10^8$ cfu/ml), or 1 ml of media for control samples, was added to $1 \times 10^7$ cells. After one hour media was replaced to remove non-adhered bacteria. Cells were harvested at 0, 1, 3, 6, 12 and 24 hours using TRI Reagent and RNA extracted accordingly.

A standardised 500 ng of extracted RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) as per manufacturer's instructions. For each cell line an RT negative reaction was used as a negative control. Primers were designed across exon-exon boundaries for four candidate genes (Additional file 1: Table S2) under the linkage peak and for the *OAZ1* housekeeping gene [25]. cDNA was amplified using a touchdown PCR protocol [15] in a volume of 11.5 μl consisting of 20 ng cDNA, a PCR master mix containing 0.85X PCR buffer, 2.6 mM $MgCl_2$, 0.45 mM dNTPs, 0.04 M betaine and 0.25 units of AmpliTaq Polymerase (Life Technologies) and 8.7 ng primer. Products were visualised on a 1.5% agarose gel stained with ethidium bromide.

### In silico comparative genomics

The presence of conserved non-coding sequences (CNS), which may harbor important regulatory elements, were examined across the *TCERG1L* and *PPP2R2D* genes (± 10 kb flanking sequence) plus the intervening intergenic region. Genomic sequences and associated annotation for human, mouse and rat were exported from Ensembl (Genome Reference Consortium Release 37, Ensembl Release 67) in FASTA and General Feature File (GFF) format, respectively. Global alignment of genomic sequences was performed in Multi-LAGAN [26,27] and the annotated alignment visualized in SynPlot [28]. CNS regions were defined as regions with a nucleotide sequence conservation level of ≥0.6 that had no associated annotation. To search for putative transcription factor binding sites (TFBS) at SNP locations we used AliBaba v2.1 [29], and MatInspector v8.0.5 [30] with a matrix similarity parameter >0.75. We also assessed selected polymorphisms (± 1000 bp flanking sequence) to determine whether they are located within a CpG island, defined using a criteria of C/G content >50%, ratio of observed to expected CpG >0.6 and length >200 bp. Repetitive elements were firstly masked using RepeatMasker [31] following which CpG islands were identified using CpGIsland Searcher [32] and CpGPlot [33]. Pairwise LD between polymorphisms in the Raine Study was calculated using Haploview 4.1 [34].

## Results

### Linkage analysis

Results of linkage analysis using the 101 multi-case families available in WAFSOM are presented in Table 1. Multi-point (peak $Z_{lr} = 2.69$; $P = 0.0036$ at D10S1770) and single-point ($Z_{lr} = 2.27$; $P = 0.012$ at D10S1770) NPL analysis provided evidence for linkage between markers at chromosome 10q26.3 and OM, contributed to by both the rAOM and COME phenotypes (Table 2). Analysis of Caucasian families alone did not reveal a substantial difference in single-point results at any region (data not shown); therefore, all multi-case families were used in subsequent analyses. Linkage analysis conditional on the linkage at 10q26.3 provided no evidence of epistatic interactions with 19q13.43 or 3p25.3 in this study population (data not shown), as previously reported [6]. Borderline evidence (multi-point $Z_{lr} = 1.65$; nominal $P = 0.05$) for replication of linkage was also observed at 10q22.3 whilst no

**Table 1 Results of multi-point and single-point non-parametric linkage analysis in the WAFSOM across chromosomal regions previously [6,7] linked to OM**

| Marker name | Genetic map location (cM) Marshfield | Physical location (Start; Mb) NCBI build 36 | Single-point $Z_{lr}$ | P value | Multi-point $Z_{lr}$ | P value | Information content |
|---|---|---|---|---|---|---|---|
| *Chromosome 3p25.3* | | | | | | | |
| D3S1304 | 22 | 6.89 | 0.00 | 0.500 | −0.93 | 0.175 | 0.88 |
| D3S3691 | 29 | 8.82 | −0.24 | 0.405 | −1.15 | 0.126 | 0.90 |
| D3S1597 | 30 | 9.34 | −0.76 | 0.225 | −1.04 | 0.149 | 0.92 |
| D3S1263 | 36 | 11.49 | −0.22 | 0.412 | −1.13 | 0.130 | 0.88 |
| D3S1259[a] | 37 | 12.07 | −0.14 | 0.444 | −0.87 | 0.192 | 0.76 |
| *Chromosome 10q22.3* | | | | | | | |
| D10S1730 | 99 | 78.60 | 1.56 | 0.060 | 1.48 | 0.070 | 0.87 |
| D10S206 | 98 | 79.45 | 1.88 | **0.031** | 1.65 | **0.050** | 0.83 |
| D10S1677 | 100 | 79.88 | 0.30 | 0.383 | 1.40 | 0.081 | 0.87 |
| D10S201 | 102 | 80.69 | 1.71 | **0.044** | 1.46 | 0.072 | 0.86 |
| *Chromosome 10q26.3* | | | | | | | |
| D10S1655 | 162 | 130.85 | 1.52 | 0.065 | 1.65 | **0.050** | 0.85 |
| D10S169 | 173 | 132.41 | 1.30 | 0.098 | 2.51 | **0.006** | 0.91 |
| D10S1770 | 169 | 132.57 | 2.27 | **0.012** | 2.69 | **0.004** | 0.92 |
| D10S212[a] | 171 | 134.29 | 1.66 | **0.048** | 2.42 | **0.008** | 0.87 |
| D10S1711 | 171 | 135.05 | 0.90 | 0.183 | 2.52 | **0.006** | 0.85 |
| *Chromosome 17q12* | | | | | | | |
| D17S1293 | 56 | 29.58 | 0.32 | 0.373 | 0.40 | 0.345 | 0.87 |
| D17S933 | 58 | 30.22 | 0.36 | 0.361 | 0.28 | 0.391 | 0.84 |
| D17S927 | 58 | 32.08 | 0.68 | 0.247 | 0.46 | 0.324 | 0.88 |
| D17S946 | 60 | 34.11 | 1.63 | 0.052 | 1.09 | 0.137 | 0.84 |
| *Chromosome 19q13.43* | | | | | | | |
| D19S572 | 89 | 58.80 | −0.23 | 0.409 | −0.22 | 0.413 | 0.81 |
| D19S210 | 100 | 61.71 | 0.01 | 0.500 | −0.85 | 0.198 | 0.94 |
| D19S887 | 100 | 62.33 | −0.60 | 0.274 | −0.13 | 0.447 | 0.96 |
| D19S254 | 101 | 62.36 | 0.19 | 0.425 | −0.22 | 0.414 | 0.95 |
| D19S214[a] | 101 | 62.47 | −1.85 | **0.032** | −0.44 | 0.330 | 0.95 |

[a]Marker at peak of linkage reported by Daly *et al.* [6].

evidence for linkage ($Z_{lr} < 1.65$; nominal $P > 0.05$) was observed at 3p25.3, 17q12 or 19q13.43.

### Association mapping of the 10q26.3 region in the Raine Study cohort

In our study, the peak of linkage at 10q26.3 is observed at 169 cM (Marshfield Genetic map)/132.57 Mb (NCBI Build 36), with evidence for linkage extending proximally to 130.85 Mb and distally to 135.05 Mb. Daly *et al.* [6] report clear evidence for linkage only at microsatellite marker D10S212, located at 171 cM/134.29 Mb, with no evidence for linkage ~126 Mb (Table 3). Although the disease locus in a linkage study might normally be found immediately under the peak of linkage [35], the true susceptibility gene can be displaced by up to 10 cM (~10 Mb) from the linkage peak, particularly in smaller samples [36,37]. Therefore, we extended our search for genes through association mapping over the region 127 Mb to 135.35 Mb (=qter).

For association mapping, genotype data from the region 127 Mb to 135.35 Mb on chromosome 10 was extracted from Illumina 660 W-Quad BeadChip data in 831 individuals (256 cases and 575 controls) from The Raine Study for whom full covariate data were available [11]. SNP locations on this Illumina chip are provided as NCBI Build 36 base pair locations. Data were available for 10,185 SNPs (2,270 genotyped, 7,915 by imputation). Logistic regression analysis under an additive model, controlling for population substructure using 2 PCs as covariates, provided evidence for several regions of association

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**Table 2 Results of multi-point and single-point non-parametric linkage analysis in the WAFSOM for the 10q26.3 region stratified by rAOM (85 families; 174 affected individuals) and COME (53 families; 115 affected individuals)**

| Marker name | rAOM | | | COME | | |
|---|---|---|---|---|---|---|
| | Multi-point $Z_{lr}$ | P value | Information content | Multi-point $Z_{lr}$ | P value | Information content |
| D10S1655 | 1.83 | **0.034** | 0.84 | 1.23 | 0.110 | 0.89 |
| D10S169 | 2.44 | **0.007** | 0.90 | 1.76 | **0.039** | 0.93 |
| D10S1770 | 2.63 | **0.004** | 0.91 | 2.00 | **0.023** | 0.95 |
| D10S212[a] | 2.20 | **0.014** | 0.86 | 1.76 | **0.039** | 0.91 |
| D10S1711 | 2.34 | **0.010** | 0.84 | 1.66 | **0.048** | 0.89 |

[a]Marker at peak of linkage reported by Daly *et al.* [6].

across the region 127 Mb to qter, with varying degrees of statistical support (Figure 1; Additional file 1: Table S3). In summary, individual top SNPs within genes/regions ($P < 10^{-3}$) are: rs7902734 intronic in *ADAM12* at 127.94 Mb ($P = 8.04 \times 10^{-4}$); rs9418832 intronic in *DOCK1* at 128.76 Mb ($P = 7.48 \times 10^{-5}$); rs2996081 intronic in *TCERG1L* at 132.86 Mb ($P = 9.15 \times 10^{-4}$) and rs7922424 intergenic between *TCERG1L* and *PPP2R2D* at 133.21 Mb ($P = 9.47 \times 10^{-6}$). Association signals across these genes/regions were robust to adjustment for independent environmental/clinical covariates (Additional file 1: Table S4).

### Testing for independent effects in genes across the chromosome 10q26 region

To evaluate whether the putative regions of association across the 127 Mb to qter region on chromosome 10q26 represented independent signals we repeated the single-point logistic regression analysis adjusting for the most significantly associated SNP (i.e. rs7922424; Figure 2). Upon adjusting for rs7922424 (Figure 2A), the significance at neighbouring SNPs within this intergenic region was lost indicating there is a single main effect across the *TCERG1L_PPP2R2D* intergenic region. However, improved significance was observed at *DOCK1* (top SNP rs9418832; $P = 4.77 \times 10^{-5}$), suggesting that these genes/regions may be independently associated with OM. All other signals were at $P > 10^{-3}$ after adjusting for rs7922424.

Given evidence of an independent effect at rs9418832 we also adjusted for this SNP (Figure 2B). When adjusting for rs9418832 significance was lost across the *DOCK1* region, again indicating a single main effect within this region, whilst improved significance was observed at rs7922424 ($P = 6.47 \times 10^{-6}$), *ADAM12* (top SNP rs7902734; $P = 2.05 \times 10^{-4}$) and *TCERG1L* (top SNP rs2996081; $P = 4.26 \times 10^{-4}$). The most significant association after adjusting for both rs7922424 and rs9418832 was observed at rs7902734 in *ADAM12* (Figure 2C; $P = 4.52 \times 10^{-4}$), indicating that *ADAM12* may also be independently associated with OM.

To formally test for independent effects between *ADAM12*, *DOCK1* and the *TCERG1L_PPP2R2D* intergenic region, we used forward stepwise logistic regression modelling. When comparing models in which the top SNPs are added to each of the other top SNPs (i.e. alternative 2-SNP model compared to the null 1-SNP model; Table 4) results show that they all add independent effects to each other ($P_{LRT} \leq 0.001$). Comparison of models containing all three SNPs compared to a null model containing only 2 of the top SNPs confirms these SNPs all add independent effects to each other ($P \leq 4.37 \times 10^{-4}$). Overall, the data suggest that variants in the intergenic region between *TCERG1L* and *PPP2R2D*, which lies at 133.2 Mb directly under the peak of linkage (Table 3), as well as in *DOCK1* (128.8 Mb) and *ADAM12* (127.9 Mb),

**Table 3 Comparison of results from the WAFSOM linkage analysis for chromosome 10q26 with those published by Daly *et al.* [6]**

| Marker name | Genetic map position (cM) Marshfield | Physical location (Start; Mb) build 36 | Spt $Z_{lr}$ | P Value | Mpt $Z_{lr}$ | P Value | Daly LOD (Spt) | Daly LOD (Mpt) | Nearest genes Proximal_Distal |
|---|---|---|---|---|---|---|---|---|---|
| D10S1656 | 149 | 126.09 | - | - | - | - | 0.0 | 0.0 | OAT |
| D10S217 | 158 | 129.54 | - | - | - | - | 0.2 | 0.2 | DOCK1_FOX12 |
| D10S1655 | 162 | 130.85 | 1.52 | 0.065 | 1.65 | **0.050** | - | - | PTPRE_MGMT |
| D10S1248 | 171 | 130.98 | - | - | - | - | 0.0 | 0.4 | MKI67_MGMT |
| D10S169 | 173 | 132.41 | 1.30 | 0.098 | 2.51 | **0.006** | - | - | GLRX3_TCERG1L |
| D10S1770 | 169 | 132.57 | 2.27 | **0.012** | 2.69 | **0.004** | - | - | GLRX3_TCERG1L |
| D10S212 | 171 | 134.29 | 1.66 | **0.048** | 2.42 | **0.008** | 3.78 | 1.64 | INPP5A |
| D10S1711 | 171 | 135.05 | 0.90 | 0.183 | 2.52 | **0.006** | - | - | PAOX_MTG1 |

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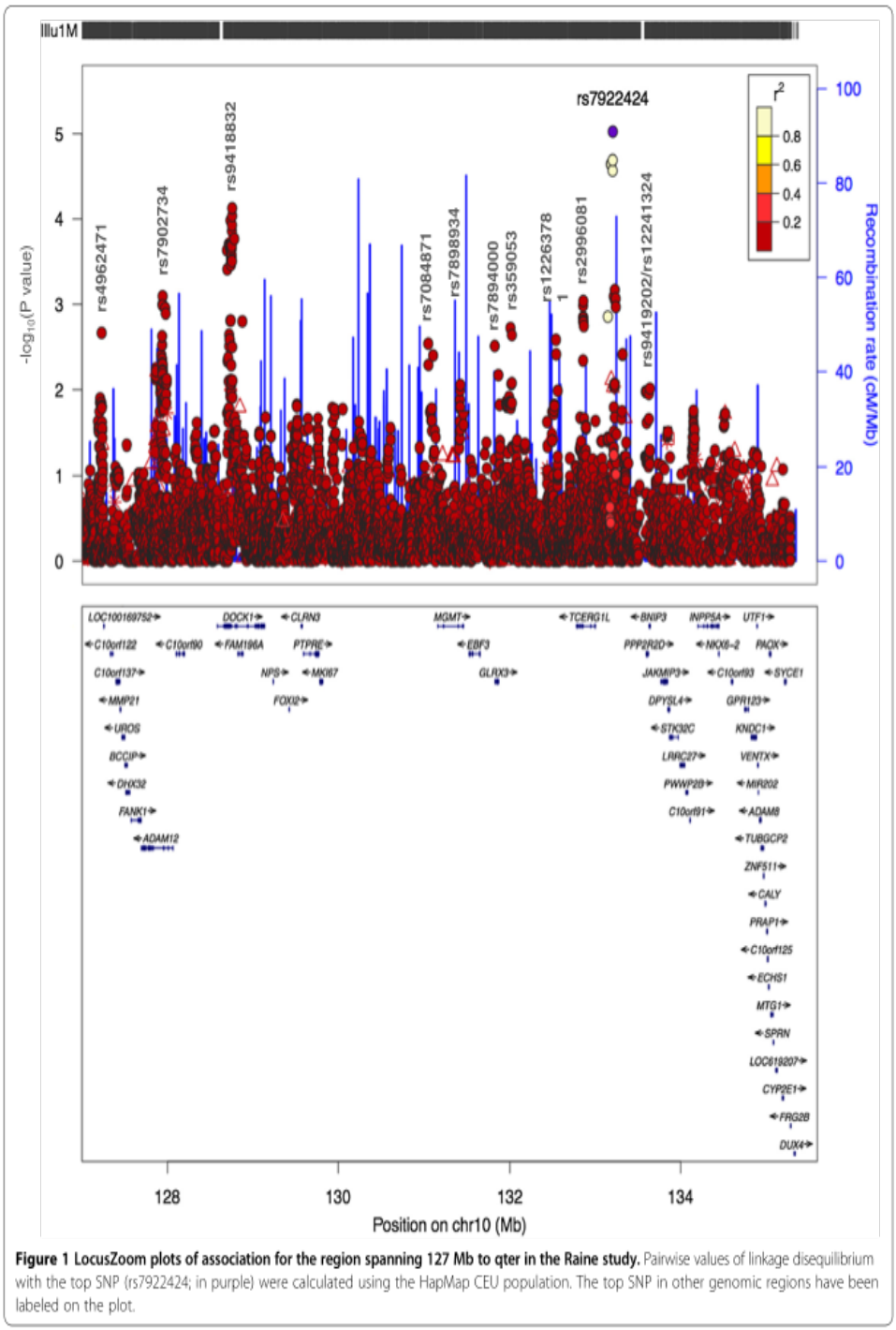


**Figure 1 LocusZoom plots of association for the region spanning 127 Mb to qter in the Raine study.** Pairwise values of linkage disequilibrium with the top SNP (rs7922424; in purple) were calculated using the HapMap CEU population. The top SNP in other genomic regions have been labeled on the plot.

may contribute independently to susceptibility to OM. Therefore, we took these genes/regions forward in evaluating further evidence for the putative etiological gene(s) for OM under the peak of linkage on chromosome 10q26.

**Expression analysis to support putative functional genes**

Information on the expression pattern of several genes in the 10q26 region is currently limited. Therefore, another way to evaluate potential candidacy of disease associated genes is to determine whether they are expressed in relevant

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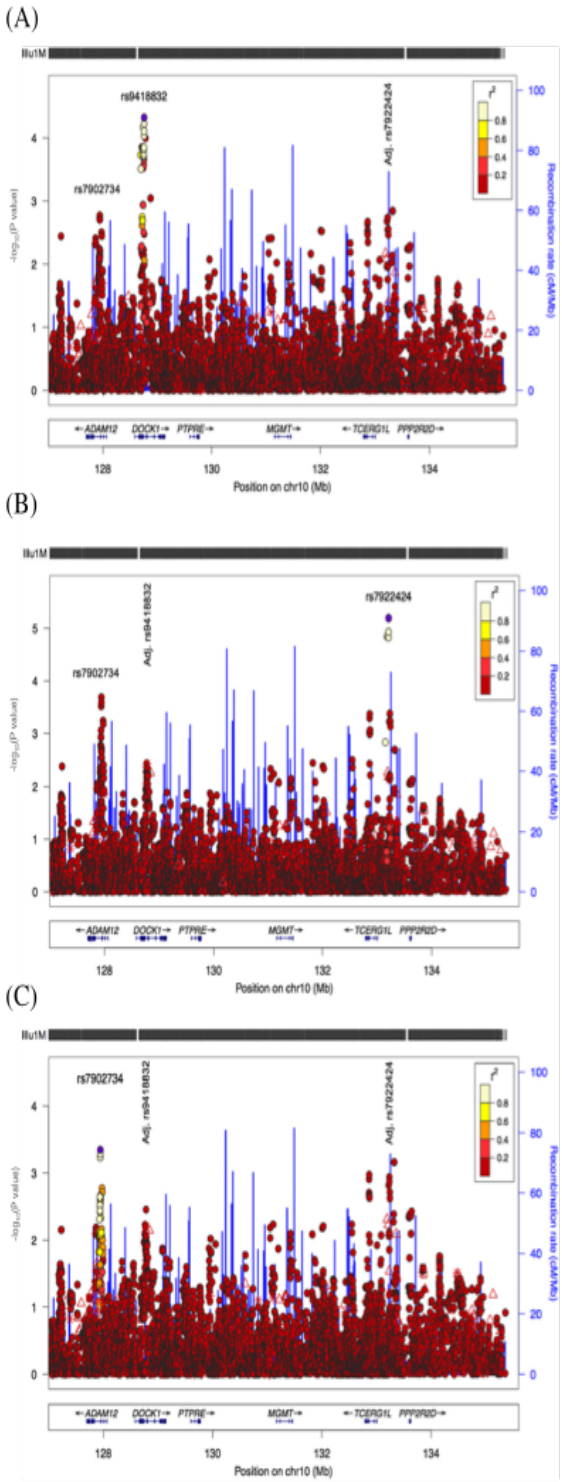


**Figure 2 LocusZoom plots of association adjusted for the top two SNPs, (A) rs7922424, (B) rs9418832 and (C) rs7922424 and rs9418832.** Significance is retained at each SNP after adjusting the analysis for the other. Pairwise values of linkage disequilibrium with the top SNP (in purple) were calculated using the HapMap CEU population.

**Table 4 Likelihood ratio tests (LRT) to determine independent effects across the 10q26.3 region**

| Null model | Alternative model[a] | LRT $\chi^2$ | $P_{LRT}$-value |
|---|---|---|---|
| | **Adding *ADAM12* SNP** | | |
| DOCK1/rs9418832 | rs9418832 + **rs7902734** | 13.89 | $1.94 \times 10^{-4}$ |
| Intergenic/rs7922424 | rs7922424 + **rs7902734** | 9.88 | 0.0017 |
| rs9418832 + rs7922424 | rs9418832 + rs7922424 + **rs7902734** | 12.37 | $4.37 \times 10^{-4}$ |
| | **Adding *DOCK1* SNP** | | |
| ADAM12/rs7902734 | rs7902734 + **rs9418832** | 18.12 | $2.07 \times 10^{-5}$ |
| Intergenic/rs7922424 | rs7922424 + **rs9418832** | 16.31 | $5.39 \times 10^{-5}$ |
| rs7902734 + rs7922424 | rs7902734 + rs7922424 + **rs9418832** | 18.80 | $1.45 \times 10^{-5}$ |
| | **Adding *TCERG1L_PPP2R2D* intergenic SNP** | | |
| ADAM12/rs7902734 | rs7902734 + **rs7922424** | 17.92 | $2.31 \times 10^{-5}$ |
| DOCK1/rs9418832 | rs9418832 + **rs7922424** | 20.11 | $7.30 \times 10^{-6}$ |
| rs7902734 + rs9418832 | rs7902734 + rs9418832 + **rs7922424** | 18.60 | $1.61 \times 10^{-5}$ |

[a]marker added to the null model in the alternative model is highlighted in bold.

tissue sites *ex vivo*, or in model culture systems *in vitro*. To address the former, we looked at expression of genes in paired adenoid and tonsil tissue from OM cases (rAOM, OME) compared to controls diagnosed with obstructive sleep disorder (OSD) or recurrent acute tonsillitis (RAT) with no concurrent OM history. The role of adenoids in OM pathogenesis is supported by a systematic review showing that adenoidectomy significantly improves resolution of MEE in OM [38] whereas tonsillectomy alone does not [39]. Figure 3A summarises the RT-PCR expression data across adenoid and tonsil samples and shows that *ADAM12*, *DOCK1*, *TCERG1L* and *PPP2R2D* are, for the most part, expressed in all adenoid samples irrespective of OM or OSD diagnosis. In tonsil samples, *ADAM12* and *DOCK1* are again expressed in all samples irrespective of diagnosis. However, *TCERG1L* and *PPP2R2D* expression is less consistent in tonsils with at least two samples showing no expression.

We also looked at epithelial (A549 & Detroit 562) and macrophage (U937, THP-1 & MM6) cell lines (Figure 3B). Again, *ADAM12* and *DOCK1* appear to be expressed in all five cell lines whilst *PPP2R2D* was seen in all cell lines except for MM6. Interestingly, *TCERG1L* was not expressed in epithelial or macrophage cell lines. *TCERG1L* expression was also not detected in any cell line following bacterial challenge with two common otopathogens (NTHi & SP; data not shown), suggesting that any role for this gene in OM would be independent of macrophage or epithelial cell inflammatory responses to otopathogens. It should be noted that this expression data is only qualitative. It is likely that quantitative RT-PCR data would add further information regarding expression levels at these candidates.

### In silico comparative genomics analysis of the TCERG1L to PPP2R2D region

While statistical and expression analyses do not formally exclude any of the genes showing association under the chromosome 10q26 linkage peak with OM, the statistical analyses favour the interval *TCERG1L* to *PPP2R2D* (Additional file 2: Figure S1), which lies directly under the peak of linkage for OM at chromosome 10q26, as the most likely candidate region carrying the etiological variants associated with OM susceptibility. Since the peak of association lies in the intergenic region between these two genes, we carried out an *in silico* analysis to determine whether the associated SNPs in this region lie in highly conserved non-coding sequences that could contain regulatory elements (Additional file 2: Figure S2). Of the 9 associated SNPs ($P < 0.001$) within the intergenic *TCERG1L* to *PPP2R2D* interval, three are located within highly conserved, non-coding regions (nucleotide conservation level (NCL) ≥ 0.6); rs7037834 (NCL = 0.61), rs7914323 (NCL = 0.61) and rs11813611 (NCL = 0.67). However, rs11813611 lies within a long interspersed nucleotide element (LINEs) repeat region and is unlikely to be functionally relevant. The top SNP, rs7922424, does not lie in a conserved non-coding region but is in strong LD with rs7087384 ($r^2 = 0.97$) and rs7914323 ($r^2 = 1.00$), both of which do fall in conserved non-coding regions (Additional file 2: Figure S3).

