Early Environmental, Genetic and Epigenetic Determinants of Acute Otitis Media in Children

Gijs van Ingen

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Early Environmental, Genetic and Epigenetic Determinants of Acute Otitis Media in Children

Vroege omgevings-, genetische en epigenetische determinanten van otitis media acuta in kinderen

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1 General introduction and outline of thesis

INTRODUCTION

Definition

Acute otitis media (AOM) is a common pediatric disease, and the most frequent reason for antibiotic treatment in children (1, 2). It is an acute infection of the middle ear and is sometimes referred to as suppurative otitis media. The middle ear is a narrow cavity between external ear canal and the inner ear that contains the ossicular chain. It is part of an aerated system that includes the nasal cavity, paranasal sinuses, the Eustachian tube, and the mastoid cells. This system is lined with thin respiratory mucosa. Events affecting one area, may induce changes throughout the system. Therefore, extension of a suppurative process from the middle ear to adjacent structures may lead to complications elsewhere such as mastoiditis, petrositis, meningitis and lateral sinus thrombosis. The normal middle ear is aerated, and the normal tympanic membrane is intact, slightly convex, translucent, and mobile.

Etiology

AOM is characterized by the presence of (purulent) fluid in the middle ear accompanied by earache, bulging of the tympanic membrane and fever, and is usually preceded by an upper respiratory tract infection (URTI) (1, 3, 4). There is a strong relationship between viral URTI and AOM (5, 6). Incidence of AOM increases during viral epidemics. In a large retrospective database analysis from Germany of over 65,000 patients demonstrating otitis media was present at a higher rate in patients with influenza compared to non-influenza-infected children (7). Detection of viruses directly from the middle ear fluid at the time of AOM has been considered convincing evidence of the capability of viruses to induce AOM (8, 9). Children are more susceptible to secondary bacterial infections during and after URTI as level of bacteria colonization of the nasopharynx increase during respiratory infection (10). At the same time, some evidence suggests that co-colonization of the middle ear with bacterial pathogens may be sufficient to trigger the cascade of events leading to AOM even in absence of URTI (11-13).

Vaccination

The three primary bacterial pathogens that cause AOM are non-typeable Haemophilus influenzae (NTHi), Streptococcus pneumoniae, and Moraxella catarrhalis. S. pneumoniae has currently licensed vaccines, the pneumococcal conjugate vaccines (PCVs) (14). PCVs have led to a reduction of AOM and of nasopharyngeal colonization by pneumococcal vaccine serotypes but have also resulted in replacement by non-vaccine pneumococcal serotypes and possibly NTHi and M. catarrhalis (2).

In the post-pneumococcal vaccination era before the age of 3 years, 60% of children will have gone through at least one episode of AOM, with 24% of children suffering 3 or more

episodes (3, 15). Every year, an estimated 709 million episodes of AOM occur worldwide, half of which are in children below 5 years of age (16). In the Netherlands, the incidence of AOM is estimated at 10 in every 1000 patients, yet, in children below the age of 5 years that number increases to 102 in every 1000 patients. This makes AOM the most common pediatric infectious disease and most common reason for medical visits, antibiotic prescriptions, and hearing impairment in children (17). Although AOM is associated with conductive hearing loss, there is limited evidence suggesting that sensorineural hearing loss (SNHL) could be a rare complication of AOM (18). Several studies investigated sensorineural hearing loss (SNHL) as a sign of permanent damage to the cochlea after chronic or recurrent AOM episodes (19-21). A large study from Taiwan showed that a history of AOM was associated with an increased risk of sudden sensorineural hearing loss later in life (22). The pathogenesis of chronic sensory hearing impairment may be related to inflammatory noxious substances that cross the round window membrane, which leads to serous labyrinthitis, or to fluid in the middle ear that impedes oxygen transport to the inner ear, or to an adverse effect of ototoxic drugs (23). Preventing even slight SNHL holds relevance for language development and school performance of children. The literature shows that children with mild to severe hearing loss have worse language development and school performance than those without, illustrated by the displayed lower scores on short-term and sequential memory, attention, language, and verbal and nonverbal IQ tests (24-26). Also, children with mild to severe hearing loss showed more emotional and behavioral problems than children with normal hearing (27). Recently it was shown that even in children with only slight to mild hearing loss, more behavioral problems and poorer school performance were observed (28). Moreover, the consequences of even mild levels of hearing loss can lead to cumulative adverse effect later in life with regards to language development, academic performance, and social functioning (26, 28-31). Untreated hearing loss can have a profound impact on interpersonal communication, psychosocial well-being, employment opportunities, and quality of life (32-35). These findings indicate the relevance of preventing slight to mild hearing loss, and, thus, in studying which children may benefit from earlier treatment of AOM.

Management

The management of AOM puts a heavy burden on health care expenditures, with annual costs in the US estimated at US$ 4.3 billion per year (36). A study on estimated health care burden and societal impact of acute otitis media in seven European countries, showed high rates of health care resource use and parental productivity losses during the most recent episode of AOM in their child, younger than 5 years (37). Antibiotic use was high, ranging from 60.8% (Germany) to 87.1% (Italy). Total costs per AOM episode ranged from € 332.00 (The Netherlands) to € 752.49 (UK). Losses in productivity accounted for 61% (France) to 83% (Germany) of the total costs (37). AOM also negatively impacts the quality

of life of children and their caregivers. Health related quality of life (QoL) can be measured by validated questionnaires such as OM-6, PAR-AOM-QoL, and EQ-5D (38-40). Several studies on QoL of children with AOM and their parents were conducted, but the level of disturbance measured in QoL varied across studies (39, 41, 42). Parental QoL was affected more if the child's AOM episode was perceived as more severe, if AOM occurred in younger children, and in those with recurrent episodes (39, 41, 42). Parents of children suffering from recurrent AOM episodes reported lower QoL judged as frequent ear pain, fever, sleepless nights, and time lost from school or work, as compared with parents of children with OME or parents with children without ear disease (43-45). A recent review of 17 qualitative studies on parental views on OM (mainly OME, but several on AOM or OM in general) found that OM may impose a significant burden on the child and for periods of time even resemble a chronic disease (46). It illustrated the concerns and psychosocial needs of the parents with a child suffering from OM, which extended to concerns regarding the child's development, absence of work, repeated antibiotic treatments, and surgical interventions.

Environmental exposures

AOM is a common disease with complex etiology which is believed to be the result of an interplay between environmental and epigenetic exposures in combination with genetic factors. Previously, many potential risk factors have been identified. Certain factors in a child's environment are related to an increase or decrease in viral pathogens, which in turn is associated with altered incidence of URTIs – and thus of ear infections. These factors include having siblings (47-50), day-care attendance (3, 49, 51-55), season-of-birth (47, 49, 56-58), pet keeping (55, 59-61), breastfeeding (3, 4, 47, 48, 51, 53-55), pacifier use (48, 62-64), and socioeconomic status (SES) in general (47, 50, 63, 65). Other child factors included gender (3, 51, 52, 65, 66), ethnicity (3, 47, 48, 58, 67), aberrant birth weight (47, 49, 56, 68), presence of meconium contaminated amniotic fluid at birth (69), family history of AOM (3), pathogenic bacterial colonization of the nose/nasopharynx (4, 70-72), and adenoid hypertrophy (48, 73, 74), and low compliance to the national vaccination program (3, 14, 54). Maternal factors with a proposed impact on childhood AOM include prenatal and postnatal (household) smoking (3, 47, 48, 65, 75-79), and maternal age (47). Although many factors have been previously associated with AOM, often results are contradictory. Some factors may be associated with an increase or decrease in AOM among children of a certain age or ethnicity in one study but are not replicated in the next. Moreover, as factors such as ethnicity, socioeconomic status and compliance to national vaccination programs may be associated with occurrence of AOM, previous results from populations elsewhere may not be generalizable to e.g. the urban, multi-ethnic population of Rotterdam, the Netherlands. Better insights into prenatal environmental exposures and exposures in early life that may affect AOM, could provide an opportunity for prevention or intervention at the time when they have their greatest effect.

Genetic and Epigenetic Susceptibility

It is well-established in sibling, twin, and family studies, that OM is a heritable trait, with the fraction of phenotype variability attributed to genetic variation (h^2) estimated between 0.22 and 0.74 (80-83). The genetic susceptibility loci for OM are not well understood. Most previous studies focused on candidate genes that were chosen based on biological plausibility of the genes and evidence from model organisms. Via this approach researchers examined the association of some relevant genes to OM and yielded significant associations for 420 genes including several interleukin (IL) genes, mucin genes, TLR4, FBXO11, TNFα (84). Results from literature are conflicting, with only a small proportion of the discoveries replicating in independent studies. In the last 10 years, a novel methodology has been introduced to study genetics of complex non-Mendelian diseases, the genome-wide associations study (GWAS). This is a large-scale agnostic discovery approach which involves scanning the genomes from many different people and looking for genetic markers (Single-nucleotide polymorphisms (SNPs)) that can be used to predict the presence of disease. Once such genetic markers are identified, they can be used to understand how genes contribute to the disease. Recently, SNPs at several loci have been reported to be associated with OM, such as those at 10q26.3, 19q13.43, 17q12, 10q22.3, 2q31.1 for association with chronic and recurrent OM (85-88), 2p23.1 (genes CAPN14, GALNT14) and 20q11.21 (BPIFA gene) for association with OM in general (89). None of these signals broke the threshold of genome-wide significance ($p>5x10^{-8}$), likely due to the small sample size and limited number of genetic variants examined. A recent study of OM via exome sequencing identified the cosegregation of a rare duplication variant in gene A2ML1 with OM in a Filipino pedigree, and additional A2ML1 variants were found in otitis-prone American children (90), but these variants were all of very low frequency which could not account for the high prevalence of OM. To date, there has been no genetic study specifically examining the susceptibility loci for AOM.

Interactions between genetics and epigenetics are complex, and epigenetic variation stands at the crossroad between genetic and environmental variance. Epigenetics refers to mitotically heritable changes to the DNA, which do not affect the DNA sequence, but can influence its function. DNA methylation is the most studied epigenetic phenomenon in large populations (91). It entails the binding of a methyl group to DNA where a cytosine is located next to a guanine, a cytosine-phosphate-guanine (CpG) site. DNA methylation at CpG sites can influence gene expression by altering the DNA's three-dimensional structure. There are approximately 28 million CpG sites in the human genome. DNA methylation is a dynamic process that can be influenced by genetic factors, as well as by environmental factors such as diet, air pollution, toxicants, or smoking, and stochastic epigenetic variation, also referred to as spontaneous epimutations (92-95). DNA methylation could link environmental exposures with the occurrence of disease, such as childhood AOM. Fetal development is a period of profound changes in DNA methylation and may, as such, be a critical period for environmentally-induced DNA methylation changes (95). Subsequently,

altered DNA methylation at birth could affect gene expression related to disease susceptibility later in life (92, 95-97). Recent advances in assays to assess DNA methylation have enable the study of methylation status of >480,000 sites (CpGs) in the genome with a good genomic coverage requiring only low amounts of DNA making its use in large cohort studies possible (98). If we could relate specific genomic regions with altered DNA methylation with susceptibility to AOM in childhood, it would help us understand the complex underlying mechanisms of environmental and genetic factors that influence AOM susceptibility.

OBJECTIVES

The major aims of this thesis are:

1.) To identify which environmental determinants are associated with childhood acute otitis media, and if such relationships exist at specific ages (Chapters 2 - 3).
2.) To identify genetic and epigenetic variants associated with acute otitis media in children (Chapters 4 - 5).
3.) To investigate the prevalence of sensorineural hearing loss in 9- to 11-year-old children and assess whether it may be attributable to recurrent acute otitis media in the first years of life (Chapters 6 - 7).

GENERAL DESIGN

The studies presented in the thesis were embedded in a population-based prospective cohort study, the Generation R Study, and international collaboration projects.

The Generation R Study

The Generation R Study is a population-based prospective birth cohort study from fetal life until young adulthood in Rotterdam, the Netherlands. The study is designed to identify early environmental and genetic causes and causal pathways leading to normal or abnormal growth, development, and health from fetal life until young adulthood. In total, 9778 mothers with a delivery date from April 2002 until January 2006 were enrolled in the study. Response at baseline was 61%, and general follow-up rates until the age of 10 years were around 80%. Data collection in children and their parents includes questionnaires, interviews, detailed physical and ultrasound examinations, behavioral observations, lung function, Magnetic Resonance Imaging and biological sampling including cord blood for genome- and epigenome-wide association screens (99, 100).

Center for Applied Genomics

We conducted a meta-analysis of genome-wide association studies on AOM in childhood with the Center of Applied Genomics (CAG) at the Children's Hospital of Philadelphia (www.caglab.org). The mission of the CAG is to develop new and better ways to diagnose and treat children affected by rare and complex medical disorders. The CAG is a specialized Center of Emphasis at the Children's Hospital of Philadelphia with the primary goal of translating basic research findings to medical innovations. It is one of the world's largest genetics research programs, and the only center at a pediatric hospital to have large-scale access to state-of-the-art high-throughput genomics technology.

PACE Consortium

We performed a meta-analysis of epigenome-wide association study results from seven population-based cohort studies collaborating in the Pregnancy And Childhood Epigenetics (PACE) consortium (91). The aim of the PACE consortium is to facilitate joint analyses of DNA methylation data in relation to a wide range of exposures and outcomes pertinent to health in pregnancy and childhood by bringing together researchers, knowledge, skill, and data (91). The studies that participated in our AOM analysis were the California Birth Cohort (CBC) (101), the Étude des Déterminants pré et postnatals du développement et de la santé de l'Enfant (EDEN) (102), the Generation R Study (99, 100), the Infancia y Medio Ambiente (INMA) study (103), two independent datasets from the Norwegian Mother, Father and Child Cohort Study (MoBa1, MoBa2) (104-106), and the Avon Longitudinal Study of Parents and Children (ALSPAC UK) (107, 108). Subjects were mainly of European descent, but the CBC study comprised two datasets with one of European and one of Hispanic descent.

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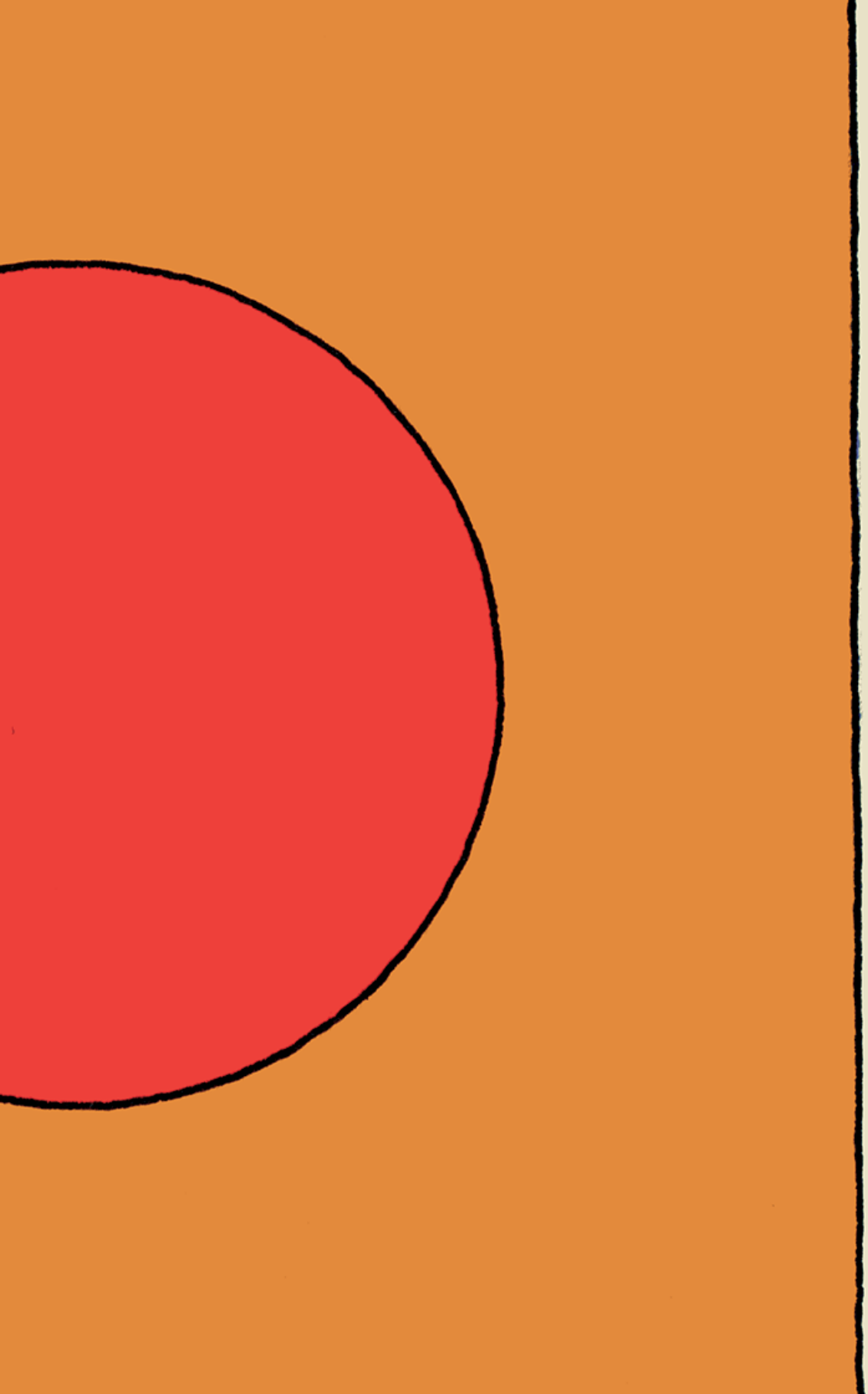
I Environmental determinants associated with acute otitis media in children



2 Environmental determinants associated with acute otitis media in children: a longitudinal study

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ABSTRACT

Background: Acute otitis media (AOM) is a common pediatric disease and frequent reason for antibiotic treatment. We aimed to identify environmental and host factors associated with AOM and assess which determinants were associated with AOM at specific ages.

Methods: This study among 7863 children was embedded in the Generation R Study: a population-based prospective cohort study from fetal life onwards. Data on outcome and possible determinants were collected using questionnaires until 6 years. We used generalized estimating equation models to examine associations with AOM with longitudinal odds at different ages, considering correlations between repeated measurements.

Results: Male gender increased odds of AOM in children at 2, 3, and 4 years but not at other ages. Postnatal household smoking, presence of siblings, and pet birds increased odds of AOM. Breastfeeding decreased AOM odds, most notably in the first 2 months of life. No association was found for season of birth, maternal age, ethnicity, aberrant birth weight for gestational age, prenatal smoking, furry pets, and daycare attendance.

Conclusions: Risk of childhood AOM varies with age. Significant association with AOM was found for gender and breastfeeding at specific ages and for household smoking, presence of siblings, and pet birds at all the studied ages.

INTRODUCTION

Acute otitis media (AOM) is a common pediatric disease, and the most frequent reason for antibiotic treatment in children. It is characterized by the presence of (purulent) fluid in the middle ear accompanied by earache, bulging of the tympanic membrane and fever, and is usually preceded by an upper respiratory tract infection (URTI) (1, 2). In the post-pneumococcal vaccination era before the age of 3 years, 60% of children will have gone through at least one episode of AOM, with 24% of children suffering 3 or more episodes (2, 3). The associated frequent consultation of physicians, school absence and consumption of antibiotics constitute a considerable societal burden (4, 5). The pathogenesis of otitis media is one of complex associations between genetic, host and environmental factors (6, 7). Previously, many potential risk factors have been identified, including – but not limited to – daycare attendance, presence of siblings, breastfeeding or lack of breastfeeding, socioeconomic status (SES), prenatal maternal smoking, postnatal exposure to household smoking, and season of birth. AOM is a common disease that afflicts nearly all children in childhood; some children, however, are more prone to it than are others, and some appear more prone to it at a younger age than are others. Likely, not only susceptibility to AOM varies with age, but also the effect of certain risk factors on AOM susceptibility. Being breastfed, for example, might lower the risk of AOM in the first year of life – but not beyond. This study aims to examine associations of potential determinants with AOM at different ages in one model, taking into account correlations between repeated measurements of AOM within the same child.

METHODS

General Study Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood in Rotterdam, the Netherlands. Details on study design, response rate and (loss to) follow-up have been published previously (8). In brief, the Generation R Study aims to identify early environmental and genetic factors affecting children's growth, development and health. Mothers with a delivery date between April 2002 and January 2006 were eligible for inclusion in the study. The study was approved by the Medical and Ethics Review Board of the Erasmus University Medical Center in Rotterdam, the Netherlands (MEC-2007-413-NL21545.078). Written informed consent was obtained from parents or legal guardians of all 7,863 subjects.

Acute Otitis Media

The following outcomes were collected through parental questionnaires: episodes of otorrhea, earache with fever, and use of eardrops prescribed by family practitioner or ear, nose and throat (ENT) surgeon. AOM was defined using these outcomes. Questionnaires were administered at the children's ages of 2, 6, 12, 24, 36, 48 and 66 months (Supplemental Figure 2.1).

Determinants

Prenatal maternal characteristics, child characteristics and postnatal exposures that might serve as environmental determinants for AOM were selected on the basis of a literature review and data availability in our present study (Supplemental Table 2.1). Upon enrolment, information was collected on maternal age, parity (nulliparous; multiparous; number of older siblings at birth), and pet keeping (yes; no – dog, cat, bird, rodent). Information on maternal smoking until pregnancy and during pregnancy was obtained by multiple questionnaires. We took maternal educational level (lower; higher) to represent SES, as maternal education level has been previously described as a consistent socioeconomic predictor in cardiovascular disease, pregnancy outcomes and early childhood behavior (9, 10). Data on postnatal household smoking were collected at 2, 24, 36 and 66 months after birth of the child. Hospital registries provided information on the child's gender, ethnicity (Western; non-Western), season of birth, gestational age at birth and birth weight. We calculated birth weight for gestational age. In the Netherlands breastfeeding is encouraged at least until the age of 6 months (http://www.rivm.nl/en). Questionnaires administered at 2, 6 and 12 months provided information on breastfeeding (yes; no) and/or daycare attendance (yes; no). The Dutch national vaccination schedule (including – but not limited to – antigens against haemophilus influenzae type B, streptococcus pneumoniae and Neisseria meningitidis) was established in 1957, and currently covers >95% of all Dutch children (http://www.rivm.nl/en). Influenza vaccination is not included, and is only administered to children with specific underlying disease such as cystic fibrosis, cardiac disease or severe early-onset asthma. Consequently, no data on vaccination status was collected.

Statistical Analysis

Statistical analyses and imputation were performed using R (www.r-project.org, R version 3.3.3, released March 2017). Screening of missing values among covariates concluded that the data set had an arbitrary missing data pattern. Missing data was imputed to reduce potential bias using the Markov chain Monte Carlo method. We used generalized estimating equation (GEE) models to examine associations with AOM of different covariates with longitudinal odds at the ages of 2, 6, 12, 24, 36, 48 and 66 months. Correlations between repeated measurements of outcome within the same child were taken into account. As part of the GEE model analysis, we used multiple linear regression analysis through the 'geeglm'

function in R to provide a way of accounting for potentially confounding variables that may have been included in the model. We checked for the potential interaction of time with each covariate, and included in our model only the interaction terms time ~ gender and time ~ breastfeeding as it increased goodness of fit. Other interaction terms between covariates were tested; that is, household smoking ~ SES; prenatal smoking ~ postnatal household smoking; breastfeeding ~ SES; breastfeeding ~ age mother; age mother ~ SES; siblings ~ daycare, but none improved the model. Nonlinearity of the association between time and gender and between time and breastfeeding were investigated by logistic regression models with natural cubic splines of three knots. As no major differences in values between imputed and non-imputed data were found (Table 2.1, and Supplemental Table 2.2), only the results from imputed analyses are presented. Odds ratio (OR) are presented with their 95% confidence intervals; level of significance was set on $p = 0.05$.

RESULTS

Study Population and Baseline Characteristics

Child and maternal baseline characteristics relating to 7,863 children are presented in Table 2.1. The children's gender distribution was equal, and the cohort included more children of Western ethnicity (66.6%) than children of non-Western descent. Most of the children were breastfed, and a small majority of children attended daycare. Cats were the most common household pet, followed in prevalence by dogs, rodents and birds in that order. A small majority of mothers were of higher SES and a small majority were nulliparous. Although 75.5% of mothers had not smoked at all during pregnancy, over 40% reported postnatal household smoking.

Overall, the prevalence of AOM in the population increased from 1.9% at 2 months of age to 37.9% at the age of 2 years. Between 3 and 4 years of age, prevalence of AOM declined to approximately 30%, before increasing once more at the school going age of 5-6 years. Thus, the probability of acquiring AOM varies over time in the first 6 years of life (Figure 2.1).

Acute otitis media and its determinants

The purported risk factors for AOM that were tested are presented in Table 2.2. Gender was not associated with altered odds of AOM when we looked at the whole population until 6 years of age. When we stratified this association with time, however, girls had slightly lower odds of acquiring AOM at 2, 3 and 4 years of age (Figure 2.2). Similarly, we stratified the association of breastfeeding (OR 0.80; $p < 0.040$) and AOM with time. Lower odds of acquiring AOM in those who were breastfed were found from 0 – 2 months of age, but not beyond (Figure 2.3). Prenatal smoking – regardless whether the mother quit smoking upon confirmation of pregnancy or continued until childbirth – was not associated with AOM

Table 2.1: Characteristics of children and their mothers based on imputed data.