We also looked for the presence of transcription factor binding sites (TFBS) that may be co-located with rs7922424 (top SNP), rs7087384 or rs7914232 (within CNS regions). Results (Additional file 1: Table S5) indicate that rs7922424 lies within the consensus sequence for the Upstream Stimulatory Factor-1 (USF1) transcription factor with the binding site abolished in the presence of the

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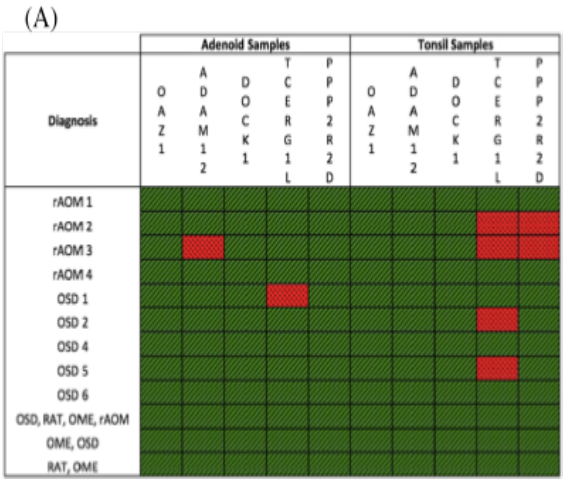


**Figure 3 RT-PCR expression of various candidate genes in (A) adenoids and tonsils of differing phenotypes and (B) in epithelial and macrophage cell lines.** OAZ1 is the housekeeping gene. A green diagonally striped box indicates expression was observed whilst a red dotted box indicates no expression was observed.

minor disease associated (A) allele. On the other hand, the minor disease associated allele (T) at rs7914323 potentially introduces a USF1 binding site. For rs7087384, the minor disease associated (A) allele disrupts a putative CREB/CREBP1 transcription factor binding site.

In addition to TFBS, we looked at the region containing rs7922424, rs7087384 and rs7914323 to determine whether these SNPs fall in regions identified as a CpG island and as such could disrupt biologically relevant DNA methylation at CpG motifs. Results from CpG Plot and CpG Island Searcher indicate that none of these polymorphisms falls at or near a CpG island (data not shown).

## Discussion

Genome-wide linkage analyses have highlighted five regions containing OM disease susceptibility loci on chromosomes 3p25.3, 10q22.3, 10q26.3, 17q12, and 19q13.43 [6-8]. Here, using a Western Australian cohort of children diagnosed with recurrent OM, we found evidence of replication of linkage at 10q26.3. We also found borderline evidence to support a region of linkage at 10q22.3 but did not find evidence for linkage at 3p25.3, 17q12, or 19q13.43, or for any region after conditioning on 10q26.3. Therefore, we focused attention on mapping the genes under the chromosome 10q26.3 linkage peak.

To identify the etiological gene/variant under the 10q26.3 peak, we performed fine-mapping using directed analysis of 2,270 genotyped SNPs (10,185 after imputation) spanning this region available on 256 cases and 575 controls within the Raine Study cohort. We focused our search in the region 127 Mb to qter, and found statistical support for variants in several genes/regions across this region contributing to the linkage peak. This is not an unusual phenomenon in complex diseases, where previous studies have also highlighted multiple genes contributing to peaks of linkage [40]. In this case, for the region of linkage at 10q26.3, at least 49 genes lie within the interval, a number of which could be

considered as potential functional candidates for OM susceptibility. Statistical support was observed for independent effects of SNPs at *ADAM12, DOCK1* and the intergenic region between *TCERG1L* and *PPP2R2D*.

The *ADAM12* (A Disintegrin and Metalloproteinase domain 12) gene is a member of the disintegrin and metalloproteinase (ADAM) family of proteins. This gene has been implicated in the epidermal growth factor receptor (EGFR) signalling pathway [41], which is upregulated in human middle ear epithelial cells in response to tobacco smoke exposure [42]. The ADAM12 protein also interacts with the muscle-specific α-actinin-2 protein, with function centred on myoblast/muscle development showing increased expression during muscle generation [43]. The DOCK1 (Dedicator of cytokinesis 1) protein has roles in phagocytosis of apoptotic cells in concert with ELMO1 during Rac signalling and cellular migration [44,45]. The *DOCK1* gene also has a role during embryogenesis in muscle development with knock-out mouse mutants having decreased skeletal and respiratory muscle tissues [46]. This suggests the apoptotic function of *DOCK1* could have a subtle role in the cell death of inflammatory factors and in apoptosis of mucous cells after an immune response to OM, whilst the ADAM12 protein interacts with pathways that could influence expression of inflammatory mediators within the middle ear.

The strongest statistical support for association was in the *TCERG1L* to *PPP2R2D* intergenic region, directly below the peak of linkage on chromosome 10q26.3. Very little is known about the function of the transcription elongation regulator like protein (*TCERG1L*) gene. In recent GWAS studies, variants at or near *TCERG1L* have been associated with fasting insulin, insulin resistance [47] and attention deficit disorder [48]. Hypermethylation of the *TCERG1L* promoter region leading to gene silencing has also been observed in colon cancer [49]. *TCERG1L* expression is documented [50] in a variety of tissues, including the brain, lung and eye. In this study, we have also demonstrated that *TCERG1L* is expressed in adenoids but not in macrophage or epithelial cell lines, either with or without otopathogen infection. Whilst expression in these cells may have been downregulated during immortalization, analogous to downregulation of this gene in cancer cells [49], the data suggest that any role *TCERG1L* may play in OM susceptibility is unlikely to occur through the innate inflammatory response to otopathogens. On balance, *TCERG1L* does not appear to be a strong candidate for OM susceptibility.

In contrast, *PPP2R2D* is a particularly interesting candidate gene for OM. *PPP2R2D* is a member of the B family of regulatory subunits of the protein phosphatase 2A (PP2A) and is widely expressed at the protein level in the brain, heart, placenta, skeletal muscle, testis and thymus [51]. This protein has a role as a modulator of the TGF-β/Activin/Nodal pathway [52], where knockdown of the protein was shown to increase nuclear accumulation and phosphorylation of Smad2. This involvement with Smad2 is of specific interest, as this gene and others in the TGFb pathway have previously been highlighted as candidate susceptibility genes for OM within the WAFSOM cohort [9]. Furthermore, a GWAS carried out in the Raine Study highlighted at least five other members of the TGFb pathway (*BMP5, GALNT13, NELL1, TGFB3* and *BPIFA1*) as candidates for OM susceptibility [11].

The strongest signal for association in our study lay within the intergenic region between the *TCERG1L* and *PPP2R2D* genes. Many association signals in complex diseases have been found to lie within intergenic regions [40], leading to a search for potential regulatory functions within those regions. Whilst the top SNP (rs7922424) in the intergenic region does not itself lie within a conserved non-coding region, it is in strong LD with rs7087384 and rs7914323, which do lie in highly conserved non-coding regions. In addition, the minor disease associated alleles at rs7922424 and rs7914323 alter putative binding sites for the upstream stimulatory factor 1 (USF1). The USF1 transcription factor is a member of the helix-loop-helix leucine zipper family and is ubiquitously expressed in a variety of cells [53]. The role of this protein is widespread, ranging from roles in embryonic development [54] to promoters for a number of activity-induced genes within neuronal nuclei [55] and as transcription factors that regulate cell-type dependent cellular proliferation [56]. However, our data favours rs7914323 as the most likely regulatory polymorphism in this region. Not only does this SNP lie within a conserved, non-coding region that could harbor regulatory elements, but our analysis shows that the presence of the minor allele potentially eliminates a TFBS for the cAMP response element binding (CREB) transcription factor and its binding protein (CREBP or CBP). This is interesting in the context of OM as the function of CBP has been linked to the TGFb pathway via the recruitment of EVI1 [57], which is mutated in a mouse model of OM [58], although the *EVI1* gene has not been associated with human susceptibility to OM to date [9,59].

Overall, analysis of conserved, non-coding regions and putative TFBS sites indicate a number of regulatory elements that lie within this intergenic region that can potentially be influenced by polymorphisms associated with OM susceptibility. It is not possible to determine from these data which gene these regulatory elements may influence. However, the TFBS consensus sequences disrupted by these polymorphisms all lie on the forward strand, upstream of the *PPP2R2D* promoter. The *TCERG1L* gene on the other hand is encoded on the reverse strand. Taken together these observations strengthen the evidence for *PPP2R2D* as the likely gene contributing to linkage at 10q26.3.

## Conclusions

Using the resources available within the family-based WAFSOM cohort the results of our study provide the first replication of linkage to OM susceptibility at chromosome 10q26.3. Subsequent association analysis using data available within the longitudinal Raine Study show that multiple genes could contribute to this linkage peak, however the weight of evidence supports *PPP2R2D* as the more likely functional candidate in this linkage region. It has previously been noted that there is phenotypic heterogeneity between WAFSOM and the Raine Study [11]. The phenotype defined for the Raine Study is biased towards the milder end of the OM spectrum, being based on yearly clinical examinations and parental reports of ≥3 AOM episodes during the first three years of life. The WAFSOM cohort on the other hand is biased towards the more severe end of the OM spectrum, being based on children recommended for grommet insertion. This suggests that *PPP2R2D* plays a role in susceptibility to OM *per se*. In summary our results point to a role for *PPP2R2D*, a TGF-β/Activin/Nodal pathway modulator, as the more likely functional candidate for OM susceptibility at chromosome 10q26.3. This contributes to the growing evidence for a role for the TGFb pathway in susceptibility to this important childhood disease.

## Additional files

**Additional file 1: Table S1.** Microsatellite markers and their respective primer sequences used for replication of previously reported linkage regions. **Table S2.** Forward and reverse primer sequences for qualitative RT-PCR expression of four chromosome 10 candidate genes. **Table S3.** Results for SNPs $P < 10^{-3}$ in the Raine Study across the region of chromosome 10 from 127 Mb to qter. **Table S4.** Raine Study results for the 10q26.3 region showing respective top SNPs for various genes/regions after adjusting for known environmental covariates using ProbABEL. **Table S5.** Details of transcription factor binding sites for top SNPs across the *TCERG1L/PPP2R2D* gene region. A transcription factor is shown only when a change in allele adds or removes a transcription factor binding site.

**Additional file 2: Figure S1.** LocusZoom plot of association (Figure 1) focused on the *TCERG1L/PPP2R2D* intergenic region. Pairwise values of LD with the top SNP (rs7922424; in purple) were calculated using the HapMap CEU population. **Figure S2.** Conserved non-coding sequence plot of the *TCERG1L/PPP2R2D* intergenic region containing the 9 associated SNPs ($P < 0.0001$). The top SNP (rs7922424) is annotated in orange, with SNPs $P < 0.0001$ in green and additional SNPs ($P < 0.001$) annotated in purple. Colours indicate the following regions; repeats (blue), genes (brown) and coding sequence (red). The three SNPs of interest are highlighted by bold text. Species are human (H), mouse (M), and rat (R). **Figure S3.** Pairwise LD between the 9 associated SNPs ($P < 0.0001$) in the Raine Study cohort, where (A) is the D′ value and (B) is the $r^2$ value. For both D′ and $r^2$, LD measures are indicated at the matrix intercept between two markers and are indicative of a decimal value. A square with no value indicated at the intercept equates to a value of 1.00 (or complete LD). For D′ measures, red and shades of red indicate a higher degree of confidence (i.e. LOD ≥2.0).

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

Conceived and designed the experiments: SEJ JMB MSR. Clinical care and characterization for WAFSOM: SV HLC. WAFSOM recruitment and preparation: ESHS SEJ MSR. Raine Study management: CEP. Adenoid/Tonsil collection and preparation: RT SV HLC. WAFSOM genotyping, cell culture, RNA preparation and RT-PCR expression: MSR. Design and maintenance of in-house genetic database for WAFSOM and bioinformatics CNS pipeline: RWF. Analyzed the data: MSR SEJ JMB. Supervised the work: SEJ JMB CEP. Wrote the paper: MSR SEJ JMB. Reviewed the manuscript. All authors read and approved the final manuscript.

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# CHAPTER 4

# TRANSLATIONAL STUDIES IN OTITIS MEDIA: CLINICAL TRIALS OF TREATMENTS AND SURGICAL INTERVENTIONS


| Paper no* | Supporting Articles | Thesis page no |
|---|---|---|
| 21 [69] - | Ototoxic eardrops and tympanic membrane perforations: time for a change? | 227 |
| 22 [71] - | Ototoxic ear drops with grommet and tympanic membrane perforations: a position statement. | 231 |
| 23 [79] - | A pilot study investigating basic fibroblast growth factor for the repair of chronic tympanic membrane perforations in pediatric patients. | 233 |


*Superscript numbers correspond to the designated general bibliography numbers – when cited in the text.

In this chapter, the author will describe studies conducted with his colleagues in interventions and treatments of middle ear diseases. These studies involve clinical trials using current and new drugs such as ciprofloxacin and Dornase alfa, respectively, and the implementation of a new surgical intervention to repair tympanic membrane perforations with the use of a genetically modified growth factor.

In the drug treatment approach, the ototoxicity study, which will be described next, helped revolutionise the way children received treatment for middle ear diseases and the clinical trial in Donase alfa has the potential to delivery a new drug to fight persistent bacterial infections related to OM.

With regards to new surgical interventions, the current trial in tissue engineered myringoplasty is investigating and validating several scaffolds in combination with basic fibroblast growth factor (b-FGF). The data of the validation trial together with a business plan will be used to develop a commercially available 'Smart Ear Fix'- Kit.

## *4.1 Clinical Trials - Drug Treatments in OM*

### 4.1.1 Ototoxicity

Ototoxicity is the permanent or temporary sensori-neural hearing loss and/or vestibular damage secondary to parenteral, oral or topical applications of medications or agents. In the case of children and adults with CSOM or ventilation tubes *in situ*, the application of potentially ototoxic ear drops, particularly aminoglycosides, can lead to absorption through the thin round

window membrane into the inner ear structures. This results in outer hair cell damage or vestibular organ injury, often permanently affecting hearing and balance function. Although believed to be uncommon, of the order of 1:1000 to 1:10,000 patients treated with potentially ototoxic ear drops, no reliable data on the ototoxic effects of prolonged use of these drops in patients with tympanic membrane perforations are available.

In the 1990's, the indicated treatment for otorrhoea in CSOM, according to the Central Australian Rural Practitioners Association (CARPA) Standard Treatment Manual (1993) was the use of ear toilet and topical antibiotics with Sofradex® ear drops for up to 16 weeks. Sofradex® is composed of dexamethasone, gramicidin and framycetin (also known as neomycin B), a significant ototoxic chemical. Ototoxicity is of special concern when treating patients with perforated tympanic membranes (as seen in CSOM or in the presence of ventilation tubes) with potentially ototoxic ear drops. At that time, little was known regarding the effects of these potentially ototoxic ear drops, and in cases where a sensori-neural hearing loss was detected, it was blamed on the underlying CSOM. It was not until 1998, when topical fluoroquinolone ear drops were introduced in the United States, offering an ototoxic risk-free option for treatment, that the current treatment with Sofradex® was re-evaluated.

In the years to follow (2002 to 2008) the author, as an Australian representative, attended a series of annual meetings of an expert committee on ear drops and middle ear disease (ENT Extensions of Excellence (EEE)) that brought together representatives from different parts of the world to discuss and evaluate the use of topical antibiotics in ear infections. Concomitantly, the author also participated in

a community-based, multicentre, double-blind randomised controlled trial ran by the National Aboriginal Community Controlled Health Organisation (NACCHO) that compared the effectiveness of different ototopical antibiotics for CSOM in Aboriginal children [11].

In this multicentre study involving 147 Aboriginal children from eight Aboriginal Communities across northern Australia, the author and colleagues compared the tympanic membrane perforation size and status prior and post-treatment with ototopical antibiotics including ciprofloxacin 0.3% (Ciloxan) and framycetin 0.5%, gramicidin and dexamethasone ear drops (Sofradex®). There was a significantly higher rate of cure in the group of children treated with ciprofloxacin ear drops compared with the group of children treated with the potentially ototoxic Sofradex® ear drops. This study was awarded the Wyeth Prize by the Medical Journal of Australia for best research in 2003 [11].

The author published a paper and letter in the Journal of Paediatric and Child Health [69,70] advocating a change from the use of potentially ototoxic ear drops to the non-ototoxic alternative (ciprofloxacin) in children suffering from CSOM or ventilation tube infections. The author advocated to the Australian Society of Otolaryngology, Head and Neck Surgery (ASOHNS) a position statement similar in scope to the 2004 American Academy of Otolaryngology, Head and Neck Surgery consensus panel recommendations, regarding potentially ototoxic antibiotics for middle ear use, resulting in ASOHNS publishing a position statement on ototoxic ear drops with ventilations tubes and tympanic membrane perforations in the Medical Journal of Australia [71].

In a subsequent article in the Australian Prescriber [72] the author and his colleagues suggested that healthcare providers in Aboriginal Communities should be given

special access to provide ototopical ciprofloxacin as first-line treatment for Aboriginal and Torres Strait Islander children with CSOM. Subsequently, changes to the Pharmaceutical Benefits Scheme (PBS) to make ciprofloxacin ear drops available to all children with otorrhoea from CSOM or ventilation tube infections occurred. Consequently, the use of non-ototoxic ear drops in children with CSOM and ventilation tube infections is now widespread best practice in Australia.

### 4.1.2 Dornase alfa Trial

There may be a future role for Dornase alfa in CSOM as well, reducing the mucosal bacterial biofilm along with mechanical and topical antibiotic therapy.

Clinical correlation of the MEE viscosity at surgery with the future need of repeat VTIs in children with OM has been undertaken in the author's practice with a 75% need for subsequent VTIs when the middle ear fluid is classified as grade 3 or grade 4 out of 4 (having a consistence akin to commercial glue). Another future possible treatment for Dornase alfa is in the severe rhinitis in the Aboriginal children with chronic bacterial adenoiditis and rhinitis.

## *4.2 Clinical Trials - Surgical Interventions in OM*

### 4.2.1 Conventional Myringoplasty

There are an estimated 60,263 Australian children under the age of 15 with CSOM, principally Aboriginal children. CSOM or chronic perforation of the tympanic membrane is the most severe of the sequelae of OM, resulting in intermittent or chronic otorrhoea, hearing loss and increased risk of cholesteatoma formation. In some communities, especially in remote regions of Western

Australia and the Northern Territory, chronic perforations of the tympanic membrane exceed 30% and can reach up to 70%.

In a Western Australian study, from 423 major ear surgeries there was a cholesteatoma rate of 10% over the 10-year period of the study [73]. In addition, more serious intracranial complications of CSOM, including meningitis, brain abscess and sigmoid sinus thrombosis continue to be a cause of morbidity and occasionally mortality in Aboriginal children. CSOM is associated typically with a 30dB hearing loss, unless there is a ossicular damage, when it will be greater [41].

In Indigenous children, as in non-Indigenous children, the main reason to close the tympanic membrane perforation is to provide better hearing, less risk of complications such as cholesteatoma, and to allow swimming without otorrhoea. Myringoplasty is the surgery of choice, utilising temporalis fascia, perichondrium or cartilage as the graft material. The success rate of myringoplasty in Aboriginal children is less than 64%, well below the 85% non-Aboriginal patients' rate. This myringoplasty failure is due to various factors, including poor nutrition, post-operative infections and loss to follow-up medical care. Conventional myringoplasty takes 30 to 90 minutes of operating time per patient.

The first large scale, prospective study of Aboriginal children undergoing myringoplasty was conducted in 2000-2001 in four rural and remote Western Australian regions with a 78% follow-up [74]. Our success rate of myringoplasty was 49% as measured by the presence of an intact tympanic membrane and improved hearing post-operatively. Full or partial closure of the tympanic membrane in 72% of the patients resulted in improved hearing in 71% of the children.

The author also participated in the Northern Territory "closing the gap" intervention in 2009 – 2010 with improved surgical techniques utilising pre-operative betadine ear washouts, intra-operative intramuscular ceftriaxone and middle ear topical ciprofloxacin ear drops [75]. The results of closure in a large series (198 cases) were 64% (personal communication Dr. Hemi Patel, 2014).

### 4.2.2 Tissue Engineered Myringoplasty

The search for an ideal method for tympanic membrane perforation that is more rapid, simple and reproducible, and that heals the tympanic membranes in three layers is still ongoing. In 2009, Kanemaru et al., displayed their initial results with a tissue engineered myringoplasty utilising basic fibroblast growth factor (b-FGF) and a gelatin foam (**Figure 4**) scaffold in 53 patients [76]. This study reported a success rate in tympanic membrane closure of 98% in the b-FGF compared with 10% in the saline control group. Although the children in the study required general anesthetic, the adults could have the procedure performed as outpatients under local anesthetic with operative time of less than 10 minutes.

Our group decided to replicate the Kanemaru research utilising initially b-FGF and gelatin foam [12,77] and subsequently trialing three other modalities (no-scaffold, collagen scaffold and silk scaffold - **Figure 5**) [13,78]. This is the first large scale trial of tissue engineered myringoplasty in the English speaking world, and to date, 40 patients have had tissue myringoplasties utilising the genetically engineered b-FGF[79].

**Figure 4:** Kanemaru Technique, utilising b-FGF and gelatin foam.

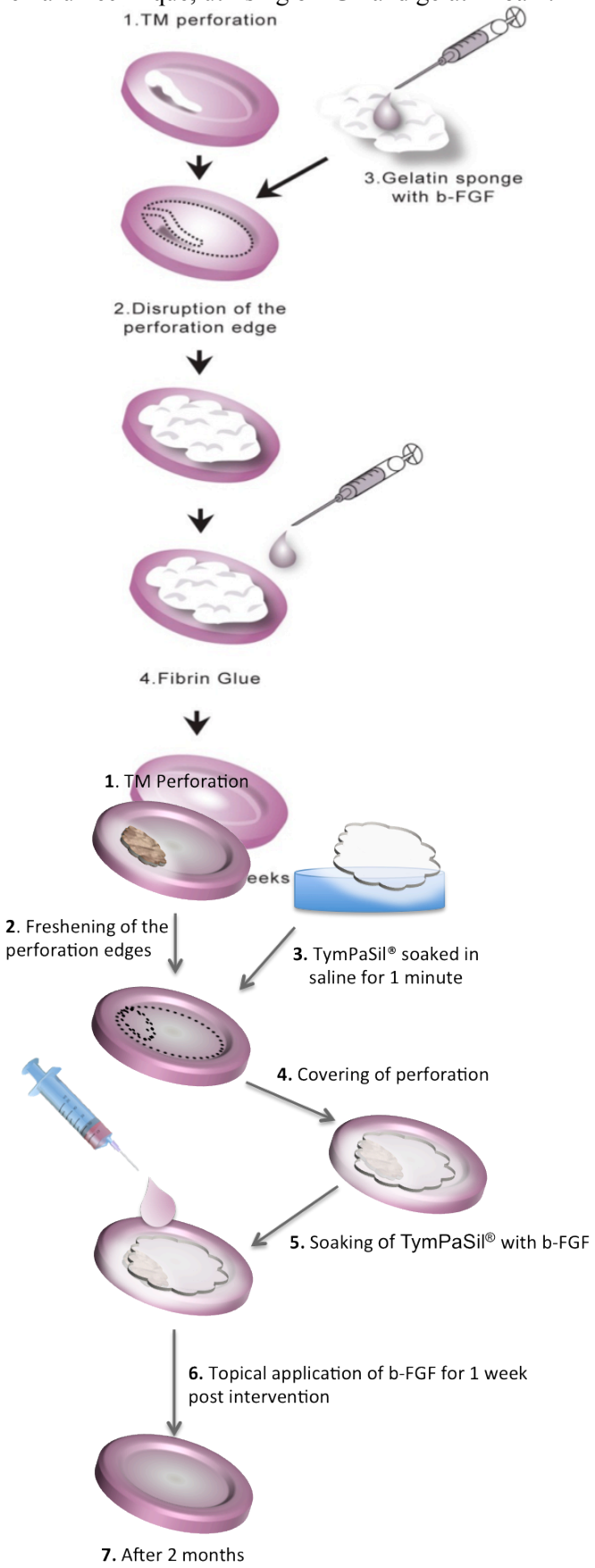


**Figure 5:** Kanemaru Technique, adapted, utilising b-FGF + gelatin foam + Silk Scaffold.

The initial results of tissue engineered myringoplasty studied in 40 patients (28 adults, 12 children) was awarded a poster prize at the July 2014 Garnett Passe and Rodney Williams Memorial Foundation Frontiers Meeting in Melbourne. The success rate in this study cohort was 83% in adults and 70% in children with extra procedures required in children secondary to water contamination and more frequent post-operative infections. The average cost was AUD$ 220 per procedure, a thousand dollars cheaper than for conventional myringoplasty. The operating time in our series was well less than 10 minutes, a significant saving when compared to the up to 90 minutes for the conventional myringoplasty. The procedure involves no incision, much less morbidity, no obvious oncogenic or cholesteatoma sequelae, and it is a relatively simple and quick procedure.

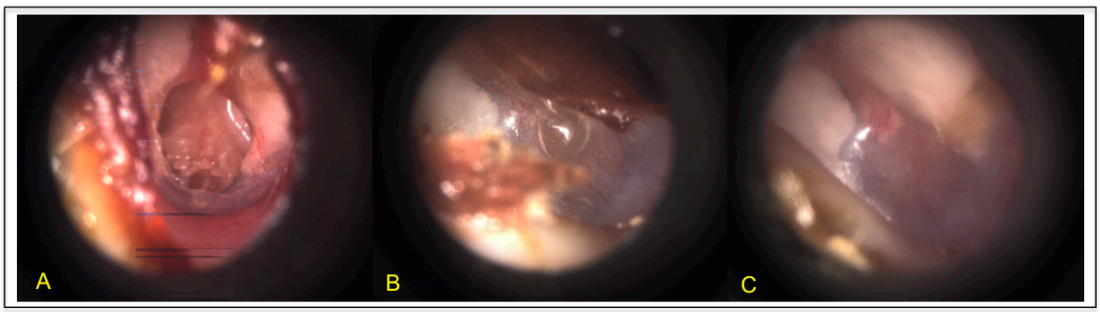


**Figure 6:** Results of a procedure utilising b-FGF + gelatin foam + Silk Scaffold. A)Pre-operatively, B) Two weeks post-operatively, C) Two months post-operatively.

The potential to reduce the long surgical waiting time for myringoplasty in Aboriginal children with CSOM in Western Australia is significant and more extensive surgical trials are planned.

To quote the editor of the Journal Otology & Neuro-otology, Dr. Robert Jackler from Stanford University: “A regenerative method of tympanic membrane repair could be the greatest advance in otology since the cochlear implant”.

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Annotation

# Ototoxic eardrops and tympanic membrane perforations: Time for a change?

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**Abstract:** Until recently the only available antibiotic eardrops for treatment of the discharging middle ear and mastoid cavity have been potentially ototoxic. With the advent of non-ototoxic fluoroquinolone eardrops, consensus panels in the USA, Canada and the UK have advocated the preferential use of these agents in the open middle ear. However, in Australia, no fluoroquinolone topical agent is approved for use with tympanic membrane perforations, and when used as an 'off label' eardrop, none is on the Pharmaceutical Benefits Scheme. This creates an ethical dilemma, particularly with best practice management of chronic suppurative otitis media in indigenous children. Despite concerns regarding resistance issues with ototopical use of systemic antibiotics, bacterial resistance has not been documented in major studies. For equity and ethical reasons, Australian regulatory authorities should consider approving a sterile non-ototoxic eardrop for use in the open middle ear.

**Key words:** aminoglycosides; eardrops; fluoroquinolones; otorrhoea; ototoxicity.

Over the last decade there has been significant and increasing awareness of the potential dangers of ototoxicity with aminoglycoside eardrops being prescribed for middle ear conditions with perforated tympanic membranes or ventilation tubes *in situ*. Although the incidence of aminoglycoside ototoxicity with eardrops is uncommon, of the order of 1:1000 to 1:10 000, the effects on the patient in terms of cochlear or vestibular damage may be significant.[1] Neomycin, gentamicin, framycetin (Neomycin B) and tobramycin, are cochleotoxic while gentamicin is more vestibulotoxic than tobramycin.[2] Ototoxic damage to the vestibule and cochlear have been noted in ototopical treatment of chronic suppurative otitis media[3–7] including neomycin-based eardrops.[8–10] In one Canadian study, 16 patients prescribed ototoxic eardrops developed cochlear and/or vestibular symptoms, with a number eventually being unable to work or being confined to a wheelchair secondary to labyrinthine symptoms. Local factors,[11] which may aggravate or ameliorate ototoxicity in the presence of a tympanic membrane perforation include: (i) size and site of the perforations; (ii) presence of thickened mucous, otorrhoea, or granulation tissue may protect the round window membrane from drops; (iii) patent eustachian tube allowing nasopharyngeal passage of drops; (iv) toxic effects of the middle ear infection by exotoxins; (v) increased thickness of the human round window membrane or the presence of webs or adhesions in the round window area; (vi) individual susceptibility to aminoglycoside ototoxicity; and (vii) patient compliance and duration of treatment.

With over a million courses of potentially ototoxic eardrops being used annually, and a significant number of these for chronic suppurative otitis media (CSOM) and infected grommets there is significant concern about potential ototoxicity.[12–16] In Australia, there is widespread use of potentially ototoxic eardrops (Table 1), accounting for 90% of ototopical prescriptions. Inevitably some patients will have tympanic membrane perforations, either overt or associated with otorrhoea (as in acute otitis media with perforation), and it is this group, especially those Aboriginal and Torres Strait Islander children with their heavy burden of CSOM, that is at risk for ototoxicity. The true incidence of vestibular or cochlear ototoxicity in Aboriginal and Torres Strait Islander children with prolonged treatment with potentially ototoxic eardrops may never be established as they seldom have pre- and post-treatment audiograms and vestibular testing in these children is not practical or advisable. Thus any hearing loss which may develop, may well be attributed to the underlying disease rather than the ototoxic medications prescribed. Medicolegal cases involving ototoxicity from eardrops treatment are on the increase in the USA, Canada and Australia.

The product information for the most commonly prescribed aminoglycoside antibiotic, framycetin sulphate gramicidin dexamethasone drops (Otodex/Sofradex) mentions contraindications for use in the patient with tympanic membrane perforation but not patent grommet. The other commonly prescribed otic drop triamcinolone acetonide neomycin, gramicidin, nystatin (Kenacomb Otic) has a contraindication for the treatment of otitis media or in the presence of a perforated tympanic membrane. In the case of ciprofloxacin hydrochloride/hydrocortisone eardrops (Ciproxin HC Ear Drops), the product information states that Ciproxin HC Ear Drops are contraindicated in patients with known or suspected perforation or where there is a risk of perforation of the tympanic membrane.[17] Similarly, the Antibiotic Guidelines[18] advocate the use of dexamethasone framycetin gramicidin drops if there is a chronic discharging otitis media associated with or without grommets for more than 6 weeks. It also comments that topical quinolones are not advised because of the limited evidence of efficacy in adults and the lack of studies in children and the high risk of selecting for resistance strains. There are specific guidelines for Aboriginal and Torres Strait Islander communities[19] and these also refer to topical aminoglycosides as first-line treatment for CSOM with fluoroquinolones as secondary management if the ear remains wet following aminoglycoside eardrop therapy.

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**Table 1** Potentially ototoxic components of eardrops used in Australia

| | |
|---|---|
| Antibiotics | |
| Chloramphenico | Chloromycetin eardrops |
| Framycetin (Neomycin B) | Sofradex, Otodex eardrops |
| Gramicidin | Sofradex, Otodex, Kenacomb Otic drops |
| Neomycin | Kenacomb Otic drops |
| Solvents | |
| Propylene glycol | |

Current prescribing statistics for antibiotic eardrops (Table 2) over a 12-month period show that in the 12-month period up to and including November 2004 there were just over 1.4 million eardrop units sold with almost 94% being for potentially ototoxic products. The table shows the individual units sold per month for the previous 12 months. This indicates that, as otitis externa is seasonal to a larger extent than chronic suppurative otitis media, if these products were not used in CSOM, you would expect to see a larger dip in the winter months.[20] There have been many studies in Australia showing that Aboriginal and Torres Strait Islander children have a heavy burden of chronic suppurative otitis media and the incidence of chronic suppurative otitis media in different aboriginal communities varies from 11 to 43%.[21–29]

The alternative lies with the recently developed fluoroquinolone group of eye and eardrops with a paradigm shift in safety, formulation and efficacy of ototopical therapy. They have been shown to be non-ototoxic in animal studies, and have few documented side-effects in humans with open middle ears. These antibiotic eardrops have broad activity against both Gram-positive and Gram-negative organisms especially Pseudomonas aeruginosa. In CSOM management the Cochrane review[30] found ototopical fluoroquinolones the most effective, a finding confirmed by the National Aboriginal Community Controlled Health Organization (NACCHO) randomized double-blind controlled trial in Australia, comparing ciprofloxacin eyedrops used as an eardrop with Sofradex (Neomycin B, gramicidin and dexamethasone) eardrops.[31] The clinical cure rate among the ciprofloxacin group was significantly higher with no evidence of ciprofloxacin resistance being noted.[31] In Australia, however, topical fluoroquinolones are approved for use only in the eye or external ear.

## GUIDELINES FOR EARDROP USE WITH TYMPANIC MEMBRANE (TM) PERFORATIONS

British,[32] Canadian[33] and most recently American[34] expert panels have suggested clinical practice guidelines. The British article[32] states 'doctors are reminded that topical treatment with aminoglycosides is contraindicated in patients with a tympanic membrane perforation'. The Canadian anti-infection review panel (2001) noted in the presence of a tympanic membrane defect, the risk of ototoxicity (primarily vestibular) with topical aminoglycosides increases with duration of therapy beyond 7 days or when the medication is placed into dry middle ear spaces. Hence these preparations should be limited to no more than 7 days (or stopped before 7 days if the discharge ceases) and should never be used in a dry middle ear with a perforation. The expert consensus panel of the American Academy of Otolaryngology – Head and Neck Surgery (AAOHNS) into efficacy and safety of topical antibiotics in the treatment of ear disease, recently produced clear, relevant and well-researched guidelines using current best evidence. The panel addressed whether potentially ototoxic eardrops should be used in open ears and mastoid cavities, the limits if they are used, the risks of ototoxicity and the efficacy of, and resistance issues with ototopical quinolones. Their recommendations were as follows:

1 Where possible use topical antibiotic products free of potential ototoxicity if the middle ear or mastoid are open. Evidence indicates that potentially ototoxic agents offer no advantage over non-ototoxic agents.
2 If potentially ototoxic products are prescribed, they should only be used in infected ears and should be discontinued shortly after the infection has resolved.
3 Patients or parents should be warned of the risk of ototoxicity if a potentially ototoxic product is prescribed when the middle ear or mastoid are open.
4 Patients should be warned of potential complications and to see their doctor if dizziness or vertigo, hearing loss or tinnitus occurs during or soon after the use of potentially ototoxic topical products.
5 There is *no* evidence that ototoxic eardrops cause any injury to the auditory or vestibular systems if the tympanic membrane is intact.[33]

The Australian Society of Otolaryngology – Head and Neck Surgery (ASOHNS) has recently formed a subcommittee of their pharmaceutical committee to develop guidelines for Australia on treatment with antibiotic eardrops and non-intact TM.

## AUSTRALIAN DILEMMA

With strong evidence-based guidelines arguing against the use of aminoglycoside eardrops in the open ear, increasing litigation and no topical fluoroquinolones approved for use in the middle ear, Australian doctors are in a difficult ethical situation regarding management of the draining middle ear. Either they prescribe 'at risk', avoid eardrops altogether or use one of the available fluoroquinolone drops 'off label'. However, the fluoroquinolone eye and eardrops used 'off label' as a private prescription are relatively expensive, particularly for use in underprivileged and indigenous children with CSOM who might require prolonged or frequent treatment. Currently available fluoroquinolone topical drops include two sterile eyedrops (ciprofloxacin and ofloxacin), which are sterile preparations suitable for use in the open middle ear. A non-sterile ciprofloxacin hydrocortisone (Ciproxin HC) eardrop approved

**Table 2** Prescribing statistics for antibiotic eardrop units over a 12-month period in Australia

| | December 2003 | January 2004 | February 2004 | March 2004 | April 2004 | May 2004 | June 2004 | July 2004 | August 2004 | September 2004 | October 2004 | November 2004 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ototoxic eardrops | 138 461 | 135 746 | 125 828 | 115 539 | 85 792 | 106 618 | 87 370 | 98 784 | 103 972 | 102 813 | 102 839 | 114 542 | 1 318 504 |
| Non-ototoxic eardrops | 7117 | 11 809 | 11 887 | 10 438 | 6572 | 5523 | 4819 | 5117 | 5098 | 5412 | 5808 | 7380 | 86 980 |
| Total | 145 578 | 147 555 | 137 715 | 125 977 | 92 364 | 112 141 | 92 189 | 103 901 | 109 070 | 108 225 | 108 847 | 121 922 | 1 405 484 |

for use in the external ear only has the disadvantage of stinging when placed in the middle ear canal and also may leave a white precipitate from the excipient.

## RESISTANCE ISSUES

In addition, there are concerns about resistance issues using a powerful systemic antibiotic agent as a topical drop. The potential risk for selecting of the emergence of resistance organisms at body sites distal to the ear infection is a theoretical issue in topical therapy. It certainly has been well-documented in systemic therapy that low concentrations of antibiotics present for relatively long periods of time throughout the body can cause the development of resistant organisms. However, only in some patients for a short period of time would orally administered ciprofloxacin pass through the eustachian tube to the pharynx and its transit time in the nasopharynx and pharynx would be measured in seconds or minutes rather than hours. So it is difficult to imagine how this short exposure time would provide a selective pressure environment sufficient to permit the emergence of resistant isolates. Klein[35] noted the chance of bacteriologic resistance developing in the upper aerodigestive tract was exceedingly rare because of the poor systemic absorption and the lack of selective pressure for developing resistant organisms in the upper airway. Resistance concerns at the middle ear site are essentially non-existent because[36–39] overwhelming concentrations of ciprofloxacin are applied directly to the infection. In a study of topical therapy treatment failures in children with acute otitis media with otorrhoea draining through a tympanostomy tube, 800 children with 1064 bacteria isolates were treated with ototopical ciprofloxacin with 68 treatment failure patients. None of the pretherapy isolates from these 68 patients were resistant to ciprofloxacin and there was no change in the susceptibility profile of any of the treatment failure isolates compared with the corresponding isolate from the initial visit. The authors concluded that the short course of topical therapy did not promote the development of resistance in the pretherapy pathogens.[40] There have been no definitive studies which showed significant bacterial resistance with ototopical fluoroquinolones. Systemic absorption of ototopical fluoroquinolones is negligible.[41,42] The NACCHO trial[31] examined ear discharge specimens for evidence of bacterial resistance to the ciprofloxacin agent used with no resistant organisms found. The AAOHNS committee examined the literature on bacterial resistance to ototopical antibiotic treatment with overall grade B evidence of no significant resistance development. Multiple international studies have shown that resistance rarely develops in ear isolates and failure of treatment has generally occurred because of persistence of original bacteria (without being resistant) or because of superinfection (of bacteria that were not resistant) as shown in the NACCHO study.[36–39]

## WHAT IS THE NEXT STEP?

Following the review by the consensus panel of ASOHNS, if the findings are in agreement with those of the USA, UK and Canada, interested groups should advocate the following:

1 Application to the Therapeutic Goods Administration (TGA) for approval of sterile fluoroquinolone eardrops for instillation into the open middle ear. This would include a sterile fluoroquinolone steroid eardrop (Ciprodex) approved in the USA for use in the middle ear, as there is evidence of facilitation of resolution of inflammation and granulation tissue with topical dexamethasone.[43]
2 Application for inclusion of these non-ototoxic eardrops on the Pharmaceutical Benefits Scheme to allow for equality and ethical issues in the populations with reduced access to these drops currently. (It is interesting to note that following the NACCHO study, several public health regions in Western Australia (Goldfields and Mid-West) have introduced ototopical fluoroquinolones as first-line treatment in CSOM in indigenous children.)
3 Continuation of research into best practice management of CSOM and ventilation tube otorrhoea, in particular with respect to duration of therapy and antibiotic resistance issues.
4 Encourage medical defence organizations to inform the medical profession of the circumstances under which they will indemnify them if topical aminoglycosides are to be used.
5 Encourage the Therapeutic Guidelines committee to revise the recommendation pertaining to management of CSOM. (The Antibiotic Therapeutic Guideline recommends Sofradex as first-line treatment for CSOM despite being contraindicated and not approved for use in the middle ear by the TGA or PBS.)

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## Finger fracture mitral valvuloplasty: a tribute to the pioneers of cardiac surgery

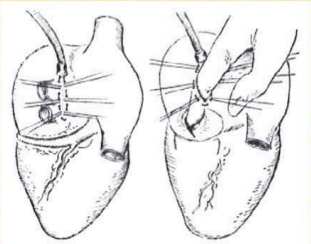
John S Murala, Hugh D Wolfenden, George S Youssef and Daniel Friedman

**To the Editor:** We report an exceptional case of a woman who underwent emergency "finger fracture valvuloplasty" (FFV) in 1954 to treat rheumatic mitral stenosis and required no further surgical intervention for 51 years.

The woman presented in 1954 with pulmonary oedema due to mitral stenosis during the first trimester of her second pregnancy. She underwent FFV at Lewisham Hospital in Sydney. She had a prolonged convalescent period, was discharged after 5 months, and delivered a healthy child. She was one of two pregnant patients reported in the *Medical Journal of Australia* by Hall and Windsor.[1]

She remained well and active until 2005, when she presented with New York Heart Association Class III symptoms of dyspnoea on exertion, and ultimately underwent mitral valve replacement that year. She made a good recovery postoperatively and remains well.

In the 1920s, 10 patients with mitral valve stenosis were treated surgically.[2] In 1923, Cutler and associates from Boston operated on seven patients using a cardiovalvulotome (through the left ventricle) and, in the same year, Duff and Evarts from Washington used a cardioscope (through the left atrium) on one patient. In 1925, Soutter from London and Pribram from Germany used a "finger fracture method" and a valvulotome, respectively, on one patient each. However, of the 10 patients, only two survived, one of Soutter's and one of Cutler's. The procedure was subsequently successfully revived in 1948 by Harken in Boston, Bailey in Philadelphia, Blalock in Baltimore, Brock in London, and others, who performed various procedures including valvuloplasty and commissurotomy. However, the so-called FFV (Harken) became the favoured procedure. It evolved from using the forefinger, to using the little finger, to eventually using a knife. Most surgeons had difficulty in using the mitral knife to divide the medial commissure and thus developed their own instrument.[2]



*Finger fracture mitral valvuloplasty technique (Hall and Windsor[1])*

There are few successful case reports of FFV in pregnancy. In the United Kingdom, Brock reported three, Logan and Turner, six, and Marshall and Pantridge, 18.[3] Hall and Windsor in Sydney performed FFV in two of seven pregnant women who were being considered for FFV, including our patient. One of the other five, who were managed conservatively, died.[1]

In 1963, Windsor said, "Eleven years' experience in the surgery of the mitral valve has brought with it a great respect for the ability of the mitral commissures to resist finger, knife and dilator". He reported follow-up of 90 patients who underwent FFV. No more than 40 patients (45%) obtained good results. Sixteen patients in this group have since been reoperated upon by the more effective transventricular route using a mechanical expanding dilator.[4]

It should be noted that mitral stenosis in young women is rarely accompanied by calcification, and this may allow a more complete and successful valvuloplasty. All the procedures mentioned above occurred before the development of cardiopulmonary bypass and open heart surgery in 1954.

Early pioneers in surgery faced many challenges and disappointments, as well as condemnation, criticism and ridicule from colleagues. Some, like Soutter and Bailey (the latter nicknamed the "butcher of Hahnemann Hospital [Philadelphia]" after his first four FFV patients died) lost their practices.[5]

We would like to pay homage to all surgical pioneers and conclude with a comment from Harken: "He who would not learn from the past is condemned to relive it".

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## Ototoxic ear drops with grommet and tympanic membrane perforations: a position statement

Robert J Black, Vince C Cousins, Peter Chapman, Zoran Becvarovski, Harvey L C Coates, Stephen J O'Leary, Christopher F Perry and Brian J Williams

**To the Editor:** Systemic ototoxicity secondary to the use of aminoglycosides is well known in clinical medicine, and appropriate monitoring measures to prevent vestibulocochlear ototoxicity are routinely performed. Less well known is the potential for topical ear drops, particularly the aminoglycoside group, to cause both vestibular and cochlear damage when introduced through a patent grommet or tympanic membrane perforation for the treatment of infection.[1] Although the incidence of aminoglycoside ototoxicity with ear drops is uncommon (for cochlear toxicity, in the order of one in 10 000 patients treated[2]), individual susceptibility and patient compliance problems may lead to inner ear damage.

Concerns with the potential ototoxicity of aminoglycoside ear drops has led to American,[3] British[4] and Canadian[5] expert committees providing guidelines on the use of potentially ototoxic ear drops in patients with tympanic membrane perforations or patent grommets. The Consensus Panel of the Australian Society of Otolaryngology Head and Neck Surgery (ASOHNS) unanimously agreed on the recommendations shown in the Box, which are based on the American guidelines.

Broadly speaking, the Consensus Panel recommends avoiding the use of ototoxic ear drops in patients with perforated tympanic membranes where possible.

The Australian National Aboriginal Community Controlled Health Organisation study showed that the non-ototoxic fluoroquinolone drops were more effective than commonly used ototoxic ear drops.[1] An application to the Therapeutic Goods Administration for introduction of cipro-

**Recommendations of the Consensus Panel of the Australian Society of Otolaryngology Head and Neck Surgery on ototoxic ear drops and tympanic membrane perforation**

- Non-ototoxic eardrops are preferable in the presence of tympanic membrane perforations or grommets.
- If potentially ototoxic antibiotic ear drops are used, they should only be used in infected ears and discontinued immediately the infection has resolved.
- If potentially ototoxic antibiotic ear drops are prescribed for use in the open middle ear or mastoid, the reason for their use and a warning to the patient/parent of the risk of ototoxicity should be given and documented.
- If potentially ototoxic antibiotics are prescribed, the patient should be specifically instructed to return to the doctor if he or she develops vertigo, hearing loss or tinnitus.
- If the tympanic membrane is known to be intact and the middle ear and mastoid are closed, then the use of potentially ototoxic preparations presents no risk of ototoxic injury. ◆

floxacin drops to the ear has recently been approved, and has been placed on the Pharmaceutical Benefits Scheme as an authority prescription for Aboriginal and Torres Strait Islander children with chronic suppurative otitis media as of February 2007.

However, clinical circumstance may dictate that potentially ototoxic agents need to be used if culture/sensitivity testing suggests that fluoroquinolone drops would not be appropriate, are unavailable, or if previous treatment with fluoroquinolone ear drops failed.

The Consensus Panel did not believe routine auditory/vestibular monitoring was warranted by the risks of ototoxicity, provided the treatment was short (5–10 days).

The full document outlining the Consensus Panel's recommendations is available from ASOHNS.

*Competing interests:* Zoran Becvarovski, Harvey Coates, Stephen O'Leary, Christopher Perry and Brian Williams received honoraria for attendance at scientific meetings sponsored by Alcon Laboratories, which maufactures ciprofloxacin ear drops.

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# A pilot study investigating basic fibroblast growth factor for the repair of chronic tympanic membrane perforations in pediatric patients

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ABSTRACT

*Objective –:* A pilot study to investigate the utility of basic Fibroblast Growth Factor (bFGF) in tympanic membrane perforation (TMP) closure in a small cohort of pediatric patients.
*Methods –:* Prospective cohort study. Suitability for inclusion in the study was confirmed by the application of defined inclusion and exclusion criteria, and informed parental consent obtained. The technique used was a modification of the bFGF-technique by Kanemaru et al. Response to treatment was monitored with serial otoscopy and audiometric outcomes were determined. Statistical analysis of the outcomes was carried out.
*Results –:* TMPs were successfully closed in 7/12 children at the first attempt (58%) and in 10/12 children overall (83%). Hearing improvement was observed in 8/10 successfully treated cases (80%). There were no complications or adverse outcomes.
*Conclusions –:* The topical bFGF regeneration technique offers a promising, minimally invasive alternative to conventional myringoplasty in pediatric patients with comparable success and reduced morbidity and cost, especially considering the option of performing repeat applications. Patients with an active infection or inflammation are not suitable for the bFGF-mediated technique.
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## 1. Introduction

Tympanic membrane perforations (TMP) especially in the context of chronic suppurative otitis media (CSOM) are a common problem affecting the pediatric population and are particularly prevalent in various rural and remote indigenous populations worldwide [1]. The resulting hearing loss is associated with impairment of social, emotional and academic development in children as well an increased absence from school, lifestyle restrictions and an increased risk of antisocial behaviour [2]. A myringoplasty procedure may be performed, however significant variation in success rates between techniques and surgeons make the outcomes unpredictable and not reliably reproducible. Furthermore the procedure is relatively expensive as it requires sophisticated theatre equipment, theatre time and personnel. Finally there is no consensus on the minimum age at which myringoplasty surgery is appropriate [3]. Delaying surgery can have a significant negative impact on a child's development thereby making earlier, less invasive and yet cost-effective intervention desirable.

Basic fibroblast growth factor (bFGF) is a polypeptide mitogen that has been shown to stimulate the proliferation of epidermal and connective tissue cells as well as promoting angiogenesis [4–6]. The application of bFGF has been shown to significantly increase the overall closure rate of TMPs and reduce the average closure time [7,8]. The bFGF regeneration technique has been reported to be safe [9,10], simple and cost-effective, with benefits over conventional surgical treatment [9,11] and high rates of success. [9,10,12].

Most studies examining the use of bFGF for tympanic membrane regeneration have investigated its use in adults. Some authors have included older children (>13 years) [8,13], there is one study that included younger children. Zhang [8] included patients as young as 3 years of age in his study however no distinction was made between children and adults in the analysis and the published results do not allow a comparison to be made.

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Furthermore, other authors used up to six repeated attempts at the treatment in quick successions where the TMP failed to completely respond to treatment [9,10] in order to investigate the efficacy of the technique. This requires significantly greater resources and is not comparable with conventional myringoplasty surgery in which the final outcome from treatment is measured 3–12 months following the surgery [3].

The aim of this study is to investigate the effectiveness of a single application of bFGF at closing TMPs in a pediatric population.

## 2. Methods

### 2.1. Patient selection

The following inclusion criteria were applied:

- Age < 16 years;
- chronic perforation (persistent for >3 months);
- central pars tensa perforation;
- size < 50% of the total tympanic membrane area;
- previous history of recurrent otorrhoea;
- no otorrhoea for minimum 3 months pre-operatively;
- no evidence of cholesteatoma or other complication of CSOM.

Exclusion criteria were:

- Marginal perforation;
- size > 50% of the total tympanic membrane area;
- failure to achieve a dry ear for 3 months pre-operatively;
- cholesteatoma or other complication of CSOM;
- inadequate view and access of the TMP;
- only hearing ear;
- failure to adhere to appropriate post-treatment care and follow-up.

A pre-operative audiogram and video-otoscopic photograph was taken for each case and the size of the perforation was measured using Image J software (NIH Bethesda MD USA).

### 2.2. Surgical technique

The surgical technique is a modification of the technique described by Kanemaru [9]. Under general anaesthesia and by a transcanal (permeatal) approach the TMP edges were freshened by excision of a rim of tissue, the size of the TMP measured and an appropriately sized piece of gelatin sponge placed in the defect. The bFGF solution (at a concentration of 21,000 IU/5 ml) was dropped onto the gelatin sponge and once this was saturated the excess solution removed. One or two drops of blood were then applied to the sponge to seal the gelfoam-bFGF-plug. Initially patients had the technique as originally described by Kanemaru et al. utilizing fibrin glue but this was subsequently modified to use of one drop of cyanoacrylate in lieu of fibrin glue. Patients were followed up regularly for a minimum of 12 months following the intervention: the minimum follow-up was at 1, 2, 3 and 4 weeks post-procedure and then at 2, 3, 6 and 12 months post-procedure. At each post-operative visit an otoscopic photograph was acquired and where there was evidence of a residual perforation the size of this was measured. A post-operative audiogram was acquired from 2 months post-treatment

### 2.3. Patients consent/ethics

Ethical approval for the study was granted by the Institutional Review Board and written information leaflets were provided to the parents of all study participants when informed consent was obtained.

### 2.4. Statistical analyses

Students *t*-test and the Wilcoxon signed rank test were utilized to analyse the audiometric outcome in the successful group. Differences were considered statistically significant when $P < 0.05$.

## 3. Results

### 3.1. Patient profiles

The parents of all patients satisfying the inclusion and exclusion criteria for the trial were invited to participate in the trial. This minimized selection bias on the part of the investigators.