Characteristic	Total study population
Children	
No. (%)	7863 (100)
AOM, yes (n [%])	
Age 2 months	148 (1.9)
Age 6 months	1005 (12.8)
Age 1 year	2508 (31.9)
Age 2 years	2978 (37.9)
Age 3 years	2282 (29.0)
Age 4 years	2379 (30.3)
Age 5-6 years	2896 (36.8)
Sex, n (%)	
Male	3965 (50.4)
Female	3898 (49.6)
Birth weight corrected for gestational age, SDS	-0.08
Ethnicity	
Western	5240 (66.6)
Non-Western	2623 (33.4)
Season of birth, n (%)	
Spring	1838 (23.4)
Summer	2138 (27.2)
Fall	2145 (27.3)
Winter	1742 (22.2)
Breastfed ever, yes (n [%])	7188 (91.4)
Daycare at 6 months, yes (n [%])	4241 (53.9)
Pet in household, yes (n [%])	
Rodent	373 (4.7)
Dog	586 (7.5)
Bird	163 (2.1)
Cat	1845 (23.5)
Mothers	
Age at intake, median (IQR)	31.0 (27.3-34.0)
Educational level, n (%)	
Higher	4264 (54.2)
Lower	3599 (45.8)
Parity, n (%)	
Nulliparous	4418 (56.2)
Multiparous	3445 (43.8)
Smoking during pregnancy, n (%)	
No	5937 (75.5)
Stopped when pregnancy was known	697 (8.9)
Yes	1229 (15.6)
Postnatal household smoking, yes (n [%])	3192 (40.6)

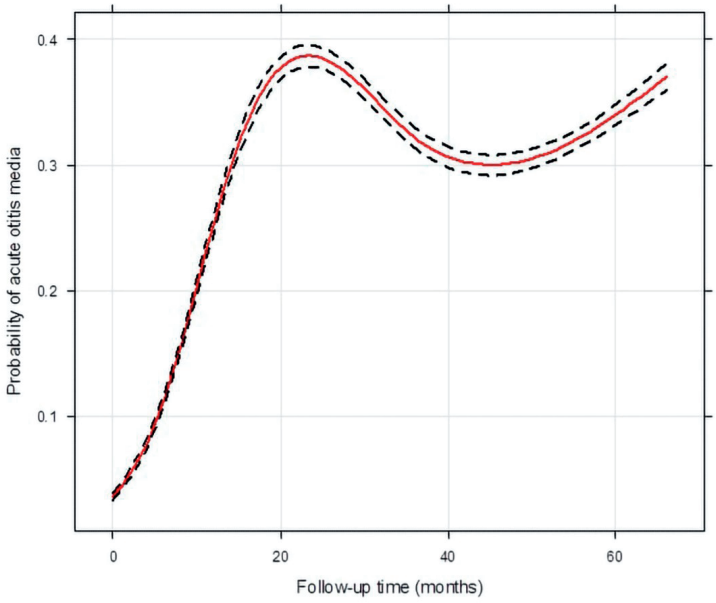

Figure 2.1: The shape of the plot illustrates the probability of developing acute otitis media in time from 2 until 66 months of age in the general population without taking into account genetic, host or environmental risk factors.

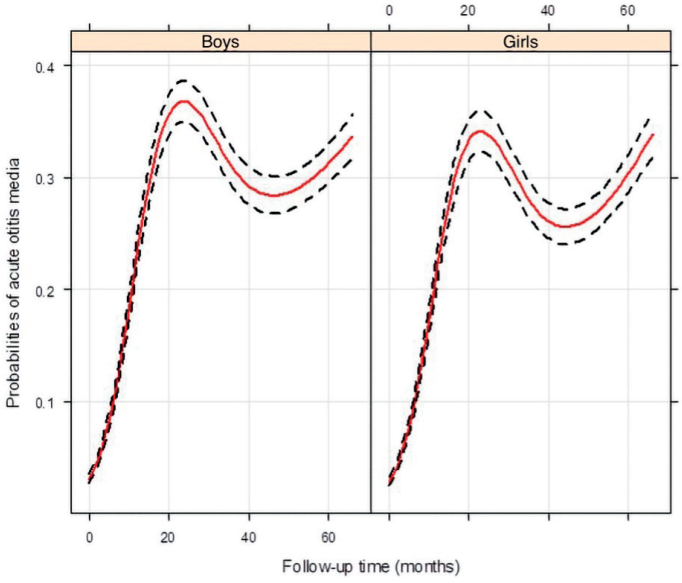

Figure 2.2: Difference in the probability of developing AOM over time for boys (left) and girls (right) when other child, maternal and environmental risk factors are similar. In this plot the subjects were born in the fall, were breastfed, attended daycare, had no siblings or pets in the household, maternal age was 31.3 years, mothers did not smoke prenatally, and the child was not exposed to household smoking after birth. The probability of developing AOM was significantly different between boys and girls at ages 24, 36 and 48 months ($p < 0.001$).

susceptibility in childhood. Postnatal household smoking, however, was associated with a higher risk of AOM among children (OR 1.12; p = 0.013). Other associations that increased a child's AOM susceptibility were with the presence of older siblings (OR 1.07 per sibling; p<0.001), in the presence of birds as household pets (OR 1.25; p<0.013). No associations with AOM were found among subjects with furry pets (dogs, cats or rodents). Season of birth, birth weight for gestational age, maternal age, ethnicity and SES were not associated with altered AOM susceptibility.

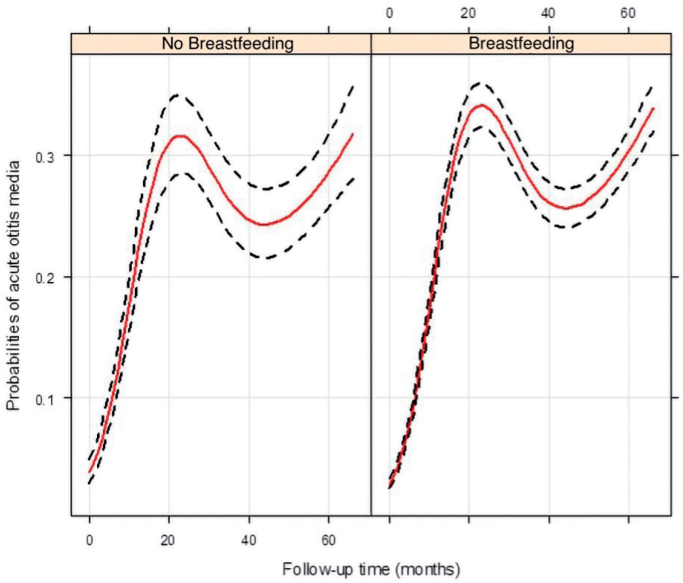

Figure 2.3: Difference in the probability of developing AOM over time for subjects who were not breastfed (left) and who were breastfed (right) when other child, maternal and environmental factors are similar. In this plot, the subjects were born in fall, attended daycare, had no siblings or pets in the household, maternal age was 31.0 years, mothers did not smoke prenatally, and the child was not exposed to household smoking after birth. The probability of developing AOM among subjects who were breastfed had significantly declined from birth to 2 months of age (p = 0.028 and p = 0.038 respectively).

DISCUSSION

While nearly all children go through AOM during childhood, some children may be more susceptible to frequent episodes of AOM than are others. The odds of acquiring AOM are influenced by many factors. This study found gender, breastfeeding or lack thereof, household smoking, number of siblings, and having pet birds to be influencing factors in the studied population. Gender and breastfeeding were associated with AOM only at specific ages.

Being a boy increased an individual's odds of acquiring AOM, specifically between 2 and 4 years of age. Results of previous studies on gender and AOM were conflicting. Studies

on AOM in populations between 0 and 3 years old also reported a higher risk of AOM among boys, although some of these studies showed this association only in boys younger than 1 year (2, 11-14). All but one previous studies that found no association with gender, concerned older children (3 – 16 years) (15-17). Design of this study with its repeatedly measured outcomes at different ages made it possible to zoom in on the specific age range gender altered odds of acquiring AOM in childhood.

Table 2.2: Determinants associated with acute otitis media in children

Determinants	OR (95% CI)[a]	P-value
Female Gender	0.93 (0.82, 1.06)	
Breastfed	**0.80 (0.64, 0.99)**	p = 0.040
Season of birth		
Spring	Ref.	Ref.
Summer	0.94 (0.87, 1.01)	
Fall	0.98 (0.92, 1.06)	
Winter	1.02 (0.95, 1.10)	
Higher maternal age	1.00 (0.99, 1.00)	
Non-Western ethnicity	1.04 (0.98, 1.10)	
Low socioeconomic class	1.02 (0.94, 1.10)	
Older siblings[b]	**1.07 (1.03, 1.11)**	p < 0.001
Below or higher than 2 SD birthweight for gestational age	1.01 (0.98, 1.03)	
Maternal smoking until pregnancy known	1.09 (0.95, 1.25)	
Maternal smoking during pregnancy	1.09 (0.92, 1.29)	
Postnatal household smoking	**1.12 (1.02, 1.22)**	p = 0.013
Pet keeping		
Dog	1.05 (0.95, 1.16)	
Cat	0.98 (0.92, 1.04)	
Bird	**1.25 (1.05, 1.50)**	p = 0.013
Rodent	0.91 (0.80, 1.03)	
Daycare attendance	0.97 (0.91, 1.03)	

a) OR (95% CI) in bold when $p < 0.05$

b) Maternal (multi)parity upon enrolment was used as a proxy for the child having siblings; OR is cumulative per sibling.

Associations between breastfeeding and AOM have been thoroughly investigated. The reported positive effects of breastfeeding included lower probability of AOM, of recurrent AOM and of otitis media with effusion; even short-term – non-exclusive – breastfeeding was found beneficial over formula feeding (18, 19). In the present study, breastfed children had lower odds of AOM before 2 months of age, but not beyond. Previous studies, however, reported lower rates of AOM up to the 2 years of age for children exclusively breastfed for at least 6 months (2, 12, 20, 21). In the Netherlands, breastfeeding is encouraged for at least 6 months. Still, maternity leave from work is only 16 weeks, of which 12 weeks are usually taken after childbirth. Our data set did not include information on whether children were

exclusively breastfed. A large proportion of mothers may have introduced formula feeding after 2 to 3 months, which could explain why the protective effect of breastfeeding in our population entailed a shorter duration.

Breastfeeding is associated with a lower rate of URTIs through the influence of secretory IgA, cytokines, and long-chain fatty acids in breastmilk, which are paramount to development of the infant's immune system (22). This might explain why in our study breastfeeding was associated with a lower risk of AOM in children. Season of birth may be associated with either an increase or decrease in the rate of URTIs, depending on the season. The amount of viral pathogens in the environment in the fall and winter is higher than that in the spring and summer. In the present study we found no evidence of altered odds of AOM in relation to season of birth. The literature on this issue is contradictory; some studies report that birth in the fall would carry higher odds of AOM, whereas other studies report lower AOM rates among subjects born in the fall (23-25).

The present study did not find an association between AOM and (number of) siblings, and between AOM and daycare attendance. While many previous studies reported higher odds of AOM for children who had siblings, two studies concurred with the present one finding no statistical difference in the likelihood of AOM between subjects with or without siblings (12, 14, 15, 18, 26, 27). One study focused on children who attended daycare – which in itself is a reason for significantly increased viral pathogen exposure that may have diminished the effect of having siblings in that particular study (15). Daycare attendance indeed has often been related to increased risk of AOM, with risk further increasing for children who attend daycare for a longer period (>12 months) (2, 21, 28). Daycare attendance rates vary widely among European countries which could perhaps account for the differences between our findings and those of other studies. Daycare attendance rates of 3-year-olds ranged from 3 – 10% of children in Iceland, Slovenia, Portugal, Poland, Italy and Denmark to 60 – 70% in Austria and the Netherlands (http://appsso.eurostat.ec.europa.eu). Where in a recent Danish study it was shown that starting attending daycare before the age of 12 months carried a higher risk of experiencing >3 episodes of AOM at 18 months, and to a lesser extent at 7 years of age, no such association was found in our studied population. Moreover, due to the relatively short maternity leave of 10 – 12 weeks after childbirth in the Netherlands, it is likely that most children in our population started attending daycare at the age of 3 months. Daycare attendance in this study was measured at 6 months. Whether early daycare attendance affected results is unclear.

Our study found no association between childhood AOM and keeping a family cat, dog or rodent, but did find higher odds of AOM in children with a pet bird at home. Keeping furry pets such as cats, dogs, rodents and rabbits, has been associated with increased rates of rhinitis and wheezing but also with decreased odds of suffering recurrent URTIs which was the case in the population we studied (27-29). This disparity has been attributed to selection

bias through parental allergy, which increases the odds of recurrence of URTIs but may also lead to parents choosing not to have a pet (28, 30).

Public health in general is influenced by health behaviors associated with socioeconomic disparities. Literature on the association between SES and AOM has been contradictory; some studies reported absence of an association and others reported a negative effect of lower SES on AOM (12, 14). The most important unhealthy behavior is smoking. We studied both prenatal maternal smoking and household smoking. We found that household smoking raised the odds of AOM which was also reported in recent literature including two meta-analyses (11, 21, 31-33) A few other studies found no convincing association (2, 12, 16, 34). We found no association between prenatal smoking and AOM which is in agreement with a recent meta-analysis (31, 32).

In the present study, we corrected birth weight for gestational age for which we found no association with AOM. In previous studies birth weight was often studied separately from gestational age. In twins where one child is OM-prone and the other is not, it has been shown that the twin with a history of OM weighed significantly less at birth (12, 26). Still, a low birth weight can be the result of preterm birth, which in itself can be related with factors that might have an effect on AOM in the child later in life; that is, SES, infections during pregnancy, and maternal (household) smoking, which is why in the present study birth weight was corrected gestational age.

Our study found no association between AOM and ethnicity. Literature on ethnicity is more difficult to compare as it encompasses society and culture in different areas on the globe. While several studies did not find a difference in AOM odds between different ethnicities, in other studies subjects of Western descent had higher odds of AOM than had subjects from Asian or African descent (2, 25, 35). The discrepancy between our results and the latter may be due to the ethnic composition of the non-Caucasian subjects in our study – Cape Verdean, Dutch Antillean, Moroccan, Surinamese-Creole, Surinamese-Hindustani, and Turkish descent versus (sub-Saharan) African and Asian descent (8).

In general, after thoroughly correcting for all covariates and taking into account repeated measurements, effect sizes found in this study were small. Perhaps the impact of individual determinants is overestimated in literature, especially when they were measured only once, or when a smaller number of environmental factors were measured for adequate correction. Still, as AOM is so common in children, even improving determinants with small effects could have an impact on public health on a larger scale.

Our study has strengths and limitations inherent to our the study design. Strengths of this study are its sample size and its appreciation of repeated measurements in a GEE model. Thus, we were able to study AOM and its determinants with respect to ageing, which in itself has an effect on AOM susceptibility. This study was limited by the availability of covariates. In addition, we used parent-reported outcomes which may carry the risk of recall bias and possibly overdiagnosis. Previous studies, however, have shown the diagnostic value of

particularly earache, fever and otorrhea in AOM with a sensitivity and specificity of 71 and 80%, respectively (36-38). In other studies specific symptoms such as ear tugging/rubbing and restless sleep were not significantly associated with occurrence of AOM (39). Still, as AOM is a painful condition often accompanied with fever and/or otorrhea, it is generally well recalled by parents. Recall accuracy was further improved by the regular administration of questionnaires. Not performing otoscopic examination at each occurrence of otorrhea did hold the moderate risk of including some otitis externa cases. Approximately 7% of all children experience an episode of otitis externa before the age of 4 years, which makes it a less common cause of otorrhea than is AOM.

Lastly, we did not address genetic factors that might influence susceptibility to AOM in children. Genetic susceptibility to AOM is not well understood. Heritability has been established in twin and family studies, in which a fraction of phenotype variability attributed to genetic variation estimated between 0.22 and 0.73 (40). Recently, the FNDC1 gene was found associated with increased risk of AOM in childhood (7). Still, this disease susceptibility variant in itself constitutes only a modestly increased risk of AOM in this likely polygenic and complex trait. Moreover, environmental factors and genetic as well as epigenetic mechanisms are interlinked. For instance, (maternal) smoking can lead to increased DNA methylation, which in turn causes decreased expression of genes likely affecting disease susceptibility. This only further emphasizes the complexity of the etiology of complex traits such as AOM in childhood.

CONCLUSION

AOM susceptibility in childhood varies with age. Male gender, breastfeeding, household smoking, and the presence of siblings and pet birds were found significantly associated with susceptibility to AOM. Gender and breastfeeding were associated with AOM only at specific ages. Although individual determinants showed small effect sizes, improving these could benefit public health on a larger scale.

Detailed acknowledgements and online resources can be found in the published article online: https://www.nature.com/articles/s41390-019-0540-3

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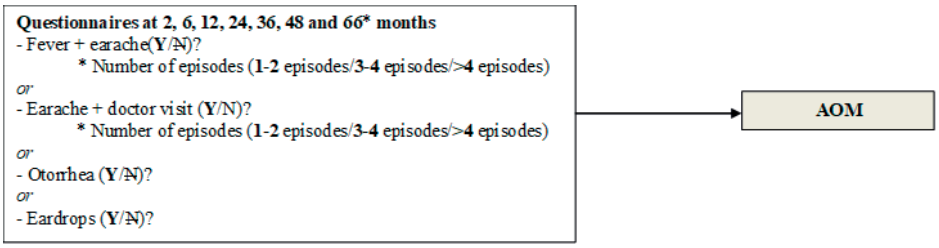
SUPPLEMENTARY MATERIAL



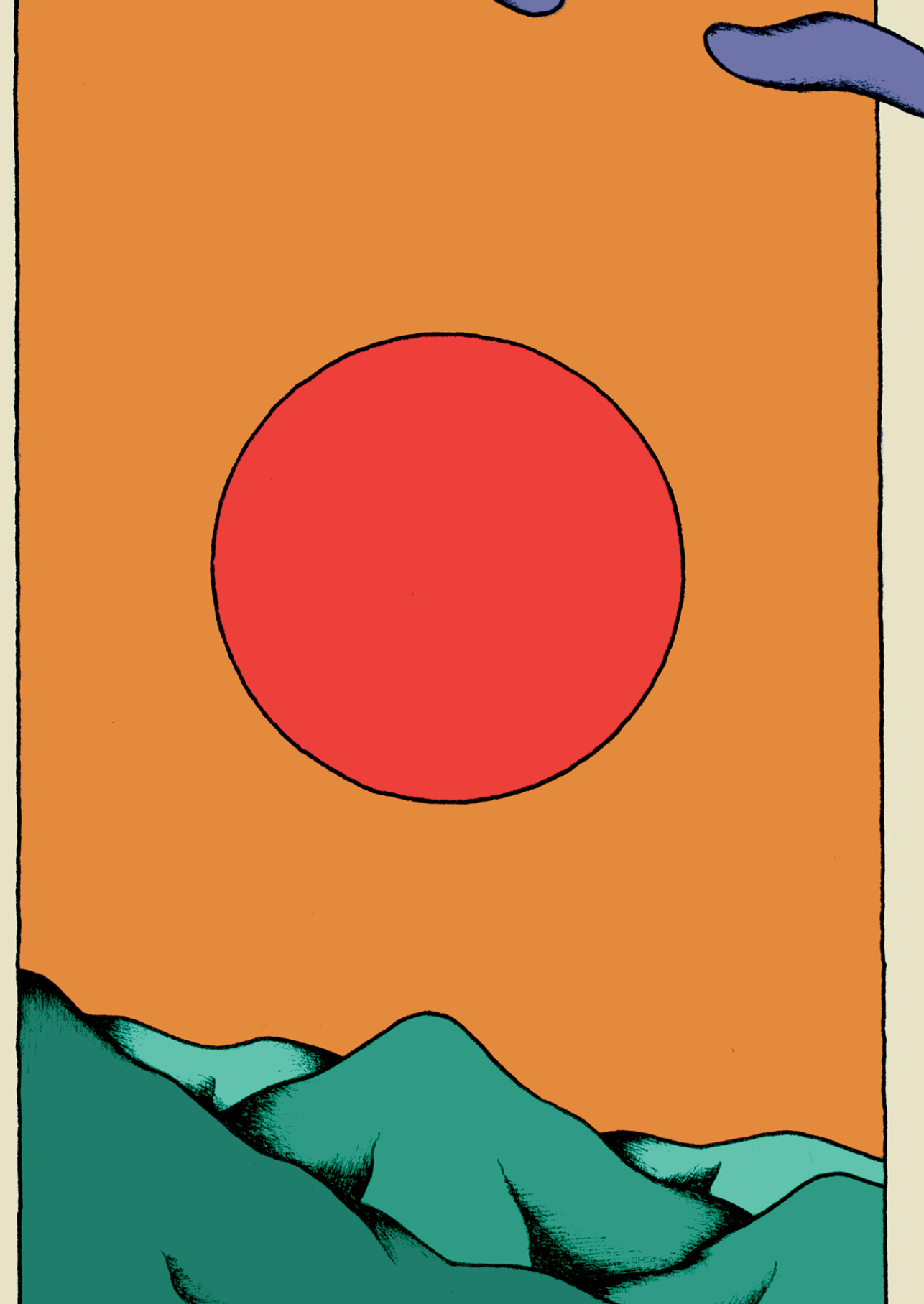
Supplemental Figure 2.1: Flow chart of questionnaire data used to define AOM.
* Questionnaire at 66 months was sent between 60 and 72 months of age with mean age of 66 months.

Supplemental Table 2.1: Determinants associated with acute otitis media, the Generation R Study.

Determinants	Data in the Generation R Study
Maternal age	Maternal age at birth in years – months
Gender child	Male/Female
Season of birth	Fall, summer, spring, winter
Birth weight	Birth weight in grams
(Lack of) breastfeeding	Yes at 6 months, No
Ethnicity	Western, non-Western
Socioeconomic class	Proxy used; education mother high/low
Siblings	Proxy used: parity
Daycare attendance	Yes at age 6 months, No
Prenatal smoking	Prenatal smoking
Postnatal household smoking	Postnatal smoking at the age of 5-6 years
Pet in household	Yes/No

Supplemental Table 2.2: Characteristics of children and their mothers based on non-imputed data.

Characteristic	Total study population
Children	
No. (%)	7863 (100)
AOM, yes (n [%])	
Age 2 months	92 (1.8)
Age 6 months	541 (12.5)
Age 1 year	1512 (29.3)
Age 2 years	1889 (35.3)
Age 3 years	1398 (28.2)
Age 4 years	1330 (27.3)
Age 5-6 years	1790 (29.7)
Sex, n (%)	
Male	3964 (50.4)
Female	3898 (49.6)
Birth weight corrected for gestational age, SDS	-0.08
Ethnicity	
Western	5123 (66.7)
Non-Western	2563 (33.3)
Season of birth, n (%)	
Spring	1303 (24.0)
Summer	1461 (26.9)
Fall	1471 (27.1)
Winter	1196 (22.0)
Breastfed ever, yes (n [%])	4260 (92.6)
Daycare at 6 months, yes (n [%])	1760 (55.3)
Pet in household, yes (n [%])	
Rodent	141 (4.4)
Dog	220 (6.9)
Bird	67 (2.1)
Cat	787 (25.3)
Mothers	
Age at intake, median (IQR)	31.0 (27.3-34.0)
Educational level, n (%)	
Higher	3533 (56.7)
Lower	2693 (43.3)
Parity, n (%)	
Nulliparous	4290 (56.4)
Multiparous	3312 (43.6)
Smoking during pregnancy, n (%)	
No	3453 (77.0)
Stopped when pregnancy was known	391 (8.7)
Yes	641 (14.3)
Postnatal household smoking, yes (n [%])	1017 (35.7)



3 Identifying distinct trajectories of acute otitis media in children: a prospective cohort study

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ABSTRACT

Objectives: To identify possibly distinct acute otitis media (AOM) trajectories in childhood, and identify determinants associated with specific AOM trajectories. To explore which child will become prone to recurrent AOM episodes, and which will not.

Design: Population-based prospective cohort study among 7,863 children from birth until 10 years and their mothers.

Methods: This study was embedded in the Generation R Study: a population-based prospective cohort study. Data on AOM and determinants were collected by repeated parental questionnaires. Distinct AOM trajectories within the population were identified with latent-class-analyses. Next, using multivariate analysis we checked if specific determinants were associated with specific trajectories.

Results: Three distinct trajectories were identified; i.e., *non-otitis-prone*, *early-AOM* – i.e. children who suffered AOM episodes until 3 years of age but not beyond, and *persistent-AOM* – i.e. children who remained otitis-prone. Male gender (OR 1.26, CI 1.11 – 1.43) and day-care attendance (OR 1.31, CI 1.06-1.60) were associated with increased odds of early-AOM. Breastfeeding was beneficial for children in both the early-AOM and persistent-AOM trajectory (OR 0.78, and 0.77 respectively). Birth in the summer or autumn as compared with birth in the spring decreased odds of AOM only in the persistent-AOM trajectory. Half of all AOM-prone children recovered after the age of 3 years.

Conclusion: Specific determinants are associated with different AOM-trajectories. Future research is needed to better predict which child will remain otitis-prone and which recovers after the age of 3 years. In this way, appropriate clinical intervention could be better tailored towards the needs of the individual child.

INTRODUCTION

Acute otitis media (AOM) is a common disease of childhood, and frequent reason for doctor visits and antibiotic treatment. This infectious disease is defined by fluid in the middle ear, and is commonly characterised by earache, fever and possibly otorrhea. It is often preceded by an upper respiratory tract infection (URTI) (1, 2). Approximately 60% of children will have gone through at least one episode before the age of 3 years, one quarter of whom will have suffered three or more episodes (2, 3). The management of AOM puts a heavy burden on health care expenditures, with annual costs in the US estimated at US$ 4.3 billion per year (4). The pathogenesis of AOM is one of complex associations between environmental, host and genetic factors (1, 5-7). Certain factors in a child's environment are related to an increase or decrease in viral pathogens, which in turn is associated with altered incidence of URTIs – and thus of ear infections. These factors include having older siblings, day-care attendance, season-of-birth, pet keeping, breastfeeding, and socioeconomic status (SES) in general (8-10). Maternal factors with a proposed impact on childhood AOM include prenatal and postnatal (household) smoking and maternal age (11). Host factors include the child's gender and ethnicity (12). Heritability of AOM is well-established in family studies, sibling and twin studies, but genetic susceptibility of AOM is as yet not well understood (13, 14). Due to the complex pathogenesis of AOM, with numerous host, environmental and genetic determinants involved, identifying at the earliest possible age whether a child will become otitis-prone is not easy. Group-based trajectory modelling is a valuable statistical approach to identify different trajectories in childhood based on AOM susceptibility.

In a prospective cohort study among 7,863 children from birth until 10 years, we aimed to identify different AOM-trajectories. Secondly, we examined if specific factors were indeed associated with a specific AOM-trajectory.

METHODS

General Study Design

This study was conducted in the framework of the Generation R Study, a population-based prospective cohort study from foetal life onwards in Rotterdam, the Netherlands (15). The Generation R Study addresses early environmental and genetic factors affecting growth, development and health of children and their mothers. Pregnant women with an expected delivery date between April 2002 and January 2006 were eligible to participate. Children born from these pregnancies form a birth cohort that is followed until young adulthood (15). The study has been approved by the Medical Ethics Review Board of the Erasmus University Medical Center in Rotterdam, the Netherlands (MEC-2007-413-NL21545.078). Written informed consent was obtained from parents or legal guardians of all 7,863 subjects.

Acute Otitis Media

Parents completed questionnaires on episodes of earache with fever, otorrhea, and use of eardrops per subscription by family practitioners or ear, nose and throat (ENT) surgeon. Data was collected at 2 and 6 months, annually between 1 and 4 years, and then by the age of 6 and 10 years (Figure 3.1). Numbers of questionnaires returned per age are shown in Figure 3.2. Children for whom fewer than three questionnaires were returned were excluded from further analyses (n = 2,009).