Thirteen children (male:female ratio of 9:4) were enrolled in the study, one of which was subsequently excluded due to failure to adhere to post-operative care instructions and failure to attend follow-up. The 12 children included for analysis (Table 1) had an age range of 6–16 years (mean 10.9 years, median 11 years). There were seven right and five left TMPs. The perforation size ranged from 6% to 40% of the tympanic membrane. Where known, the aetiology for the TMP was secondary to insertion of ventilation tube or previous recurrent acute otitis media. One patient had

**Table 1**
Patient profiles.

| Patient | Side | Gender | Aetiology of TMP | Subjective hearing loss? | History of otorrhoea? | Previous attempt at closure? | Age at Surgery (years) | Pre-treatment FFA (dBHL) | Post-treatment FFA (dBHL) | Successful? |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | L | M | VT | Yes | Yes | No | 15 | 30 | 30 | No |
| **1(r)** | L | M | VT | Yes | Yes | Yes | 16 | 30 | 17.5 | Yes |
| **2** | R | M | AOM | Yes | Yes | Yes | 6 | 31.25 | 3.75 | Yes |
| **3** | L | M | VT | No | Yes | No | 13 | 27.5 | 22.5 | Yes |
| **4** | L | F | AOM | No | Yes | No | 7 | 17.5 | 7.5 | Yes |
| **5** | R | M | U | No | Yes | No | 13 | 20 | 16.25 | Yes |
| **6** | R | M | U | No | Yes | No | 14 | 12.5 | 12.5 | Yes |
| **7** | R | F | AOM | Yes | Yes | No | 6 | 27.5 | 10 | Yes |
| **8** | L | M | VT | No | Yes | No | 8 | 10 | 16.25 | No |
| **8(r)** | L | M | VT | No | Yes | Yes | 8 | 16.25 | 15 | No |
| **9** | R | M | VT | Yes | Yes | No | 12 | 12.5 | 18.75 | No |
| **10** | L | M | U | Yes | Yes | No | 12 | 27.5 | 27.5 | No |
| **10(r)** | L | M | U | Yes | Yes | Yes | 13 | 27.5 | 16.25 | Yes |
| **11** | R | M | VT | No | Yes | No | 10 | 18.75 | 20 | No |
| **11(r)** | R | M | VT | No | Yes | Yes | 11 | 20 | 5 | Yes |
| **12** | R | F | VT | No | Yes | No | 10 | 7.5 | 5 | Yes |

(r) = revision procedure, R = right, L = left, M = male, F = female, VT = ventilation tube, AOM = acute otitis media, U = unknown, TMP = tympanic membrane perforation, FFA = four frequency average air-conduction audiometry (air conduction thresholds at 500 Hz, 1 KHz, 2 KHz, 4 KHz).

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previously undergone an attempt at conventional underlay myringoplasty.

### 3.2. Closure rates and time to achieve closure

Seven out of the 12 primary procedures resulted in a successful closure of the TMP, with success being defined as an intact tympanic membrane 12 months post-treatment. Two out of the five cases of initial failure resulted from external causes independent of the treatment procedure: in one case the patient went swimming in the early post-treatment period (patient 10) and in the other the patient contracted a severe upper respiratory tract infection in the early post-treatment period (patient 11). Both these patients underwent a second attempt at the procedure with a successful outcome. In two other cases of initial failure (patient 1 and patient 8) it is felt that this was likely due to rapid onset otorrhoea as a result of liquefaction of the fibrin glue cap, prompting a modification of the surgical technique as described earlier. Both these patients underwent a second attempt at the procedure, one of which was successful (patient 1).

Thus successful closure was achieved in 7/12 children (58%) at their *first* attempt. If the outcome is considered by *patients treated*, the procedure was successful in 10 out of the 12 children (83%). In those children who were successfully treated, the mean number of treatments required to achieve success was 1.3 (including the two cases in which initial failure resulted from external causes).

Among the successful cases the time for complete closure to be confirmed ranged from 2 weeks to 3 months post-treatment.

In the two children whose treatments were unsuccessful (patient 8 and patient 9) the perforation size and position was not statistically significantly different from the successful cases. Patient 8 was younger than the mean/median age but not the youngest in the cohort; three children younger than him had a successful outcome from their first treatment. Both of patient 8's treatments involved the use of a fibrin glue cap and rapid liquefaction of this cap is felt to have contributed to failure of the treatments. Following the two procedures, the size of his TMP had not changed significantly. Patient 9 was identified to have a residual perforation at 3 months following his treatment.

### 3.3. Audiometric outcome

The four-frequency average air conduction threshold (FFA, at 0.5 KHz, 1 KHz, 2 KHz and 4 KHz) improved in eight out of the 10 patients who had their TMP closed successfully. The mean improvement in the FFA amongst the successful closures was 9.1 dB HL with a range of 0–27.5 dB HL. This improvement was statistically significant using both the *t*-test ($P = 0.024$) and the Wilcoxon signed rank test ($P = 0.025$).

## 4. Discussion

This is the first study examining the effect of bFGF on regeneration of chronic tympanic membrane perforations specifically in the pediatric population. In our study the trial technique proved successful in the two youngest patients treated (each aged 6 years at the time of surgery) indicating that this technique is equally appropriate for the management of younger and older children with TMP. This is in contrast to the findings of previous studies that have indicated a lower likelihood of success in TMP repair among younger patients [3]. Given that one aim of TMP repair is to reduce morbidity and hearing loss at a young age, the finding that this technique is successfully applicable to children as young as 6 years of age is an important one.

Frequently conventional myringoplasty results in a 2-layer repair rather than a *regeneration* of the 3-layer morphology of the healthy, normal tympanic membrane. The healed tympanic membrane does not recover its complete tensile strength [14] and this can be associated with increase predisposition to retraction and associated complications. The bFGF technique results in a 3-layer *regeneration* of the tympanic membrane [15] thereby avoiding these potential problems and presenting an additional benefit of this technique over conventional myringoplasty techniques.

Perforations up to 50% of the total tympanic membrane surface area were treated in this pilot study. The effect of this treatment on larger perforations in the pediatric population remains to be determined although in the adult population the treatment has been shown to be successful for perforations exceeding 2/3 of the tympanic membrane, albeit frequently requiring more than one application. [9]

Four of the treatments were in children who had undergone previous attempts at TMP repair, either by conventional underlay myringoplasty or by the trial bFGF technique. The bFGF technique was successful in three of these patients, demonstrating that the technique employed is suitable as both a primary and secondary treatment option. In the case of treatment failures the use of the bFGF technique does not preclude subsequent attempts at repair using the bFGF technique or conventional myringoplasty techniques.

Four-frequency-average audiometry improved in 80% of the successfully treated patients. This improvement has been demonstrated to be significant using both the Student's *T*-test ($P = 0.024$) and the Wilcoxon signed rank test ($P = 0.025$). Thus it can be concluded from this small study that there is a change towards improvement in the hearing thresholds. Given that one of the aims of treatment in children is to facilitate normal hearing function in their environment, thus promoting academic and social development, the fact that the hearing can be improved is an important secondary potential benefit to this treatment option.

The bFGF procedure is a considerably shorter procedure than a conventional myringoplasty (average 7 min compared with 45 min) and requires considerably less sophisticated equipment and instrumentation. Cost analysis demonstrates that in the study centre the cost of the trial procedure is less than a third (29%) of the cost of a conventional underlay myringoplasty (see Table 2). Furthermore the cost of the instrument tray that is required for the trial procedure is less than 20% of the cost of the instrument tray required for a conventional underlay myringoplasty (see Table 2).

The 83% success rate demonstrated in this study is comparable with published conventional myringoplasty series [3], but is lower than that demonstrated by other authors utilizing bFGF [8,9,16]. One

**Table 2**
Operating theatre cost analysis between conventional underlay myringoplasty procedure and trial FGF procedure (all figures in Australian dollars).

| Cost item | Conventional underlay procedure (per patient) | Trial procedure (per patient) | Cost saving (per patient) |
|---|---|---|---|
| Instrument tray | $9185 | $1700 | (Not applicable) |
| Operating theatre time | $1800 (45 min at $40 per minute) | $280 (7 min at $40 per minute) | $1520 |
| Processing (sterilization) | $71 | $61 | $10 |
| Consumables | $40 | $219 | −$179 |
| Total | $1911 | $560 | $1351 |

Please cite this article in press as: A.N. Acharya, et al., A pilot study investigating basic fibroblast growth factor for the repair of chronic tympanic membrane perforations in pediatric patients, Int. J. Pediatr. Otorhinolaryngol. (2015), http://dx.doi.org/10.1016/j.ijporl.2014.12.014

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significant difference between these studies and ours is that in our study, chronic TMP were treated, whereas other studies looked at traumatic TMP which have a known high closure rate. A second difference lies in the number of treatments utilized: while our study examined the response of a TMP perforation to a single attempt and a single revision attempt using this treatment modality, other authors investigating bFGF for TMP repair have repeated the treatment up to six times in quick succession in any individual patient [9,10]. A final difference is in the length of time for which the perforation should have remained free of otorrhoea prior to intervention. Opinions vary on this with requirements of up to 3 years reported by some authors [9]. Our study investigated patients with a much shorter length of time for which the perforation remained dry: 3 months.

There were no adverse outcomes to treatment in our study group. Most authors who have investigated the use of bFGF in TMP repair reported no adverse outcomes. Hakuba et al. [13] reported a 5% incidence of cholesteatoma formation with five out of the six cholesteatomas occurring in association with perforations that involved the anterior half of the tympanic membrane. The incidence of iatrogenic cholesteatoma following overlay myringoplasty is 3–7%, [13] indicating that cholesteatoma formation is not particular to this regenerative technique. In Hakuba's study [13] cholesteatoma was first identified up to 17 months post-treatment suggesting that long-term follow-up is indicated for patients undergoing this technique. Lou and Wang [8] reported the development of myringitis in 52% of cases in whom gelatin sponge was utilized, however CT imaging confirmed the absence of any middle ear or mastoid inflammation in these cases and the myringitis did not have any effect on hearing outcome or patient symptoms. None of our patients treated with bFGF and gelfoam developed the myringitis described by Lou and Wang.

## 5. Weaknesses

One significant weakness in this study is the small number of participants recruited. This has a direct impact on the likelihood of achieving statistically significant results and is inherent to pilot studies. Another important weakness is that this is not a randomized trial and is without a control arm: the benchmark standard for success against which this technique has been compared is the success rate for a standard myringoplasty procedure as published in the literature.

## 6. Conclusions

Results from our pilot study indicate that the use of bFGF in a single application for the repair of TMPs in children shows promise. The technique is faster, cheaper and more readily delivered than classic myringoplasty techniques, with rates of success that are higher than with published conventional myringoplasty literature with minimal morbidity. Ongoing studies with regular postoperative bFGF application, different scaffolds and long-term follow-up are underway to further investigate the utility of this treatment modality to ascertain whether this technique is appropriate for widespread general use.

## Conflict of interest

None to declare.

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# CHAPTER 5

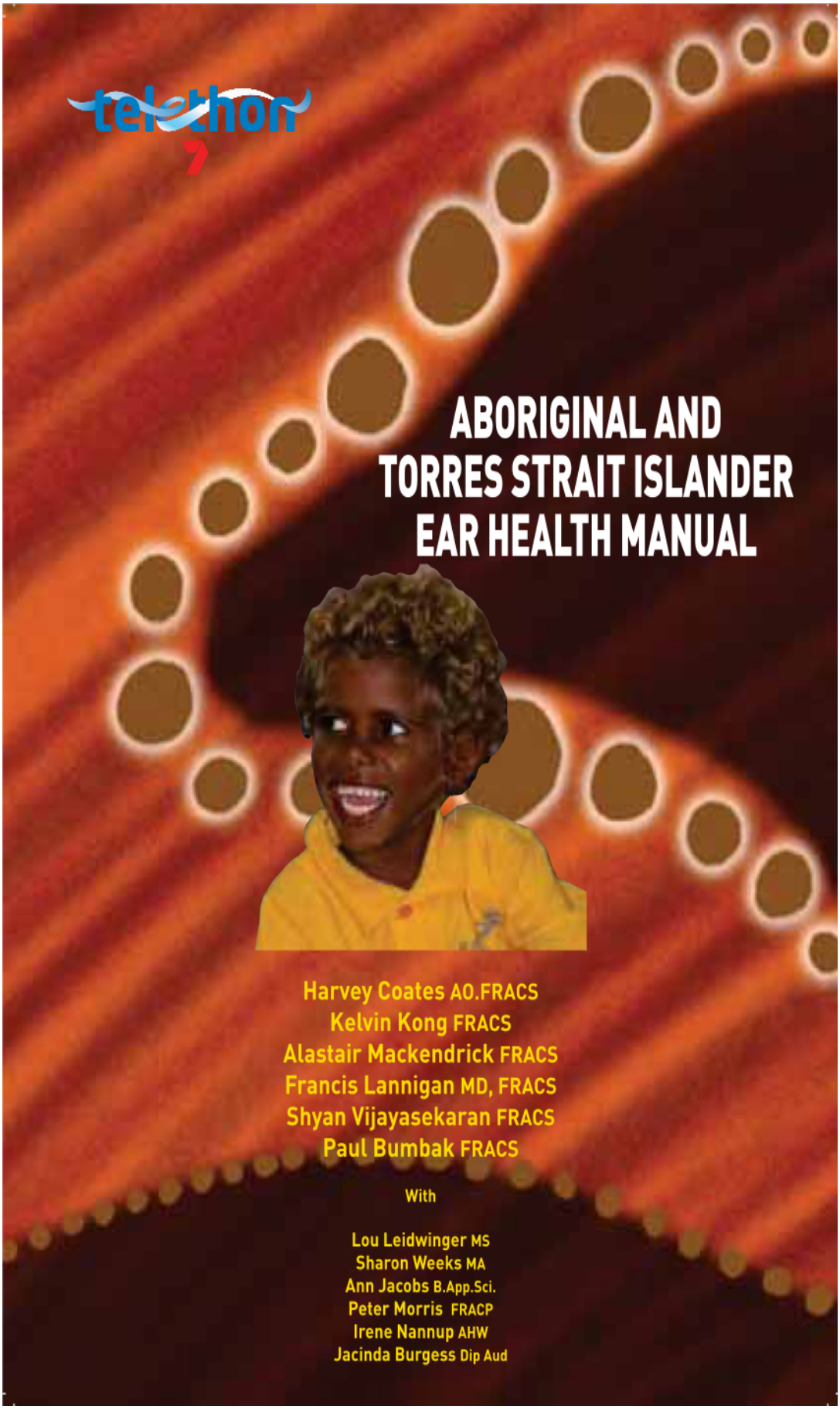
# ADVOCACY FOR ABORIGINAL EAR HEALTH MANUAL

| Paper no* | Supporting Articles | Thesis page no |
|---|---|---|
| 24 [32] - | Aboriginal and Torres Strait Islander Ear Health Manual | 240 |

*Superscript numbers correspond to the designated general bibliography numbers – when cited in the text.

In 1981 the author obtained a research grant from the Telethon Foundation to demonstrate the efficacy of intensive otitis media treatment in Aboriginal children in Western Australia. At the conclusion of the trial of the efficacy of frequent suction toilets under operating microscope control by trained Aboriginal health workers, the reduction in admission to the Roebourne Hospital for children with CSOM for management of their otorrhoea reduced from 19 children, admitted for a total of 108 days in the previous 12 months prior to the trial to 1 child being admitted for 3 days during the 12 months period of the trial. Regardless of these results showing that intensive intervention for the draining ears of children with CSOM was effective, no further action was taken at a health governmental level. This study was not reported but was an impetus for the author to advocate for change in the focus, delivery methods and treatment efficacy of OM in Aboriginal children during subsequent years. Translating this advocacy into action, strategies were initiated, including participation in the development of new advocacy groups, including the Paediatric Otolaryngology Study Group, Otitis Media Australia (OMOZ) membership, Australian Council for Hearing and Education (ACHE) membership and chairmanship of the Aboriginal subcommittee of the Australian Society of Otolaryngology, Head and Neck surgery (ASOHNS). Internationally, the author is a member of the Society for Middle Ear Diseases (SMED), which is an advocacy group for middle ear infections, The International Society for Otitis Media (ISOM), a member of the International Federation of Otolaryngology Societies (IFOS) Ad-Hoc paediatric sub committee, which advises the World Health Organization (WHO). In addition, the author was the Australian representative for eight years of the ENT extensions of Excellence

(EEE) group, which was a panel of experts meeting annually, to discuss ototopical antibiotics, ototoxicity and research developments in the field. In 2002 the author was a co-author of an editorial in the Medical Journal of Australia, entitled "Otitis media in Aboriginal children: tackling a major health problem" [80] and in 2009 the author was coordinating editor of a supplement in the Medical Journal of Australia entitled: "Otitis media 2009 – an update" [81]. In 2004, with colleagues we published the first Aboriginal ear health manual, aimed at facilitating diagnosis and management of otitis media by Aboriginal health workers and other medical professionals. This was followed by a second, and more recently (2012), third "Aboriginal and Torres Strait Islander Ear Health Manual" [32] with wide national and international distribution.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ABORIGINAL AND TORRES STRAIT ISLANDER EAR HEALTH MANUAL
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# Introduction

The state of a nation's health can be measured by the prevalence of children with chronic suppurative otitis media (CSOM). Otitis media (OM) in all of its forms is a disease of poverty. When the World Health Organization (WHO) defines a 4% prevalence rate of CSOM as being a major public health problem, then the current rates of many times that in some Aboriginal communities is a serious indictment of the poor living conditions in these communities. The effect of up-to-32 months of conductive hearing loss in childhood has a life-long impact on the child's speech and language development and subsequent educational and vocational outcomes.

It is in light of these concerns that public health initiatives to improve the general health of Aboriginal communities by improving housing and access to water, nutrition and medical care have been advocated. In addition, publications such as the "Systemic review of existing evidence and primary care guidelines on the management of otitis media in Aboriginal and Torres Strait Islander populations" from the Office of Aboriginal and Torres Strait Islander Health have become standard reference protocols for management of otitis media. We also thank Australian Hearing for the use of some of their teaching materials. The purpose, then, of this manual is to supplement these guidelines with background teaching material and resources.

The authors are grateful to Kimberley Health and the Health Department of Western Australia for permission to base the manual on the excellent second edition of The Kimberley Ear Health Manual. We appreciate the updated 2010 Recommendations for Clinical Care Guidelines on the Management of Otitis Media in Aboriginal and Torres Strait Islander Populations. We also acknowledge the organizational skills of Ms Jan Zach.

*The preparation of this Aboriginal Ear Health Manual was made possible by a seeding grant from the American Society of Paediatric Otolaryngology. The funding for the production for this ear health manual was made possible by a grant from the Telethon Foundation in Perth, Western Australia.*

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# Foreword

The Hon Richard Court AC, Former Premier of Western Australia, Chairman, The Channel 7 Telethon Trust.

The Channel 7 Telethon Trust has a charter to financially support the medical and social welfare of children and young people, and fund the research into children's diseases.

Funding the production of the Aboriginal and Torres Strait Islander Ear Health Manual which is a practical guide to assist in tackling hearing issues, is a very worthwhile initiative for the Channel 7 Telethon Trust.

We would like to thank the authors and the Government agencies responsible for this publication and we are eager to support its widespread distribution.

Hon Richard Court AC
Chairman
Channel 7 Telethon Trust

## FOREWORD TO THE FIRST EDITION

Hearing problems and ear infections are some of the most common problems managed at the primary health care level throughout Australia. How these problems affect the Aboriginal and Torres Strait Islander population has, for some reason, received scant attention in teaching programs. Almost everyone can benefit from teaching material written in plain language and that is the advantage of this Manual. It explains the basics and the essentials of ear health, has superb drawings and photographs, and its sections are compact and readable.

This Manual has been in the making for many years and is a credit to Professor Coates and the health professionals that have contributed to its development. Everyone involved in it has enormous expertise, a strong passion, and have been advocates for improved ear health within the Aboriginal and Torres Strait Islander population for a long time.

This Manual will certainly help address practice uncertainty, but the vital ingredient for effective programs and services will always be their development in partnership with Aboriginal peoples and their representative agencies. I encourage readers to join us in this journey.

Mr Henry Councillor
Chair,
National Aboriginal Community Controlled Health Organisation

***Dedicated to Sarah***

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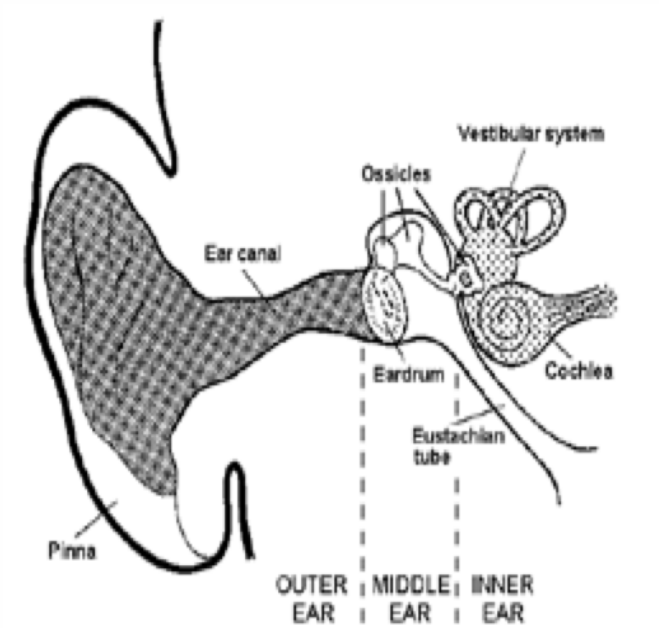
# Anatomy and Function of the Ear

Study of the structure and function of various parts of the ear is needed to understand the different types of hearing loss and the methods used in preventing or treating hearing loss. The ear can be divided into three parts, which work together, they are the outer ear, the middle ear and the inner ear.

## Basic Anatomy of the Ear




### The Outer Ear

The outer ear consists of the pinna and the ear canal ending in but not including the eardrum. The outer ear works like a funnel. It directs sound down the ear and helps localize sound. The ear canal is about 24mm (1 inch) long. It has two bends and ends at the ear drum. The outer two thirds of the canal is formed of cartilage and the rest is bone.

The ear canal is lined with skin. The skin of the outer two thirds is the same as on the rest of the body. The skin contains sweat glands and special glands, which produce wax (cerumen). The wax is sticky and antibacterial and helps waterproof the canal. This prevents small particles from entering the canal and keeps a healthy ear free from infection. The skin is covered with hairs that help move dirt out of the canal. The ear canal ends at the ear drum.

The eardrum has three layers. The outer layer is skin; the middle layer is fibre which gives the ear drum its bounce; and the inner layer of mucous membrane which is part of the lining of the middle ear.

## The Middle Ear

The middle ear is an air filled cavity, bridged by the ossicular chain. It passes sound from the eardrum to the inner ear. The ossicular chain is made up of three small bones, the hammer, (malleus) anvil (incus) and stirrup (stapes) .The hammer is attached to the eardrum. The stapes footplate rests on the membrane-covered oval window of the inner ear. When sound waves move the eardrum, they are transferred mechanically through the ossicular chain to the inner ear.

The middle ear is connected to the back of the nose by the Eustachian tube. This connection allows for pressure to be equalized during swallowing, yawning and sneezing. It also acts as a drain. The Eustachian tube is shorter in children and is more likely to get contaminated from the back from the nose. It is because of the difference in the shape and function that makes young children prone to ear disease.

## The Inner Ear

The inner ear is a very delicate mechanism. For protection it is located deep in the skull behind the eyes. The inner ear is filled with fluid and contains two sensory systems, the balance and the hearing systems. The back section of the inner ear consists of three canals which give us our balance and help in the stabilisation of eye movement. These are the semi circular canals and are known as the vestibular system.

The hearing system is contained in the cochlea and the auditory nerve. The purpose of the cochlea is to change the signal from a sound message to an electrical message. These electrical messages are sent to the brain by the auditory nerve. The cochlea is a fluid filled snail shell like structure. In it there are about 30,000 tiny nerve endings called hair cells and all these connect to the fibres of the auditory nerve which transmits the sound message to the brain.

Otoscopic features of the eardrum showing the ossicular chain

# Causes of Hearing Loss

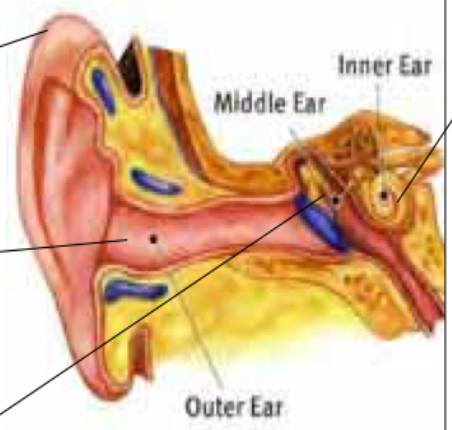
The main causes of hearing loss in Aboriginal and Torres Strait Islander children is otitis media or middle ear infections and its complications including otitis media with effusion (glue ear) and chronic suppurative otitis media.

**CONDUCTIVE HEARING LOSS**

Congenital Atresia

Wax obstructions
Foreign Bodies

Otitis media with effusion; chronic suppurative otitis media; ossicular problems




**SENSORINEURAL HEARING LOSS**

Congenital

Genetic
Syndromic
Non-syndromic

Non-Genetic

Acquired

# Hearing loss – types and effects

There are three types of hearing loss. Below is a brief description of them.

**Sensorineural**
Sensorineural hearing losses are caused by damage to the hair cells of the inner ear, auditory nerve or brain. There are several causes of sensorineural hearing losses such as noise exposure, ageing, meningitis, genetic factors, certain drugs, certain pre-natal conditions, some viruses, etc. Most, but not all, sensorineural hearing losses are permanent and do not get better. A majority of people with sensorineural hearing losses can be helped with amplification (hearing aids and /or cochlear implant). Often people with sensorineural hearing loss will experience tinnitus, or ringing in the ear.

**Conductive**
Conductive hearing losses are caused by some physical blockage or mechanical problem, which interferes with sound transmission through the outer or middle ear. Causes of conductive hearing loss include wax blockage, perforated ear drum, the various forms of otitis media, otosclerosis, a break in one of the middle ear ossicles, etc. Most, but not all, conductive hearing losses can be medically and/or surgically treated, resulting in improved hearing, frequently to near normal levels. Some people with conductive hearing losses use amplification.

**Mixed**
A mixed hearing loss has both conductive and sensorineural components to the loss. As in pure conductive hearing losses, medical and/or surgical intervention with the conductive component can often improve the hearing.

**The effect of hearing loss**

To understand the effects of hearing loss it is important to understand the need for good hearing. Good hearing is essential for the development of normal language and communication. Even a *mild*, fluctuating conductive hearing loss, which can be associated with otitis media, can have a negative effect on language and learning in many children.

If a child has a hearing loss it will cause problems in the development of various skills. The amount of problems that happen will depend on two main aspects of the hearing loss:

- The age of onset - the earlier in the child's life, the greater these skills are impacted.
- The degree of hearing loss - the greater the hearing loss, the greater these skills are affected.

## These are some of the effects of different degrees of hearing loss on learning and classroom performance:

***Slight (16 - 25 dB)***

Understand conversation at 3 m
Speech/language development may be affected because of hearing loss
Should understand most classroom discussion
A few children may require some degree of amplification

***Mild (26 - 40 dB)***

Understand conversation at 1 - 1.5 m
May have delayed speech/language development
Will miss up to 50% classroom discussion
Will need amplification, hearing aids or FM system
May need special education attention

***Moderate (41 - 55 dB)***

Understand conversation at 0.5m
Will have difficulty at school
Likely will have speech/language delay
Will require hearing aids and/or FM systems
Will need special education assistance and probably special training for listening
Uses vision for additional cues to understand spoken messages

***Moderately Severe (56 – 70 dB)***

May understand speech at ~25 cm
Will hear moderately loud environmental sounds
Will have delayed speech/language
Requires use of hearing aids and FM systems
Will need special education assistance and special training for listening

***Severe (71 - 90 dB)***
May understand some speech at <15 cm
Hears loud environmental sounds
Will have delayed speech / language
Requires use of hearing aids and FM systems
Requires auditory training
Uses vision for additional cues to understand spoken messages
Speech / language will not develop spontaneously if loss present before 1 year old
May be a candidate for cochlear implantation

***Profound (> 91 dB)***

May only be aware of very loud sounds
Speech and language will be defective
Visual and gestural cues essential for learning
Needs full time special education assistance
Requires use of a hearing aids and FM systems
May be a candidate for a cochlear implant

# Hearing and the development of language

A normal human ear can hear a wide range of sounds ranging in frequencies between 20 and 20,0000 Hz. Human hearing is most sensitive to the frequencies between 500 to 4000 Hz, which are the most important for understanding speech.

A normal human ear is also capable of detecting very, very soft sounds around 0 dB (decibels) in intensity. It can also tolerate very loud sounds up to 120 dB. Above 120 dB sounds become painfully loud.

When babies are born, their most highly developed sense is hearing. For the first few months of life, babies without middle ear disease or a profound hearing loss will exhibit a stereotyped startle response to sudden loud sounds, the Moro reflex. A sudden loud sound will cause the baby to extend its arms and legs and throw its head back. This particular reflex normally will disappear by about 4 months of age. After 4 months of age, a baby will simply "jump" at sudden loud sounds, much as an adult will do.

From a very early age (days), babies can distinguish subtle cues in speech sounds, for example, the difference between a spoken "ba" and "pa", or "ba" and "da. By one month of age, a baby will lay still in response to certain sounds, particularly the mother's voice.

Between 4 and 6 months babies begin to locate sound by shifting their eyes or turning their heads towards the sound. This behaviour is a reflex at this stage.

From about 6 months of age, babies will turn their head towards a sound.

Also at around 6 months of age, babies will produce speech-like sounds. Within a short time, a couple of months, the baby will then only produce speech sounds of the language(s) spoken around it.

In addition, around 6 months of age, babies will smile at soothing sounds, and cry at harsh or angry sounds. They begin to recognize sounds like the rattle of the spoon in a plate. They will localize sounds more directly and can follow the conversation of familiar people by turning and watching as each person speaks.

By 12 to15 months, the child will start using single words with meaning, and is able to understand much of the conversation around it. The child should now be following simple instructions.

At 18 to 20 months of age, a child should start to put 2 words together in basic sentences.

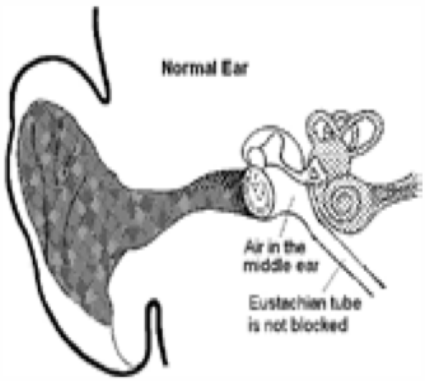
By 2 years of age a child has learned to interpret all sorts of sounds and the words and actions of people in their environment. The child may have 50 words and talk in 3 word sentences. From here language continues to rapidly expand so that by 3 years of age it is possible to have an intelligible conversation with the child. By 6 years of age, most children have developed language skills to a high level. They use these language skills in many ways:

1. To communicate and socialize with others
2. To think (cognitive skills)
3. To learn through reading, writing and listening

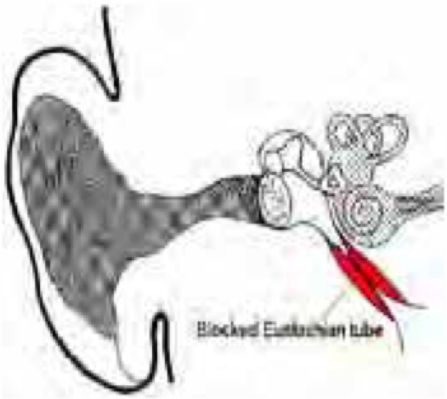
# Questions about hearing

| AGE | QUESTIONS TO ASK CAREGIVERS |
|---|---|
| Newborn to 4 months | • Do sudden, loud noises wake the baby?<br>• Does the baby cry at very loud noises?<br>• Does an awake baby jump at sudden, loud noises like a door slamming or a dog barking nearby? |
| 3 to 4 months | • Does the baby sometimes turn its eyes or start to turn its head to see where a noise comes from?<br>• Is the baby distracted from feeding by moderately loud noises close by? |
| 4 to 7 months | • Does the baby frequently turn straight to sounds?<br>• Does the baby make a variety of babbling sounds?<br>• Does the baby enjoy playing with noisy toys or objects?<br>• Can you soothe the baby with your voice? |
| 7 to 9 months | • Does the baby turn to find things heard but not seen?<br>• Does the baby gurgle, coo or babble to unseen sources of voices or other sounds? |
| 9 to 24 months | • Does the baby show pleasure when hearing sounds like the bath running, food being prepared or kids coming home?<br>• Does the baby copy words and sounds?<br>• Does the baby by about 15 months use some single words spontaneously?<br>• Does the baby respond when you call from another room? |

# The Development of Otitis Media

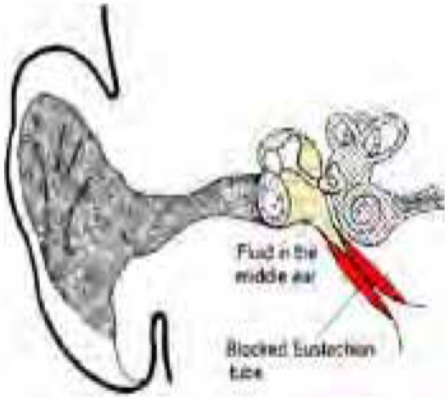



In a normal ear there is air in the middle ear. The Eustachian tube opens when yawning and swallowing to let air get into the middle ear.



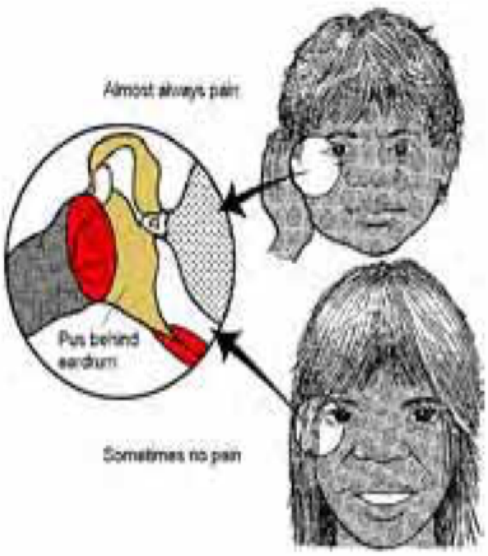

The Eustachian tube can swell and become blocked. Air cannot get into the middle ear and fluid cannot drain out of the middle ear.

Keeping the nose clean and doing the Breathing, Blowing and Coughing Program can help unblock the Eustachian tube.

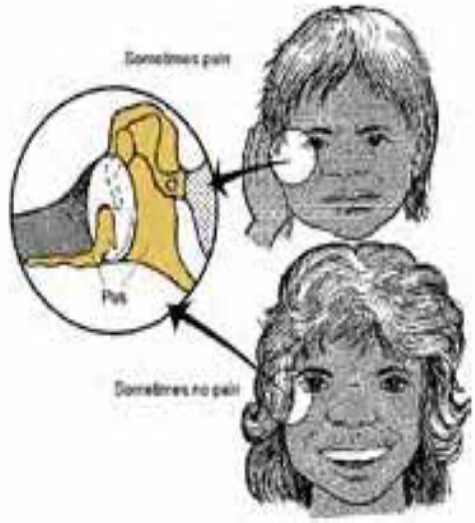



Fluid can build up behind the eardrum. At this point the ear is not infected. This is called otitis media with effusion. If the fluid is present for 3 months or more, it is called glue ear.

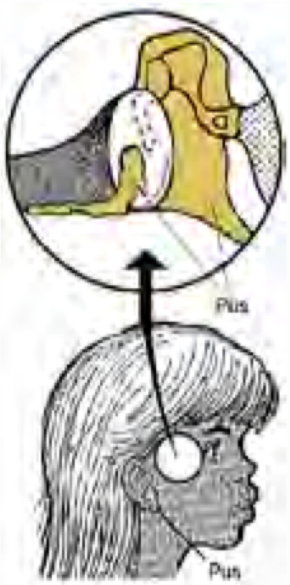
Keeping the nose clean and doing the Breathing, Blowing and Coughing Program can help the fluid go away.



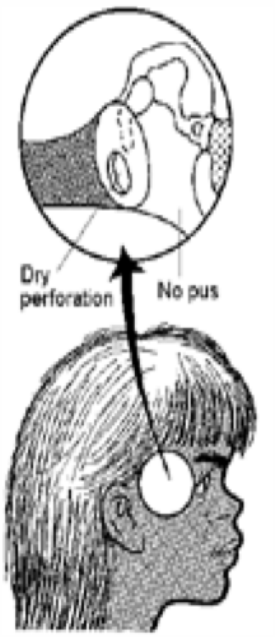

The ear can become infected and the fluid turns to pus. The eardrum becomes red and bulges out. There is almost always much pain. This condition is called acute otitis media.




The eardrum can burst and the pus drains out. After the eardrum bursts, the pain usually goes away. Sometimes the eardrum will heal up on its own, and sometimes it will not.




If the pus keeps draining from the ear for a long time, it is called chronic suppurative otitis media, or runny ear.




If the runny ear is syringed and treated with antibiotic ear drops, the pus can go away and the ear becomes dry. Sometimes the eardrum will heal up on its own. If it does not, and the hearing is bad, the ear surgeon can put a patch or graft on the eardrum.

If the pus does not go away with syringing and antibiotic ear drops, there may be a cholesteatoma or 'skin cyst" in the middle ear. The ear surgeon will need to examine the ear.

# Preventative otology
# Preventing ear disease and hearing loss

Preventative otology is the utilization of public health, medical or surgical management to prevent or reduce the manifestations of otological disease and hearing loss. Preventative otology uses strategies that aim to prevent the development of middle ear disease, or prevent or reduce some of the signs and symptoms of middle ear disease and hearing loss. Strategies and interventions include environmental, medical, surgical, audiological and educational initiatives.

It is important that prevention strategies should be culturally appropriate for Aboriginal and Torres Strait Islander communities, and that community consultation is essential before the introduction of prevention programs.

Amongst the indigenous communities of the world, there are higher rates of chronic suppurative otitis media and associated hearing loss. This is felt in part to be due to poor hygiene, diet and housing. It results in negative schooling, behavioural and health outcomes in an already disadvantaged group in the community. Improved public health measures play an important role in reducing the prevalence of hearing loss, ear infection rates, and improving quality of life and social outcomes. Early medical and surgical interventions are needed to treat the disease during the critical periods of development.

**Environmental prevention strategies:**

Improve nutrition
- Less "junk food"
- More fresh, healthy food

Improve the home environment
- Clean water for showering and washing
- Avoiding overcrowding – a bed for each child

- Keep flies away from ears, nose and throat

Increase breast feeding

- Protection against infection

Reduce passive smoking

- Don't smoke around babies or children

Regular health checkups
Nose blowing
Breathe Blow Cough (BBC) Program

**Medical and surgical prevention strategies:**

Vaccination:
Pneumococcal conjugate vaccination available to all Indigenous children particularly those under the age of two years and can help reduce the incidence of otitis media, pneumonia and meningitis caused by the pneumococcus bacteria. In children over two years of age and adults the pneumococcal polysaccharide vaccine is available and also helps reduce these infections but is not recommended for those under the age of two years.

Access to Specialist Healthcare:
Timely referral to otolaryngologists for assessment and surgical intervention can improve hearing outcomes

**Audiological prevention strategies:**

Regular hearing tests
Audiological rehabilitation is critical once hearing loss has been diagnosed:

- Ongoing education about effective communication strategies
- Appropriate use of devices to assist hearing -
  1. standard hearing aids and bone conductors
  2. classroom devices such as soundfield amplification systems (which provide uniform soundfield throughout the classroom and increase the speech-signal: noise ratio) and FM systems (a form of personal amplification whereby an FM signal from a microphone worn by the teacher is picked up by a receiver worn by a child with hearing loss)

**Educational prevention strategies:**

Encourage regular visits to healthcare providers
Provide information to parents and other caregivers about middle ear disease and hearing loss
Provide information on the effects of middle ear disease and hearing loss on school performance
Encourage parents and other caregivers to use strategies to develop speech and language skills
Inform teachers or carers when a child has a hearing problem

# Otoscopy
# Examination of the ear

This section indicates the steps in carefully and safely examining a child's ear, including the all important history from the parents or caregiver.

**1. History**

Ask the child or parents about the ear and/or the infection:

- Is the ear sore?
- How long has it been sore for?
- Has any pus been coming out of the ear?
- How long has it been coming out for?
- Has the child been swimming, and where?
- Can the child hear as well as before?

**2. Ear examination**

Sit on a chair when looking at the child's ear so that your eye is at about the same level as the child's ear. For very young children, have them sit on someone's lap.

a. Look at the ear from the outside noting any pus coming out etc

b. Show the child the otoscope and explain what you are going to do.

c. Using the otoscope

For children, gently pull the pinna (outer ear) up and back to straighten the ear canal. Hold the otoscope in such a way that you can brace your little finger against the child's head. In this way if the child moves suddenly, the otoscope will not be jammed into their ear.

For infants, gently pull down and back on the ear lobe to straighten the ear canal.

d. Looking in the ear

The following figure depicts normal right and left eardrums. The major landmarks are the long process of the malleus, the cone of light reflected from where the long process of the malleus connects to the ear drum (umbo), and the short process of the malleus. Some eardrums are so transparent that other features behind the eardrum can be seen.

However, a large number of children may have abnormal appearing eardrums due to a history of repeated bouts of otitis media. Consequently, when looking at eardrums it is important to report one of the following attributes and record them accordingly.

Is the eardrum:

- Normal?
- Intact?
- Perforated?
  - dry, wet or with pus
  - small, medium or large perforation
- Foreign object?
- Healed perforation?
- Excessive wax?
- Unsure?
- Unsure

| Normal Ear | Retraction pocket | AOM |
|---|---|---|
| | | |

| CSOM | OME |
|---|---|
| | |

# Pneumatic Otoscopy

Pneumatic otoscopy is a subjective measure of tympanic membrane and middle ear status. Although there is a shortage of evidence based literature on its use it remains a common diagnostic procedure. It is used to diagnose possible eustachian tube dysfunction or otitis media.

The pneumatic otoscope is a hand held instrument similar to an otoscope but with the ability to seal the ear canal with the speculum. It has a light source with a pneumatic tube attached to the otoscope which allows a puff of air to be placed into the ear canal. By squeezing a bulb connected to the pneumatic tube the pressure can be changed in the ear canal. The puff or air allows for the observation of how the ear drum responds to changes in ear pressure. The pressure required to move a normal tympanic membrane ranges from 10-15mm of water pressure. However the pressure needed to move a tympanic membrane with a significant middle ear effusion may be from 40-116mm.

Pneumatic otoscopy requires that the pneumatic otoscope is correctly functioning, that is, the battery should be fully charged and there should be a strong light source to allow for optimal visualization. The pneumatic seals should also be checked to ensure they are functioning.

The use of the pneumatic otoscope requires some practise. Observation of the movement in the posterior superior part of the tympanic membrane is most indicative of the middle ear status. To perform the test the child should remain quite still. The pneumatic otoscope is inserted into the ear canal with appropriate sized speculum to obtain a seal. There is often reported pain and discomfort to patients during the test and as a result the test may not be used frequently. This discomfort is due to the pressure being introduced exceeding that which is required to move the tympanic membrane. The presence of cerumen partially or completely obstructing the ear canal may need to be removed and reviewed to obtain a clear view of the tympanic membrane.

If the tympanic membrane moves well then the eardrum is intact and there is no middle ear fluid. If the eardrum has restricted movement or no movement at all and the tympanic membrane is intact, then the child may have a middle ear effusion.

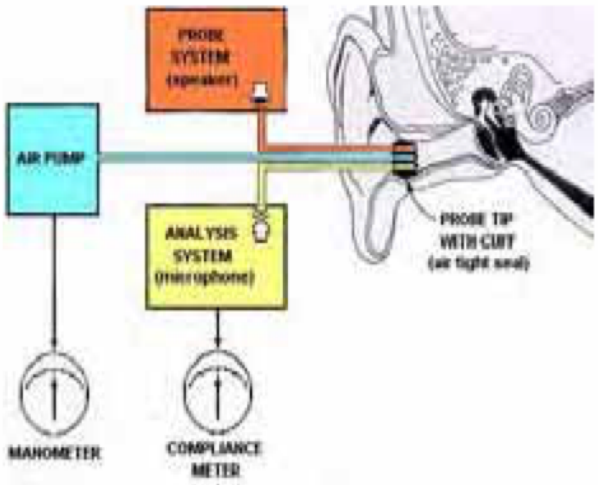
# Tympanometry

**What is tympanometry?**

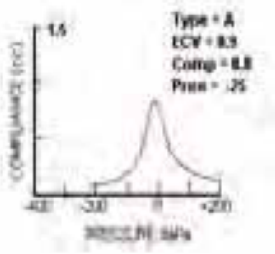
Tympanometry is a technique used to look at the function of the middle ear. It is NOT a hearing test. It is a test used together with otoscopy (looking into the ears) and audiometry (testing the hearing) and is useful in helping to determine how the middle ear is functioning.

**How does a tympanometer work?**

The procedure of tympanometry involves inserting a probe into the outer ear canal and creating an air-tight seal. The probe contains a tiny speaker, a microphone and an air pump. The air pump changes the air pressure range (typically +200 daPa to –400 daPa) in the ear canal. The speaker introduces a calibrated tone into the ear canal, which changes in frequency and loudness. Some of the sound produced by the speaker will be passed through the middle ear, while some of the sound will be reflected back off of the tympanic membrane. The microphone measures the amount of reflected sound in the ear canal. The "compliance" of the eardrum and middle ear (i.e. how well this system responds to sound) is then determined by the tympanometer as the air pressure changes.



**What is a tympanogram?**
A tympanogram is a chart which can tell us how well the middle ear is functioning.



**What does a tympanogram tell us?**

Tympanograms are classified into types according to the shape of the tympanometric trace ("peak"), which is dependent upon the middle ear pressure, and the middle ear compliance.

A tympanogram can provide three helpful pieces of information:

**Middle ear pressure**
This is the air pressure of the air contained within the middle ear. It is shown by where the "peak" of the tympanometric trace falls along the pressure axis.

Middle ear pressure values ranging from +50 daPa to –200 daPa for children, and +50 daPa to –50 daPa for adults is generally considered normal.

**Compliance**
The compliance of the middle ear system is a measure of how well the system responds to sound. This is shown by the height of the "peak".
Middle ear compliance values from 0.3 to 1.5 cc are usually considered normal.

**Equivalent volume of the ear canal**
Normative ear canal volumes vary as a function of age. Typically for children a volume range of 0.5 to 1.5 cc is typically considered normal, while for adults the range is 0.5 to 2.00 cc. This value is reported by the tympanometer, but not shown on the tympanogram graph.

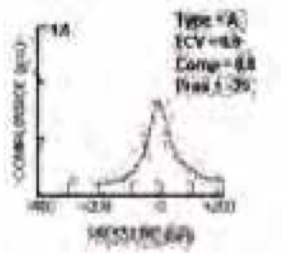
In general, classification of tympanograms is as follows:

| | |
|---|---|
| Type A tympanograms | normal middle ear pressure<br>Peak between +50 daPa to –200 daPa for children |
| Type C tympanograms | abnormally low middle ear pressure<br>Peak less than –200daPa for children |
| Type B tympanograms | no pressure peak |

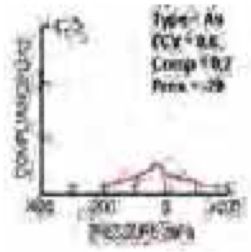
Compliance values and equivalent ear volume allow classification into subtypes, and provide valuable information for diagnosis.

**Type A:**

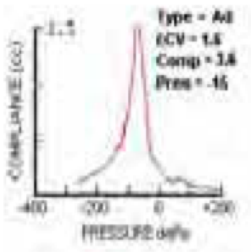
There are three sub-types of Type A tympanograms, A, As and Ad. All Type A tympanograms have normal middle ear pressure peaks. Following are illustrations of the Type A sub-types:



A standard **Type A** tympanogram, with normal middle ear pressure (Pres = -25) and normal compliance (Comp = 0.8), is indicative of a properly functioning Eustachian tube. Generally this tympanogram would be described as representing normal middle ear function.



**Type As** tympanograms, with normal middle ear pressure (Pres = -20) but reduced compliance (Comp = 0.2) can represent normal middle ear function, or a stiff middle ear system suggestive of some form of ossicular fixation (otosclerosis or tympanosclerosis affecting the ossicles) with normal Eustachian tube function.



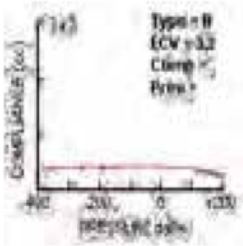
**Type Ad** tympanograms, with normal middle ear pressure (Pres = -15) but increased compliance (Comp = 3.6) indicate a flaccid, or hyper-mobile, middle ear system. This could represent an ossicular subluxation (a partial or full ossicular discontinuity), or a healed tympanic membrane perforation.

**Type B:**

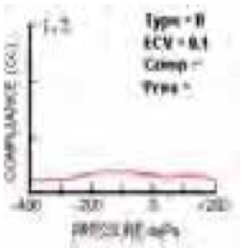
Type B tympanograms exhibit no air pressure peaks and are generally described as "flat" tympanograms. There are several middle ear conditions, which can result in Type B tympanograms.
Type B subtypes are

- those with normal equivalent ear canal volume
- those with high equivalent ear canal volume
- those with low equivalent ear canal volume.

A **Type B** tympanogram with normal ear canal volume (ECV = 0.5 cc) typically means a middle ear effusion. However, some people with very thickened tympanic membranes, or perforations may also exhibit a tympanogram of this type.



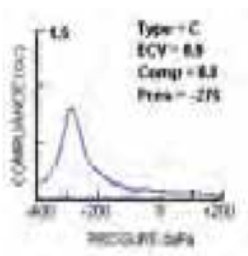
**Type B tympanogram with a high ear canal volume** (ECV = 3.2), consistent with a tympanic membrane perforation, a patent grommet or T tube.



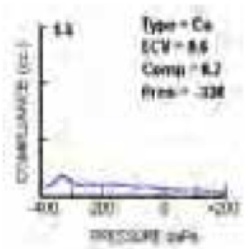
**Type B tympanogram with a low ear canal volume** (ECV = 0.1) indicating that the probe is blocked, either by wax in the ear canal, or the probe tip is against the side of the ear canal wall.

**Type C:**

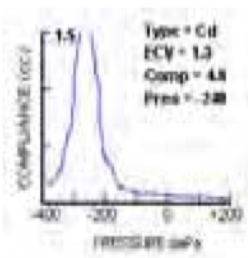
Type C tympanograms generally have similar compliance values and shapes as Type A tympanograms. What sets them aside from Type A tympanograms is that the middle ear pressure is abnormally low indicating Eustachian tube dysfunction.



This **Type C** tympanogram, with normal compliance (Comp = 0.8) but low middle ear pressure (Pres = -275) typically means a Eustachian tube dysfunction without the presence of middle ear effusion.



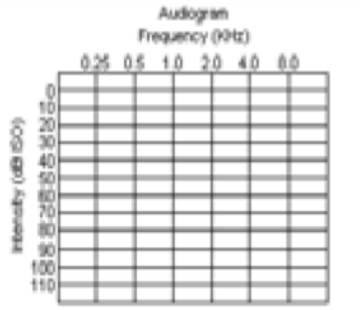
This **Type Cs** (shallow) tympanogram, with reduced compliance (Comp = 0.2) and low middle ear pressure (Pres = -330), in addition to indicating a Eustachian tube dysfunction, most likely also means there is some fluid in the middle ear as well as some air.



This **Type Cd** (deep) tympanogram, with increased compliance (Comp = 4.8) and low middle ear pressure (Pres = -240), also suggests either an ossicular subluxation, or healed tympanic membrane perforation (similar to Type Ad) with Eustachian tube dysfunction.