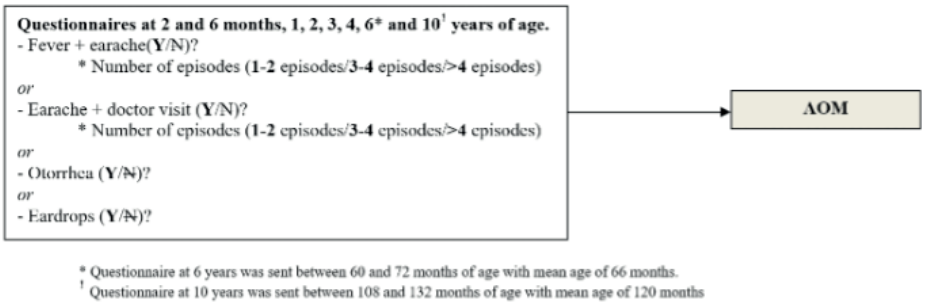

Figure 3.1: Flow chart of questionnaire data used to define AOM phenotype and control status.

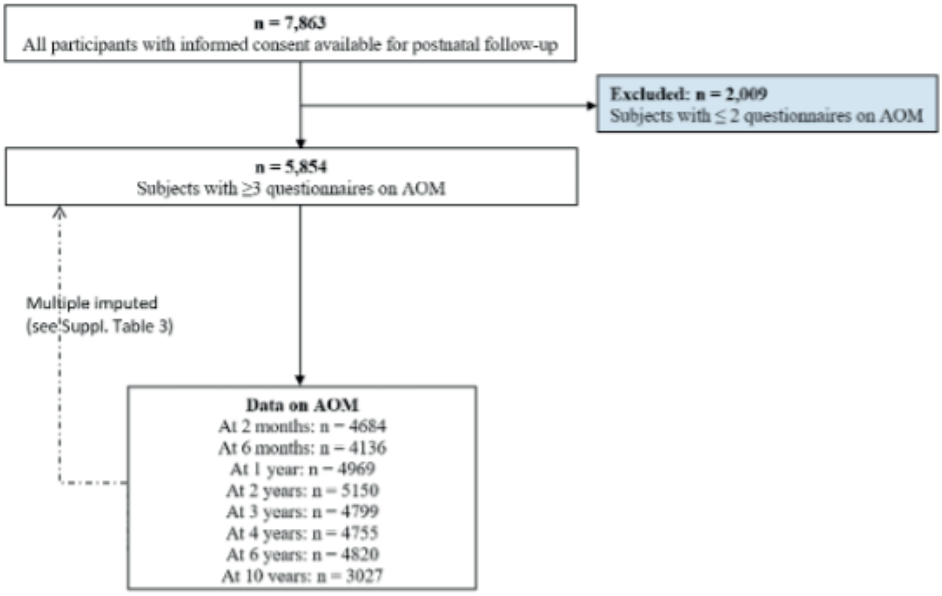

Figure 3.2: Flow chart of the study population.

Determinants

Questionnaires were used to collect data on prenatal maternal characteristics, child characteristics and environmental determinants (Supplemental Table 3.1). Information on maternal age at birth, parity (nulliparous; multiparous; number of older siblings at birth), and pet keeping (yes; no – dog, cat, bird, rodent) had been collected upon enrolment in the Generation R Study during pregnancy. Maternal education level has been previously described as a consistent socioeconomic predictor (16). Data on maternal education level (lower; higher) was used as a proxy for SES, and was collected at the child's age of 6 years. Information on maternal smoking until pregnancy and during pregnancy was obtained from multiple questionnaires. Information on gender, birth weight, and gestational age at birth was retrieved from hospital registries. Birth weight for gestational age was calculated. Data on ethnicity (Western; non-Western) and postnatal smoking was collected through parental questionnaires. Postnatal smoking was assessed at the child's ages of 2 months, and 2, 3 and 6 years. In the Netherlands, breastfeeding is encouraged at least until the age of 6 months (http://www.rivm.nl/en). Questionnaires at 6 and 12 months provided information on breastfeeding (yes; no) and/or day-care attendance (yes; no).

Statistical Analysis

The first aim of the analysis was to identify and describe distinct subgroups within the population that follow a specific AOM trajectory. Different trajectories were identified by latent-class-analyses (LCA) using Mplus version 8 (https://www.statmodel.com/index.shtml) on the basis of repeated measurements of AOM outcome. LCA was used to classify subjects into homogeneous trajectories after imputation. Number of latent classes was determined on the minimum Bayesian Information Criterion first, and minimum size of at least 5% of the population next. The latter criterion was introduced to eliminate small groups of outliers of limited clinical relevance. We created 2, 3, 4, 5 and 6 latent classes, compared goodness of fit and BIC, and produced plots of trajectories of each model (Supplemental Figure 3.1a-e, and Supplemental Table 3.2). Clinical relevance of different (number of) trajectories was taken into account when a further subdivision of latent classes would only slightly increase goodness of fit.

The second aim of the analysis was to assess whether any determinants were associated with membership of specific AOM trajectories. First, screening of missing values among determinants concluded an arbitrary missing data pattern. To reduce potential bias, missing data was imputed using the Markov chain Monte Carlo method (Supplemental Table 3.3). IBM SPSS Statistics software (version 24.0.0.1 for Windows) was used for imputation. No major differences were observed between imputed and non-imputed data (Table 3.1, and Supplemental Table 3.4); thus, only results based on imputed data are presented. Next, univariate analyses of determinants associated with membership of a specific trajectory was performed. The non-otitis-prone trajectory served as reference category. Upon examina-

tion of the correlation matrix, no multicollinearity between determinants was detected. Determinants associated with a trajectory in univariate analysis with p<0.05, were selected for inclusion in the multivariate model (Supplemental Table 3.5). Multivariate analysis was performed; odds ratios (ORs) and their 95% confidence intervals are presented (Table 3.2). Determinants that showed subthreshold association in univariate analyses with p>0.05 and p<0.15 were added to the multivariate model, but this did not alter outcome (Supplemental Table 3.6). Data of subjects for whom fewer than three questionnaires had been returned – and were excluded from analyses – were compared with data of the entire study population to assess whether values were missing at random (Supplemental Table 3.7).

Table 3.1: Characteristics of children and their mothers based on imputed data.

Characteristic	Non-otitis-prone	Early-AOM	Persistent-AOM	Total study population
Children				
No.	3067	1335	1452	5854
Gender, n (%)				
Male	1477 (48.2)	719 (53.9)	724 (49.9)	2920 (49.9)
Female	1590 (51.8)	616 (46.1)	728 (50.1)	2934 (50.1)
Birth weight for gestational age, SDS	-0.06	0.01	-0,02	-0.03
Ethnicity				
Western	2226 (72.6)	976 (73.1)	1041 (71.7)	4242 (72.5)
Non-Western	841 (27.4)	359 (26.9)	411 (28.3)	1612 (27.5)
Season of birth, n (%)				
Spring	712 (23.2)	349 (26.1)	396 (27.3)	1457 (24.9)
Summer	849 (27.7)	334 (25.0)	348 (24.0)	1531 (26.2)
Autumn	839 (27.4)	367 (27.5)	381 (26.2)	1587 (27.1)
Winter	667 (21.7)	285 (21.3)	327 (22.5)	1279 (21.8)
Breastfed at 6 months, yes (n [%])	1100 (35.9)	411 (30.8)	438 (30.2)	1949 (33.3)
Day-care at 6 months, yes (n [%])	2045 (66.7)	977 (73.2)	948 (65.3)	3971 (67.8)
Pet in household, yes (n [%])				
Rodent	168 (5.5)	69 (5.2)	60 (4.1)	298 (5.1)
Dog	238 (7.8)	94 (7.0)	128 (8.8)	459 (7.8)
Bird	82 (2.7)	39 (2.9)	55 (3.8)	175 (3.0)
Cat	740 (24.1)	330 (24.7)	379 (26.1)	1448 (24.7)
Mothers				
Age at intake, median (IQR)	31.1 (28.1-34.1)	31.5 (28.6-34.4)	31.2 (28.2-34.2)	31.2 (28.2-34.2)
Educational level, n (%)				
Higher	1878 (61.2)	839 (63.0)	844 (58.1)	3560 (60.8)
Lower	1189 (38.8)	496 (37.0)	608 (41.9)	2294 (39.2)
Parity, n (%)				
Nulliparous	1795 (58.5)	752 (56.3)	824 (56.7)	3370 (57.6)
Multiparous	1272 (41.5)	583 (43.7)	628 (43.3)	2484 (42.4)
Smoking during pregnancy, n (%)				
No	2388 (77.9)	1041 (78.0)	1124 (77.4)	4553 (77.8)
Yes, until pregnancy was known	275 (9.0)	131 (9.8)	118 (8.1)	524 (9.0)
Yes, continued smoking in pregnancy	404 (13.1)	163 (12.2)	210 (14.5)	777 (13.3)
Postnatal household smoking, yes (n [%])	996 (32.5)	422 (31.6)	527 (36.3)	1945 (33.2)

Data after multiple imputation represents the pooled results derived from 10 imputed data sets.

RESULTS

Study Population and Baseline Characteristics

Characteristics of the study population are presented in Table 3.1. The population totalled 5,854 children and their mothers. There was a light preponderance of girls (50.1%), and 72.5% of all children were of Western descent. One third of the population had been breastfed until at least 6 months of age, and two thirds went to day-care at 6 months of age. Birth rates in the winter were lower than birth rates in the other three seasons, and cats were the most common family pet (24.7%). Sixty-eight percent of mothers had higher SES, 57.6% was pregnant of their first child upon inclusion; 22.3% smoked when pregnancy was discovered, and almost 60% of these women continued smoking throughout pregnancy. One third of all subjects grew up exposed to household smoking.

Three distinct AOM trajectories could be distinguished (Figure 3.3). One trajectory comprised non-otitis-prone children; the second consisted of children prone to AOM only in the first 3 years of life (early-AOM); and the third concerned children who remained otitis-prone throughout (persistent-AOM). When in LCA the number of trajectories was increased from three to four, goodness of fit did marginally improve, but this only subdivided the early-AOM trajectory into two smaller fractions without creating a new distinct trajectory, and thus not of clinical relevance (Supplemental Figure 3.1b-c). Further increase of the number of trajectories provided only small groups of outliers comprising <5% of the population (Supplemental Table 3.2).

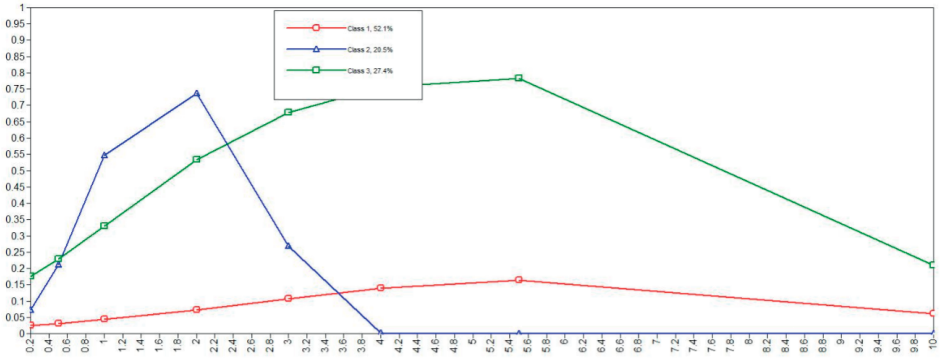

Figure 3.3: Trajectories of AOM susceptibility in childhood: The plot shows three distinct trajectories of childhood AOM, knowing the Non-otitis prone (red), Early-AOM (blue), and Persistent-AOM (green) trajectories.

The non-otitis-prone trajectory totalled 3067 subjects (52.4%). Children in this trajectory had a lower birth rate in the spring and more were breastfed compared with children in the other trajectories. In the early-AOM trajectory (n=1335, 22.8% of total population) there was a preponderance of boys (53.9%) and slightly more subjects were of Western descent than of non-Western descent. More children attended day-care (73.2%) compared

to children in the other trajectories (66.7% and 65.3%), and a higher proportion of subjects grew up in a household of higher SES. Persistent-AOM held for 24.8% (n=1452) of the population. Compared with the other trajectories, a higher proportion of these children was exposed to household smoking; and grew up in a household of lower SES with more often a cat, dog or both.

Multivariate analysis showed which specific determinants altered odds of acquiring AOM per trajectory, compared with subjects who were non-otitis-prone (Table 3.2). Both male gender (OR 1.26, p=0.001) and day-care attendance (OR 1.31, p=0.015) were associated with increased odds of early-AOM. Having been breastfed until at least 6 months of age showed decreased odds of acquiring AOM in all otitis-prone children, with OR 0.78 (p=0.003) and OR 0.77 (p=0.002) in early- and persistent-AOM, respectively. Birth in the summer (OR 0.74, p=0.002) and autumn (OR 0.82, p=0.04) was associated with decreased odds of acquiring AOM in persistent-AOM, but this effect was not found in early-AOM. Exposure to household smoking, and having siblings had a negative effect in univariate analyses in the persistent-AOM trajectory. This effect disappeared, however, in multivariate analyses. Similarly, potential effects of SES, maternal age and aberrant birth weight for gestational age were not found in multivariate analysis (Supplemental Tables 3.5 and 3.6).

Determinants	Early-AOM		Persistent-AOM	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Male Gender	**1.26 (1.11-1.43)**	p=0.001	1.07 (0.94-1.21)	
Aberrant birth weight for gestational age[a]	1.06 (1.00-1.13)		1.05 (0.99-1.12)	
Higher maternal age[b]	1.01 (1.00-1.03)		1.01 (0.99-1.02)	
Season of birth				
Spring	Ref.		Ref.	
Summer	0.81 (0.65-1.01)		**0.74 (0.62-0.90)**	p=0.002
Autumn	0.89 (0.73-1.09)		**0.82 (0.68-0.99)**	p=0.04
Winter	0.89 (0.72-1.09)		0.88 (0.73-1.07)	
Breastfeeding at 6 months	**0.78 (0.66-0.91)**	p=0.003	**0.77 (0.66-0.90)**	p=0.002
Day-care attendance at 6 months	**1.31 (1.06-1.60)**	p=0.015	0.92 (0.79-1.09)	

Table 3.2: Multivariate analysis of associations between determinants and membership of different childhood AOM trajectories.
Reference category for trajectories: Non-otitis-prone trajectory.
Significant ORs in bold, p<0.05.
a) Below or above 2 SD birth weight for gestational age.
b) Increase per year of in maternal age at birth of study subject

DISCUSSION

Trajectories of Acute Otitis Media in Childhood

This study is the first to reveal three distinct trajectories of AOM in childhood: not being prone to it, indeed being prone to it, and being prone to it only in the first 3 years of life. We

found that gender and day-care, which have previously been described in association with childhood AOM, in our studied population were associated only with membership of the early-AOM trajectory. This strengthens the idea that children in this subgroup perhaps have a different phenotype from children who remain AOM-prone.

Gender

Male gender generally is associated with increased odds of acquiring AOM in childhood (2, 12, 17, 18). The present study shows increased odds of AOM among boys only in the early–AOM subgroup. Recent studies among subjects below 12, 18 and 24 months showed a similar negative association among boys, with OR 2.6, 1.3 and 1.8, respectively (2, 6, 18). Several studies among older subjects described no significant difference between boys and girls aged from 3 to 16 years (19, 20). These findings suggest that gender may perhaps serve as a predictor for AOM in the first years of life, but not beyond the age of three years.

Day-care Attendance

Certain environmental factors are related to an increase in viral pathogens, which in turn are associated with increased incidence of URTIs – and thus ear infections (8-10, 21). It is well established that day-care attendance exposes a child to a higher amount of viral pathogens. Day-care attendance has indeed often been related to increased risk of AOM, with risk further increasing for children who attend day-care for a longer period (>12 months) (2, 17, 18, 21). In the Netherlands, maternity leave consists of 16 weeks for the mother, of which at least 12 weeks are taken after childbirth. Of our population, approximately 70% of children attended day-care at the early age of 6 months, and 77.3% at 12 months. Day-care attendance rates of three-year-old children in European Union member states vary greatly from 3 – 28% of children in Iceland, Slovenia, Portugal, Poland, Italy and Denmark to 60 – 70% in Austria and the Netherlands (http://appsso.eurostat.ec.europa.eu). A recent study in a Danish population found that children who had started day-care attendance before the age of 12 months had an increased probability risk of experiencing more than three episodes of AOM at 18 months, and to a lesser extent at 7 years of age (6). These findings may explain in part why in our study the percentage of day-care attendance was highest among children prone to early-AOM, and that only this subgroup had increased odds of acquiring AOM.

Breastfeeding

The positive effect of breastfeeding on AOM susceptibility is widely accepted (1, 2, 5, 17). Possible explanations of this positive effect include a lower rate of URTIs through the effects of secretory IgA, cytokines, and long-chain fatty acids in breastmilk, which are paramount to development of the infant's immune system (7). The greatest protection of breastfeeding on AOM has been described in children breastfed for at least 6 months (2, 5). Shorter duration of breastfeeding induced higher AOM rates, both below 2 years of age and later in

childhood (6, 20, 21). This is in concurrence with findings in our studied population, where breastfeeding at 6 months was associated with decreased odds of acquiring AOM in both the early- and persistent-AOM trajectories.

Season-of-Birth

Season-of-birth may be associated with either an increase or decrease in the rate of URTIs, depending on the season. This study found a beneficial effect of being born in the summer and autumn as compared to being born in the spring in the persistent-AOM trajectory, yet interpreting this result is difficult. Literature on the possible relationship between AOM and season-of-birth is contradictory; some studies report that birth in the autumn would carry higher odds of AOM, whereas other studies report lower AOM rates among subjects born in the autumn (9, 10, 22). Moreover, definitions of seasons vary across countries, which limits generalizability of these results.

Strengths and limitations

This study has several strengths and weaknesses inherent to its design. A strength lies in its large population-based study sample with repeated measurements and extensive data on determinants. Medical records or otoscopy were not available in our dataset. Outcome was parent-reported which holds the risk of introducing recall bias. An episode of AOM is often a painful experience for a young child, one that parents may not easily forget. The diagnostic value of parent-reported earache, fever, and otorrhea in AOM was shown previously with a sensitivity and specificity of 71% and 80%, respectively (23). Yet, in other studies specific symptoms such as ear tugging/rubbing and restless sleep were not significantly associated with occurrence of AOM (24). A further limitation of this study is that it lacked data on surgical history pertaining adenoidectomy or insertion of ventilation tubes. Still, in our experience even after ventilation tube insertion the otitis-prone child will likely go through episodes of (mild) otorrhea after URTIs or swimming. To check for possible non-response bias, we compared characteristics of children lost to follow-up with those of the studied population. The characteristics compared well between these groups, supporting that non-response values are missing at random (Supplemental Table 3.7). A further limitation was that data on vaccination status – a possible intermediate – was not available in this study. The Netherlands national immunization program was established in 1957, and currently holds coverage of >95% of all Dutch children (http://www.rivm.nl/en). The most frequently involved bacterial pathogens in AOM are Streptococcus pneumoniae, non-typeable Haemophilus influenzae and Moraxella catarrhalis. Widespread use of pneumococcal conjugate vaccines (PCVs) have decreased incidence of AOM since its introduction. It has been shown that PCVs can prevent early episodes of disease associated with vaccine serotypes, resulting in a reduction of subsequent complex cases caused by non-vaccine serotypes and other otopathogens, which contribute considerably to the disease burden (25). PCV-7 was

introduced in the Netherlands immunization program in June 2006 shortly after inclusion of subjects in this study had been completed. It is likely that many subjects in our dataset were subsequently vaccinated.

Our analyses provide information on altered odds of acquiring AOM within a specific trajectory. As such, results of this study cannot be extrapolated to form a prediction model. We trust that results from this study still contribute to the understanding of the complex aetiology of childhood AOM, and that increased knowledge of its aetiology will ultimately lead to earlier identification of otitis-prone children.

CONCLUSION

Half of all AOM-prone children in the studied population recovered after the age of 3 years. Future research is needed to better predict which child will remain otitis-prone and which recovers after 3 years of age. In this way, appropriate clinical intervention could be better tailored towards the needs of the individual child.

Detailed acknowledgements and online resources can be found in the published article online: https://onlinelibrary.wiley.com/doi/10.1111/coa.13736

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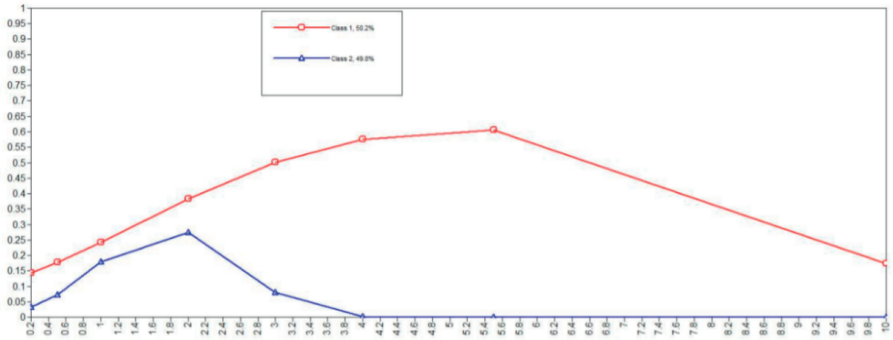
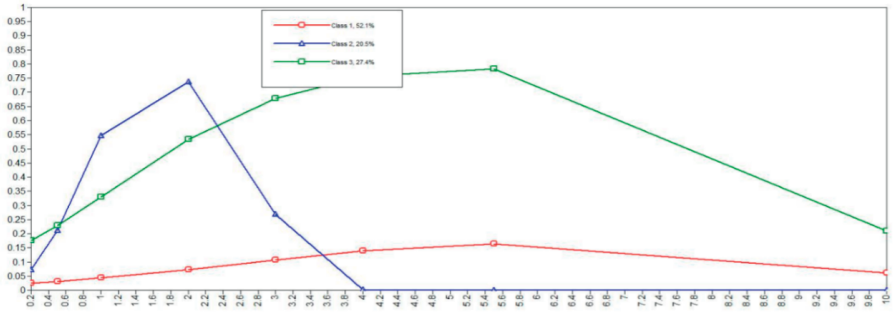
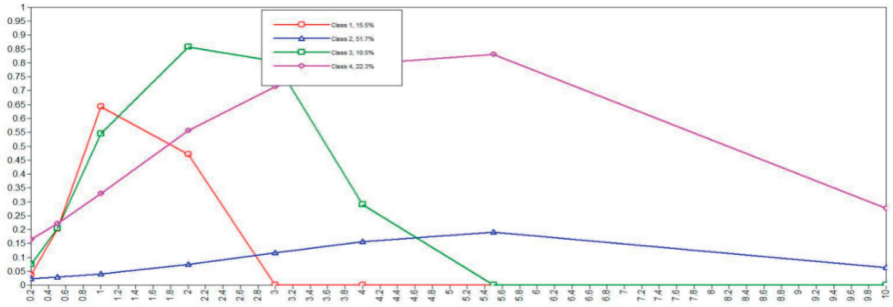
SUPPLEMENTARY MATERIAL



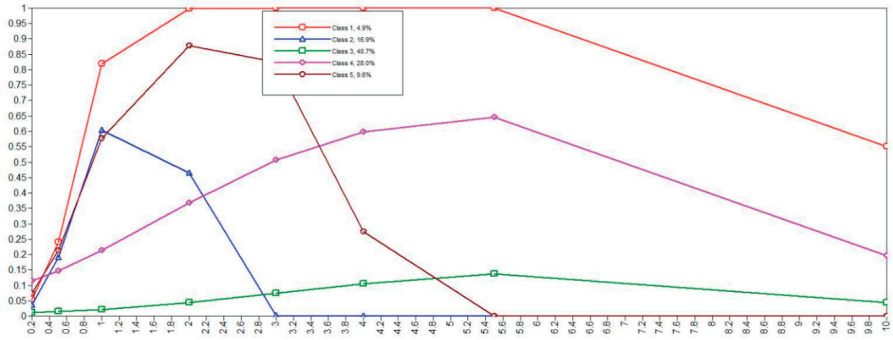
Supplemental Figure 3.1a: Potential trajectories of AOM susceptibility in childhood: Two trajectories.



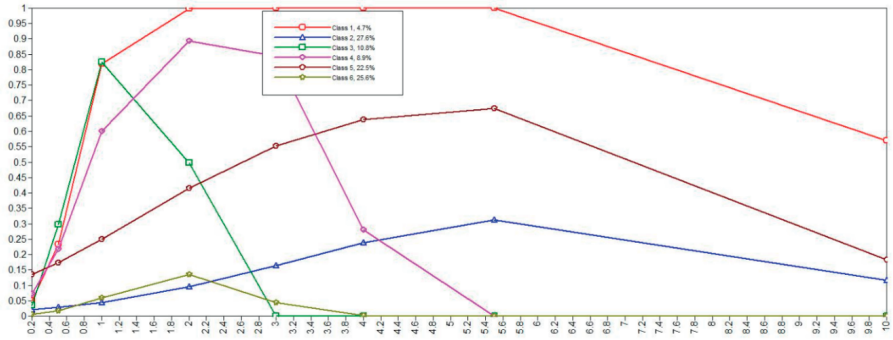
Supplemental Figure 3.1b: Potential trajectories of AOM susceptibility in childhood: Three trajectories.



Supplemental Figure 3.1c: Potential trajectories of AOM susceptibility in childhood: Four trajectories.



Supplemental Figure 3.1d: Potential trajectories of AOM susceptibility in childhood: Five trajectories.



Supplemental Figure 3.1e: Potential trajectories of AOM susceptibility in childhood: Six trajectories.

Supplemental Table 3.1: Determinants associated with AOM, available in the Generation R Study.

Determinants	Data in the Generation R Study
Maternal age	Maternal age at birth in years – months
Gender child	Male/Female
Season of birth	Autumn, summer, spring, winter
Birth weight	Birth weight in grams
(Lack of) breastfeeding	Yes at 6 months, No; Yes at 12 months, No
Ethnicity	Western, non-Western
Socioeconomic class	Proxy used; education mother high/low
(older) Siblings	Proxy used: parity
Day-care attendance	Yes at age 6 months, No; Yes at 12 months, No
Prenatal smoking	Yes until pregnancy was known, Yes throughout pregnancy, No
Postnatal household smoking	Postnatal smoking at the age of 5-6 years
Pet in household	Yes/No; Cat, Dog, Rodent, Bird

Supplemental Table 3.2: Latent class growth analysis with growth terms used in model: intercept, (linear) slope, and quadratic term. Participants with >2 time points (n = 5854)

Number of classes	BIC	Minimum n per class
2	34,875	2605 (49.8%)
3	33,876	1199 (20.5%)
4	33,638	616 (10.5%)
5	33,412	286 (4.9%)
6	33,401	277 (4.7%)

Supplemental Table 3.3: Details of the multiple imputation modelling.