# Audiology
# The Measurement of Hearing

Hearing is measured by determining how well a person can hear a range of frequencies of sounds. There is a specific procedure used to gather this information, which then is generally plotted on a graph called an audiogram. Below is a sample audiogram form:




Across the top of the audiogram are numbers representing various frequencies of sounds that are typically tested. Low frequency sounds are represented on the left side of the audiogram, while high frequencies are on the right. Frequencies are measured in hertz (Hz), or kilohertz (KHz). This audiogram form depicts frequencies in KHz. The range of frequencies on the audiogram corresponds to the range of frequencies most needed for verbal communication purposes. Occasionally intermediate frequencies of 0.75, 1.5, 3 and 6 KHz may also be tested and plotted.

Going from the top to the bottom of the audiogram is the intensity scale in decibels (dB). "ISO" refers to the International Standards Organization which is a worldwide association that sets standards for a wide range of physical measurements. Softer sounds are represented at the top of the audiogram, while loud sounds are at the bottom. The actual values plotted on an audiogram at the different frequencies are called thresholds. A threshold is defined as the intensity at which a person can hear a specific sound 50% of the time. There is a standardized procedure to determine thresholds.

Generally an audiogram will show the hearing results for both the right and left ears. As such there are different symbols used to differentiate the hearing results for each ear. However, before proceeding with this topic, two other concepts need to be introduced.

***Air and Bone Conduction Testing:***

Air and bone conduction testing is used to establish the type of hearing loss present. A hearing loss can be sensorineural, conductive or mixed.

Air conduction testing is done using standard earphones, although sometimes an insert phone is used. Basically, air conductive testing assesses the outer, middle and inner ear as a unit. On the other hand, bone conduction testing uses a bone vibrator, which is placed on the mastoid prominence behind the pinna. The bone vibrator transmits sounds to the bones of the skull, which in turn stimulate the inner ear directly. Basically, bone conduction testing assesses inner ear function. Comparison of the air and bone conduction thresholds is used to determine the type of hearing loss.

***Masking:***

There are certain circumstances that arise during air and bone conduction testing where the non-test ear may hear the stimulus presented to the test ear. This situation can result in an erroneous interpretation of the test results. Masking of the non-test ear is a technique used to eliminate the possibility of the non-test ear hearing the stimulus. Masking is necessary if there is a 40 dB difference in the air conduction threshold of the test ear and the bone conduction threshold of the non-test ear. An unmasked signal of a high enough intensity presented to the test ear can "cross over" and be heard by the non-test ear. Masking is almost always necessary when testing bone conduction. Bone conducted sounds are transmitted equally by the bones of the skull to both ears. There are a few situations when masking for bone conduction does not need to be used, or cannot be used. It is beyond the scope of this manual to engage in a comprehensive discussion of masking. The masking concept is introduced in order for one to recognize masked audiometric signals plotted on an audiogram and to be able to interpret the audiogram accordingly.

***Audiometric Symbols:***

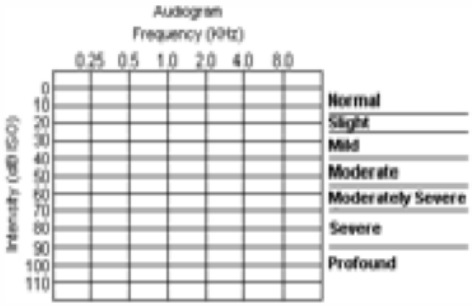
In order to differentiate test results from the right and left ears, and whether plotted thresholds have been masked or not, a series of standardised audiometric symbols have been developed. Occasionally the symbols will also be colour coded, with red representing the right ear, and blue the left.

| | Air Conduction | | Bone Conduction | |
|---|---|---|---|---|
| | unmasked | masked | unmasked | masked |
| Right | O | or △ | < | [ |
| Left | X | or □ | > | ] |

Any of the above symbols with an arrow, down and to the left for the right ear, ↙, or down and to the right for the left ear, ↘, attached to it means that there was no response at that frequency. Most audiograms will contain a key to the audiometric symbols.

### *Audiogram Interpretation:*

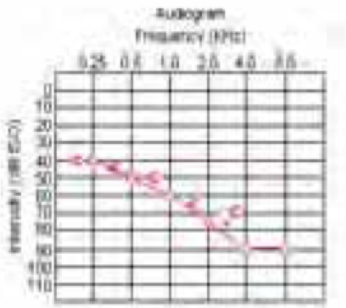
The information contained on an audiogram allows the determination of the degree and type of hearing loss. For verbally reporting the degree of hearing loss, there are standardized descriptive terms that are used. The following figure illustrates these terms and ranges of hearing loss associated with them:




There are three types of hearing loss, sensorineural, conductive and mixed. By definition, a sensorineural hearing loss is present if there is less than a 15 dB difference between the air and bone conduction thresholds of a given ear, and the air and bone conduction thresholds are worse than 15 dB. A conductive hearing loss is defined by bone conduction thresholds being 15 dB or better than the air conduction thresholds, and the bone conduction thresholds are 15 dB or less. And a mixed loss will have both conductive and sensorineural components in the loss.

Following are sample audiograms illustrating sensorineural, conductive and mixed hearing losses.

**Sensorineural hearing loss:**



Moderate to severe sensorineural hearing loss

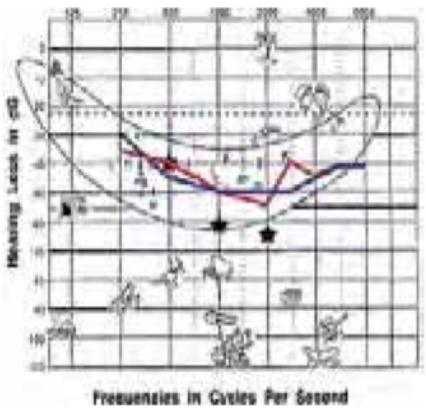
**Conductive hearing loss:**

Mild to moderate conductive loss

**Mixed hearing loss:**

Moderately severe to profound mixed loss

**Speech Banana**




Ref: http://www.hearingaidforums.com/showthread.php?t=5668

# Middle Ear Disease and School

All children who suffer from middle ear disease face difficulties with learning. In the case of Aboriginal children, learning may be further complicated by:

- English being a second language
- English being the language used for instruction in the classroom

Otitis media in the early childhood years may mean that young Aboriginal children may have a poor grasp of their own first language by the time they enter school. Children who do not hear properly will find it difficult to pick up the sounds of their own language, and even harder to then learn a second language. In addition, there are a number of sound differences between English and Aboriginal languages.

A hearing loss, even a mild fluctuating loss caused by otitis media, will affect learning. Some difficulties that may occur include:

- Telling the difference between sounds
- Putting sounds in words together
- Sequencing and remembering sounds
- Listening for sounds in noisy conditions
- Developing word usage due to poor vocabularies
- Remembering and explaining words
- Understanding questions properly
- Replying to questions properly

When children go to school, they have to rely on their hearing a lot more. At home when they don't hear something properly, they can always figure out what is going on by looking, or asking or waiting till someone tells them what is going on. At school, teachers often only give instructions once and they do not like the children to be looking around or asking someone else what they should be doing. This is very frustrating for the child and he/she can find him/herself always in trouble for not concentrating and disturbing others.

The other problem that children with a conductive hearing loss will find is that their auditory processing system is not developed enough for them to learn to read and write easily. The combination of hearing problems in the classroom and a history of poor hearing, means that school can be very hard, even for bright children.

**Central Auditory Processing Disorder (CAPD)**

"Central Auditory Processing Disorder (CAPD), also known as Auditory Processing Disorder (APD), is an umbrella term for a variety of disorders that result in a breakdown in the hearing process. In short, our brain cannot make sense of what our ears hear because the auditory signal is distorted in some way" *(Australian Hearing).*

As a result, one of the biggest problems experienced by individuals with CAPD is difficulty listening in background noise. It cannot be explained on the basis of a normal peripheral hearing loss. Children that have central auditory processing problems may have one or more of the following symptoms; be distractible, constantly asking for repeats, have difficulty understanding speech in background noise, be sensitive to loud sounds, have difficulty following complex instructions, or have poor memory for chains of digits or words. The child may also have reading spelling and other academic difficulties or display behaviour problems. The most common cause for CAPD is long standing conductive hearing loss such as glue ear or chronic suppurative otitis media that later causes inefficiency in the brain processing of the electrical output from the ear.

The condition can be assessed with specialized testing particularly in children over the age of 6 years. If the diagnosis is confirmed there are several classroom strategies that the audiologist may recommend and in some cases further treatment may also be suggested such as speech therapy, occupational therapy or computer based learning using one of the several programs available.

Sometimes children do not understand that their problems are caused by a hearing loss - they think that they are stupid. It is extremely important that all children are educated about middle ear disease so they can tell the Health Worker or Teacher and adjustments can be made to the program.

**Social Emotional Aspects of Hearing Loss**

For anyone suffering long-term hearing loss in early life and childhood (and this is not exclusive to Indigenous people), it can have life long negative consequences in the following areas;

- Language development
- Socialization
- Education - hence literacy and numeracy
- Social/emotional wellbeing and self esteem
- Training and employment opportunities
- Mental health and self harm
- Domestic violence
- And as a consequence of the above factors, can be a reason for the over representation of Indigenous people in the criminal justice system

The effects of auditory sensory deprivation from hearing loss especially in the first 5 years of life can lead to abnormal brain development that is manifested as central auditory processing disorders, or more simply termed, learning problems or listening problems. Even if a person's hearing becomes normal at a later age, these listening problems remain. Consequently they can have significant impacts on adult education, training, and employment. Hence, the need to address hearing loss as early as possible.

# Children with a Hearing Loss
## *General strategies that teachers can use in classrooms*

- Be aware of the factors in the environment which affect listening:
  - noise from people talking and moving
  - general background noise e.g. traffic, air conditioners
- Write the key vocabulary on the board
- Use pictures or objects to illustrate a point and maintain interest
- Ask a peer to adopt a child and give prompts
- Inform parents or clinic staff about children who have sore ears
- Remind children and parents of the importance of healthy ears to learning
- Make sure the role model is speaking clearly at all times
- When the students want to talk to the teacher, make sure they are quiet and facing you

- Keep the students close to the teacher
- Keep plenty of light on your face when talking
- Make sure that you keep your hand away from your face when you are talking
- Use non-verbal gestures to help give meaning
- Use language that is accessible and explaining difficult terms if they are used
- Implement a blow, breathe and cough (BBC) program daily in the classroom
- Provide the students with tissues on a needs basis to frequently clear noses

Suggestions for teachers for children with Central Auditory Processing Disorder (CAPD). Some children will appear to be helped by most suggestions but some will be difficult to help no matter what is tried.

1. Reduce distractions – this involves getting the child's attention before giving instructions.
2. Preferential seating – seat the child away from known distractions such as open windows, pencil sharpeners, doors, air conditioners.
3. Delivery style – avoid multiple commands, give instructions in the most simple form possible.
4. Speak at a slower rate than normal and clearly as research has shown that background noise is often equal to or louder than the teacher's voice.
5. Instructional transitions – utilizing review of the previous material before beginning new lessons to give the student a feeling of success.
6. Utilization of words such as "Listen" "Ready" and "Remember" seem to be effective for signaling an important message.
7. Visual aids include overheads, opaque projectors and computers can be utilized to supplement the teachers oral presentations as well as to provide an alternative mode to the auditory channel. Combining the visual and auditory modes of learning may benefit all students in the classroom.
8. Some children may benefit from the child having an FM system and the teacher wearing a microphone during class.

# Common Conditions

*Features, Diagnosis, Audiogram, Tympanogram, Management, Complications for;*

## Acute Otitis Media (AOM)

Acute otitis media without perforation (AOM) - Presence of middle ear fluid with symptoms or signs of suppurative infection, which may include, otalgia, fever, irritability, vomiting or diarrhoea.

Acute otitis media with perforation - Acute suppurative infection with recent discharge from the middle ear (within the last 7 days).

## Chronic suppurative Otitis Media (CSOM)

Persistent discharge from the middle ear through a tympanic membrane perforation for more than 6 weeks.

## Otitis Media with Effusion (OME or Glue Ear)

Presence of middle ear fluid without symptoms or signs of suppurative infection.

## Otitis Externa (Swimmer's Ear)

Infection in the ear canal.

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ACUTE OTITIS MEDIA (AOM)
FEATURES
MOST COMMON SYMPTOMS
Otalgia (ear pain) or pulling of the ear
Irritability
Fever
OTHER SYMPTOMS
Anorexia
Vomiting
Diarrhoea
Otorrhoea (runny ears)
May be relatively "silent"
DIAGNOSIS
Bulging red ear drum
Decreased mobility with pneumatic otoscopy
Ear drum may rupture → discharge in ear canal
AGGRAVATING FACTORS
Upper respiratory tract infection (cold)
Stopping breast feeding early
Reflux
Passive cigarette smoke exposure
Overcrowding
Poor hygiene
Poor nutrition
Bulging eardrum of AOM
AUDIOLOGY
Mild Conductive hearing loss
TYMPANOMETRY
Usually Type B Tympanogram
PREVENTATIVE MEASURES
Continue breast feeding until 12 months
Avoiding smoking around babies and children
Avoid dummies after 12 months
Avoid prop-feeding, with the baby lying flat and the bottle "propped up"
Vaccination including Prevnar
Early treatment of ear infection to avoid
Aboriginal Ear Health Manual • Page 39

# ACUTE OTITIS MEDIA (AOM)

**TREATMENT**

- Antibiotics e.g. Amoxicillin 90mg/kg/day for 10 days
- If allergic to Penicillin – Rulide D/Septrin
- Analgesia e.g. paracetamol
- Local anaesthetic ear drops (if no perforation)
- If no improvement in 48 hours, Augmentin/Cefaclor
- Treatment of otorrhoea
  - Non-ototoxic topical antibiotic ear drops e.g. Ciproxin HC/Ciloxan ear drops

**COMPLICATIONS**

- Otorrhoea (if perforated ear drum)
- Chronic otitis media
- Otitis media with effusion
- Perforation
- Acute mastoiditis
- Facial nerve palsy
- Cholesteatoma
- Meningitis
- Sensorineural hearing loss

**INDICATIONS FOR REFERRAL**

- Persistent otitis media
- If complications are suspected
- Persistent otorrhoea after one week of treatment
- Recurrent otitis media
  - 3 attacks in 6 months
  - 4-6 attacks in 12 months
  - presence of middle ear effusion for 3 months or longer
  - balance problems, speech/language delay
- Suspected cholesteatoma



*Acute perforated eardrum.*

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CHRONIC SUPPURATIVE OTITIS MEDIA (CSOM)
FEATURES
MOST COMMON SYMPTOMS
Otorrhoea (discharge from ear)
Itchy ear
Hearing loss
AUDIOLOGY
Mild Conductive hearing loss
TYMPANOMETRY
Usually Type B Tympanogram
PREVENTATIVE MEASURES
Continue breast feeding until 12 weeks
Avoiding smoking around babies and children
Avoid prop-feeding, with the baby lying flat and the bottle "propped up"
Vaccination including Prevnar
Early treatment of ear infection to avoid chronic discharge.
Avoid contaminated water entering ear
Moist moderate Tympanic membrane perforation
Small perforation will produce discharge
DIAGNOSIS
Otorrhoea (discharge from ear) with perforation of tympanic membrane
Perforated tympanic membrane
Ear drum may rupture → discharge in ear canal
OTHER AGGRAVATING FACTORS
Contaminated water entering the ear
Upper respiratory tract infection (cold)
Stopping breast feeding early
Reflux
Passive cigarette smoke exposure
Overcrowding
Poor hygiene
Poor nutrition
Aboriginal Ear Health Manual • Page 41

# CHRONIC SUPPURATIVE OTITIS MEDIA

**TREATMENT**

- Betadine ear toilets
- Antibiotics – topical fluoroquinolones eg. Ciloxan eardrops
- Culture of middle ear fluid
- Myringoplasty to close perforation (once ear is dry)

**COMPLICATIONS**

- Hearing loss
- Granulation tissue and polyps
- Cholesteatoma
- Chronic mastoiditis
- Meningitis
- Intracranial abscess

**INDICATIONS FOR REFERRAL**

- Hearing loss
- Discharge that fails to resolve despite adequate medical treatment
- Non-healing perforation
- Any of the listed complications



Cholesteatoma

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OTITIS EXTERNA (SWIMMER'S EAR)
FEATURES
Pain and tenderness of the ear canal (may spread to the outer ear)
Discharge
Itching
Swollen ear and ear canal
Occasionally reduced hearing
Sometimes noises in the ear (tinnitus)
DIAGNOSIS
Otoscopy – appearance of skin of outer ear canal
red scaled and peeling
"soggy" cardboard like appearance
Ear canal may be inflamed and swollen and painful to touch
Swab
OTHER AGGRAVATING FACTORS
Swimming
Hot and humid climates
Eczema
Cotton bud use
TREATMENT
Dry ear toilet
Antibiotic drops (Ciproxin HC as ear drops)
Use of otowicks for swollen ear canal
Swab for microscopy
Classical Otitis Externa showing infected debris and slight swelling of ear canal
Severe otitis externa and swelling
COMPLICATIONS
Narrowing of the ear canals
Facial cellulitis
Malignant otitis externa leading to osteomyelitis of the temporal bone
Fungal infection
INDICATIONS FOR REFERRAL
Failure to resolve after 1 –2 weeks of treatment
PREVENTATIVE MEASURES
Avoid water entering ear
Ear protection when swimming and bathing
Avoid use of cotton buds or inserting other objects into the ear
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OTITIS MEDIA WITH EFFUSION (OME OR GLUE EAR)
AUDIOLOGY
Mild Conductive hearing loss
TYMPANOMETRY
Type B or Type C Tympanogram
PREVENTATIVE MEASURES
Treatment of colds and upper respiratory tract infections
Avoid smoking around babies and children
Early treatment of acute otitis media
Regular ear health checks
Diffusely retracted ear with OME
OME with attic retraction pocket
FEATURES
SYMPTOMS IN YOUNG CHILDREN (0-3)
Balance problems
Delayed speech development
Hearing loss (fluid in the middle ear blocks the conduction of sound)
Irritability and pulling at ear
SYMPTOMS IN OLDER CHILDREN (3-7)
Hearing loss
Difficulty at school
Behaviour problems
Delayed speech and language (may affect reading development)
DIAGNOSIS
Retracted ear drum
May show fluid levels or bubbles
Decreased mobility on pneumatic otoscopy
Different colours – from red to white to yellow
OTHER AGGRAVATING FACTORS
Upper respiratory tract infection (cold)
Reflux
May follow acute otitis media (AOM)
Blocked Eustachian tube
Prematurity/low birth weight
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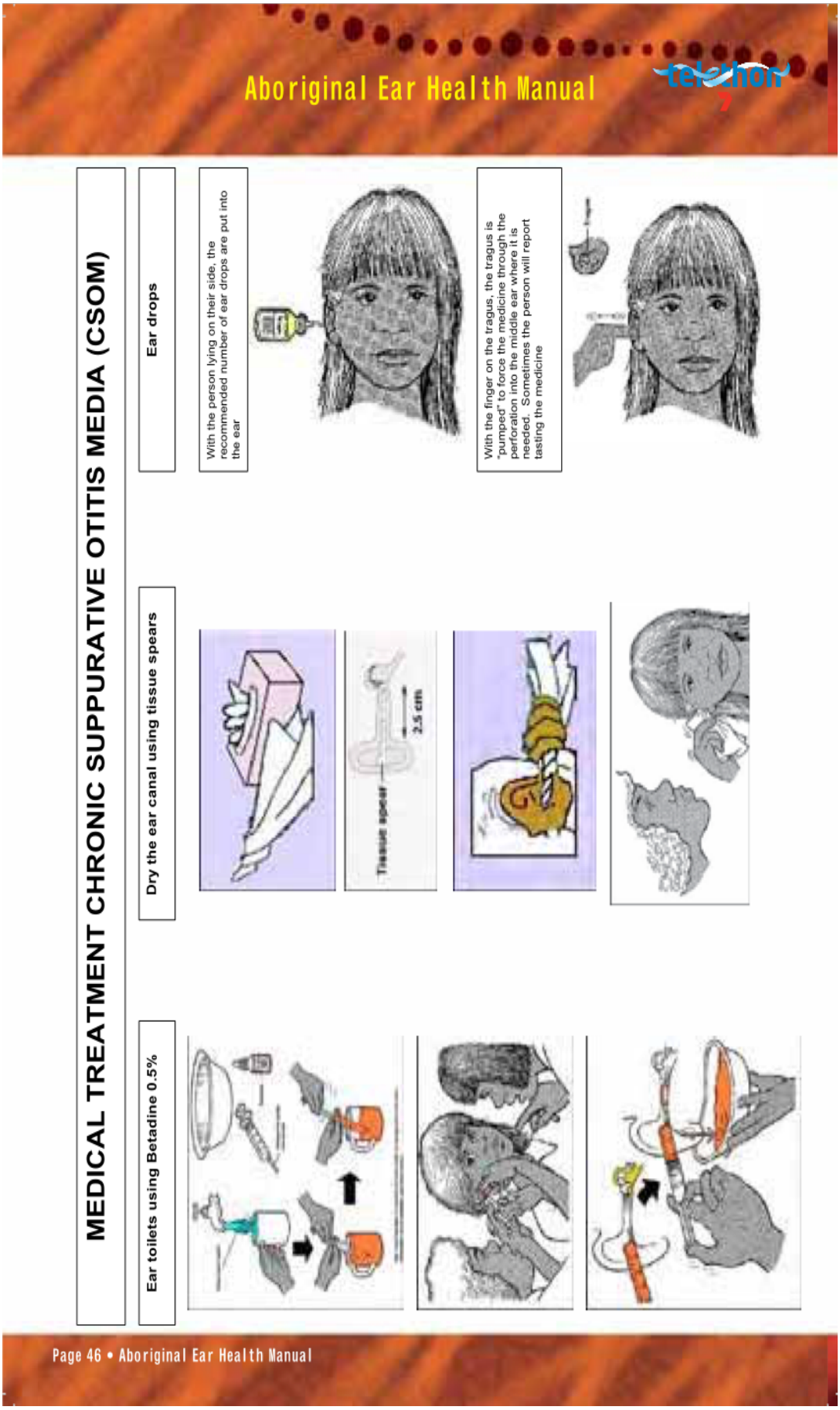
OTITIS MEDIA WITH EFFUSION (OME OR GLUE EAR)
TREATMENT
• Antibiotics
• BBC program
• Steroid nasal spray if allergic child
• Hearing aids
• Grommets (ventilation tubes)
See below: various types of grommet tubes in the tympanic membrane
COMPLICATIONS
• Acute otitis media
• Cholesteatoma
• Ossicular problems
• Language problems/reading difficulties
• Central auditory processing disorder
• Hearing loss in background noise as adult
INDICATIONS FOR GROMMETS
• 3 attacks of otitis media in 6 months
• 4 attacks of otitis media in 12 months
• handicapping hearing loss for 3 months
Different types of grommet tubes in tympanic membrane
First image of granulation polyp obscuring grommet.
Aboriginal Ear Health Manual • Page 45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MEDICAL TREATMENT CHRONIC SUPPURATIVE OTITIS MEDIA (CSOM)
Ear drops
With the person lying on their side, the recommended number of ear drops are put into the ear
With the finger on the tragus, the tragus is "pumped" to force the medicine through the perforation into the middle ear where it is needed. Sometimes the person will report tasting the medicine
Dry the ear canal using tissue spears
Tissue spear
2.5 cm
Ear toilets using Betadine 0.5%
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Aboriginal Ear Health Manual
telethon
MEDICAL TREATMENT CHRONIC SUPPURATIVE OTITIS MEDIA (CSOM)
Ear toilets using Betadine 0.5%
Ear syringing is done to wash pus due to suppurative otitis media out of the ear canal and middle ear.
Getting rid of the pus then allows the instillation of antibiotic ear drops to treat the infection.
Syringing: The senior author does not believe this is as effective for clearing discharge as Betadine ear toilets. But it is included because it is current practise in some areas in Australia
PROCEDURE
Dilute 5ml (one teaspoon) of 10% solution of Betadine with 100ml of clean warm water
Use a 10 or 20ml disposable plastic syringe. To syringe an ear, a short piece of tubing from a scalp vein needle is attached to the end of the syringe. Whenever refilling the syringe, remove the tubing to keep it from contaminating the solution or container. Attach the cut off end of a scalp vein needle firmly. This will provide a soft tip.
With infants or children with small ear canals, the solution can be dropped into the ear with a pipette, with the infant or child lying on their side. The pipette is then used to suck the solution out of the ear canal.
With older children, with one hand the pinna is grasped and pulled up and back. The syringe is aimed toward the top part of the eardrum and gentle syringing begins.
Hold the syringe up to the ear and point the tip so that the solution does not directly hit the ear drum
The person having their ear syringed, or care giver, should hold a kidney dish or bowl under the ear to catch the solution that comes out of the ear. A towel should be placed over the shoulder
Syringing is continued until no more pus comes out. The ear is then dried with tissue spears.
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telethon

## MEDICAL TREATMENT of CHRONIC SUPPURATIVE OTITIS MEDIA (CSOM)

### Ear drops

- Use of topical antibiotic corticosteroid combination ear drops helps with otorrhoea and reduces the risk of developing secondary otitis externa.
- The use of non-ototoxic ear drops e.g. Ciproxin HC or Ciloxan ear drops, has become the treatment of choice.
- With a perforation in the ear drum there is the risk that potentially ototoxic ear drops, e.g. Sofradex, may enter the middle ear and inner ear and cause permanent hearing loss.

**PROCEDURE**

**Clean out the discharge using irrigation with Betadine**
**Use a tissue spear to dry the ear**
**Instill the drops as shown**
**Treatment is three drops twice a day for three to five days**

With the person lying on their side, the recommended number of ear drops are put into the ear

With the finger on the tragus, the tragus is "pumped" to force the medicine through the perforation into the middle ear where it is needed. Sometimes the person will report tasting the medicine

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# MEDICAL TREATMENT OF CHRONIC SUPPURATIVE OTITIS MEDIA (CSOM)

## Drying the ear canal using tissue spears

- Tissue spearing is a technique used to either remove pus from the ear canal, or to dry the ear canal following ear syringing.