Software used	IBM SPSS version 24.0.0.1 for Windows
Imputation method	Fully conditional specification (Markov chain Monte Carlo method)
Maximum Iterations	20
Number of imputed data sets	10
Variables to be imputed	Breastfeeding at 6 months; breastfeeding ever; season of birth; aberrant birthweight for gestational age; prenatal smoking; postnatal household smoking; ethnicity; pet cat; pet dog; pet rodent; pet bird; day-care at 6 months; day-care at 12 months; maternal education level; siblings
Variables added as predictor	Trajectory; maternal age, singleton birth, gender
Treatment of non-normally distributed variables	Predictive mean matching
Treatment of binary/categorical variables	Logistic regression

Supplemental Table 3.4: Characteristics of all subjects based on original (non-imputed) data.

Characteristic	Non-otitis-prone	Early-AOM	Persistent-AOM	Total study population
Children				
No.	3067 (100)	1335 (100)	1452 (100)	5854 (100)
Gender, n (%)				
Male	1477 (48.2)	719 (53.9)	724 (49.9)	2920 (49.9)
Female	1590 (51.8)	616 (46.1)	728 (50.1)	2934 (50.1)
Missing %	–	–	–	–
Birth weight for gestational age, SDS	-0.06	0.01	-0,02	-0.03
Missing %	0.5%	0.5%	0.6%	0.5%
Ethnicity				
Western	2211 (72.6)	970 (73.2)	1033 (71.8)	4214 (72.6)
Non-Western	833 (27.4)	355 (26.8)	406 (28.2)	1594 (27.4)
Missing %	0.7%	0.7%	0.9%	0.8%
Season of birth, n (%)				
Spring	538 (23.2)	260 (26.0)	298 (27.1)	1096 (24.8)
Summer	645 (27.8)	253 (25.3)	259 (23.6)	1157 (26.2)
Autumn	636 (27.4)	275 (27.5)	290 (26.4)	1201 (27.2)
Winter	501 (21.6)	213 (21.3)	252 (22.9)	966 (21.9)
Missing %	24.4%	25.0%	24.3%	24.5%
Breastfed ever, yes (n [%])	2099 (92.4)	914 (92.6)	1002 (93.0)	4015 (92.6)
Missing %	26.0%	26.1%	25.8%	25.9%
Breastfed at 6 months, yes (n[%])	776 (35.6)	297 (30.9)	304 (30.2)	1377 (33.2)
Missing %	29.0%	28.0%	30.7%	29.2%
Day-care at 6 months, yes (n [%])	806 (69.8)	457 (78.1)	347 (66.5)	1610 (71.2)
Missing %	62.4%	56.2%	64.0%	61.4%
Day-care at 12 months, yes (n[%])	1300 (76.1)	731 (82.0)	604 (74.5)	2635 (77.3)
Missing %	44.3%	33.2%	44.1%	41.7%
Pet in household, yes (n [%])				
Cat	399 (24.8)	181 (24.6)	189 (26.5)	769 (25.1)
Missing %	47.5%	44.9%	51.0%	47.8%
Dog	116 (7.0)	46 (6.1)	54 (7.4)	216 (6.9)
Missing %	46.3%	43.4%	49.7%	46.5%
Bird	30 (1.8)	16 (2.1)	21 (2.9)	67 (2.2)
Missing %	46.7%	43.7%	47.6%	47.0%
Rodent	80 (4.8)	34 (4.5)	26 (3.5)	140 (4.5)
Missing %	46.2%	43.1%	47.6%	46.3%
Mothers				
Age at intake, median (IQR)	31.1 (28.1-34.1)	31.5 (28.6-34.4)	31.2 (28.2-34.2)	31.2 (28.2-34.2)
Missing %	–	–	–	–
Educational level, n (%)				
Higher	1644 (63.5)	735 (65.1)	757 (60.6)	3136 (63.1)
Lower	947 (36.5)	394 (34.9)	492 (39.4)	1833 (36.9)
Missing %	15.5%	15.4%	14.0%	15.1%
Parity, n (%)				
Nulliparous	1756 (58.8)	731 (56.4)	799 (56.8)	2386 (57.7)
Multiparous	1231 (41.2)	566 (43.6)	608 (43.2)	2405 (42.3)
Missing %	2.6%	2.8%	3.1%	2.8%

Supplemental Table 3.4: Characteristics of all subjects based on original (non-imputed) data. (continued)

Characteristic	Non-otitis-prone	Early-AOM	Persistent-AOM	Total study population
Smoking during pregnancy, n (%)				
No	1555 (78.4)	676 (78.8)	695 (77.5)	2926 (78.3)
Yes, until pregnancy was known	180 (9.1)	82 (9.6)	74 (8.2)	336 (9.0)
Yes	248 (12.5)	100 (11.7)	128 (14.3)	476 (12.7)
Missing %	35.3%	38.2%	38.2%	36.1%
Postnatal household smoking, yes (n [%])	445 (31.4)	192 (29.8)	230 (36.0)	867 (32.1)
Missing %	53.8%	51.8%	56.0%	53.9%

Supplemental Table 3.5: Univariate analysis of association between determinants and membership of different childhood AOM trajectories.

Determinants	Early-AOM OR (95% CI)	Persistent-AOM OR (95% CI)
Male Gender	**1.26 (1.11-1.43)**	1.07 (0.95-1.21)
Aberrant birth weight for gestational age[a]	**1.07 (1.00-1.14)**	1.05 (0.98-1.11)
Western ethnicity	1.03 (0.89-1.19)	0.96 (0.83-1.10)
Season of birth		
Spring	Ref.	Ref.
Summer	♦0.80 (0.64-1.01)	**0.74 (0.61-0.89)**
Autumn	0.89 (0.73-1.09)	**0.81 (0.67-0.99)**
Winter	0.87 (0.71-1.07)	0.88 (0.73-1.07)
Breastfeeding ever	1.03 (0.78-1.36)	1.07 (0.80-1.43)
Breastfeeding at 6 months	**0.80 (0.68-0.94)**	**0.77 (0.66-0.91)**
Day-care attendance at 6 months	**1.33 (1.08-1.62)**	0.93 (0.79-1.09)
Day-care attendance at 12 months	♦1.36 (1.00-1.85)	0.92 (0.74-1.15)
Pet keeping		
Cat	1.03 (0.85-1.25)	1.11 (0.84-1.46)
Dog	0.90 (0.64-1.25)	1.15 (0.85-1.56)
Bird	1.12 (0.50-2.44)	1.46 (0.83-2.58)
Rodent	0.94 (0.64-1.39)	0.74 (0.46-1.20)
Higher maternal age[b]	**1.02 (1.00-1.04)**	1.00 (0.99-1.02)
Higher socioeconomic class	1.07 (0.93-1.24)	♦0.88 (0.77-1.01)
Older siblings[b]	1.02 (0.94-1.11)	♦1.06 (0.92-1.14)
Prenatal smoking		
No	Ref.	Ref.
Yes, until pregnancy known	1.09 (0.82-1.44)	0.91 (0.70-1.19)
Yes, continued during pregnancy	0.93 (0.72-1.19)	1.11 (0.90-1.37)
Postnatal household smoking	0.96 (0.81-1.15)	♦1.18 (0.97-1.44)

Reference category for trajectories: Non-otitis-prone trajectory.
Significant ORs in bold, p<0.05.
♦ ORs with p>0.05 and p<0.15.
a) Below or above 2 SD birth weight for gestational age.
b) Increase per year of in maternal age at birth of study subject.
c) Increase per older sibling at birth of study subject.

Supplemental Table 3.6: Multivariate analysis of associations between determinants and membership of different childhood AOM trajectories.

Determinants	Early-AOM		Persistent-AOM	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Male Gender	**1.26 (1.11-1.43)**	p=0.001	1.07 (0.94-1.21)	
Aberrant birth weight for gestational age[a]	1.06 (1.00-1.14)		1.05 (0.99-1.12)	
Higher maternal age[b]	1.01 (1.00-1.03)		1.01 (1.00-1.03)	
Season of birth				
Spring	Ref.		Ref.	
Summer	0.81 (0.65-1.02)		**0.74 (0.61-0.89)**	p=0.002
Autumn	0.89 (0.73-1.09)		**0.82 (0.68-1.00)**	p=0.048
Winter	0.89 (0.72-1.09)		0.88 (0.73-1.08)	
Breastfeeding at 6 months	**0.78 (0.66-0.92)**	p=0.004	**0.79 (0.67-0.92)**	p=0.004
Day-care attendance at 6 months	**1.31 (1.06-1.62)**	p=0.018	0.96 (0.81-1.13)	
Postnatal household smoking	1.01 (0.83-1.23)		1.15 (0.93-1.42)	
Higher socioeconomic class	1.00 (0.84-1.18)		0.93 (0.79-1.08)	
(number of) older siblings	1.00 (0.92-1.10)		1.03 (0.95-1.12)	

Reference category for trajectories: Non-otitis-prone trajectory.
Significant ORs in bold, p<0.05.
a) Below or above 2 SD birth weight for gestational age.
b) Increase per year of in maternal age at birth of study subject.

Supplemental Table 3.7: Characteristics of study population compared with subjects with ≤ 2 questionnaires returned and thus excluded from analyses based on original (non-imputed) data.

Characteristic	Total study population	Excluded from analyses
Children		
No.	5854	2009
Gender, n (%)		
Male	2920 (49.9)	1044 (52.0)
Female	2934 (50.1)	964 (48.0)
Missing %	–	0%
Birth weight for gestational age, SDS	-0.03	-0.20
Missing %	0.5%	2.3%
Ethnicity		
Western	4214 (72.6)	896 (47.7)
Non-Western	1594 (27.4)	982 (52.3)
Missing %	0.8%	6.5%
Season of birth, n (%)		
Spring	1096 (24.8)	207 (20.5)
Summer	1157 (26.2)	304 (30.1)
Autumn	1201 (27.2)	270 (26.7)
Winter	966 (21.9)	230 (22.7)
Missing %	24.5%	49.7%
Breastfed ever, yes (n [%])	4015 (92.6)	245 (92.1)
Missing %	25.9%	86.8%
Breastfed at 6 months, yes (n[%])	1377 (33.2)	29 (19.9)
Missing %	29.2%	92.7%

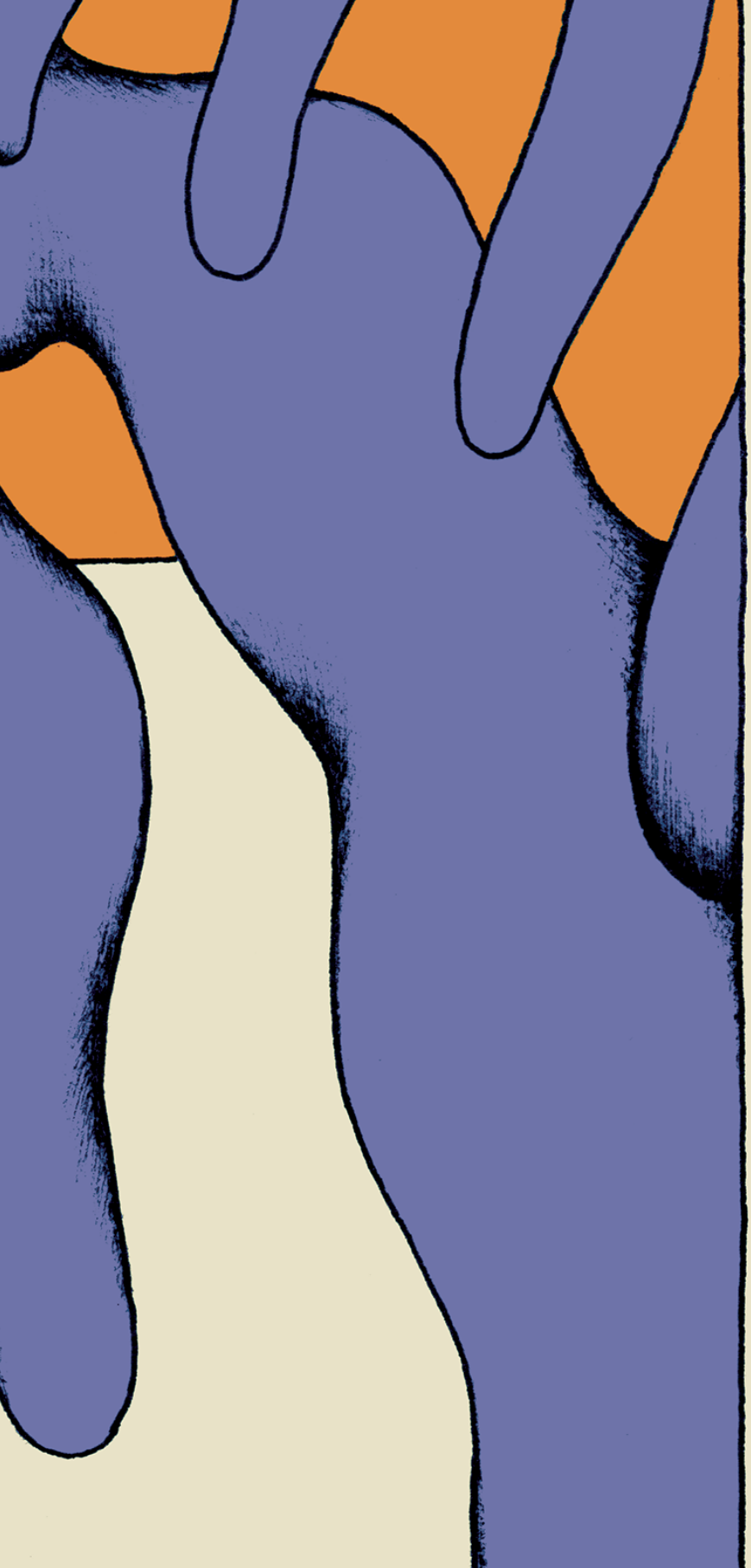
Supplemental Table 3.7: Characteristics of study population compared with subjects with ≤ 2 questionnaires returned and thus excluded from analyses based on original (non-imputed) data. (continued)

Characteristic	Total study population	Excluded from analyses
Day-care at 6 months, yes (n [%])	1610 (71.2)	10 (50.0)
Missing %	61.4%	99.0%
Day-care at 12 months, yes (n[%])	2635 (77.3)	51 (71.8)
Missing %	41.7%	96.5%
Pet in household, yes (n [%])		
Cat	769 (25.1)	9 (20.9)
Missing %	47.8%	97.9%
Dog	216 (6.9)	4 (8.9)
Missing %	46.5%	97.8%
Bird	67 (2.2)	0 (0)
Missing %	47.0%	97.8%
Rodent	140 (4.5)	1 (2.2)
Missing %	46.3%	97.7%
Mothers		
Age at intake, median (IQR)	31.2 (28.2-34.2)	28.8 (24.3-32.5)
Missing %	–	0.0%
Educational level, n (%)		
Higher	3136 (63.1)	860 (68.4)
Lower	1833 (36.9)	397 (31.5)
Missing %	15.1%	37.4%
Parity, n (%)		
Nulliparous	2386 (57.7)	1004 (52.5)
Multiparous	2405 (42.3)	907 (47.5)
Missing %	2.8%	4.9%
Smoking during pregnancy, n (%)		
No	2926 (78.3)	527 (70.5)
Yes, until pregnancy was known	336 (9.0)	55 (7.4)
Yes	476 (12.7)	165 (22.1)
Missing %	36.1%	62.8%
Postnatal household smoking, yes (n [%])	867 (32.1)	150 (100)
Missing %	53.9%	92.5%
% AOM at specific age		
At 2 months, %	1.6%	4.0%
Missing %	20%	78.7%
At 6 months, n	12.1%	22.0%
Missing %	29.3%	90.7%
At 1 year, n	29.1%	34.2%
Missing %	15.1%	90.4%
At 2 years, n	35.3%	36.5%
Missing %	12.0%	90.2%
At 3 years, n	28.3%	30.8%
Missing %	18.0%	93.5%
At 4 years, n	26.9%	42.4%
Missing %	18.8%	94.1%
At 6 years, n	29.0%	32.3%
Missing %	17.7%	39.3%

Supplemental Table 3.7: Characteristics of study population compared with subjects with ≤ 2 questionnaires returned and thus excluded from analyses based on original (non-imputed) data. (continued)

Characteristic	Total study population	Excluded from analyses
At 10 years, n	8.5%	9.4%
Missing %	43.5%	75.9%

II Genetic and epigenetic susceptibility to acute otitis media in children



4 Genome-wide association study for acute otitis media in children identifies FNDC1 as disease contributing gene

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ABSTRACT

Acute otitis media (AOM) is among the most common pediatric diseases, and the most frequent reason for antibiotic treatment in children. Risk of AOM is dependent on environmental and host factors, as well as a significant genetic component. We identify genome-wide significance at a locus on 6q25.3 (rs2932989, $P_{meta} = 2.15 \times 10^{-09}$), and show that the associated variants are correlated with the methylation status of the *FNDC1* gene (cg05678571, $P = 1.43 \times 10^{-06}$), and further show it is an eQTL for FNDC1 ($P = 9.3 \times 10^{-05}$). The mouse homologue, Fndc1, is expressed in middle ear tissue and its expression is upregulated upon lipopolysaccharide treatment. In this first GWAS of AOM and the largest OM genetic study to date, we identify the first genome-wide significant locus associated with AOM.

INTRODUCTION

Otitis media (OM) is among the most common pediatric diseases, and the most frequent reason for antibiotic treatment, in children. Interrelated phenotypes include acute OM (AOM), recurrent AOM and OM with effusion. Among OM phenotypes, AOM is defined by the presence of (purulent) fluid in the middle ear accompanied by earache, bulging of the tympanic membrane and fever, usually preceded by an upper respiratory tract infection (1-3), which is the type of OM most frequently encountered by pediatricians, and constitutes the largest number of OM patients. The etiology of OM is one of complex associations between environmental, pathogen, host and genetic risk factors (4). Heritability is well-established in sibling, twin and family studies, with the fraction of phenotype variability attributed to genetic variation (h^2) estimated between 0.22 and 0.74 (5-8).

The genetic susceptibility loci for OM are not well understood. Candidate gene studies, based on biological plausibility of the genes and evidence from model organisms, have examined the association of some relevant genes to OM and yielded significant associations for 420 genes including several interleukin (IL) genes, mucin genes, *TLR4*, *FBXO11*, *TNFα* (reviewed by Rye et al (9)). However, as with most candidate gene studies, results are conflicting with only a small proportion of the discoveries replicating in independent studies. Recently, agnostic discovery approaches have been adopted to identify the genetic susceptibility loci underlying OM. Single-nucleotide polymorphisms (SNPs) at several loci have been reported to be associated with OM, such as those at 10q26.3, 19q13.43, 17q12, 10q22.3, 2q31.1 for association with chronic and recurrent OM (10-13), 2p23.1 (genes *CAPN14*, *GALNT14*) and 20q11.21 (*BPIFA* gene) for association with OM in general (14). None of these signals broke the threshold of genome-wide significance ($P > 5 \times 10^{-08}$), likely due to the small sample size and limited number of genetic variants examined. A recent study of OM via exome sequencing identified the cosegregation of a rare duplication variant in gene *A2ML1* with OM in a Filipino pedigree, and additional *A2ML1* variants were found in otitis-prone American children (15), but these variants were all of very low frequency which could not account for the high prevalence of OM. In addition, there has been no genetic study specifically examining the susceptibility loci for AOM.

We perform genome-wide association studies (GWAS) to identify susceptibility loci for AOM. In the meta-analysis of two discovery cohorts including 825 cases, 7,936 controls and 1,219 cases, 1067 controls respectively, we identify a genome-wide significant locus at on 6q25.3. We subsequently replicate the association in an additional cohort of 293 cases of AOM and 1,719 controls. We further reveal significant correlation between the associated variant and the methylation status and expression levels of the fibronectin type III domain containing 1 (*FNDC1*) gene. Additionally, we demonstrate that the mouse homologue Fndc1 is expressed in the middle ear tissue and is being upregulated under proinflamma-

tory conditions, such as lipopolysaccharide treatment, suggesting *FNDC1* is the causal gene underlying the association.

METHODS

Statement of ethics

This study was conducted at the Center for Applied Genomics (CAG) at the CHOP, and in the Generation R Study (GenR) at Erasmus University Medical Center, The Netherlands. The study was approved by the Institutional Review Board at the CHOP, and the Medical and Ethical Review Committee of the Erasmus University Medical Center. Written informed consent was obtained from all participants.

CHOP population and phenotype definition

All children in the CAG biobank recruited from the Greater Philadelphia area with both phenotypic and genotypic data available were eligible for this study. Case-status for AOM was defined using ICD-9 codes (Supplementary Fig. 4.1). Exclusion criteria were established, defined by specific diseases with strong correlation to AOM due to specific defects in anatomy and/or strongly related to susceptibility to upper respiratory tract infections (Supplementary Table 4.1). Subjects in the CAG database with no history of middle ear disease were labelled as controls (Supplementary Table 4.2). DNA samples at CHOP were obtained via whole blood venipuncture or saliva samples collected at CHOP and associated health centers in the Philadelphia metropolitan area.

The Generation R Study population and phenotype definition

The Generation R Study is a population-based prospective cohort study from fetal life until adulthood (16). We defined cases and controls using survey data on OM, fever with earache, otorrhea, use of eardrops per subscription by family practitioner of ear, nose and throat (ENT) surgeon. Questionnaires were sent at the ages of 2, 6, 12, 24 and 36 months. As such, case status was established for the phenotype of AOM up to the age of three years, and control status (Supplementary Fig. 4.2, and Supplementary Table 4.2). Only children of European ancestry were considered, and exclusion criteria were applied (Supplementary Table 4.1). At Generation R, whole-blood samples were collected postpartum from the umbilical cord, or at 5 years from venipuncture.

Discovery phase genotyping and quality control

gDNA, extracted following the phenol-chloroform protocol, was genotyped using Illumina HumanHap550-V1/V3 or HumanHap610 or 660-Quad DNA Bead Chips at CHOP and the Generation R Study. Quality control was performed through PLINK (software release v1.07;

http://pngu.mgh.harvard.edu/purcell/plink/) (17). Samples with a call rate below 98% at CHOP and below 97.5% at Generation R, or, at either center, with ambiguous sex detected by PLINK or excess of autosomal heterozygosity (F < mean -4 s.d.), were excluded. Only SNPs that were common to all chip types were included in analysis. SNPs with call rate <95% were excluded, as well as SNPs with MAF <0.01, and Hardy-Weinberg equilibrium P values < 10^{-05}. PLINK was used for PCA at the Generation R Study, whereas, at CHOP, PCA was conducted using software Eigenstrat (18). We excluded subjects that were not of European ancestry and repeated the PCA in the remaining samples to derive the principal components to use as covariates for the association analysis as described below. Genome-wide identity-by-descent analysis was performed using PLINK. Duplicated or cryptically related samples were detected as PI_HAT ≥ 0.1875; and one from each pair was excluded from further analysis.

Association analysis and meta-analysis

After quality control filtering, a total of 825 cases, 7,936 controls and 507,300 SNPs were left for association analyses at CHOP. At Generation R, 469,664 SNPs were available after quality control filtering, a total of 1,219 cases, 1,067 controls. Association analysis was performed at the two sites separately. At both sites, logistic regression analysis under the additive model was performed to assess the association between SNP genotype and AOM. Principal components (PC) were implemented in our model: AOM 1⁄4 SNP þ PC1 þ PC2 þ PC3 þ sex. Association analyses were carried out via PLINK at CHOP, and using GRIMP (19, 20) at the Generation R Study. We completed a GWAS at each center and meta-analyzed SNPs shared by the two centers. As such, ~460,000 genotyped SNPs were available for meta-analysis. We performed sample-sized weighted meta-analyses using METAL software package (21).

Replication analysis

To increase power, we included all AOM cases that passed the phenotype selection criteria and were genotyped on the HumanOmniExpress-12v1 array to form the replication cohort. Similarly, controls were genotyped on the same array type and had no history of middle ear disease. Samples underwent the same quality control as the discovery cohorts. Logistic regression analysis was conducted including the first three PCs and sex as covariates.

Regional imputation

The CHOP discovery and replication cohorts were prephased using SHAPEIT (22, 23) version 2, followed by genotype imputation of chromosome 6 using IMPUTE 2 (24, 25) against the 1,000 Genome Phase 3 reference (https://mathgen.stats.ox.ac.uk/impute/1000GP%20Phase%203%20haplotypes%206%20October%202014.html) Association testing of the imputed genotypes was carried out in SNPTEST (24) V2 using the missing data likelihood

score test, including sex and the first three principal components as covariates. We excluded SNPs with info score <0.8 or with Hardy-Weinberg equilibrium-test P value <1 x 10^{-06}.

Analysis of methylation data

Genome-wide methylation profiles were generated on a total of 854 subjects recruited by the CAG on the Infinium HumanMethylation450 BeadChip Kit according to the manufacturers' protocols. gDNA was isolated from peripheral blood mononuclear cells. Methylation data were exported from the Illumina GenomeStudio, as GenomeStudio output text files containing probe level summarized information. The GenomeStudio output files were loaded into the R statistical package (r-project.org) using the lumi package (26, 27). The lumi package was used for preprocessing of data, which involved quantile colour balance adjustment and background level correction and simple scaling normalization (ssn). M-value density and CpG-site intensity were plotted after normalization, and two aberrant chips were removed. Principle component analysis identified 425 subjects of European ancestry, 374 African Americans, 20 East Asians, and 24 Hispanics among these subjects. Methylation probes known to overlap with common SNPs, were identified and removed using the IMA R package (28). M-values (the log2 ratio between the methylated and unmethylated probe intensities) were extracted and stored as a matrix. Additive genotypes at rs2932989 for subjects of European ancestry were extracted from existing genotyping data using PLINK. There are a total of 402 subjects of European ancestry without missing genotype at rs2932989 and extreme outlier values of methylation M-values (≥median M-value of the genotype group ±3 s.d.). Methylation data in gene FNDC1 were analyzed as the response variable in a linear regression, with genotype at rs2932989 as the predictor variable among these 402 subjects. Sex, age, and 10 genotype-derived principal components were included as covariates. Linear regression and generation of boxplots was performed using base packages in R.

Animals

The Institutional Animal Care and Use Committee of the CHOP approved all animal studies. C57/BL6 female mice were used in the study. Mice were randomly distributed into two groups and deeply anesthetized, using ketamine (100 mg per kg) and xylazine (10 mg per kg) via intraperitoneal injection. The experimental group received lipopolysaccharide (2 mg per ml) in PBS, 20 ul per ear derived from Salmonella typhimurium (Sigma-Aldrich), and 0.01 M PBS was injected into the middle ears of the control group. Mice were killed at third day after injection of lipopolysaccharide or PBS.

Western blot analysis

For western blot analysis, mice middle ear tissue, including the epithelial lining was removed from animals after decapitation and sonicated in ice-cold NP40 lysis buffer (Invitrogen) containing protease inhibitor (Calbiochem). Proteins extracts were boiled for 5 min in SDS–

polyacrylamide gel electrophoresis sample buffer (0.125 M Tris-HCl, 20% glycerol, 4% SDS, and 0.005% bromo-phenol blue) for denaturation. A 20-µg protein sample was separated on a 4–12% NuPAGE Bis-Tris gel (Novex), transferred overnight to a nitrocellulose membrane (Invitrogen), and blocked overnight with 3% BSA tris-buffered saline with Tween (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20). The membrane was cut in half with the upper half of the membrane incubated with rabbit anti-FNDC1 polyclonal antibody (H-65; sc-382009) at 1:1000 dilution and the lower half of the membrane probed with rabbit anti-b-Actin polyclonal antibody (N-21; sc-130656) at 1:1000 dilution at room temperature for one hour. Subsequently, the membranes were washed for three times and incubated in a 1:10,000 dilution of horseradish peroxidase– conjugated anti-rabbit secondary antibody (Promega) for 1 h at room temperature and washed again three times. WesternBright enhanced chemiluminescence (ECL) detection system (Advansta) was used to detect bound antibody. Then the band intensities were measured with Image J software (NIH Shareware).

RESULTS

GWAS identifies AOM susceptibility locus

We performed a GWAS on AOM with age of onset < 3 years old. Both patients and controls were recruited and their genomic DNA (gDNA) was genotyped at the Children's Hospital of Philadelphia (CHOP) and the Generation R Study (Supplementary Figs 4.1 and 4.2, Supplementary Tables 4.1 and 4.2). In the discovery phase, all samples were genotyped using Illumina HumanHap550-V1/V3 or HumanHap610 or 660-Quad DNA Bead Chips. After quality control filtering, a total of 825 cases and 7,936 controls of European descent retained for analysis at CHOP together with 1219 cases and 1,067 controls at Generation R. Logistic regression analysis under an additive model was performed to assess the association between SNP genotype and AOM at each individual site, including the first three principal components and sex as covariates (Supplementary Fig. 4.3). The residual genomic inflation factor was 1.00 for analysis at CHOP and 1.01 at the Generation R Study (Supplementary Fig. 4.4). The site-specific GWAS were subsequently meta-analyzed to combine the 460,000 exclusively non-imputed SNPs (minor allele frequency (MAF) > 0.01) shared by the two centers, using a sample-size based fixed-effect model. One variant on 6q25.3 with MAF 0.13 in controls (rs2932989, $P_{meta} = 4.36 \times 10^{-08}$) (Table 4.1, Supplementary Fig. 4.5) surpassed the genome-wide significance threshold ($P < 5 \times 10^{-08}$) following the meta-analysis. The associated locus was subsequently imputed using the 1,000Genome data set as a reference. A total of 77 genotyped and imputed SNPs in this region showed suggestive association with P values $< 5 \times 10^{-05}$ (Figure 4.1a, Supplementary Table 4.3).

We subsequently investigated whether the association signal could be replicated in an independent cohort. An additional 293 cases of AOM and 1,719 controls were recruited at

CHOP and genotyped for this purpose, using the Illumina HumanOmniExpress-12 v1 chip (Supplementary Table 4.2). The replication samples were mainly of European and African American ancestry with a minority of subjects of Asian and Hispanic ancestry (Supplementary Fig. 4.3). The first three principal components and sex were included as covariates in the association analysis. The resulting genomic inflation factor was 1.01 (Supplementary Fig. 4.4), suggesting population stratification was well controlled. While the top SNP from the discovery cohort, rs2932989 is not directly genotyped on the HumanOmniExpress chip, two proxy SNPs in strong linkage disequilibrium (r^2 =1) with rs2932989 both showed association with AOM (P < 0.05) (Supplementary Table 4.4) in the replication cohort and approached genome-wide significance in the discovery cohort based on imputed genotype data (P = 7.49 x 10^{-07} for rs578217 and P = 9.79 x 10^{-07} for rs419009, Supplementary Table 4.3). Direct interrogation of rs2932989 following imputation of the replication cohort confirmed the association P = 0.0155 (Table 4.1). Further meta-analysis of the discovery cohorts and the replication cohort yielded a P value of 2.15 x 10^{-09} for top SNP rs2932989. We, therefore, demonstrate the first genome-wide significant association of common variation with susceptibility to AOM and replicate the finding in an independent pediatric AOM cohort.

Table 4.1: Genome-wide significant association of 6q25.3 with acute otitis media.

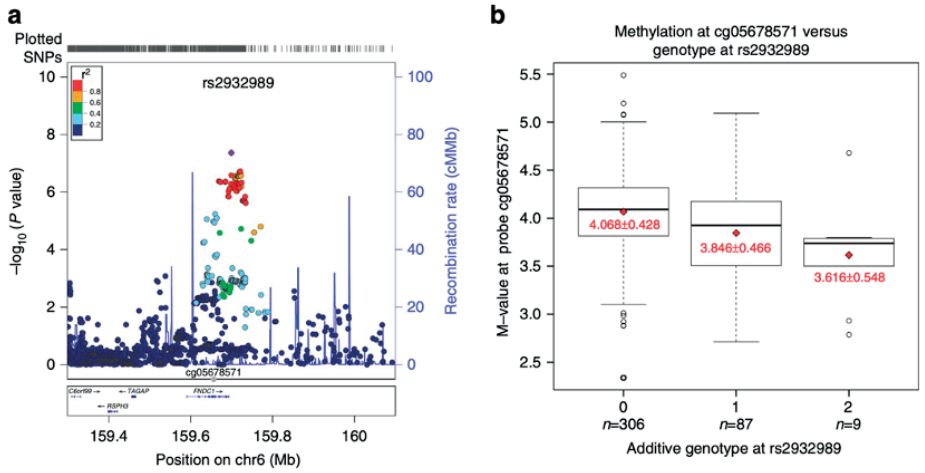
SNP	Chr	Pos (hg19)	Gene	A1/A2	MAF cases/controls	Stage	OR_{CHOP} (95% CI)	P_{CHOP}	OR_{GenR} (95% CI)	P_{GenR}	P_{meta}
rs2932989	6	159699284	*FNDC1*	T/G	0.17/0.13	Discovery	1.41 (1.23, 1.62)	$1.46e^{-06}$	1.25 (1.05, 1.48)	$1.02e^{-02}$	$4.36e^{-08}$*
						Replication	1.35 (1.05, 1.73)	$1.55e^{-02}$			
						Combined					$2.15e^{-09}$†

A1, minor allele; A2, major allele; Chr, chromosome; CI, confidence interval; MAF, minor allele frequency; OR, odds ratie; P, *P* value; Pos, position; SNP, single-nucleotide polymorphism.
*P value of meta-analysis at discovery stage.
†P value of meta-analysis of all cohorts.

Test statistics for suggestive loci and candidate genes

In addition to the genome-wide significant association at 6q25.3 locus, we also observed suggestive evidence for association at eight additional loci (5 x10^{-08} < P value < 5 x 10^{-05}; Supplementary Fig. 4.6 and Supplementary Table 4.5), among which, *KIF21B*, *CACNA1S*, *CRHR2*, *BDKRB2* and *TPM4* have been reported to be involved in pathogenesis of autoimmune disease, inflammatory disease or response to bacterial infection (29-35). Interrogating our GWAS data set for candidate genes that have been previously proposed to be involved in pathogenesis of OM we found 45 out of 82 genes demonstrated evidence of nominally significant association, with intragenic SNPs or nearby SNPs of P value < 0.05 (Supplementary Table 4.6), such as *SMAD2*, *SMAD4*, *NELL1*. *BMP5*, *GALNT13* which are involved in transforming growth factor (TGF)-beta signalling, pro-inflammatory cytokine IL1B and its inhibitor IL1RN, receptor for fibroblast growth factor (FGFR1). TGF-beta, IL-1, fibroblast growth factor (FGF) all have been reported to regulate *FNDC1* expression (36, 37).

Figure 4.1: The association statistics of locus 6q25.3 with acute otitis media and correlation with methylation status in gene *FNDC1*.



(**a**) The regional association plot at locus 6q25.3. The SNP chromosomal location on genome build hg19 is indicated on the x axis and the negative log10 of P value for each SNP is plotted on the left-hand y axis. Association statistics were from the meta-analysis of the two cohorts in the discovery phase. The most associated SNP (rs2932989) is shown as purple dot and the other SNPs including both genotyped SNPs and imputed SNPs are colored according to their linkage disequilibrium with SNP rs2932989. The recombination rates are shown as blue lines. The plot was generated with software LocusZoom (70). The position of the methylation probe cg05678571 is indicated by a grey dot. (**b**) M-values for methylation probe cg05678571 are plotted against the additive genotype at SNP rs2932989. Dark horizontal lines in the boxplots represent the median of the group, the boxes represent the 25–75% quantiles, and the whiskers of the boxplot extend beyond those quartiles to 1.5 times the interquartile range. Data outside those ranges are represented by points (open circles). Red diamonds indicate the means of each group, and the red text is the mean ±s.d. of each group. The number of individuals with each additive genotype of minor allele T is indicated below the x axis.

FNDC1 is expressed in mouse middle ear tissue

The associated variants at 6q25.3 locus map to a linkage disequilibrium block that contains one gene, the *FNDC1* (Figure 4.1). Expression data from both mouse and human tissues (http://www.informatics.jax.org; http://www.eurexpress.org; GTEx database (38)) indicate *FNDC1* is expressed in different tissue types with high levels in mesenchymal stem cells, synovial membrane, fetal cartilage, thyroid gland, adipose tissue and others (Supplementary Fig. 4.7; Supplementary Date 1 which can be accessed at https://www.nature.com/articles/ncomms12792#Sec21). In addition, Fndc1 expression was also found in pharyngeal tissues including the oral epithelium, next to esophagus tissue, lips, palate and salivary glands (Supplementary Table 4.7). Database 'Bgee: Gene Expression Evolution' (http:// bgee.unil.ch/) (39) included three studies detecting the expression of Fndc1 in mice ears and one study showed its expression in inner ears (40-42); additional data from the Shared Harvard Inner-Ear Laboratory database (https://shield.hms.harvard.edu) indicate Fndc1 expression is present in both cochlea and utricle. We further conducted experiments to examine the expression of Fndc1 in mouse middle ears. By western blotting, we observed not only the expression of Fndc1 in C57/BL6 mice middle ears but also the upregulation of Fndc1 upon

lipopolysaccharide treatment compared with control treated with PBS alone (Supplementary Fig. 4.8).

AOM variants correlate with FNDC1 expression and methylation

In addition to the expression of *FNDC1* in tissues of relevance to the phenotype, data from the GTEx database (38) also indicates the AOM associated SNP, rs2932989, is an expression quantitative trait locus (eQTL) for *FNDC1* in esophageal smooth muscle (Supplementary Fig. 4.9) ($P = 9.3 \times 10^{-05}$).

Evidence of an association between the AOM-associated variants and *FNDC1* expression was further strengthened following the analysis of methylation data. We found significant association between methylation probe cg05678571 (chr6: 159,660,813-159,660,813, Figure 4.1a) and rs2932989 (beta = -0.200, $P = 1.43 \times 10^{-06}$, Figure 4.1b) and even a stronger association between cg05678571 and SNP rs2501175 (beta = -0.31, $P = 7.27 \times 10^{-15}$; rs2501175 meta-analysis $P = 2.89 \times 10^{-07}$) (Supplementary Fig. 4.10 and Supplementary Table 4.3). The genomic distance between cg05678571 and rs2932989 is 38.5 kb and that between cg05678571 and rs2501175 is 53.6 kb, both of which are <1 Mb, the average size of topologically associating domains and gene co-expression clusters in human (43). Checking the 3D Genome Browser (http://www.3dgenome.org) (44-46), we found that the above two GWAS SNPs and the methylation probe are indeed located in the same topologically associating domain (Supplementary Fig. 4.11), suggesting plausible interactions between these loci.

As expected (47), the eQTL and methylation data were inversely correlated. The minor allele (T) at rs2932989 was positively correlated with the *FNDC1* expression levels (Supplementary Fig. 4.9) and negatively correlated with methylation status of probe cg05678571 in *FNDC1* (Figure 4.1b) indicating that methylation of CpG islands in *FNDC1* results in reduced expression. Finally, we used Haploreg (48) to determine if any of the 77 AOM-associated SNPs at the locus overlaps with protein binding domains. Two of the SNPs were found to have CTCF and EGR1 bound based on the ENCODE data (49) (Supplementary Table 4.8). The same binding motifs were also predicted at the location of the rs2932989-associated methylation probe (cg05678571 CTCF at chr6:159660813-159660831 and EGR1 at chr6:159660869- 159660882) suggesting a role for these proteins in the modulation of *FNDC1* expression.

DISCUSSION

In summary, we have performed the first GWAS of AOM and the largest genetic study of OM to date, the most common medical problem encountered by general pediatric practitioners. We discovered genome-wide significance at a locus on 6q25.3 that contains the *FNDC1* gene and replicated the association in an independent pediatric cohort. We further show

that the mouse homologue of this gene is expressed in the middle ear and is upregulated upon lipopolysaccharide treatment. In addition to the tissue expression patterns, the eQTL and methylation data also implicate *FNDC1* as the causal gene underlying the association.

FNDC1 gene encodes a fibronectin type III domain containing protein. Fibronectin is an important type of extracellular matrix proteins, which interacts with integrins and is involved in cell adhesion, migration, proliferation and differentiation (50). Only a few studies have been reported regarding the function of *FNDC1*, which suggest that *FNDC1* is involved in multiple cellular processes. *FNDC1* was expressed in heart tissue, with the role of activating G-protein signalling by interacting with Gβγ (51). This interaction also affects CX43 function and cell permeability, thus sensitizing cells to hypoxic stress in rat neonatal cardiomyocytes and playing an essential role in hypoxia induced apoptosis (52).

While the biological function of *FNDC1* has not been well studied, it has been shown that *FNDC1* has a role in inflammation. *FNDC1* was first identified as a differentially expressed mRNA from human dermal fibroblasts. Its expression level is increased following treatment with TGF-β, IL-1 and TNF-α (36). Partially unfolded type III fibronectin module has been shown to induce the expression of IL-8 and TNF-α via activation of the NF-κB signalling pathway, suggesting that fibronectin matrix remodeling may impact cytokine expression (53, 54). Both tissue remodeling and increased levels of cytokines are involved in AOM (55, 56). In line with these observations, in our study, the minor allele (T allele) of SNP rs2392989 confers a higher risk of AOM and is correlated with a lower level of methylation at cg05678571 in *FNDC1*, as well as higher expression of *FNDC1*. In addition, Fndc1 expression in mouse middle ear was upregulated upon lipopolysaccharide treatment, which is known to be a potent inducer of inflammation, stimulating TGF-β, TNF-α and IL-1 signalling (57-59). Consistent with the association of *FNDC1* with OM and its expression being modulated by lipopolysaccharide, several important players involved in these signalling pathways of inflammatory responses also showed at least nominal association with OM in our GWAS. FNDC1 is also one of the differentially expressed genes in primary central nervous system lymphoma compared with normal lymph node tissues (60), with possible involvement in lymphocyte production. Therefore, the cumulative evidence from literature and the results from our GWAS and mouse experiments suggest that *FNDC1* may have a role in the pathogenesis of OM, likely through altered immune or inflammatory response.

The diverse signalling processes that *FNDC1* is involved in are inter-related. In an environment of inflammation, hypoxia is typical, with decreased level of oxygen and glucose, as well as increased level of inflammatory cytokines (61). Hypoxia and hypoxia-inducible factor have an important role in chronic OM and myringotomy reduces hypoxia and inflammation, which are demonstrated by mouse model (62, 63). Hypoxia-inducible factor is of well-known function in hypoxia induced apoptosis (64). Hypoxia signalling is linked to innate immunity, adaptive immunity and infections. Genes functioning in hypoxia signalling interact with players of NF-κB pathway (reviewed by Eltzschig and Carmeliet

(65)) G protein signalling is of critical roles in various immune functions (66). The *FNDC1* interactor Gβγ has important functions in immune response. For example, it activates PI3Kgamma, regulating inflammation through neutrophil recruitment (67, 68). It also activates RhoGef, involved in lymphocyte chemotaxis and actin polymerization (69). It would be interesting to examine whether *FNDC1* is involved in AOM pathogenesis through any of these signalling pathways.

Among the genes that have been previously proposed to be involved in OM pathogenesis, we found more than half of them exhibited nominally significant association, suggesting consistency between genetic studies and/or with results from model organism studies. It is not surprising that some of the candidate genes did not show significant association, considering the following possible reasons. In our GWAS, we focused on a more defined phenotype of early-onset AOM, which is different from the phenotypes examined in many of the previous OM genetic studies including OM in general, chronic OM or OM with effusion. In addition, different ethnicity of the study population is another influential factor to consider. Furthermore, polymorphisms of candidate genes, proposed based on evidence from differential gene expression, molecular, cellular functions and rodent model studies, may not always present an effect large enough to be captured by GWAS.

The strength of the study lies in the largest sample size and dense genome-wide SNP coverage. Furthermore, we have balanced power and optimal quality control. Compared with previous efforts, this study applied more stringent quality control and more robust correction for population substructure. Indeed, by performing principal-component analysis (PCA) to identify participants of European ancestry, followed by a second PCA on this selected population, we were able to more thoroughly correct for population stratification. By a well-defined phenotype in both populations we were able to combine data from two studies of different designs. Limitation herein is severity of disease, as in one population cases consisted of children who were specifically diagnosed with AOM, likely the more severe episodes, whereas the other population used extensive questionnaires, discovering even the mildest of episodes. These differences were resolved by the increased power of combining the studies, underlined by results showing the same direction of effect and similar effect size between both. Furthermore, the P value of homogeneity test in our meta-analysis is >0.9 for the genome-wide significant SNP rs2932989, which indicates that there is no suggestion of between-study heterogeneity.

CONCLUSION

This study provides evidence of a genetic component influencing susceptibility to AOM in children. By gaining better understanding of the complex polygenetic pathogenesis of AOM, we hope to be able to develop more specific therapies and to ultimately learn which

children are most susceptible to this disease, where earlier clinical intervention may have the most impact.

Supplementary Methods, Figures and Tables can be found in this thesis following References. Detailed Acknowledgements and Supplementary Data 1 can be found in the published article online: https://www.nature.com/articles/ncomms12792#Sec21

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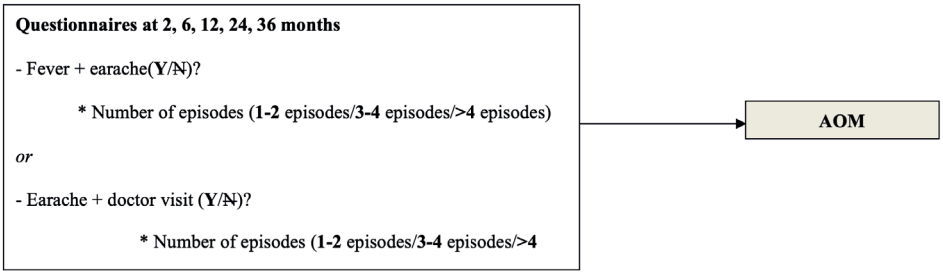
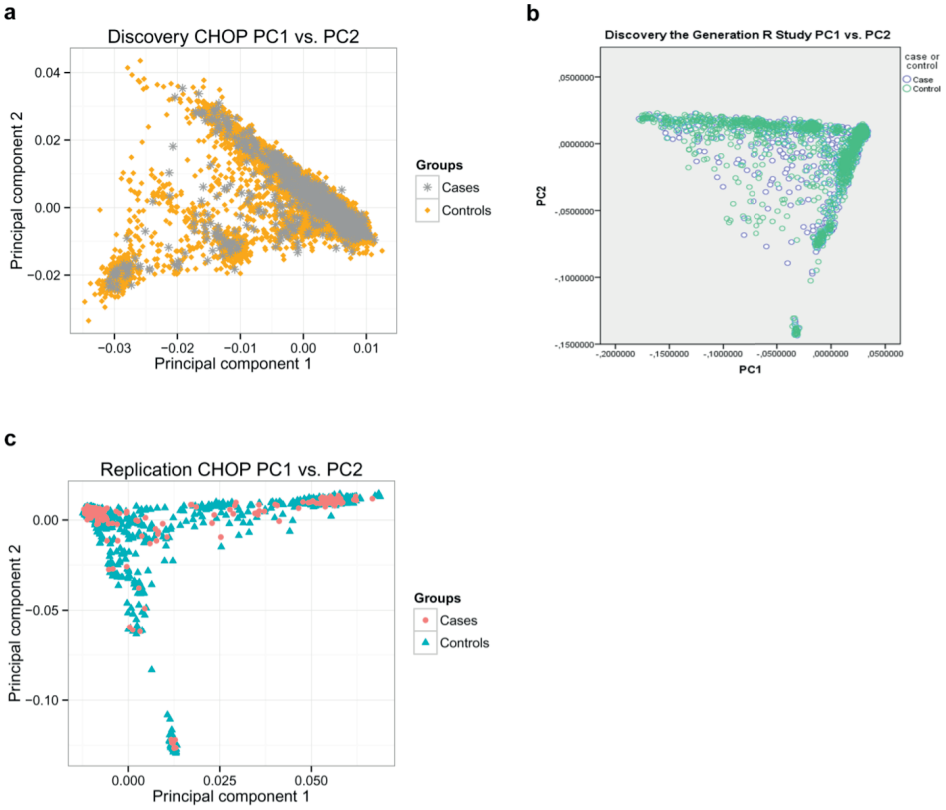
SUPPLEMENTARY MATERIAL

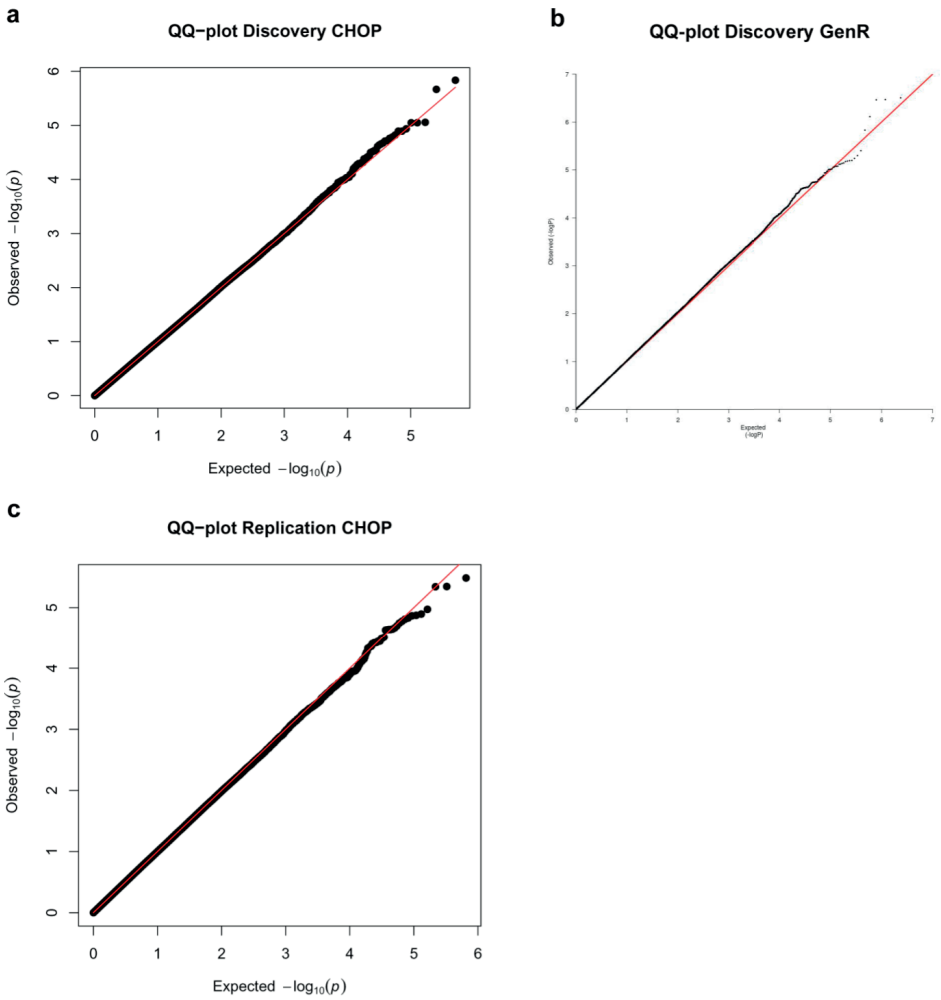
Supplementary Figure 4.1: Flow chart of ICD-9 or procedure codes used as search term in CAG database (CHOP) to define AOM phenotype.
% is a wildcard character used in our database search, representing the inclusion of all subtypes of this ICD-9 code



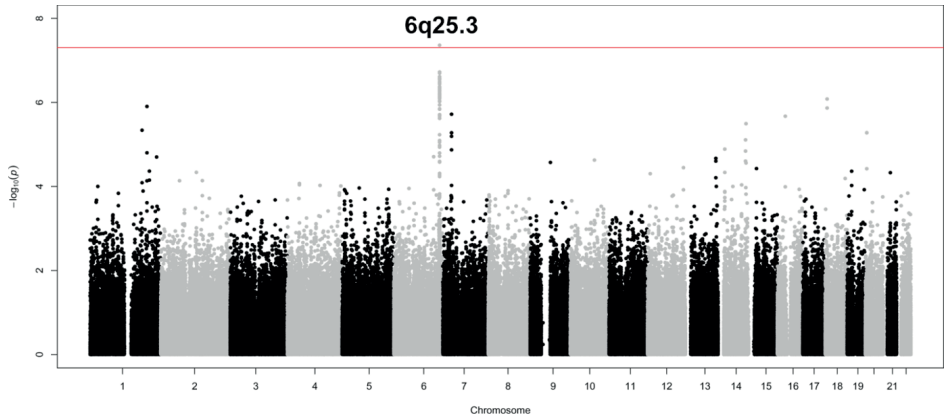
Supplementary Figure 4.2: Flow chart of questionnaire data used to define AOM phenotype at the Generation R Study.



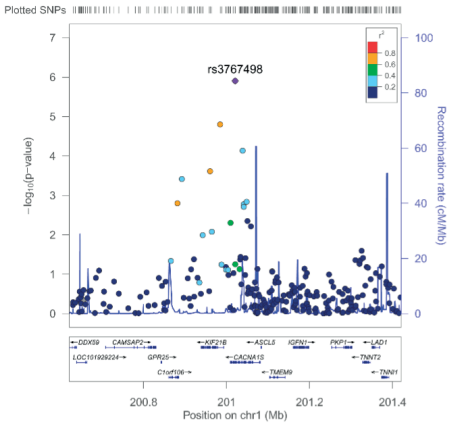
Supplementary Figure 4.3: Principal component analysis (PCA) of subjects at CHOP and the Generation R Study. Principal component 1 (PC1) is shown on the X-axis and principal component 2 (PC2) is shown on the Y-axis. (a) PCA results of CHOP discovery cohort; (b) PCA results of the Generation R Study discovery cohort; (c) PCA results of CHOP replication cohort.