**PROCEDURE**

**A tissue spear is made by twisting a square of toilet tissue or facial tissue into a "rat's tail".**
**The tissue spear is then inserted into ear by rotating it.**
**The tissue spear is inserted to about 2.5 cm, or until the child blinks.**
**The tissue spear is left in place 1-2 minutes.**
**Remove and repeat until the tissue is dry.**

# Telemedicine

Telemedicine and specifically teleotology, allow the transmission either "live " or of stored images of the ear drum to specialists for diagnostic and treatment recommendation. A video otoscope (the Welch–Allyn macro view is a commonly used hand held video otoscope) with a built in digital camera which allows the image of the eardrum (tympanic membrane) to be shown on a computer to the patients/carers. In addition the live images can be transferred to a central telemedicine unit often with a whole room view allowing patients/carers to talk to the ENT specialist and discuss their treatment options. On occasion the computer image can be stored and later sent to the ear surgeon for comment (store and send) along with history, audiometry and tympanometry. This whole process of telemedicine allows the Aboriginal Health Worker, nurse or doctor in rural and remote regions to obtain specialist opinion via email or live telemedicine conference.

**How to use a video otoscope**

Using the video otoscope is surprisingly easy to those who have otoscopic skills with conventional otoscopes--- the main problem for the viewer is usually the presence of wax/debris, infection in the ear canal obscuring their view of the eardrum. It is crucial to remove the wax/infection either by syringing or by direct removal with a wax hook and headlight by those trained in that technique. It is important to dry the ear canal with a tissue spear if it is wet – otherwise the video otoscope lens may fog up.

The video otoscope is held upright and a finger propped against the patient's head to steady the instrument and prevent hurting the patient's ear canal if they move suddenly. The viewer's other hand gently pulls the outer ear upward and backwards to straighten the ear canal to allow the introduction of the video otoscope in the ear canal. If the viewer needs to "store and send" the image, then it can be captured by pressing either a camera button or in some cases a foot pedal. On occasions the video otoscope is used to capture a video of the eardrum's movement when the patient holds their nose and "pops" (valsalvas) their ear or the examiner blows air into the sealed ear canal (pneumatic otoscopy) to see if the eardrum is mobile. There is a

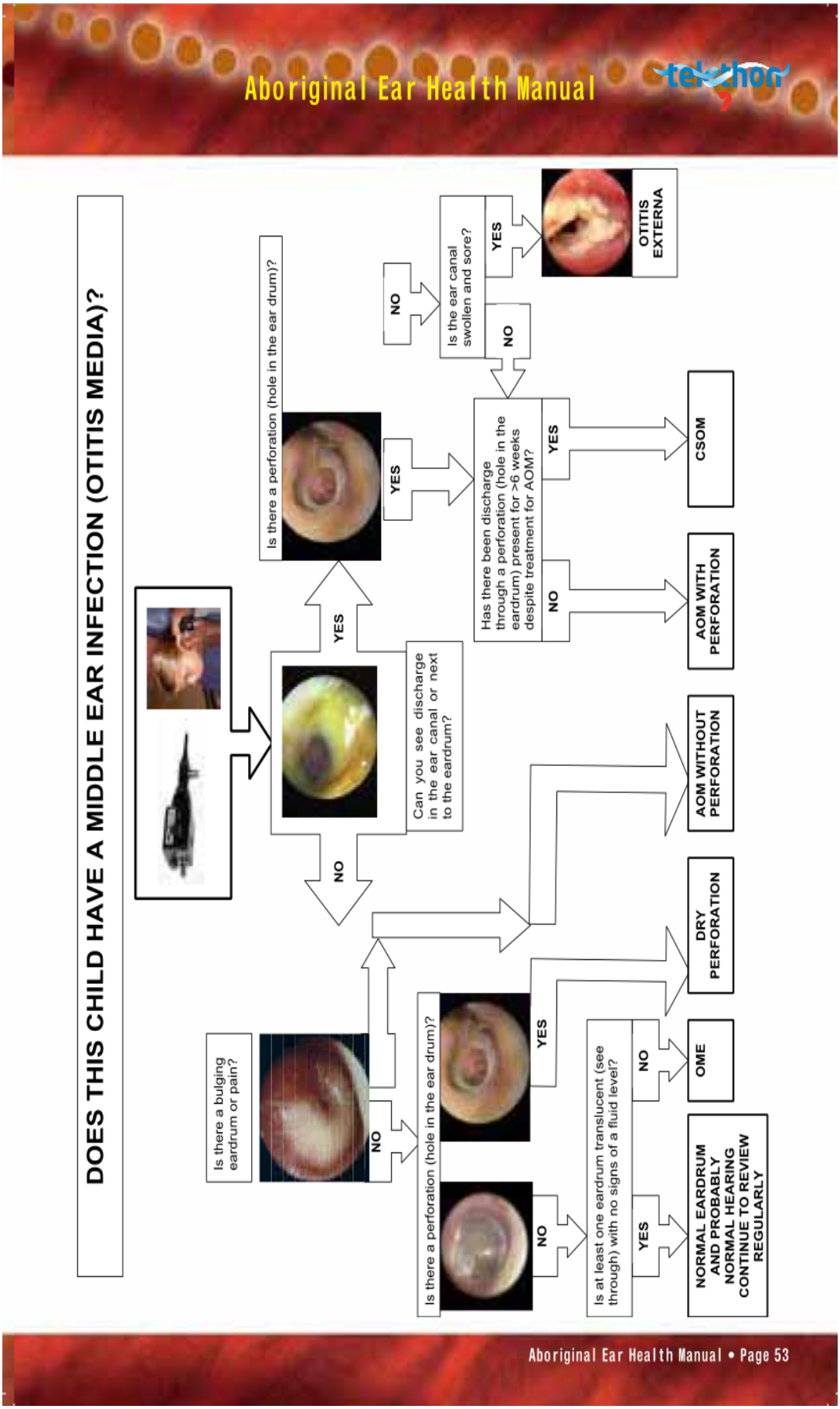
learning curve with the use of the video otoscope but with patience and practice will be rewarded with every improving images of the eardrum.

**Videoconferencing**

"Live view" telemedicine allows the Aboriginal Health Worker, nurse, doctor and patient/carer to directly discuss with the Ear Specialist history and treatment options for their ear condition. The viewer screen at each end of the teleconference can have multiple images of the patient's eardrum, of the audiogram and of the patient/carer and medical attendants. This enables the patient/carer to be fully assessed by the ear Specialist and for medical and surgical treatments to be discussed and arranged, saving much time for the patient/carer who might otherwise have months to wait to see the visiting specialist.

# Algorithms for Treatment

- Does this child have a middle ear infection?
- Management of OME.
- Management of AOM with perforation.
- Management of AOM without perforation.
- Management of CSOM.
- Management of dry perforation.
- Hearing loss due to otitis media.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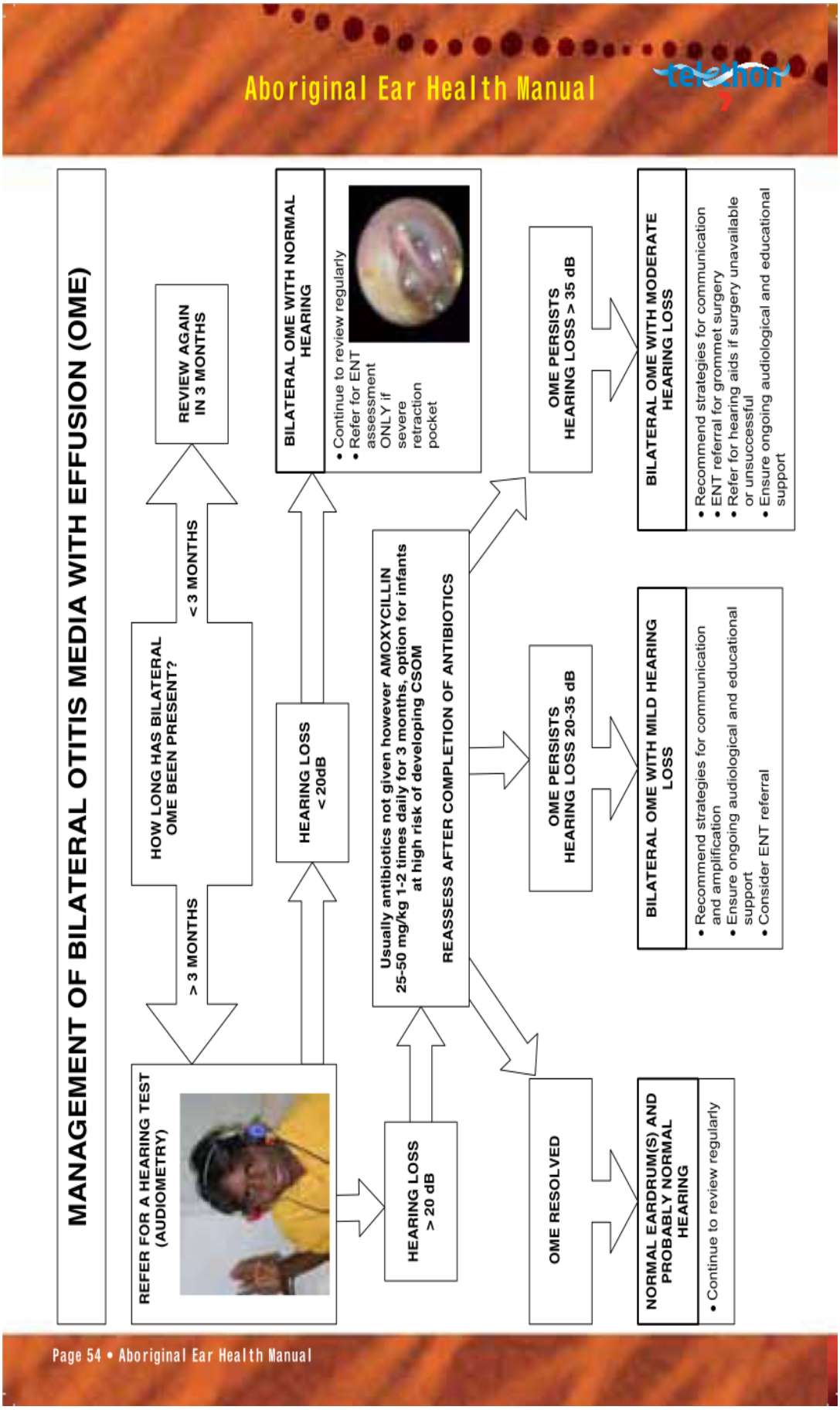
DOES THIS CHILD HAVE A MIDDLE EAR INFECTION (OTITIS MEDIA)?
Is there a perforation (hole in the ear drum)?
YES
NO
Is the ear canal swollen and sore?
YES
NO
OTITIS EXTERNA
Has there been discharge through a perforation (hole in the eardrum) present for >6 weeks despite treatment for AOM?
YES
NO
CSOM
AOM WITH PERFORATION
Can you see discharge in the ear canal or next to the eardrum?
YES
NO
AOM WITHOUT PERFORATION
Is there a bulging eardrum or pain?
NO
Is there a perforation (hole in the ear drum)?
YES
NO
DRY PERFORATION
Is at least one eardrum translucent (see through) with no signs of a fluid level?
YES
NO
NORMAL EARDRUM AND PROBABLY NORMAL HEARING CONTINUE TO REVIEW REGULARLY
OME
Aboriginal Ear Health Manual • Page 53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MANAGEMENT OF BILATERAL OTITIS MEDIA WITH EFFUSION (OME)
REFER FOR A HEARING TEST (AUDIOMETRY)
HOW LONG HAS BILATERAL OME BEEN PRESENT?
< 3 MONTHS
> 3 MONTHS
REVIEW AGAIN IN 3 MONTHS
HEARING LOSS < 20dB
BILATERAL OME WITH NORMAL HEARING
• Continue to review regularly
• Refer for ENT assessment ONLY if severe retraction pocket
HEARING LOSS > 20 dB
Usually antibiotics not given however AMOXYCILLIN 25-50 mg/kg 1-2 times daily for 3 months, option for infants at high risk of developing CSOM
REASSESS AFTER COMPLETION OF ANTIBIOTICS
OME RESOLVED
NORMAL EARDRUM(S) AND PROBABLY NORMAL HEARING
• Continue to review regularly
OME PERSISTS HEARING LOSS 20-35 dB
BILATERAL OME WITH MILD HEARING LOSS
• Recommend strategies for communication and amplification
• Ensure ongoing audiological and educational support
• Consider ENT referral
OME PERSISTS HEARING LOSS > 35 dB
BILATERAL OME WITH MODERATE HEARING LOSS
• Recommend strategies for communication
• ENT referral for grommet surgery
• Refer for hearing aids if surgery unavailable or unsuccessful
• Ensure ongoing audiological and educational support
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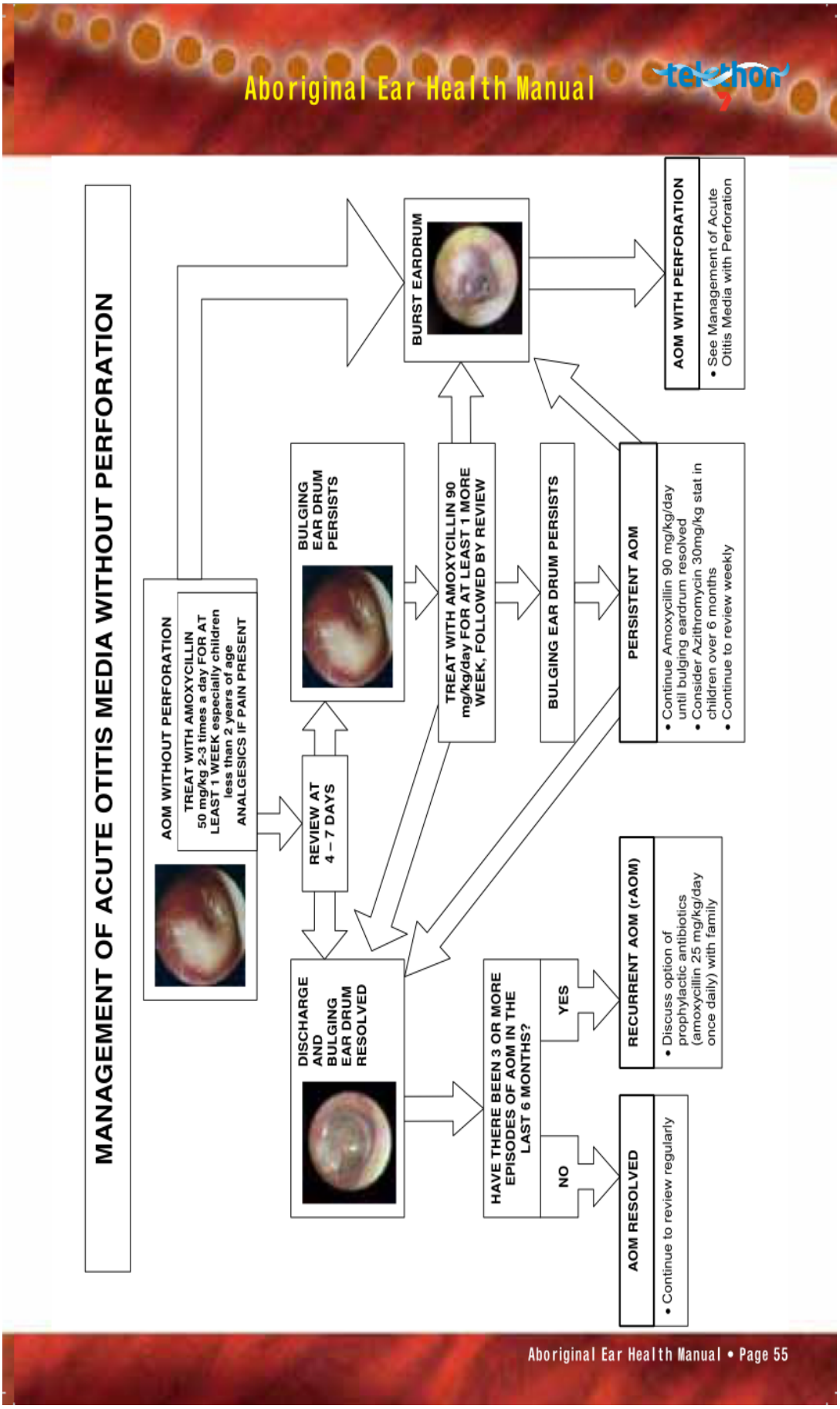
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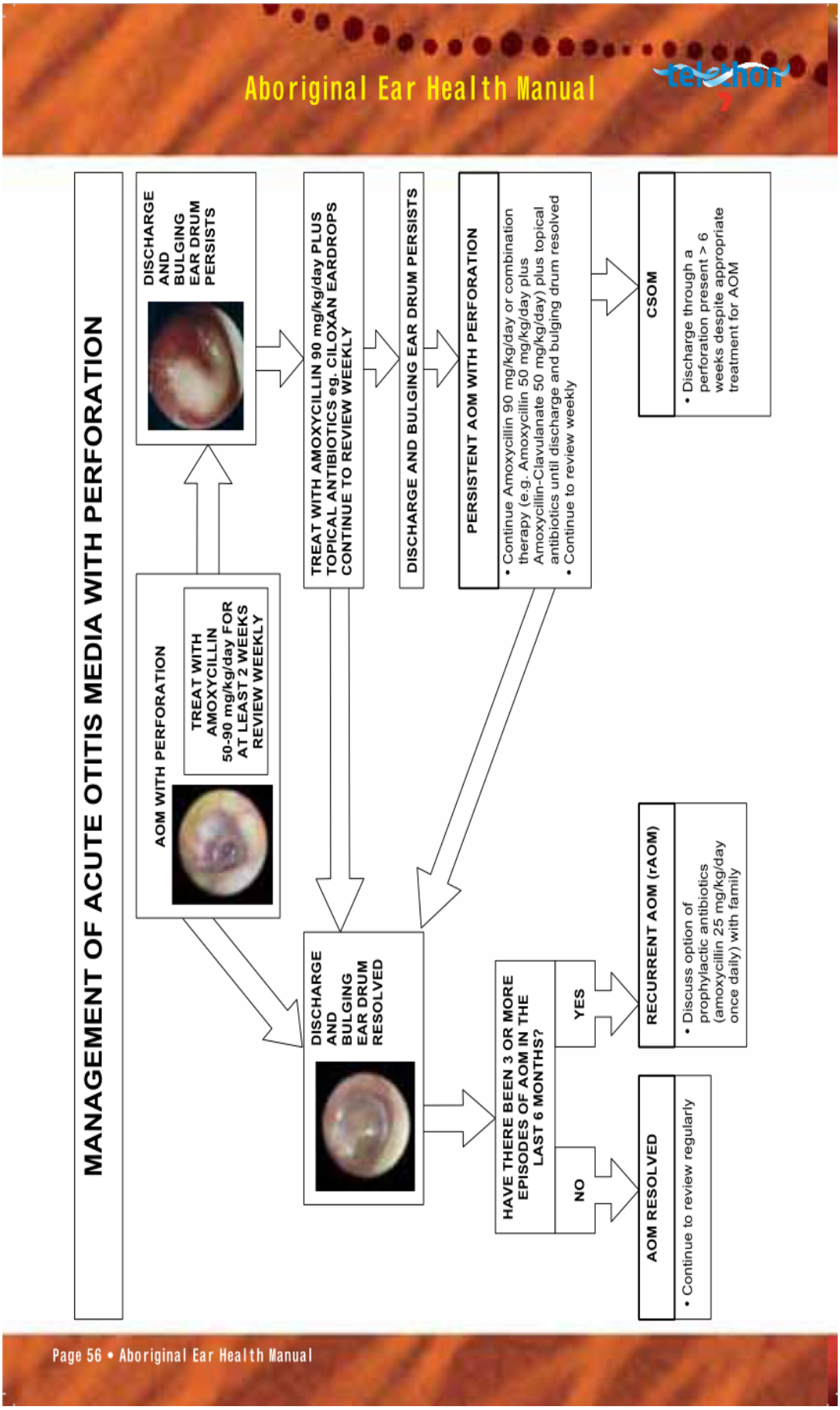
MANAGEMENT OF ACUTE OTITIS MEDIA WITHOUT PERFORATION
AOM WITHOUT PERFORATION
TREAT WITH AMOXYCILLIN 50 mg/kg 2-3 times a day FOR AT LEAST 1 WEEK especially children less than 2 years of age ANALGESICS IF PAIN PRESENT
REVIEW AT 4 – 7 DAYS
DISCHARGE AND BULGING EAR DRUM RESOLVED
BULGING EAR DRUM PERSISTS
TREAT WITH AMOXYCILLIN 90 mg/kg/day FOR AT LEAST 1 MORE WEEK, FOLLOWED BY REVIEW
BULGING EAR DRUM PERSISTS
PERSISTENT AOM
• Continue Amoxycillin 90 mg/kg/day until bulging eardrum resolved
• Consider Azithromycin 30mg/kg stat in children over 6 months
• Continue to review weekly
BURST EARDRUM
AOM WITH PERFORATION
• See Management of Acute Otitis Media with Perforation
HAVE THERE BEEN 3 OR MORE EPISODES OF AOM IN THE LAST 6 MONTHS?
YES
RECURRENT AOM (rAOM)
• Discuss option of prophylactic antibiotics (amoxycillin 25 mg/kg/day once daily) with family
NO
AOM RESOLVED
• Continue to review regularly
Aboriginal Ear Health Manual • Page 55