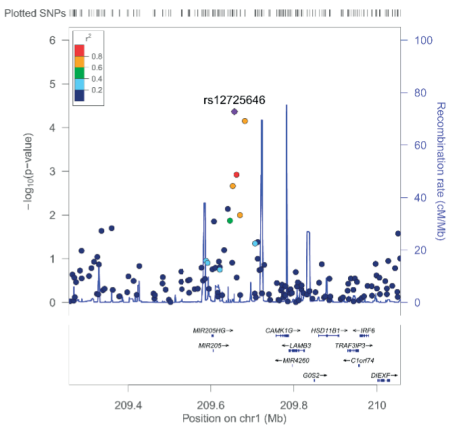


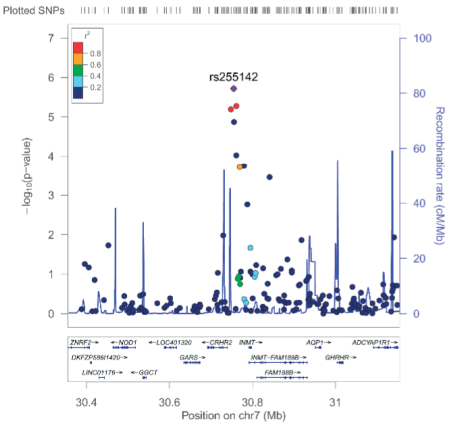
Supplementary Figure 4.4: Quantile-quantile plot of observed versus expected P-values for each cohort. (a) Discovery CHOP cohort; (b) Discovery cohort of the Generation R Study; (c) Replication CHOP cohort.
Line y = x is shown in red. The genomic inflation factor λ for each cohort is 1.00, 1.01 and 1.01 respectively.

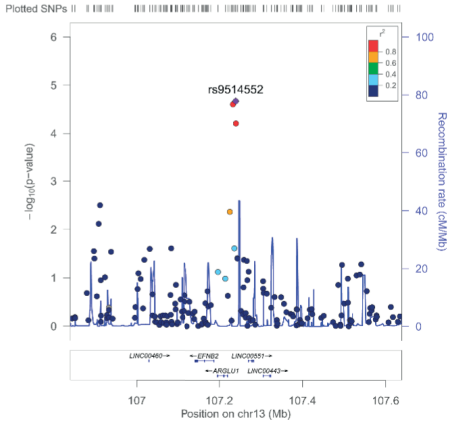


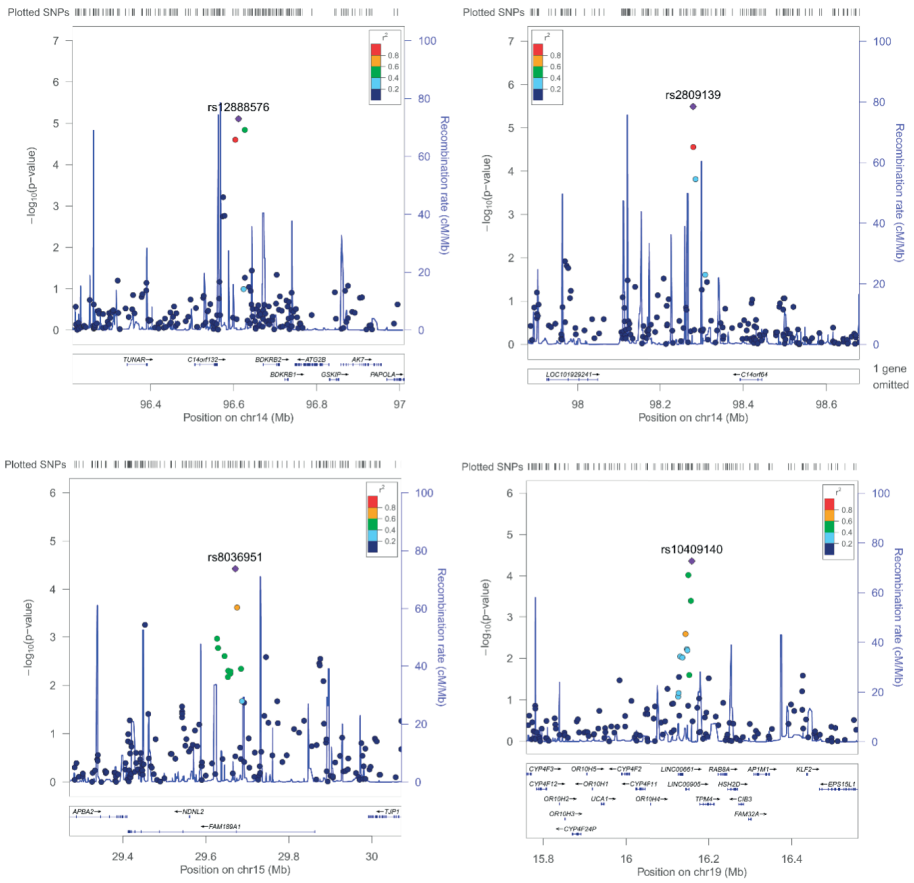
Supplementary Figure 4.5: Manhattan plot of the association testing statistics showing the AOM associated loci. The –log10(P-value) of each SNP (Y-axis) is plotted against the SNP genomic position (X-axis). Association statistics were from the meta-analysis of the two cohorts in the discovery phase. The genome-wide significance threshold of $P=5x10^{-8}$ is indicated by the horizontal red line. Both genotyped SNPs and imputed SNPs at 6q25.3 are plotted.


Plotted SNPs
rs3767498
−log10(p-value)
Recombination rate (cM/Mb)
Position on chr1 (Mb)


Plotted SNPs
rs12725646
−log10(p-value)
Recombination rate (cM/Mb)
Position on chr1 (Mb)

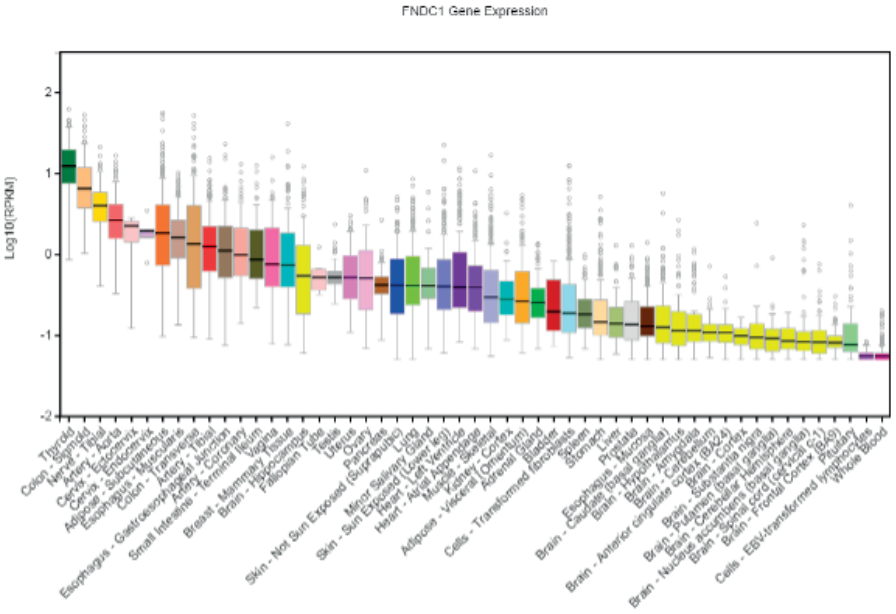

Plotted SNPs
rs255142
−log10(p-value)
Recombination rate (cM/Mb)
Position on chr7 (Mb)


Plotted SNPs
rs9514552
−log10(p-value)
Recombination rate (cM/Mb)
Position on chr13 (Mb)

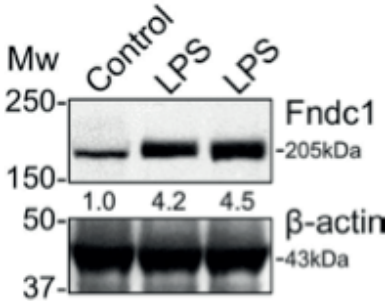


Supplementary Figure 4.6: Regional association plots of loci showing suggestive evidence for association ($5x10^{-8}$ < *P*-value < $5x10^{-5}$).

The regional plots were generated using the software LocusZoom (1). The X-axis indicates the genomic position of the genotyped SNPs, and the Y-axis shows the $-\log_{10}$*P*-value of these SNPs in our logistic regression analysis. The most strongly associated SNP at each locus is indicated by the purple color, and the colors of the other SNPs represent the strength of linkage disequilibrium with the index SNP. The color coding legend is shown in each plot and the recombination rate is shown by the vertical blue lines.

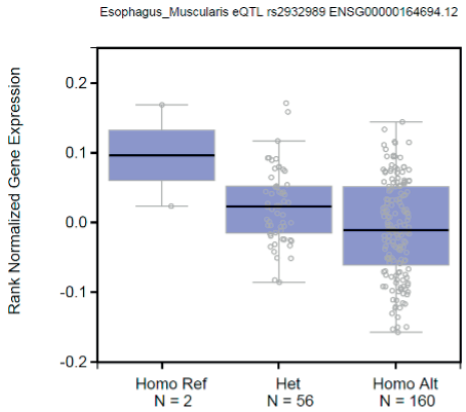


Supplementary Figure 4.7: Box plot showing the ranking of gene *FNDC1* expression level in different human tissue/cell types. Tissue/cell types are shown on the X-axis, and the log10 based RPKM (Reads Per Kilobase of transcript per Million mapped reads) values, representing normalized gene expression level, are shown on the Y-axis. The median expression level is shown by the dark black line in each box. The first quartile and the third quartile of the log10(RPKM) are represented by the bottom and the top border of each box, respectively. The lowest and the highest datum within the 1.5 interquartil range (IQR) of the lower quartile and the upper quartile are shown by the end of the lower whisker and that of the upper whisker, respectively. The data point of each individual is represented by the small grey circle. The plot was generated via GTEx (2) online portal.

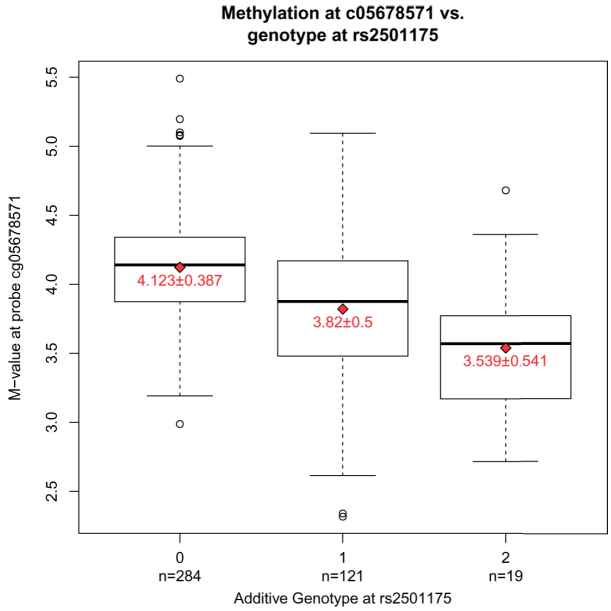


Supplementary Figure 4.8: The expression of mouse homologue Fndc1 in middle ears and its upregulation upon LPS treatment.

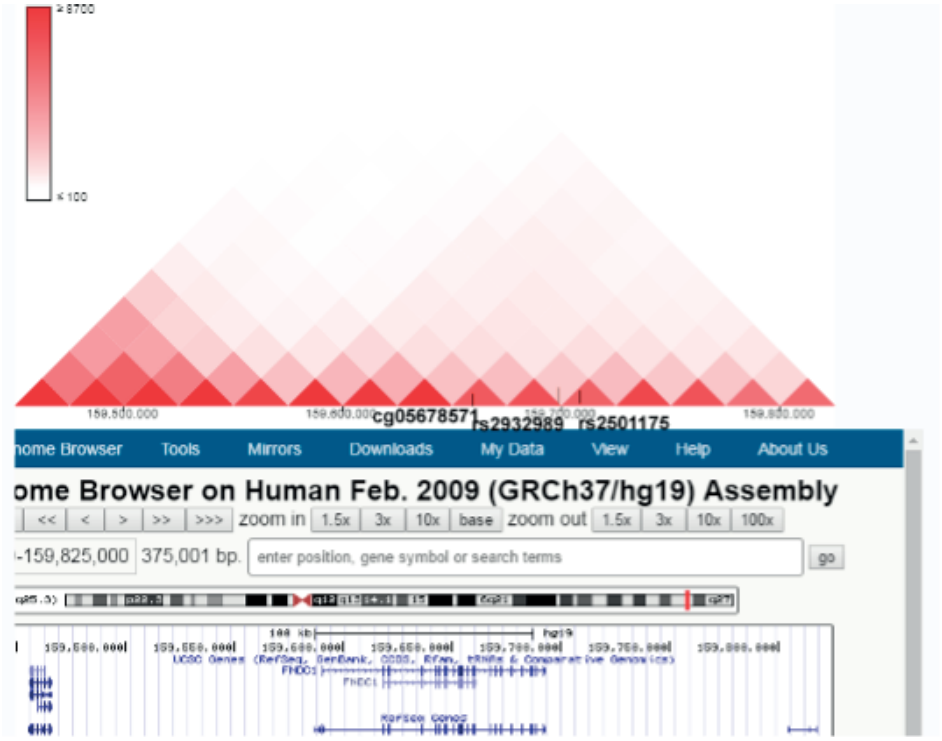
C57/BL6 mice were randomly distributed into two groups. After deep anesthetization, the experimental group received injection into the ears of Lipopolysaccharide (LPS) (2mg per ml) in PBS, 20ul per ear derived from Salmonella typhimurium, and the control group received 0.01M PBS injection. Mice were sacrificed at 3rd day after injection and protein extracts from mice middle ear tissue were separated on a 4–12% NuPAGE Bis-Tris gel and transferred onto nitrocellulose membrane. The upper half of the membrane was probed with rabbit anti-FNDC1 polyclonal antibody; and the lower half of the membrane was probed with rabbit anti-β-Actin polyclonal antibody. Band intensities were measured using Image J software and normalized to β-Actin loading control. A three-fold increase in Fndc1 expression was observed in the experimental group with LPS treatment compared to the control group.



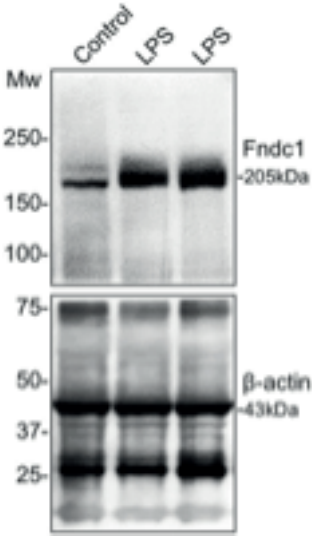
Supplementary Figure 4.9: The association between SNP rs2932989 genotype and gene *FNDC1* expression level in tissue esophagus muscularis.
The relationship between SNP rs2932989 genotype and gene *FNDC1* expression level was assessed in GTEx Portal (2). The different genotype groups of rs2932989 are indicated on the X-axis. In this figure, the reference allele is T and the alternative allele is G. The Rank Normalized gene expression level of *FNDC1* is indicated by the Y-axis. The median expression level is shown by the black line in the box plot. The first quartile and the third quartile of the rank normalized gene expression are represented by the bottom and the top border of each box, respectively. The lowest and the highest datum within the 1.5 interquartil range (IQR) of the lower quartile and the upper quartile are shown by the end of the lower whisker and that of the upper whisker, respectively. The data point of each individual is represented by the small grey circle.
P=9.3x10^{-5}



Supplementary Figure 4.10: The correlation between SNP rs2501175 and methylation probe cg05678571 in gene *FNDC1*.
M-values for methylation probe cg05678571 are plotted against the additive genotype at SNP rs2501175. Dark horizontal lines in the boxplots represent the median of the group, the boxes represent the 25%-75% quantiles, and the whiskers of the boxplot extend beyond those quartiles to 1.5 times the interquartile range. Data outside those ranges are represented by points (open circles). Red diamonds indicate the means of each group, and the red text is the mean ± standard deviation of each group. The number of individuals with each additive genotype of minor allele T is indicated below the x-axis.



Supplementary Figure 4.11: The topologically associating domains (TAD) at gene *FNDC1* and surrounding region (chr6:159450000-159825000). Heatmap shows pairwise interaction frequencies. The genomic location of SNPs rs2932989, rs2501175 and methylation probe cg05678571 is indicated by the black vertical lines.



Supplementary Figure 4.12: Uncropped image of Western blotting in Supplementary Figure 4.8.

Supplementary Table 4.1: Phenotype exclusion criteria in case selection of the genome-wide association study. OM cases were excluded from the study if they have any of the following diagnosis.

Exclusion criteria
Obstruction of Eustachian tube
Patulous Eustachian tube
Cystic fibrosis*
Primary ciliary dyskinesias
Trisomy 21
Cleft palate
Anomalies of skull and face

*or positive CF gene carrier status; sor situs inversus;

Supplementary Table 4.2: The characteristics of study subjects in each cohort after quality control filtering.

Cohort		Control	AOM <3 years
CHOP Discovery	N	7,936	825
	♂	4,168 (53%)	504 (61%)
	Age-1st episode♂	*	1.3
Generation R	N	1,067	1,219
	♂	538 (50%)	656 (54%)
	Age-1st episode♂	*	1.2
CHOP Replication	N	1,719	293#
	♂	905 (53%)	176 (60%)
	Age-1st episode♂	*	NA

*not applicable; ♂age in years; #AOM; NA – not available

Supplementary Table 4.3: Association statistics for all SNPs at the locus of 6q25.3 with suggestive evidence of association with AOM in the discovery cohort ($P<5x10^{-05}$).

SNP	Pos (hg19)	A1/A2	OR (95% CI) (CHOP)	P (CHOP)	OR (95% CI) (GenR)	P (GenR)	Direction	P (Meta)	Format*
rs2932989	159699284	T/G	1.41 (1.23, 1,62)	1.46e^{-06}	1.25 (1.05, 1.48)	1.02e^{-02}	++	4.36e^{-08}	genotyped
rs1394232	159718990	C/G	1.36 (1.19, 1.56)	3.71e^{-06}	1.22 (1.03, 1.44)	1.81e^{-02}	++	2.04e^{-07}	imputed
rs2221389	159719183	T/G	1.36 (1.19, 1.56)	3.71e^{-06}	1.22 (1.03, 1.44)	1.81e^{-02}	++	2.04e^{-07}	imputed
rs1553482	159722147	T/G	1.33 (1.17, 1.51)	1.85e^{-06}	1.17 (1.00, 1.36)	4.46e^{-02}	++	2.46e^{-07}	imputed
rs449789	159699125	C/G	1.35 (1.18, 1.56)	6.15e^{-06}	1.24 (1.04, 1.46)	1.32e^{-02}	++	2.55e^{-07}	imputed
rs2340806	159722806	A/G	1.33 (1.17, 1.51)	2.07e^{-06}	1.17 (1.00, 1.37)	4.35e^{-02}	++	2.67e^{-07}	imputed
rs10806706	159721945	A/C	1.33 (1.17, 1.51)	2.02e^{-06}	1.17 (1.00, 1.36)	4.47e^{-02}	++	2.68e^{-07}	imputed
rs4708817	159720640	A/G	1.33 (1.17, 1.51)	2.02e^{-06}	1.17 (1.00, 1.36)	4.48e^{-02}	++	2.69e^{-07}	imputed
rs9355713	159722548	G/A	1.33 (1.17, 1.51)	2.07e^{-06}	1.17 (1.01, 1.36)	4.41e^{-02}	++	2.71e^{-07}	imputed
rs591953	159716746	G/A	1.33 (1.17, 1.51)	2.10e^{-06}	1.17 (1.00, 1.36)	4.49e^{-02}	++	2.80e^{-07}	imputed
rs529281	159708505	T/C	1.33 (1.17, 1.50)	2.19e^{-06}	1.17 (1.01, 1.36)	4.39e^{-02}	++	2.85e^{-07}	imputed
rs579533	159715203	C/T	1.33 (1.17, 1.50)	2.19e^{-06}	1.17 (1.01, 1.36)	4.39e^{-02}	++	2.85e^{-07}	imputed
rs1875814	159720266	G/A	1.33 (1.17, 1.51)	2.15e^{-06}	1.17 (1.00, 1.36)	4.49e^{-02}	++	2.86e^{-07}	imputed
rs1875816	159720314	A/G	1.33 (1.17, 1.51)	2.14e^{-06}	1.17 (1.00, 1.36)	4.52e^{-02}	++	2.87e^{-07}	imputed
rs578714	159715118	G/T	1.33 (1.17, 1.50)	2.18e^{-06}	1.17 (1.00, 1.36)	4.44e^{-02}	++	2.87e^{-07}	imputed
rs553197	159714625	G/T	1.33 (1.17, 1.50)	2.19e^{-06}	1.17 (1.00, 1.36)	4.45e^{-02}	++	2.89e^{-07}	imputed
rs534519	159711929	A/C	1.33 (1.17, 1.50)	2.19e^{-06}	1.17 (1.00, 1.36)	4.45e^{-02}	++	2.89e^{-07}	imputed
rs2501175	159714412	C/T	1.33 (1.17, 1.50)	2.19e^{-06}	1.17 (1.00, 1.36)	4.45e^{-02}	++	2.89e^{-07}	imputed
rs606833	159717776	T/A	1.33 (1.17, 1.50)	2.19e^{-06}	1.17 (1.00, 1.36)	4.48e^{-02}	++	2.91e^{-07}	imputed
rs687314	159716262	G/C	1.33 (1.17, 1.50)	2.19e^{-06}	1.17 (1.00, 1.36)	4.50e^{-02}	++	2.92e^{-07}	imputed
rs1687980	159710460	A/T	1.33 (1.17, 1.50)	2.19e^{-06}	1.17 (1.00, 1.36)	4.99e^{-02}	++	3.24e^{-07}	imputed
rs473536	159709843	C/G	1.33 (1.17, 1.50)	2.19e^{-06}	1.17 (1.00, 1.36)	5.25e^{-02}	++	3.42e^{-07}	imputed
rs531091	159708697	G/C	1.33 (1.17, 1.50)	2.19e^{-06}	1.16 (1.00, 1.36)	5.64e^{-02}	++	3.69e^{-07}	imputed
rs294909	159668662	G/T	1.34 (1.16, 1.54)	2.16e^{-05}	1.28 (1.10, 1.49)	5.00e^{-03}	++	4.20e^{-07}	imputed

Supplementary Table 4.3: Association statistics for all SNPs at the locus of 6q25.3 with suggestive evidence of association with AOM in the discovery cohort ($P<5x10^{-05}$). (continued)

SNP	Pos (hg19)	A1/A2	OR (95% CI) (CHOP)	P (CHOP)	OR (95% CI) (GenR)	P (GenR)	Direction	P (Meta)	Format*
rs824140	159684911	T/C	1.33 (1.15, 1.54)	$2.96e^{-05}$	1.29 (1.09, 1.54)	$3.37e^{-03}$	++	$4.35e^{-07}$	imputed
rs633880	159684584	T/C	1.33 (1.15, 1.54	$2.96e^{-05}$	1.29 (1.09, 1.54)	$3.41e^{-03}$	++	$4.39e^{-07}$	imputed
rs294902	159671636	T/C	1.34 (1.16, 1.54)	$2.25e^{-05}$	1.28 (1.08, 1.51)	$5.05e^{-03}$	++	$4.42e^{-07}$	imputed
rs294896	159673246	A/G	1.34 (1.16, 1.54)	$2.25e^{-05}$	1.28 (1.08, 1.51)	$5.08e^{-03}$	++	$4.44e^{-07}$	imputed
rs534329	159711857	T/C	1.34 (1.16, 1.55)	$1.27e^{-05}$	1.24 (1.05, 1.47)	$1.15e^{-03}$	++	$4.73e^{-07}$	imputed
rs2102244	159724297	G/A	1.34 (1.16, 1.55)	$1.15e^{-05}$	1.24 (1.05, 1.47)	$1.33e^{-02}$	++	$4.83e^{-07}$	imputed
rs2340804	159713536	C/G	1.34 (1.16, 1.55)	$1.21e^{-05}$	1.24 (1.05, 1.46)	$1.34e^{-02}$	++	$5.11e^{-07}$	imputed
rs594613	159717362	G/C	1.34 (1.16, 1.55)	$1.27e^{-05}$	1.24 (1.05, 1.47)	$1.33e^{-02}$	++	$5.35e^{-07}$	imputed
rs372415	159698312	T/C	1.34 (1.16, 1.55)	$1.65e^{-05}$	1.25 (1.06, 1.49)	$9.74\ e^{-03}$	++	$5.38e^{-07}$	imputed
rs7760427	159719239	G/A	1.34 (1.16, 1.54)	$1.30e^{-05}$	1.24 (1.05, 1.47)	$1.32e^{-02}$	++	$5.45e^{-07}$	imputed
rs7766678	159705443	T/C	1.34 (1.16, 1.55)	$1.29e^{-05}$	1.24 (1.04, 1.47)	$1.43e^{-02}$	++	$5.77e^{-07}$	imputed
rs625284	159703373	G/T	1.31 (1.16, 1.49)	$5.32e^{-06}$	1.18 (1.01, 1.37)	$4.03e^{-02}$	++	$6.15e^{-07}$	imputed
rs10455775	159723721	C/T	1.32 (1.16, 1.49)	$5.03e^{-06}$	1.17 (1.00, 1.37)	$4.27e^{-02}$	++	$6.17e^{-07}$	imputed
rs568372	159702765	G/T	1.31 (1.16, 1.49)	$5.32e^{-06}$	1.18 (1.01, 1.37)	$4.04e^{-02}$	++	$6.17e^{-07}$	imputed
rs625687	159703294	G/A	1.31 (1.16, 1.49)	$5.32e^{-06}$	1.18 (1.01, 1.37)	$4.05e^{-02}$	++	$6.18e^{-07}$	imputed
rs543547	159702351	A/G	1.31 (1.16, 1.49)	$5.32e^{-06}$	1.17 (1.01, 1.37)	$4.05e^{-02}$	++	$6.19e^{-07}$	imputed
rs575681	159703534	G/A	1.31 (1.16, 1.49)	$5.32e^{-06}$	1.17 (1.01, 1.37)	$4.07e^{-02}$	++	$6.21e^{-07}$	imputed
rs12529478	159704722	T/C	1.31 (1.16, 1.49)	$5.32e^{-06}$	1.17 (1.01, 1.37)	$4.30e^{-02}$	++	$6.56e^{-07}$	imputed
rs7765794	159704925	T/C	1.32 (1.16, 1.49)	$5.32e^{-06}$	1.17 (1.01, 1.37)	$4.32e^{-02}$	++	$6.58e^{-07}$	imputed
rs1687979	159710299	G/A	1.31 (1.16, 1.49)	$5.29e^{-06}$	1.17 (1.01, 1.36)	$4.39e^{-02}$	++	$6.66e^{-07}$	imputed
rs592212	159711083	G/C	1.31 (1.16, 1.49)	$5.29e^{-06}$	1.17 (1.01, 1.36)	$4.42e^{-02}$	++	$6.69e^{-07}$	imputed
rs2451988	159714390	G/A	1.31 (1.16, 1.49)	$5.29e^{-06}$	1.17 (1.00, 1.36)	$4.45e^{-02}$	++	$6.74e^{-07}$	imputed
rs498223	159707419	A/G	1.34 (1.16, 1.54)	$1.61e^{-05}$	1.24 (1.05, 1.46)	$1.33e^{-02}$	++	$6.78e^{-07}$	imputed
rs9346784	159703757	G/T	1.31 (1.16, 1.49)	$6.26e^{-06}$	1.17 (1.01, 1.37)	$4.12e^{-02}$	++	$7.36e^{-07}$	imputed

Supplementary Table 4.3: Association statistics for all SNPs at the locus of 6q25.3 with suggestive evidence of association with AOM in the discovery cohort ($P<5x10^{-05}$). (continued)

SNP	Pos (hg19)	A1/A2	OR (95% CI) (CHOP)	P (CHOP)	OR (95% CI) (GenR)	P (GenR)	Direction	P (Meta)	Format*
rs1394233	159719575	T/C	1.33 (1.16, 1.54)	1.77e^{-05}	1.24 (1.05, 1.47)	1.33e^{-02}	++	7.46e^{-07}	imputed
rs578217	159716957	C/T	1.33 (1.16, 1.54)	1.77e^{-05}	1.24 (1.05, 1.47)	1.33e^{-02}	++	7.49e^{-07}	imputed
rs369018	159694722	A/G	1.33 (1.15, 1.53)	2.50e^{-05}	1.26 (1.06, 1.50)	8.66e^{-03}	++	7.51e^{-07}	imputed
rs529273	159708499	T/C	1.33 (1.16, 1.54)	1.87e^{-05}	1.24 (1.04, 1.46)	1.36e^{-02}	++	8.05e^{-07}	imputed
rs509833	159711515	A/G	1.33 (1.15, 1.53)	2.19e^{-05}	1.24 (1.05, 1.46)	1.30e^{-02}	++	9.09e^{-07}	imputed
rs650456	159713117	T/C	1.33 (1.17, 1.50)	2.19e^{-06}	1.13 (0.96, 1.33)	0,132	++	9.50e^{-07}	imputed
rs419009	159692596	T/C	1.32 (1.14, 1.53)	4.74e^{-05}	1.28 (1.08, 1.53)	5.14e^{-03}	++	9.79e^{-07}	imputed
rs1394234	159732990	A/G	1.31 (1.14, 1.51)	2.02e^{-05}	1.21 (1.02, 1.43)	2.43e^{-02}	++	1.43e^{-06}	imputed
rs434578	159693220	C/T	1.32 (1.14, 1.52)	4.25e^{-05}	1.25 (1.06, 1.49)	9.83e^{-03}	++	1.44e^{-06}	imputed
rs389546	159691657	T/C	1.32 (1.14, 1.53)	6.89e^{-05}	1.28 (1.08, 1.53)	5.11e^{-03}	++	1.45e^{-06}	imputed
rs7767146	159728226	T/C	1.31 (1.14, 1.50)	2.35e^{-05}	1.20 (1.02, 1.41)	2.79e^{-02}	++	1.88e^{-06}	imputed
rs4709297	159731869	G/A	1.31 (1.14, 1.50)	2.35e^{-05}	1.20 (1.02, 1.41)	2.83e^{-02}	++	2.05e^{-06}	imputed
rs7774311	159726752	G/A	1.31 (1.14, 1.50)	2.57e^{-05}	1.20 (1.02, 1.41)	2.79e^{-02}	++	2.05e^{-06}	imputed
rs1394235	159728693	G/A	1.31 (1.14, 1.50)	2.53e^{-05}	1.20 (1.02, 1.41)	2.84e^{-02}	++	2.05e^{-06}	imputed
rs1504255	159731221	A/G	1.31 (1.14, 1.50)	2.53e^{-05}	1.20 (1.02, 1.40)	2.91e^{-02}	++	2.10e^{-06}	imputed
rs905684	159733694	T/G	1.34 (1.17, 1.53)	2.45e^{-05}	1.19 (1.01, 1.39)	3.44e^{-02}	++	2.36e^{-06}	genotyped
rs4077944	159734491	T/C	1.30 (1.13, 1.50)	2.99e^{-05}	1.20 (1.02, 1.41)	2.86e^{-02}	++	2.44e^{-06}	imputed
rs76252099	159659971	G/A	1.35 (1.15, 1.59)	1.22e^{-04}	1.32 (1.06, 1.65)	1.48e^{-02}	++	5.88e^{-06}	imputed
rs76096006	159663831	C/G	1.33 (1.14, 1.59)	1.82e^{-04}	1.33 (1.06, 1.66)	1.33e^{-02}	++	8.19e^{-06}	imputed
rs79695409	159663703	T/C	1.33 (1.14, 1.59)	1.83e^{-04}	1.33 (1.06, 1.66)	1.33e^{-02}	++	8.25e^{-06}	imputed
rs381568	159639482	T/C	1.26 (1.13, 1.40)	3.87e^{-05}	1.12 (0.98, 1.27)	8.82e^{-02}	++	8.84e^{-06}	genotyped
rs77131924	159655516	T/C	1.33 (1.14, 1.59)	2.15e^{-04}	1.32 (1.06, 1.65)	1.45e^{-02}	++	1.05e^{-05}	imputed
rs59701391	159656107	G/A	1.33 (1.14, 1.56)	2.15e^{-04}	1.32 (1.06, 1.65)	1.46e^{-02}	++	1.05e^{-05}	imputed
rs73799724	159658029	C/G	1.33 (1.14, 1.56)	2.48e^{-04}	1.32 (1.06, 1.65)	1.35e^{-02}	++	1.15e^{-05}	imputed

Supplementary Table 4.3: Association statistics for all SNPs at the locus of 6q25.3 with suggestive evidence of association with AOM in the discovery cohort ($P<5x10^{-05}$). (continued)

SNP	Pos (hg19)	A1/A2	OR (95% CI) (CHOP)	P (CHOP)	OR (95% CI) (GenR)	P (GenR)	Direction	P (Meta)	Format*
rs7744848	159770787	G/A	1.36 (1.18, 1.57)	$1.74e^{-05}$	1.10 (0.93, 1.30)	0.282	++	$1.60e^{-05}$	genotyped
rs76029050	159723266	C/T	1.37 (1.14, 1.64)	$4.33e^{-04}$	1.35 (1.07, 1.71)	$1.19e^{-02}$	++	$1.89e^{-05}$	imputed
rs75391241	159671268	A/G	1.35 (1.14, 1.61)	$5.67e^{-04}$	1.34 (1.06, 1.69)	$1.26e^{-02}$	++	$2.57e^{-05}$	imputed
rs7746188	159754888	A/G	1.30 (1.13, 1.50)	$3.54e^{-05}$	1.11 (0.93, 1.31)	0.248	++	$2.57e^{-05}$	imputed
rs1875819	159747743	T /C	1.29 (1.14, 1.47)	$8.07e^{-05}$	1.10 (0.94, 1.29)	0.227	++	$4.90e^{-05}$	genotyped

SNP – single nucleotide polymorphism; Pos – position; A1 – minor allele; A2 – major allele; OR – odds ratio; CI – confidence interval; P - association P-value per cohort and in meta-analysis; Direction – direction of effect in both discovery analyses; Format*: SNPs imputed in regional imputation analysis are indicated as "imputed".

Supplementary Table 4.4: Replication cohort association results for SNPs that are in strong LD with rs2932989 and are on chip HumanOmniExpress-12 v1.

SNP	Chr	Pos (hg19)	A1/A2	OR	95% CI	P	r^2
rs419009	6	159692596	T/C	1.39	1.07, 1.80	0.012	1
rs578217	6	159716957	C/T	1.38	1.06, 1.79	0.016	1

SNP – single nucleotide polymorphism; Chr – chromosome; Pos – position; A1 – minor allele; A2 – major allele; OR – odds ratio; CI – confidence interval; P – association P-value in CHOP replication cohort; r^2 – LD measurement between each SNP and rs2932989 in 1000Genome CEU dataset.

Supplementary Table 4.5: Summary statistics of index SNP at each loci with suggestive evidence for association (5×10^{-8} < P-value < 5×10^{-5}).

SNP	CHR	Pos (hg19)	Nearby Genes	A1/A2	P_{meta}	Direction
rs3767498	1	201020727	*KIF21B, CACNA1S, ASCL5*	A/G	$1.25\text{x}10^{-06}$	++
rs12725646	1	209656906	intergenic region near *MIR205HG*	C/T	$4.33\text{x}10^{-05}$	++
rs255142	7	30754949	*CRHR2, INMT*	A/G	$1.91\text{x}10^{-06}$	++
rs9514552	13	107239714	*ARGLU1*	G/A	$2.14\text{x}10^{-05}$	--
rs12888576	14	96611391	*BDKRB2*	G/A	$7.79\text{x}10^{-06}$	++
rs2809139	14	98279189	intergenic region near *C14orf177*	C/T	$3.21\text{x}10^{-06}$	++
rs8036951	15	29671295	*FAM189A1*	G/A	$3.76\text{x}10^{-05}$	++
rs10409140	19	16159493	*TPM4*	A/G	$4.35\text{x}10^{-05}$	--

SNP – single nucleotide polymorphism; Chr – chromosome; Pos – position (hg19); Distance – distance to closest gene in base-pairs; A1 – minor allele; A2 – major allele; P_{meta} - association P-value in meta-analysis; Direction – direction of effect in both discovery analyses.

Supplementary Table 4.6: Association statistics of candidate genes previously proposed to be involved in pathogenesis of otitis media.
Each SNP in our GWAS is mapped to its closest gene. For genes the best SNP of which is located outside of its transcript and upstream/downstream 50kb region, the top SNP within this defined gene boundary is also shown. Nominally significant *P*-values are highlighted.

Gene	Best SNP	Chr	Pos (hg19)	Distance	A1/A2	P_meta	Direction	Study type	Reference
*TP73**	rs3765731	1	3611892	0	A/G	**0.0274**	--	Candidate gene	3
CCDC27	rs1181876	1	3681681	0	T/C	0.0724	--	GWAS	4
LIN28	rs11581746	1	26744087	0	T/C	**0.0429**	--	GWAS	4
*CLCA1**	rs2734700	1	86950320	0	A/G	**0.00383**	--	Candidate gene	5
OVGP1	rs1777833	1	111956473	464	G/A	0.176	-+	Candidate gene	6
MUC1	rs4072037	1	155162067	0	G/A	0.904	-+	Candidate gene	7
IL10	rs3024505	1	206939904	1044	T/C	0.277	--	Candidate gene	8
PARP1	rs1136410	1	226555302	0	C/T	**0.0162**	-+	Candidate gene	9
GALNT14	rs4245766	2	31242194	0	C/T	**0.0170**	++	GWAS	4
CAPN14	rs17010826	2	31396729	0	A/G	**0.0343**	+-	GWAS	4
FBXO11(*)	rs4952896	2	48116126	0	G/A	0.244	++	Candidate gene	10, 11
FBXO11(*)	rs999036	2	48217552	84738	G/A	**0.0466**	++	Candidate gene	10, 11
IL1A	rs17042407	2	113558914	15943	C/T	0.492	--	Candidate gene	12
IL1B	rs2723167	2	113621210	26854	T/C	**0.0283**	--	Candidate gene	13
IL1RN	rs315952	2	113890304	0	C/T	**0.0475**	--	Transcriptome analysis	14
GALNT13	rs13414624	2	154904730	0	A/C	**0.0141**	--	GWAS	4
CDCA7	rs6751320	2	174264424	30706	T/C	**0.0464**	++	GWAS	15
CDCA7	rs6714145	2	174454044	220326	C/T	**0.00257**	--	GWAS	15
SP3	rs4972618	2	174769344	3914	T/C	**0.0299**	--	GWAS	15
IRAK2	rs6442159	3	10243304	0	A/G	0.147	+-	Candidate gene	16, 17
HRH1	rs443137	3	11253721	0	T/C	**0.0373**	++	Genome scan	17
*MYD88**	rs7744	3	38184021	0	G/A	0.147	+-	Candidate gene	18
LTF	rs17078876	3	46500978	0	T/C	0.403	++	Candidate gene	19

Each SNP in our GWAS is mapped to its closest gene. For genes the best SNP of which is located outside of its transcript and upstream/downstream 50kb region, the top SNP within this defined gene boundary is also shown. Nominally significant *P*-values are highlighted. (continued)

Gene	Best SNP	Chr	Pos (hg19)	Distance	A1/A2	P_meta	Direction	Study type	Reference
IL12A	rs550662	3	159663317	43312	A/C	**0.00396**	++	Candidate gene	20
*EVI1**	rs10513653	3	168746566	54721	A/G	**0.00966**	++	Candidate gene	21
*EVI1**	rs6798744	3	168791596	9691	C/T	0.09187	--	Candidate gene	21
BCL6	rs2229362	3	187446211	0	A/G	0.227	--	Candidate gene	22
BCL6	rs2889879	3	187657216	193741	C/T	**0.0181**	++	Candidate gene	22
MUC4	rs2259419	3	195505417	0	G/A	**0.0115**	--	Candidate gene	7
*IDUA**	rs3755955	4	994414	0	T/C	0.191	--	Candidate gene	23
MUC7	rs6600832	4	71353508	4794	C/T	0.122	--	Candidate gene	6
IL8	rs12506479	4	74592161	14114	C/T	0.259	--	Candidate gene	24
GRID2	rs977955	4	93966480	0	A/G	**0.00168**	--	GWAS	4
*IL2**	rs10857092	4	123389219	11569	A/G	0.577	-+	Candidate gene	25
TLR2	rs1816702	4	154609523	0	T/C	0.288	--	Candidate gene	26
TPPP	rs413666	5	673408	0	T/C	0.376	+-	GWAS	15
DNAH5	rs1910092	5	13781480	0	T/C	**0.0241**	++	Candidate gene	27
*ISL1**	rs2961811	5	50413703	265255	A/C	**0.00269**	++	exome sequencing	28
*ISL1**	rs10939961	5	50738478	47915	A/C	0.319	++	exome sequencing	28
IL13	rs1295686	5	131995843	0	A/G	**0.0266**	--	Candidate gene	29
CD14	rs778584	5	140005212	6105	T/C	0.0661	-+	Candidate gene	30
IL12B	rs2569253	5	158750993	0	C/T	**0.0152**	--	Candidate gene	20
*SH3PXD2B**	rs10059733	5	171908794	27267	G/A	0.110	++	Candidate gene	31
*SH3PXD2B**	rs7713320	5	172024642	143115	T/C	**0.0117**	++	Candidate gene	31
TNF	rs3093662	6	2846777	0	G/A	0.974	--	Candidate gene	32
*SPDEF**	rs13194133	6	34532922	8831	G/T	0.121	--	Candidate gene	5

Each SNP in our GWAS is mapped to its closest gene. For genes the best SNP of which is located outside of its transcript and upstream/downstream 50kb region, the top SNP within this defined gene boundary is also shown. Nominally significant *P*-values are highlighted. (continued)

Gene	Best SNP	Chr	Pos (hg19)	Distance	A1/A2	P_meta	Direction	Study type	Reference
BMP5	rs9475400	6	55638258	0	T/C	**0.0119**	--	GWAS	4
*EYA4**	rs211619	6	133727321	0	A/G	**0.00226**	++	Candidate gene	33
*PLG**	rs6935921	6	161108536	14738	C/T	0.236	++	Candidate gene	34
*DNAH11**	rs1859107	7	21859436	0	C/A	**0.0166**	--	Candidate gene	35
IL6	rs10261484	7	22583326	183492	G/A	**0.0416**	++	Candidate gene	32
IL6	rs6949149	7	22749157	17661	T/G	0.0877	--	Candidate gene	32
*GUSB**	rs12698511	7	65474919	27673	A/C	0.150	--	Candidate gene	36
DEFB1	rs2702839	8	6722082	6017	A/G	**0.0133**	--	Candidate gene	37
CTSB	rs12898	8	11701198	0	C/T	0.306	--	Candidate gene	38
FGFR1(*)	rs6996321	8	38322346	0	A/G	**0.00163**	++	Candidate gene	39, 40
TLR4(*)	rs4986790	9	120475302	0	G/A	0.0912	++	Candidate gene	41, 42
TLR4(*)	rs955259	9	120904985	425219	C/T	**0.0111**	++	Candidate gene	41, 42
ADAMTS13	rs685523	9	136310908	0	A/G	**0.0347**	-+	Case report	43
MBL2	rs2120132	10	54526040	0	C/T	**0.0213**	--	Candidate gene	44
MBL2	rs1962474	10	54914951	383491	C/T	**0.0133**	++	Candidate gene	44
SFTPA1	rs1650198	10	81417214	42012	C/T	0.495	-+	Candidate gene	45
SFTPD	rs1885551	10	81712353	3492	C/T	0.125	+-	Candidate gene	41
SFTPD	rs1932571	10	81761489	52628	T/C	**0.0346**	++	Candidate gene	41
*FAS**	rs9325604	10	90775756	214	G/A	**0.00419**	-+	Candidate gene	46
*FAS**	rs9325604	10	90775756	214	G/A	**0.00419**	-+	Candidate gene	46
ADAM8	rs1131718	10	135085754	0	C/T	0.595	--	Candidate gene	17
MUC6	rs11246381	11	1019194	0	G/A	**0.0105**	--	Candidate gene	6
MUC2	rs4077759	11	1105976	1559	C/T	0.0789	++	Candidate gene	47

Each SNP in our GWAS is mapped to its closest gene. For genes the best SNP of which is located outside of its transcript and upstream/downstream 50kb region, the top SNP within this defined gene boundary is also shown. Nominally significant *P*-values are highlighted. (continued)

Gene	Best SNP	Chr	Pos (hg19)	Distance	A1/A2	P_meta	Direction	Study type	Reference
MUC5AC	rs868903	11	1242690	0	T/C	0.357	-+	Candidate gene	48
MUC5AC,MUC5B	rs2075859	11	1250488	1	T/C	0.295	--	Candidate gene	48
MUC5AC,MUC5B	rs2075859	11	1250488	0	T/C	0.295	--	Candidate gene	48
NELL1	rs11026076	11	21436232	0	T/C	**0.00132**	--	GWAS	4
A2ML1	rs16917857	12	9020680	0	A/G	**0.0319**	--	Exome sequencing	49
IFNG	rs1548653	12	68360943	187607	G/A	0.114	--	Candidate gene	50
IFNG	rs2193045	12	68534520	14030	A/G	0.352	+-	Candidate gene	50
*OXGR1**	rs10851069	13	97628279	9694	T/C	0.0591	++	Candidate gene	51
TGFB3	rs3917192	14	76431674	0	A/G	0.311	++	GWAS	4
SMAD3	rs266377	15	67229636	128559	G/T	0.0507	--	Candidate gene	52
SMAD3	rs893473	15	67438091	0	T/C	0.102	++	Candidate gene	52
TICRR	rs8041127	15	90135196	0	A/G	0.407	-+	GWAS	15
KIF7	rs1110060	15	90190048	0	A/G	0.771	--	GWAS	15
MMP2	rs243866	16	55511537	1544	A/G	**0.0234**	++	Candidate gene	53
*E2F4**	rs11700	16	67232684	0	C/T	0.315	-+	Candidate gene	54
*E2F4**	rs11700	16	67232684	0	C/T	0.315	-+	Candidate gene	54
*CCL3**	rs1851503	17	34412202	3401	G/T	0.878	+-	Candidate gene	55
*RPL38**	rs6501659	17	71771387	428408	T/C	**0.035**	++	Candidate gene	56
*RPL38**	rs7211140	17	72173910	25885	A/G	0.0916	-+	Candidate gene	56
SMAD2	rs1942159	18	45484940	27428	G/A	**0.000919**	--	Candidate gene	57
SMAD4	rs948588	18	48586344	0	A/G	**0.0342**	--	Candidate gene	57
*TICAM1**	rs8120	19	4816160	0	G/T	**0.0367**	++	Candidate gene	58
TGFB1	rs8110090	19	41845872	0	G/A	0.41	+-	Candidate gene	59

Each SNP in our GWAS is mapped to its closest gene. For genes the best SNP of which is located outside of its transcript and upstream/downstream 50kb region, the top SNP within this defined gene boundary is also shown. Nominally significant *P*-values are highlighted. (continued)

Gene	Best SNP	Chr	Pos (hg19)	Distance	A1/A2	P_meta	Direction	Study type	Reference
*FOXA2**	rs199820	20	22323038	238604	A/C	**0.0022**	--	Candidate gene	60
*FOXA2**	rs6075924	20	22512529	49113	T/C	0.122	--	Candidate gene	60
MMP9	rs13038175	20	44624097	13450	A/G	0.384	++	Candidate gene	53
*SALL4**	rs6063719	20	50440054	21006	T/G	**0.0391**	+-	Candidate gene	61
*TBX1**	rs4819843	22	19765182	0	A/G	0.396	++	Candidate gene	62
*NF2**	rs2530664	22	30038152	0	C/A	**0.0342**	++	Candidate gene	63
*CBY1**	rs4821797	22	39016182	36476	C/T	0.429	--	Candidate gene	64

* the rodent model homologue of which has been implicated in otitis media

Best SNP: The SNP of the lowest P-value in each gene; Chr: chromosome; Pos(hg19): the position on human genome build hg19; Distance: the distance between the SNP and the gene transcript; A1: effect allele; A2: reference allele; P_meta: meta-analysis P-value; Direction: the direction of effect for the effect allele; Study type: the type of the study reported in the reference.

Supplementary Table 4.7: Summary of mouse gene Fndc1 tissue expression pattern. Data are from http://www.informatics.jax.org/

Structure	Total number of assays	Number of assays Detected	Level	References
TSTS21: eye	1	1	NA	[65]
TSTS22: conjunctival sac	1	1	NA	[65]
TSTS23: conjunctival sac	4		Strong	[66]
TSTS23: cornea epithelium	5	5	Strong	[66]
TSTS23: oral epithelium	16	16	Strong	[66]
TSTS23: sublingual gland primordium	5	5	Strong	[66]
TSTS23: submandibular gland primordium	7	7	Strong	[66]
TSTS23: dermis	24	24	Strong	[66]
TSTS23: lower lip	7	7	Strong	[66]
TSTS23: upper lip	9	9	Strong	[66]
TSTS23: mesenchyme	24	24	Strong	[66]
TSTS24: conjunctival sac	1	1	NA	[65]
TSTS26: conjunctival sac	1	1	NA	[65]
TSTS27: conjunctival sac	1	1	NA	[65]

NA: not reported.

Supplementary Table 4.8: Proteins bound to significant SNPs at locus 6q25.3. We input all SNPs of suggestive significant association with AOM ($P<5x10^{-5}$) into Haploreg search (67). Two of them were found to have proteins bound in ENCODE ChIP assay (68).

SNP	Chr	Pos (hg19)	Proteins bound	Cell lines
rs1394234	6	159732990	CTCF	AG10803 GM06990 GM12878 NHEK
rs1553482	6	159722147	EGR1	K562

SNP – single nucleotide polymorphism; Chr – chromosome; Pos – position; AG10803 – abdominal skin fibroblasts; GM06990 – B-lymphocyte, lymphoblastoid; GM12878 – lymphoblastoid cell line; NHEK – Normal Human Epidermal Keratinocytes; K562 – leukemia.

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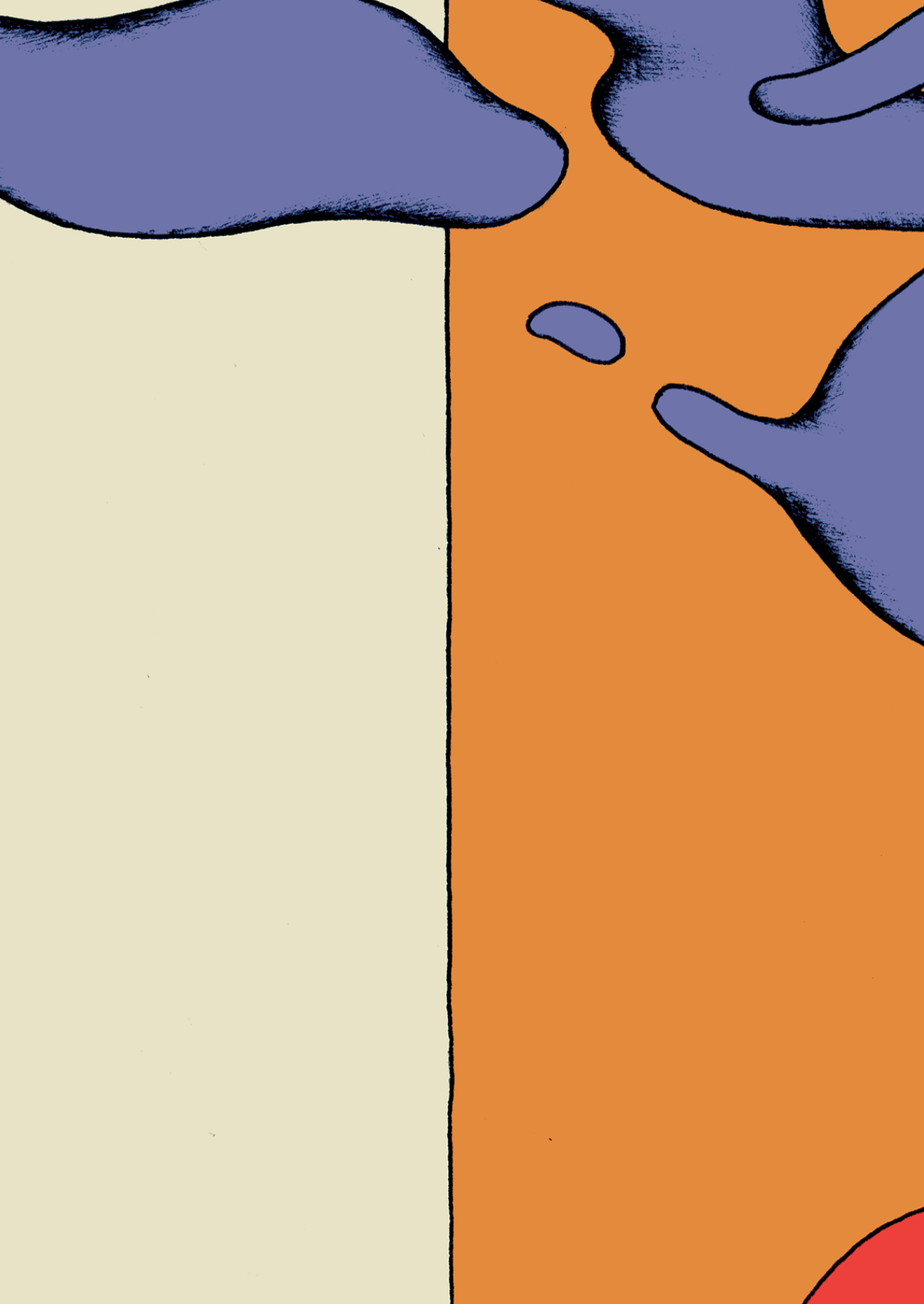

5 Newborn DNA methylation and risk of acute otitis media in childhood: meta-analysis of epigenome-wide association studies

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ABSTRACT

Background: Acute otitis media (AOM) is a common pediatric disease with complex pathogenesis. DNA methylation may be a biological mechanism underlying associations of early-life exposures and susceptibility to AOM.