Aboriginal Ear Health Manual
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MANAGEMENT OF ACUTE OTITIS MEDIA WITH PERFORATION
AOM WITH PERFORATION
TREAT WITH AMOXYCILLIN 50-90 mg/kg/day FOR AT LEAST 2 WEEKS REVIEW WEEKLY
DISCHARGE AND BULGING EAR DRUM PERSISTS
TREAT WITH AMOXYCILLIN 90 mg/kg/day PLUS TOPICAL ANTIBIOTICS eg. CILOXAN EARDROPS CONTINUE TO REVIEW WEEKLY
DISCHARGE AND BULGING EAR DRUM PERSISTS
PERSISTENT AOM WITH PERFORATION
• Continue Amoxycillin 90 mg/kg/day or combination therapy (e.g. Amoxycillin 50 mg/kg/day plus Amoxycillin-Clavulanate 50 mg/kg/day) plus topical antibiotics until discharge and bulging drum resolved
• Continue to review weekly
CSOM
• Discharge through a perforation present > 6 weeks despite appropriate treatment for AOM
DISCHARGE AND BULGING EAR DRUM RESOLVED
HAVE THERE BEEN 3 OR MORE EPISODES OF AOM IN THE LAST 6 MONTHS?
YES
RECURRENT AOM (rAOM)
• Discuss option of prophylactic antibiotics (amoxycillin 25 mg/kg/day once daily) with family
NO
AOM RESOLVED
• Continue to review regularly
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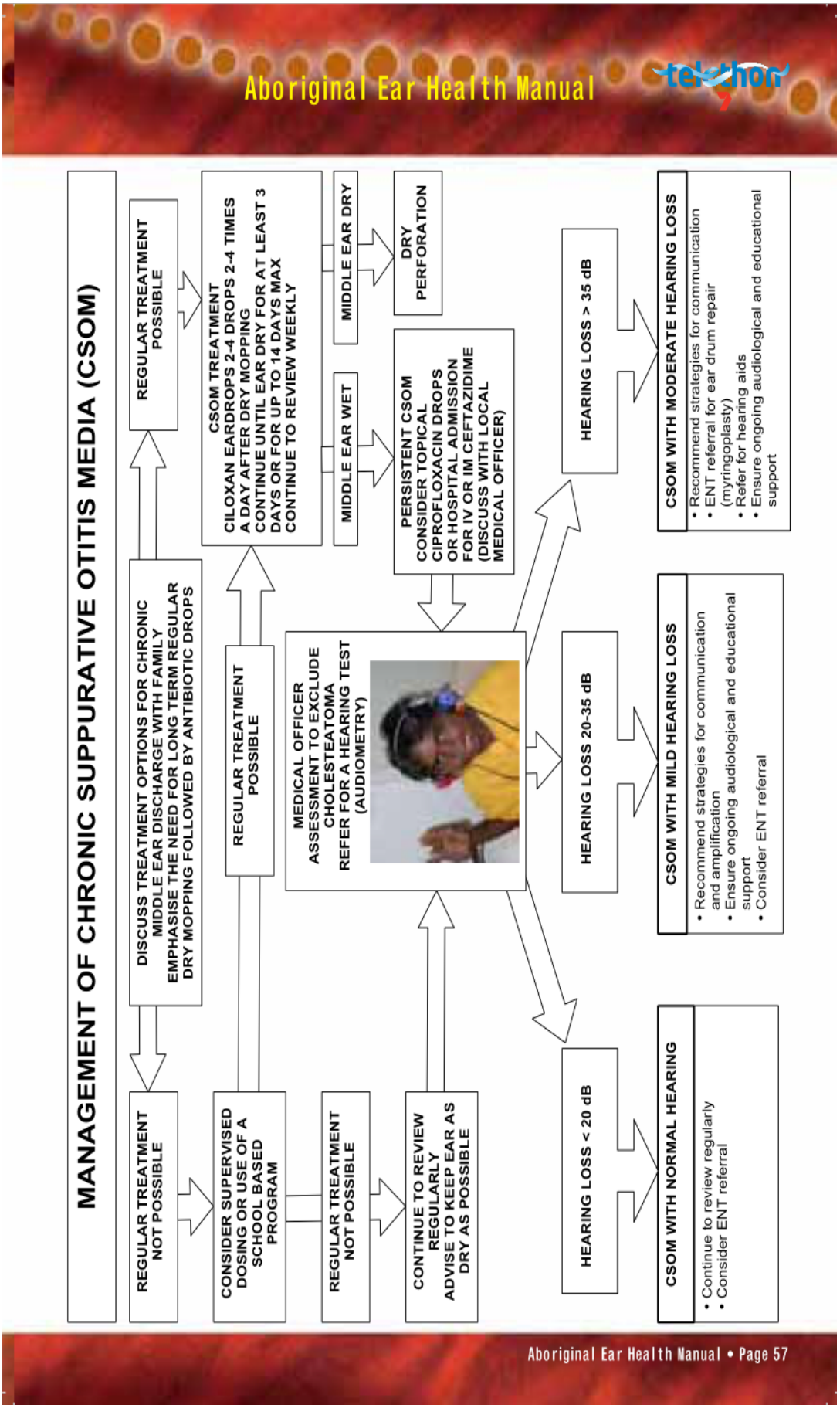
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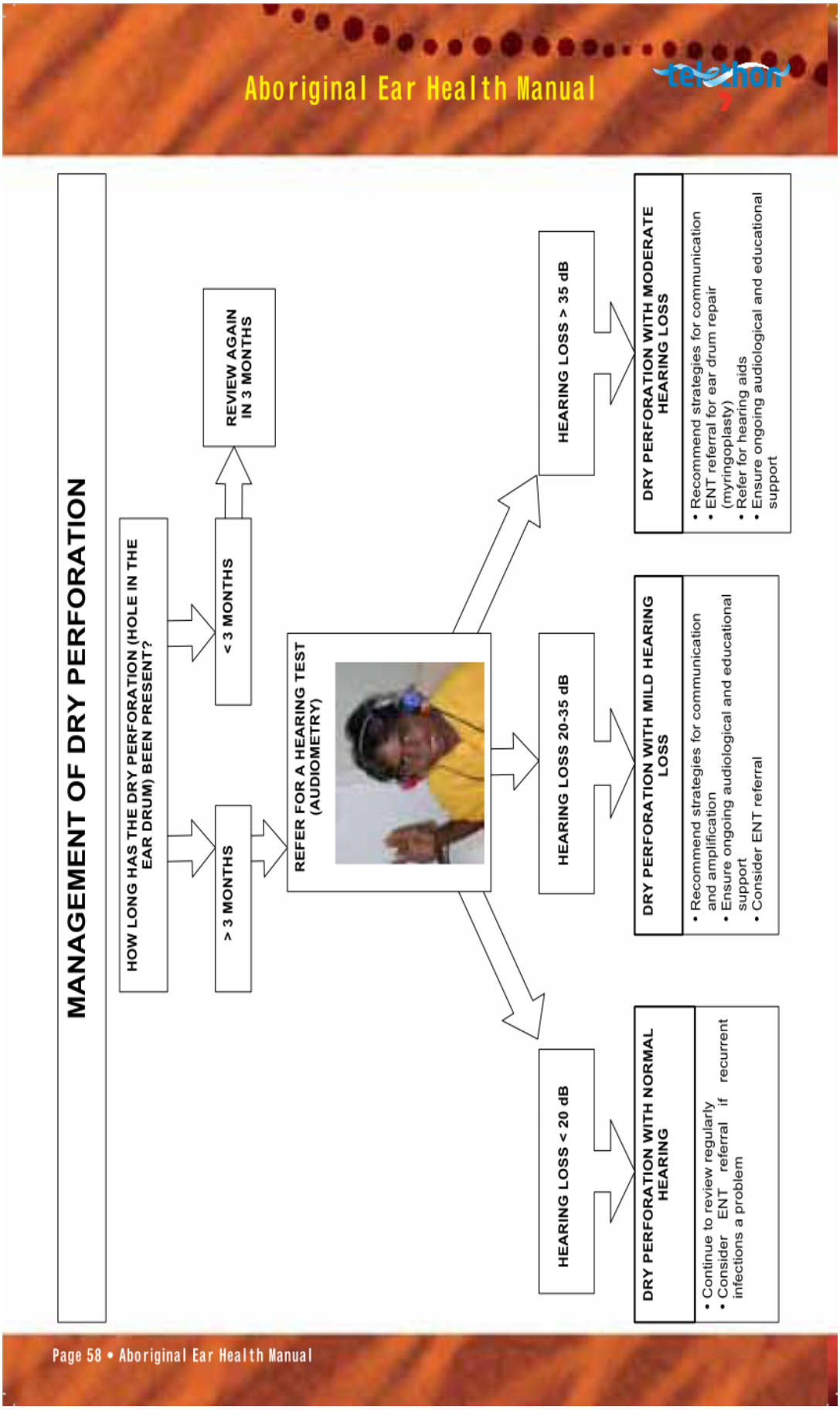
MANAGEMENT OF CHRONIC SUPPURATIVE OTITIS MEDIA (CSOM)
DISCUSS TREATMENT OPTIONS FOR CHRONIC MIDDLE EAR DISCHARGE WITH FAMILY EMPHASISE THE NEED FOR LONG TERM REGULAR DRY MOPPING FOLLOWED BY ANTIBIOTIC DROPS
REGULAR TREATMENT POSSIBLE
CSOM TREATMENT CILOXAN EARDROPS 2-4 DROPS 2-4 TIMES A DAY AFTER DRY MOPPING CONTINUE UNTIL EAR DRY FOR AT LEAST 3 DAYS OR FOR UP TO 14 DAYS MAX CONTINUE TO REVIEW WEEKLY
MIDDLE EAR DRY
DRY PERFORATION
MIDDLE EAR WET
PERSISTENT CSOM CONSIDER TOPICAL CIPROFLOXACIN DROPS OR HOSPITAL ADMISSION FOR IV OR IM CEFTAZIDIME (DISCUSS WITH LOCAL MEDICAL OFFICER)
REGULAR TREATMENT NOT POSSIBLE
CONSIDER SUPERVISED DOSING OR USE OF A SCHOOL BASED PROGRAM
REGULAR TREATMENT POSSIBLE
REGULAR TREATMENT NOT POSSIBLE
CONTINUE TO REVIEW REGULARLY ADVISE TO KEEP EAR AS DRY AS POSSIBLE
MEDICAL OFFICER ASSESSMENT TO EXCLUDE CHOLESTEATOMA REFER FOR A HEARING TEST (AUDIOMETRY)
HEARING LOSS > 35 dB
HEARING LOSS 20-35 dB
HEARING LOSS < 20 dB
CSOM WITH MODERATE HEARING LOSS
• Recommend strategies for communication
• ENT referral for ear drum repair (myringoplasty)
• Refer for hearing aids
• Ensure ongoing audiological and educational support
CSOM WITH MILD HEARING LOSS
• Recommend strategies for communication and amplification
• Ensure ongoing audiological and educational support
• Consider ENT referral
CSOM WITH NORMAL HEARING
• Continue to review regularly
• Consider ENT referral
Aboriginal Ear Health Manual • Page 57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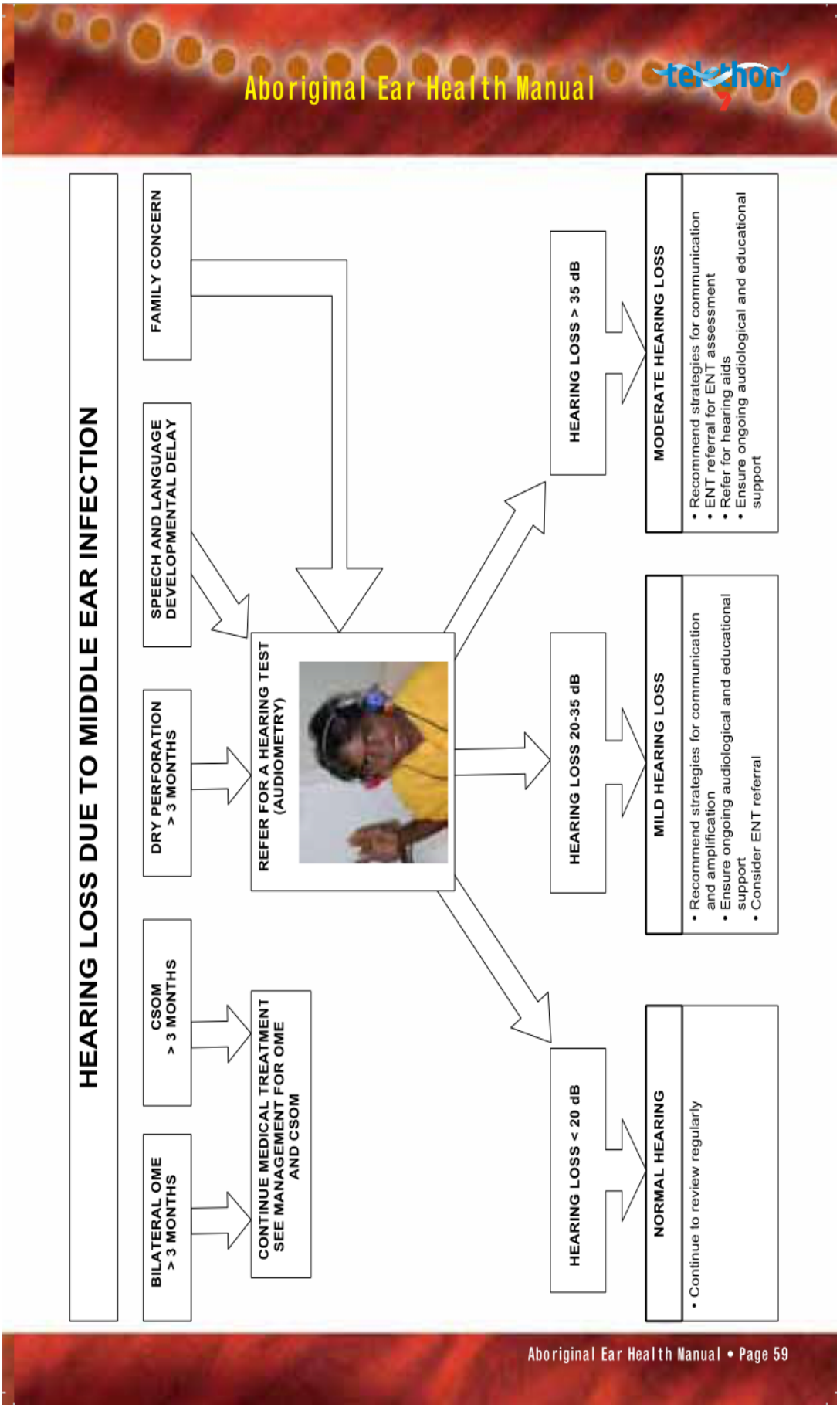
MANAGEMENT OF DRY PERFORATION
HOW LONG HAS THE DRY PERFORATION (HOLE IN THE EAR DRUM) BEEN PRESENT?
< 3 MONTHS
REVIEW AGAIN IN 3 MONTHS
> 3 MONTHS
REFER FOR A HEARING TEST (AUDIOMETRY)
HEARING LOSS > 35 dB
DRY PERFORATION WITH MODERATE HEARING LOSS
• Recommend strategies for communication
• ENT referral for ear drum repair (myringoplasty)
• Refer for hearing aids
• Ensure ongoing audiological and educational support
HEARING LOSS 20-35 dB
DRY PERFORATION WITH MILD HEARING LOSS
• Recommend strategies for communication and amplification
• Ensure ongoing audiological and educational support
• Consider ENT referral
HEARING LOSS < 20 dB
DRY PERFORATION WITH NORMAL HEARING
• Continue to review regularly
• Consider ENT referral if recurrent infections a problem
Page 58 • Aboriginal Ear Health Manual


Aboriginal Ear Health Manual
telethon
HEARING LOSS DUE TO MIDDLE EAR INFECTION
FAMILY CONCERN
SPEECH AND LANGUAGE DEVELOPMENTAL DELAY
DRY PERFORATION > 3 MONTHS
CSOM > 3 MONTHS
BILATERAL OME > 3 MONTHS
CONTINUE MEDICAL TREATMENT SEE MANAGEMENT FOR OME AND CSOM
REFER FOR A HEARING TEST (AUDIOMETRY)
HEARING LOSS > 35 dB
HEARING LOSS 20-35 dB
HEARING LOSS < 20 dB
MODERATE HEARING LOSS
• Recommend strategies for communication
• ENT referral for ENT assessment
• Refer for hearing aids
• Ensure ongoing audiological and educational support
MILD HEARING LOSS
• Recommend strategies for communication and amplification
• Ensure ongoing audiological and educational support
• Consider ENT referral
NORMAL HEARING
• Continue to review regularly
Aboriginal Ear Health Manual • Page 59

# Surgical Procedures
# Ventilation Tubes (grommets)

**Why is the operation needed?**
Grommets (ventilation tubes) are usually inserted for treatment of otitis media with effusion (glue ear), or recurrent middle ear infection. Grommets may be needed in these cases:

- Four to six separate attacks of otitis media in 12 months
- Three separate attacks of otitis media in 6 months
- A handicapping hearing loss, which affects the child for 3 months
- A mild hearing loss for 12 months or greater
- Speech and language delay
- Balance problems

**How is the operation performed?**
The procedure can be done either by itself or with another procedure. If it is done by itself, sometimes the child can go home the same day (same day care unit). If done with another procedure then the child may spend a night in hospital.

**How long do grommets stay in?**
Grommets usually remain in place for between 3 and 9 months, at which time they come out of the eardrum by themselves into the ear canal. A second set of grommets is required in approximately 20%of children.

**What will I notice after the operation?**
There should be no significant pain for more than twenty four hours and usually there should be little in the way of discharge or bleeding from the ear. Often there will be antibiotic drops given post operatively to help keep the grommet from blocking or getting infected.

**What should I do after the operation?**
Do not let water go into the ear. To stop water getting into the ear, use:

- silicon ear putty
- "blue tac"
- ear plugs

Children should not do "bombies" or dive or swim underwater, as water may enter the ear despite the ear protection. Children are reviewed every 6 - 9 months while the tubes are in place, to make sure that no discharge or problems have occurred, and to check the child's hearing after the operation. Children are reviewed once the tubes have come out as well, to check on the healing of the eardrum and to make sure that no fluid is behind the eardrum.

**What if water gets into the ear?**
If water does get into the ear, or the patient has a cold, there may be some drainage from the ear This can be easily treated with antibiotic eardrops. The treatment for discharging grommets is the application of 5 drops of Ciloxan ear drops 2 times a day for 5 days. Occasionally ear washouts are necessary using Betadine 0.5% solution and a plastic syringe followed by the use of the drops as described.

**What complications may happen after the operation?**
Complications may include discharge (as mentioned above), polyps and bleeding, or development of retraction pockets or cholesteatoma at the site of the operation. In rare cases, where there is infection that does not respond to eardrops and oral antibiotics, admission to hospital for IV antibiotics and removal of the tube may be needed. In 1.6%-10% of cases, a hole (or perforation) may remain once the grommet has come out. A further operation to repair the hole in the eardrum (myringoplasty) may be needed.

Photograph of Ventilation Tube (grommet) in situ

# Surgical Procedures
# Myringoplasty

## (repair of holes in the eardrum using a special graft)

**How do holes in the eardrum occur?**
A hole in the eardrum, or perforation, may come from a previous ear infection or if the eardrum does not heal after grommets (ventilation tubes) have come out.

**Why is the operation needed?**
Repair of the eardrum (myringoplasty) is an operation that is performed to reconstruct the eardrum if there is a perforation. Material is grafted over the hole in the eardrum allowing the eardrum to heal. This improves the hearing, allows the child to swim and prevents skin at the edges of the perforation growing into the middle ear (cholesteatoma).

**What is used to repair the hole?**
The graft material varies, but is always taken from the patient. The graft material used is either subcutaneous tissue/fat/fascia, or a tragal cartilage graft. Graft material is sometimes taken from behind the ear or from the front of the ear (tragus). This requires a small cut and occasionally a larger cut behind the ear.

**What will I notice after the operation?**
There will be blood stained discharge draining from the ear for up to four weeks. If the discharge becomes smelly, please see a doctor. There may be some "popping" sounds. Long-lasting pain is not usual, and you should see a doctor. If you become very dizzy after leaving hospital, also see a doctor.

**What should I do after the operation?**
Water should be kept off the small cut to remove the graft material for one week. Do not let water go into the ear. To stop water getting into the ear, use:

- silicon ear putty
- "blue tac"
- ear plugs
- Vaseline and cotton wool

**How successful is the operation?**
The success of the operation varies according to the size of the hole, and the presence or absence of infection both before and after the operation. Success rate varies between 50-80%.

**What complications may happen after the operation?**
Complications include infection and rejection of the graft, hearing loss (uncommon), ringing in the ears (tinnitus) and dizziness and loss of balance (vertigo), and very rarely (1 in 10,000) facial nerve palsy.

Photo of myringoplasty.

**Tissue Engineered Regeneration of Ear Drum**
Exciting recent developments in Japan by Professor Kanemaru have shown a method of regenerating the eardrum in people with chronic ear drum perforations by a short procedure under local anaesthetic in adults and under general anaesthetic in children. This uses a tissue growth factor which stimulates the growth of the three components of the tympanic membrane or ear drum, to grow along a gelatine foam scaffold to successfully repair the perforations in a significant number of patients in pilot studies in Japan. This technique has been performed in Perth for the first time in Australia with success. It is hoped in the future that this procedure will be available throughout Australia if Australian pilot programs show success rates equivalent to conventional myringoplasty.

# Complications of Otitis Media

**Which patients to refer for urgent treatment?**
Despite the common use of antibiotics, there is still the risk of complications of otitis media and chronic suppurative otitis media of infection involving the middle ear, mastoid, inner ear and intracranial cavity.

**Mastoiditis**
Acute mastoiditis involves the development of an abscess within the mastoid bone behind the external ear, often accompanied by systemic symptoms and a tender lump between the external ear and the mastoid region, which may cause the outer ear to stick out. In acute mastoiditis the crease behind the ear is usually blunted or absent.

**Suspected cholesteatoma**
Cholesteatoma occurs when the normal lining skin of the eardrum accumulates in the middle ear or other parts of the temporal bone. Usually it develops from a retraction pocket in the eardrum or migration of skin through a defect of the eardrum such as a perforation. Clinically, the diagnosis is made by the visualisation of a white mass in the tympanic membrane or middle ear. Usually surgical management is necessary for this condition.

**Intracranial complications (associated meningitis or brain abscess)**
Acute otitis media and chronic suppurative otitis media may lead to meningitis or inflammation of the lining of the brain or an abscess in the brain or thrombosis of one of the great blood vessels in the region (lateral sinus thrombosis).

**Facial paralysis**
Facial nerve paralysis can be caused by otitis media, either in its acute or chronic form as well as with cholesteatoma. The child will have an inability to move one side of the face, and this condition requires acute intervention by the otolaryngologist.

**Labyrinthitis**
Labyrinthitis occurs when there is spread of infection from the middle ear or mastoid into the vestibular and cochlear organs. This may cause a permanent hearing loss and dizziness and, in the presence of fever and an acute infection, is a medical emergency.

# Resources

Recommendations for Clinical Care Guidelines on the Management of Otitis Media in Aboriginal and Torres Strait Islander Populations. Updated April 2010.

*Prepared by The Darwin Otitis Guideline Group in collaboration with the Office for Aboriginal and Torres Strait Islander Health Medical Technical Advisory Group for the Office for Aboriginal and Torres Strait Islander Health, Australian Government Department of Health and Ageing. Canberra ACT.*

Indigenous EarInfoNet. A web resource and yarning place about ear health and hearing issues among Indigenous Australian
www.earinfonet.org.au

**Otitis media in Aboriginal children: tackling a major health problem**
Harvey L Coates, Peter S Morris, Amanda J Leach and Sophie Couzos
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Victorian Aboriginal Health Service
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# CHAPTER 6

# CONCLUSIONS

Otitis media continues to be a major cause of chronic morbidity in Australia, particularly in Aboriginal children, and has been recognised as such by the "Closing the Gap" legislation.

This thesis has addressed multiple points of pathogenesis and potential intervention in the causal pathways to OM. Although we are aware that this disease of poverty needs consistent improvements in the social determinants of health, slow but steady progress of ear health in susceptible groups is already visible.

This work has added new insights into the causal pathways to OM, including the role of smoking and overcrowding in the pathogenesis of OM, whilst the swimming pool study broke new ground in the role of environmental manipulation on a multitude of medical and social conditions.

Our discoveries of the three modalities of bacterial preservation in the human middle ear, namely bacterial biofilm, intracellular colonization and NETs, has advanced our knowledge and provided understanding for potential treatment of chronic middle ear infections in children.

The tissue engineering surgical advances pioneered by Kanemaru have been extended by our team's surgical trials, involving different scaffolds in conjunction with b-FGF, to regenerate new tympanic membranes in the relatively large population of patients in Australia with chronic tympanic membrane perforations.

Our collaborative genetics research into the causation of otitis media has lead to the discovery of a susceptibility gene FBX011 for severe OM. Further studies showed that SLC11A1 is a novel candidate gene for OM susceptibility. The early identification of these genes in children at risk for OM might identify those more likely to benefit from early adenoidectomy and grommet insertion.

Further directions in OM research and treatment include the further refinement of our genetic, microbiologic and epidemiology, while the treatment protocols utilising evidence based outcomes from national research studies will ensure best practice. The future for tissue engineering in middle ear surgery including regeneration of ossicles is bright, and new vaccines, antimicrobials and active agents such as Dornase Alfa portend a new era in OM treatment.

The exciting work on the genetics of rAOM, the multiple discoveries regarding bacterial preservation in middle ear infections and the enormous promise of our simple, quick and effective tissue engineered myringoplasty all offer hope that innovative, preventive and therapeutic interventions may ameliorate the severity and persistence of this disease in the future.

# CHAPTER 7

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* Superscript numbers correspond to the designated general bibliography numbers – when cited in the text.

## 7.1.2 Author's Contribution

**Table legend:** **AC**: Author's Contribution **P**: Primary **S**: Secondary **M**: Minor **C**: Conceptualization **SD**: Study Desi
Analysis **MD**: Manuscript Draft **MER**: Manuscript Editing/Reviewing

| Chapter | Page | Paper/ Ref. No | Citation | Author's Contribution | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | AC | C | SD | PR | S | A | MD | MER |
| 2 | 24 | 1 [27] | The effect of passive smoking on the risk of otitis media in Aboriginal and non-Aboriginal children in the Kalgoorlie-Boulder region of Western Australia. | M | | ✔ | | ✔ | ✔ | ✔ | ✔ |
| | 29 | 2 [28] | Absent otoacoustic emissions predict otitis media in young Aboriginal children: a birth cohort study in Aboriginal and non-Aboriginal children in an arid zone of Western Australia. | S | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ |
| | 40 | 3 [01] | The Kalgoorlie Otitis Media Research Project: rationale, methods, population characteristics and ethical considerations. | M | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ |
| | 52 | 4 [33] | Association between early bacterial carriage and otitis media in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia: a cohort study | M | | ✔ | | | | | ✔ |
| | 61 | 5 [08] | Middle ear disease in Aboriginal children in Perth: analysis of hearing screening data, 1998-2004. | S | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ |
| | 64 | 6 [29] | Benefits of swimming pools in two remote Aboriginal communities in Western Australia: intervention study. | M | ✔ | ✔ | | ✔ | ✔ | ✔ | ✔ |
| | 69 | 7 [05] | Natural history, definitions, risk factors and burden of otitis media. | S | ✔ | ✔ | | | | ✔ | ✔ |
| | 74 | 8 [04] | Cost of treating otitis media in Australia. | M | | | | | ✔ | ✔ | ✔ |
| 3 | 94 | 9 [23] | Chronic Suppurative otitis media without cholesteatoma. | P | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ |
| | 101 | 10 [42] | Biofilm, intracellular bacteria and neutrophil extracellular traps in otitis media. | S | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ |
| | 127 | 11 [25] | The role of chronic infection in children with otitis media with effusion: evidence for intracellular persistence of bacteria. | P | ✔ | ✔ | ✔ | ✔ | | ✔ | ✔ |
| | 131 | 12 [50] | Multi-species bacterial biofilm and intracellular infection in otitis media. | M | ✔ | ✔ | ✔ | ✔ | | ✔ | ✔ |
| | 141 | 13 [26] | Neutrophil extracellular traps and bacterial biofilms in middle ear effusion of children with recurrent acute otitis media--a potential treatment target. | M | | | ✔ | ✔ | | ✔ | ✔ |
| | 151 | 14 [54] | Predominance of nontypeable Haemophilus influenzae in children with otitis media following introduction of a 3+0 pneumococcal conjugate vaccine schedule. | M | | | ✔ | ✔ | | ✔ | ✔ |

| | Page | Paper/ Ref No | Citation | Author's Contribution | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | AC | C | SD | PR | S | A | MD | MER |
| 3 | 159 | 153 | IgG responses to Pneumococcal and Haemophilus influenzae protein antigens are not impaired in children with a history of recurrent acute otitis media. | M | | | ✔ | ✔ | | ✔ | ✔ |
| | 168 | 158 | High pneumococcal serotype specific IgG, IgG1 and IgG2 levels in serum and the middle ear of children with recurrent acute otitis media receiving ventilation tubes. | M | | | ✔ | ✔ | | ✔ | ✔ |
| | 175 | 165 | FBXO11, a regulator of the TGFbeta pathway, is associated with severe otitis media in Western Australian children. | M | | | ✔ | ✔ | | ✔ | ✔ |
| | 183 | 163 | Genome-wide association study to identify the genetic determinants of otitis media susceptibility in childhood. | M | | | ✔ | ✔ | | ✔ | ✔ |
| | 195 | 167 | Genetic and functional evidence for a role for SLC11A1 in susceptibility to otitis media in early childhood in a Western Australian population. | M | | | ✔ | ✔ | | ✔ | ✔ |
| | 203 | 168 | Genetic and functional evidence for a locus controlling otitis media at chromosome 10q26.3. | M | | | ✔ | ✔ | | ✔ | ✔ |
| 4 | 227 | 169 | Ototoxic eardrops and tympanic membrane perforations: time for a change? | P | ✔ | ✔ | | | | ✔ | ✔ |
| | 231 | 171 | Ototoxic ear drops with grommet and tympanic membrane perforations: a position statement. | S | ✔ | ✔ | | | | ✔ | ✔ |
| | 233 | 179 | A pilot study investigating basic fibroblast growth factor for the repair of chronic tympanic membrane perforations in pediatric patients. | S | ✔ | ✔ | ✔ | | | | ✔ |
| 5 | 240 | 232 | Aboriginal and Torres Strait Islander ear health manual | P | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ |

**Table legend:** **AC**: Author's Contribution **P**: Primary **S**: Secondary **M**: Minor **C**: Conceptualization **SD**: Study Desi
Analysis **MD**: Manuscript Draft **MER**: Manuscript Editing/Reviewing

### 7.1.3 Citations Index

Citations from published papers in which the author has Primary or Secondary role contributions, as described in aurhor's contribution table pages 314,315.

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papers numbered 1 [27], 3 [01], 4 [33], 6 [29], 8 [04] and 12 [50] to 20 [68] (according to the list on pages 310 -313) have been cited a total of 145 times altogether.
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