Methods: We meta-analyzed results from epigenome-wide association studies of AOM in six cohorts participating in the Pregnancy And Childhood Epigenetics (PACE) Consortium, totaling 2377 children. DNA methylation levels were measured in cord blood using the Illumina Infinium HumanMethylation450 Beadchip. Data on AOM during the first seven years of life were collected using parental questionnaires. CpGs were annotated to the nearest gene, and associations with gene expression and enriched biological processes were explored. We looked-up top results in an independent cohort study on otitis media with effusion (OME) in 811 children.

Results: DNA methylation levels in cord blood were not associated with AOM in early childhood, either at Bonferroni or FDR adjusted significance levels. Nineteen CpGs showed subthreshold associations ($p<1.0x10^{-4}$) with AOM, of which two, cg11201229 and cg06838215, were significantly associated with OME in the look-up analysis. Only cg06838215 showed a similar direction of effect among all studies. Meta-analysis of cg06838215 using all data on AOM and OME strengthened the association, although it did not reach the Bonferroni threshold ($p=6.31 \times 10^{-7}$).

Conclusions: We did not find evidence to support an association between DNA methylation at birth and AOM in early childhood. Joint analysis of data on AOM and OME showed some potential differences in DNA methylation between cases and controls, which should be further explored using larger sample sizes.

INTRODUCTION

Acute otitis media (AOM) is a common pediatric disease and the most frequent reason for antibiotic administration in children (1, 2). AOM is a bacterial infection of the middle ear, secondary to an upper respiratory tract infection (URTI), and is characterized by earache, fever, and bulging of the tympanic membrane due to the presence of purulent fluid in the middle ear (3, 4). In general, AOM is associated with high health care expenditures, with annual costs in the United States estimated at US$ 4.3 billion per year (5). Part of the reason why the AOM burden cannot be easily reduced is the incomplete understanding of its pathogenesis, which is one of complex associations between environmental, host, and genetic factors. Environmental and host factors are best understood. Factors related to infection pressure, such as having siblings, day-care attendance, season-of-birth, and other factors like pet-keeping, breastfeeding, and socioeconomic status are related to exposure to viral pathogens, which in turn is associated with incidence of URTIs – and thus of ear infections (6-8). Maternal factors with a proposed impact on childhood AOM include prenatal and postnatal (household) smoking and maternal age (9). Host factors include child's sex and ethnicity (10, 11). Heritability of AOM is well-established in family studies, sibling, and twin studies, with the fraction of phenotype variability attributed to genetic variation (h^2) estimated between 0.22 and 0.74, but genetic susceptibility of AOM is as yet not well understood (12-15). To date, 21 genetic loci have been implicated in 5 genome-wide association studies (GWAS) on otitis media (OM), including AOM, from which only 5 were replicated in an independent OM dataset (16-20). These include the *FNDC1* gene variant rs2932989, which was positively correlated with *FNDC1* expression, and negatively correlated with DNA methylation status of cg05678571 (19). Whether AOM was associated with DNA methylation at cg05678571 was not analyzed. Epigenetic mechanisms such as DNA methylation could link environmental exposures with the occurrence of childhood AOM. DNA methylation is an epigenetic mechanism that is dynamic and may affect gene expression. It is associated with health-related outcomes and is influenced by environmental, genetic, and stochastic factors (21-25). We hypothesized that differential DNA methylation measured in cord blood DNA of newborns is associated with susceptibility to AOM in early childhood. To test our hypothesis, we meta-analyzed results from six epigenome-wide association studies (EWAS) of cord blood DNA methylation levels and AOM in childhood using data from a total of 2377 children.

METHODS

Study design and data sources

This meta-analysis of epigenome-wide association studies included results from six population-based cohort studies collaborating in the Pregnancy And Childhood Epigenetics (PACE) Consortium (26), with a combined sample size 2377 children: the California Birth Cohort (CBC) (27), the Étude des Déterminants pré et postnatals du développement et de la santé de l'Enfant (EDEN) (28), the Generation R Study (29, 30), the Infancia y Medio Ambiente (INMA) study (31), and two independent datasets from the Norwegian Mother, Father and Child Cohort Study (MoBa1, MoBa2) (32-34). Subjects were mainly of European descent, but the CBC study comprised two datasets with one of European and one of Hispanic descent (Table 5.1). Local Medical Ethics Committees approved all studies, and informed consent was obtained for all participants (Supplemental Material).

DNA methylation

DNA was extracted from cord blood samples, and bisulfite conversion was performed using the EZ-96 DNA Methylation kit (Zymo Research Corporation, Irvine, CA, USA). DNA methylation was measured using the Illumina Infinium® HumanMethylation450 BeadChip assay (Illumina, San Diego, California, USA) followed by cohort-specific quality control and data normalization (Supplemental Material) (35). Untransformed normalized beta-values of individual Cytosine-phosphate-Guanine (CpG) sites were used as exposure variables. To exclude extreme outlying DNA methylation beta values, the outer fences of the CpG distributions were trimmed: values < (25th percentile – 3*interquartile range (3IQR)) and values > (75th percentile + 3IQR) were excluded.

Otitis media

AOM case description was established through parental reports and questionnaires on episodes of earache, with one or more accompanying symptoms such as fever, otorrhea, or use of eardrops as prescribed by a doctor. Participants were defined as controls if no history of middle ear disease was present. See Supplemental Methods for cohort-specific details on AOM phenotype definition.

Covariates and estimation of cell-type proportions

Each cohort adjusted for the following covariates: maternal age, maternal educational level, maternal smoking during pregnancy (never smoked versus any smoking during pregnancy), parity (multiparous versus nulliparous), and gestational age at birth, sex, technical covariates and estimated cord white blood cell proportions (Supplemental Methods, Supplemental Table 5.1). These cell-type proportions included B-cells, CD8+ T-cells, CD4+ T-cells, granulocytes, NK-cells, and monocytes, and were estimated using the Houseman

method with the Reinius reference implemented in the minfi package in R (17, 36-38). After meta-analysis was performed, a cord blood-specific reference panel and method were published (39). We compared the results of the main model adjusted for cell-type proportions estimated using the Houseman method with those adjusted using the cord blood-specific cell-type proportions within the Generation R Study, which showed a high correlation (r = 0.97) (Supplemental Fig. 5.1). Batch effects were adjusted for using cohort-specific methods (Supplemental Methods).

Statistical analysis

According to a predefined analysis plan, each cohort performed cohort-specific analyses of associations of cord blood DNA methylation with AOM independently. Each cohort used generalized linear models in R to evaluate associations of DNA methylation levels at each CpG site individually with AOM, adjusting for the confounders mentioned above. Results are shown as odds ratios (OR) with 95% confidence intervals. The two ethnic subgroups from the CBC study were analyzed separately, and both results files were included in the meta-analysis.

Meta-analysis

After quality control of the study-specific results at the meta-analysis center, results from individual studies were combined using inverse variance-weighted fixed-effect meta-analysis using METAL (40, 41). Researchers from two sites, the Generation R Study and CBC, independently performed all meta-analyses and then compared them. Results were identical. Probes that mapped to X- and Y-chromosomes and probes that co-hybridized to alternate sequences (cross-reactive probes) were excluded (42, 43). We included 429,960 probes for the meta-analysis. Probes that mapped to DNA containing a SNP, repetitive sequence elements, or DNA harboring an INDEL were not excluded, but flagged in the results (42, 43). Multiple testing was accounted for using Bonferroni correction, which gave a threshold for significance of $p<1.16x10^{-7}$ to account for 429,960 tests, and using the Benjamini-Hochberg false discovery rate (FDR) (44). Heterogeneity across studies was calculated (I^2) and shown graphically in Forest plots. I^2 estimates for each CpG the proportion of variation in the effect estimate that is due to between-study differences rather than random/sampling variation. We considered values of >50% as strongly heterogeneous. Next, we performed leave-one-out analyses for CpGs with $p<5.0x10^{-5}$. In this step, we repeated the main meta-analysis for these CpGs several times, leaving out each of the six studies one by one. Results were examined to determine if one or more studies affected the meta-analysis results unduly (Supplemental Fig. 5.2a-j). We performed a look-up in our dataset of all 30 CpGs that mapped to *FNDC1*, including cg05678571, which had previously been associated with AOM (Supplemental Table 5.2). The threshold for significant association with AOM in this look-up was $p<0.05/30 = 1.67x10^{-3}$.

Gene expression

To aid functional interpretation, we associated differential DNA methylation with gene expression. Top CpGs with at least $p<1.0x10^{-4}$ were considered for this analysis. Gene expression was assessed for each CpG in a region of +/- 250 kb in blood samples. We had one dataset available for this analysis, which used mRNA gene expression (Affymetrix Human Transcriptome Array 2.0) and 450K methylation data on whole-blood samples from 110 children at 4 years of age from the INMA study in Spain (see Supplemental Methods for further details). Further, we explored whether differentially expressed genes were expressed in human lung tissue of the Genotype-Tissue Expression (GTEx) database as lower airway mucosa most resembles upper airway and middle ear mucosa, which were not available in GTEx (45).

Exploration of biological processes

CpGs were annotated to the nearest gene using the UCSC Genome Browser. All of the annotations used the human February 2009 (GRCh37/hg19) assembly. Next, we explored possible underlying biological processes using GeneMANIA with the genes to which top CpGs ($p<1.0x10^{-4}$) mapped as input. GeneMANIA finds other genes that are related to a set of input genes through protein and genetic interactions, pathways, co-expression, co-localization, and protein domain similarity (46). Functional enrichment analysis was performed on the constructed interaction network genes against Gene Ontology (GO) terms using g:Profiler (47). We used the Online Mendelian Inheritance in Man (OMIM) database and the Universal Protein Resource (UniProt) to explore whether annotated genes have been previously related to OM.

Look-up in Independent Cohort on Otitis Media with Effusion

We performed a look-up of top results (CpGs with $p<1.0x10^{-4}$) from the main model in an independent birth cohort with 450K methylation data in cord blood samples and information on the AOM-related phenotype of otitis media with effusion (OME) in children of European ancestry from the Avon Longitudinal Study of Parents and Children (ALSPAC UK) (48, 49). It comprised 811 subjects, of which 150 subjects were defined as cases (Table 5.1). OME was established through tympanometry and video otoscopy. An EWAS on OME was performed using the same pre-specified analysis plan used by the six studies on AOM (Supplemental Methods). A Bonferroni corrected $p < 0.05/$ (number of top CpGs) was used to define significance.

Table 5.1: Characteristics of participating cohorts*

Cohort		Location	Birth years	Age participants in years	AOM diagnosis	No. of available CpGs	No. of participants (cases)
CBC	- Hispanic - White	U.S.A.	1994-2007	0 - 1	Parent reported	484,972	172 (77) 188 (93)
EDEN		France	2003-2004	0 - 3	Parent reported	439,306	148 (118)
Generation R		Netherlands	2002-2006	0 - 6	Parent reported	436,013	773 (488)
INMA[#]		Spain	2004-2007	0 - 4	Parent reported	439,306	175 (111)
MoBa1		Norway	2000-2009	0 - 7	Parent reported	473,844	921 (614)
MoBa2		Norway	2000-2009	0 - 7	Parent reported	473,748	416 (296)
ALSPAC (Replication cohort)		U.K.	1991-1992	0 - 9	Tympanometry / Video otoscopy	471,193	811 (150)

*Additional information on design and other characteristics of each cohort are in included in **Supplementary Material**. N/A: not available. # INMA had 110 participants with blood gene expression data.

RESULTS

General characteristics of the participants are presented in Table 5.1, and detailed characteristics and covariates are presented in Supplemental Table 5.1. Results of the meta-analysis are presented in Table 5.2. We did not find genome-wide significant associations of DNA methylation with AOM at any CpGs. DNA methylation at two CpGs, cg26638266 and cg04373106, was associated with AOM at subthreshold significance ($P<5.0x10^{-6}$). These CpGs map to the genes *VIT* and *OTOP3, respectively.* A further 17 CpGs, mapping to 13 genes: *CD2AP*, *NCOR2*, *MIR497*, *MIR195*, *MACF1*, *GPR44*, *CEP104*, *PIK3C2A*, *HOXB-AS3*, *HOXB5*, *DNAJC15*, *PTGIR* and *SH3PXD2A*, had p-values between $p>5.0x10^{-6}$ and $p<1.0x10^{-4}$. The largest effect size was found for cg1120129 mapping to *PIK3C2A* with an odds ratio (OR) of 0.26 per 10% increase in DNA methylation. These 19 CpGs with $p<1.0x10^{-4}$ were used in further exploratory analyses.

Gene Expression

Associations of DNA methylation levels at the 19 CpGs with gene expression in whole blood cells were examined in 110 children from the INMA study. Eighteen of 19 CpGs passed QC. Increased DNA methylation at one CpG, cg09677945, was significantly associated with decreased expression of the *DNAJC15* gene ($p=1.38x10^{-14}$) (Table 5.3). Moreover, *DNAJC15* was also expressed in human lung tissue of the Genotype-Tissue Expression (GTEx) database.

Look-up of Findings

Results of the look-up of the 19 CpGs in relation to OME are presented in Table 5.4. Two CpGs, cg11201229 and cg06838215, were significantly associated with OME [$p<2.63x10^{-3}$

Table 5.2: Results (p<1.0x10-4) from meta-analysis of EWAS of children who suffered from AOM in early childhood.

CpG	Closest gene	Chr	Bp position	Location in gene	Relation to UCSC CpG Island	OR	95% CI	p-value	Heterogeneity (I^2)	Flagged probe
cg26638266	*VIT*	2	36,923,086	TSS1500	Open sea	1.51	1.27, 1.79	2.24×10^{-6}	0	SNP at CpG
cg04373106	*OTOP3*	17	72,932,992	Body	S-Shore	1.99	1.48, 2.67	4.91×10^{-6}	0	-
cg20172563	*CD2AP*	6	47,487,173	Body	Open sea	2.10	1.49, 2.95	2.05×10^{-5}	0	Repeat sequence
cg27518631	-	16	2,846,005	-	N-Shelf	1.79	1.37, 2.33	2.22×10^{-5}	21.2	SNP at CpG, repeat sequence
cg06242243	*NCOR2*	12	124,992,461	5'UTR	Open sea	2.26	1.55, 3.31	2.67×10^{-5}	0	SNP at CpG
cg21040575	*MIR497; MIR195*	17	6,921,465	TSS200; TSS1500	N-Shelf	1.47	1.23, 1.76	2.78×10^{-5}	27.9	-
cg00352106	*MACF1*	1	39,864,197	Body	Open sea	1.92	1.41, 2.60	3.03×10^{-5}	0	Repeat sequence
cg13077745	*GPR44*	11	60,623,592	TSS200	S-Shelf	2.12	1.48, 3.02	3.52×10^{-5}	43.7	-
cg02576892	-	1	2,878,813	-	S-Shelf	3.34	1.88, 5.91	3.59×10^{-5}	40.4	-
cg26782169	-	4	146,679,918	-	N-Shelf	2.54	1.62, 3.96	4.25×10^{-5}	33.2	-
cg10009875	*CEP104*	1	3,740,050	Body	Open sea	0.44	0.30, 0.65	5.03×10^{-5}	0	-
cg11201229	*PIK3C2A*	11	17,191,235	1stExon	Open sea	0.26	0.13, 0.50	5.10×10^{-5}	0	-
cg20184247	*HOXB-AS3; HOXB5*	17	46,672,052	Body; TSS1500	N-Shore	1.73	1.32, 2.28	7.49×10^{-5}	43.9	SNP at CpG
cg09677945	*DNAJC15*	13	43,597,657	5'UTR; 1stExon	Island	0.77	0.67, 0.88	7.90×10^{-5}	0	-
cg14157549	-	6	168,393,963	-	S-Shelf	1.47	1.22, 1.79	7.95×10^{-5}	21.5	-
cg13644715	*PTGIR*	19	47,127,041	Body	Island	1.86	1.37, 2.54	8.03×10^{-5}	0	-
cg06780777	-	8	28,246,464	-	S-Shelf	2.45	1.57, 3.82	8.07×10^{-5}	0	-
cg06838215	*SH3PXD2A*	10	105,389,403	Body	Open sea	2.22	1.49, 3.30	8.94×10^{-5}	0	SNP at CpG
cg04561120	-	12	7,798,450	-	Open sea	0.74	0.64, 0.86	9.62×10^{-5}	0	SNP at CpG, repeat sequence

Analysis was adjusted for maternal age, maternal educational level, maternal smoking during pregnancy, parity (multiparous versus nulliparous), and gestational age at birth, sex, technical covariates and estimated white blood cell proportions. Odds ratios (OR) are presented per 10% increase in methylation level. Confidence Intervals (95% CI) were not adjusted for multiple testing. Bp position showed genetic location of CpG. Location in gene: location of the CpG in the epigenome. N-shelf: North shelf. N-Shore: North shore. S-Shore: South shore. Flagged probe: if probe mapped to DNA containing a SNP, to repetitive sequence elements, or to DNA harboring an INDEL.

(0.05/19)]. Cg06838215 showed a similar direction of effect in AOM and OME. Further, meta-analysis of cg06838215 combining the analyses of AOM and OME strengthened the association ($p=6.31x10^{-7}$, OR=2.17 per 10% increase of DNA methylation, $I^2=0$), although it fell just short of epigenome-wide significance. Other CpGs with similar direction of effect in AOM and OME and $I^2=0$ included cg13077745 and cg09677945.

Table 5.3: Association (false discovery rate corrected p<0.05) between gene expression in whole blood cells and methylation in a cohort of 110 children (INMA) calculated for CpGs with subthreshold association ($p<1.0x10^{-4}$) with AOM in children in the meta-analysis. For 18 CpGs that passed QC a total of 359 regions with expression were tested. One CpG showed significant association between DNA methylation and gene expression.

CpG	Probe ID	Expressed Gene	Beta	SE	p-value	FDR
cg09677945	TC13000157.hg.1	*DNAJC15*	-3.50	0.39	1.38×10^{-14}	4.97×10^{-12}

FDR – false discovery rate corrected p-value.

Table 5.4: Look-up of most significant CpG sites from the meta-analysis on AOM, in an independent cohort on children with OME (ALSPAC).

CpG	Meta-EWAS on AOM		Look-up in EWAS on OME		Meta-analysis of discovery and look-up[#]			
	OR	p-value	OR	p-value	OR	p-value	Direction	I^2
cg26638266	1.51	2.24×10^{-6}	0.99	0.95	1.30	1.87×10^{-4}	+-	88.1
cg04373106	1.99	4.91×10^{-6}	1.12	0.97	1.87	1.10×10^{-5}	++	37.9
cg20172563	2.10	2.05×10^{-5}	1.28	0.23	1.71	5.40×10^{-5}	++	69.7
cg27518631	1.79	2.22×10^{-5}	1.01	0.90	1.22	0.012	++	91.4
cg06242243	2.26	2.67×10^{-5}	1.01	0.68	1.57	0.001	++	86.0
cg21040575	1.47	2.78×10^{-5}	1.01	0.95	1.34	2.20×10^{-4}	++	74.5
cg00352106	1.92	3.03×10^{-5}	1.19	0.17	1.43	2.30×10^{-4}	++	82.6
cg13077745	2.12	3.52×10^{-5}	1.89	0.50	2.11	2.79×10^{-5}	++	0
cg02576892	3.34	3.59×10^{-5}	1.57	0.19	2.44	6.33×10^{-5}	++	64.4
cg26782169	2.54	4.25×10^{-5}	1.12	0.62	1.68	0.001	++	84.8
cg10009875	0.44	5.03×10^{-5}	0.89	0.34	0.74	0.004	--	88.9
cg11201229	0.26	5.10×10^{-5}	4.13	**0.0025***	0.65	0.12	-+	95.7
cg20184247	1.73	7.49×10^{-5}	0.83	0.039	1.03	0.68	+-	94.9
cg09677945	0.77	7.90×10^{-5}	0.55	0.10	0.76	2.98×10^{-5}	--	0
cg14157549	1.47	7.95×10^{-5}	0.88	0.26	1.18	0.024	+-	91.5
cg13644715	1.86	8.03×10^{-5}	1.05	0.49	1.34	0.032	++	91.3
cg06780777	2.45	8.07×10^{-5}	1.13	0.46	1.49	0.003	++	86.6
cg06838215	2.22	8.94×10^{-5}	2.11	**0.0021***	2.17	6.31×10^{-7}	++	0
cg04561120	0.74	9.62×10^{-5}	-		-			

I^2 heterogeneity: >50% was considered too heterogeneous to compare results. *: significantly associated with OME. Both the meta-EWAS on AOM and the EWAS on OME were adjusted for maternal age, maternal educational level, maternal smoking during pregnancy, parity (multiparous versus nulliparous), and gestational age at birth, sex, technical covariates and estimated white blood cell proportions. Odds ratios (OR) are presented per 10% increase in methylation level.

Related biological processes

We used the 15 genes to which the 19 CpGs with $p<1.0x10^{-4}$ were mapped as input in an exploratory functional interaction network analysis. We found an additional 17 genes related to the input genes through protein and genetic interactions, pathways, co-expression, co-localization, and protein domain similarity. Twelve significantly enriched GO terms (after Bonferroni correction) were observed for these 32 genes, related to several biological processes, including lipid signaling, cell signaling, membrane trafficking, endocrine resistance, and interactions related to bacterial invasion of epithelial cells (Table 5.5, Supplemental Fig. 5.3, and Supplemental Fig. 5.4a-b).

Table 5.5: Enriched gene ontology (GO) terms identified in functional network analysis related to biological processes*. Only significantly enriched GO terms after Bonferroni correction were presented here.

GO ID	Description	p-value
GO:0036092	Phosphatidylinositol-3-phosphate biosynthetic process	1.98×10^{-8}
GO:0046854	Phosphatidylinositol phosphorylation	3.28×10^{-5}
GO:0046834	Lipid phosphorylation	1.12×10^{-4}
GO:0036089	Cleavage furrow formation	1.63×10^{-4}
GO:0006661	Phosphatidylinositol biosynthetic process	1.35×10^{-3}
GO:0014065	Phosphatidylinositol 3-kinase signaling	7.07×10^{-3}
GO:0046488	Phosphatidylinositol metabolic process	1.35×10^{-2}
GO:0048015	Phosphatidylinositol-mediated signaling	1.88×10^{-2}
GO:0048017	Inositol lipid-mediated signaling	2.07×10^{-2}
GO:0033031	Positive regulation of neutrophil apoptotic process	3.01×10^{-2}
GO:0046474	Glycerophospholipid biosynthetic process	3.80×10^{-2}
GO:0032506	Cytokinetic process	4.10×10^{-2}

*Additional information on gene ontology terms in functional analysis related to other processes are in included in **Supplementary Figures 4a-b**). GO ID: Gene ontology identification number.

DISCUSSION

We hypothesized that differential DNA methylation measured in cord blood would be associated with AOM in early childhood. Our EWAS meta-analysis did not yield epigenome-wide significant CpGs associated with AOM in children. We found 19 CpGs, mapping to 15 genes, with a suggestive association $p<1.0x10^{-4}$, and used these in a number of exploratory follow-up analyses.

A look-up of these 19 CpGs in an EWAS on the AOM-related phenotype of OME showed that only cg06838215, mapping to SH3PXD2A, was significantly associated with OME. A further meta-analysis using data from both AOM and OME did not convincingly prove an association, despite showing the same direction of effect. SH3PXD2A has a role in the extracellular matrix organization. It was not previously associated with OM, but has been

suggested to play a role in non-syndromic cleft lip with or without cleft palate in a GWAS among 1497 subjects (285 cases) of European descent (50). Subjects with a cleft palate have an increased risk of OME due to aberrant attachment of musculature of the Eustachian tube leading to its dysfunction (51).

Methylation is known to be associated with gene expression (52). One differentially methylated CpG with subthreshold association with AOM in the meta-analysis was strongly associated with gene expression of DNAJC15. DNAJC15 encodes an inner mitochondrial membrane protein with a role in mitochondrial protein import, mitochondrial complex assembly, and electron transport (53). In knock-out mice, it was shown that Dnajc15 serves as a regulator of the respiratory chain, and loss of expression leads to alterations in metabolism (54). In humans, although no data exist on middle ear mucosa, gene expression for DNAJC15 is found in lung tissue which resembles that of the upper airway and middle ear mucosa.

We performed exploratory network and enrichment analysis to facilitate functional interpretation of our top results. Most enriched gene ontology (GO) terms were related to biological processes, including lipid signaling, cell signaling, membrane trafficking, endocrine resistance, and interactions related to bacterial invasion of epithelial cells through neutrophil homeostasis. The role in neutrophil homeostasis through regulation of neutrophil apoptosis is essential in infectious diseases such as AOM (55). Neutrophils make up the majority of white blood cells and form an essential part of the innate immune system, which carries out part of the inflammatory response to bacterial infection. Neutrophils, in the middle ear and elsewhere in the human body, are the first line of host inflammatory cell response to infection, eliminating bacteria by phagocytosis (56).

Although this study comprised all available studies to date on AOM with epigenome-wide data and its sample size was thus as large as it could be, it was still limited. Therefore, it is possible that actual signals may have gone unnoticed due to insufficient statistical power. All studies on AOM used a parent-reported outcome such as earache with fever or fever and otorrhea. Parent-reported outcomes carry the risk of recall bias. However, previous studies have shown the diagnostic value of particularly earache, fever and otorrhea in AOM with a sensitivity and specificity of 71 and 80%, respectively (57-59). Otorrhea can be a symptom of otitis externa as well as AOM. One could argue that using otorrhea as a symptom to identify children with AOM could carry the risk of including children in the study that had, in fact, otitis externa instead of AOM. However, otitis externa is rare among children. Only 7% of children will experience an episode of otitis externa before the age of 4 years (60). Therefore, all available cohorts were included in the meta-analysis to maximize power to detect new associations. Homogeneity between all AOM cohorts was high and checked through leave-one-out analyses supporting the robustness of the reported results. Still, several probes among the top results mapped to DNA containing a SNP or a repetitive sequence element should be interpreted with some caution, as genetic effects may play a role. We used an independent cohort with data on OME as look-up study. OME and AOM

are interrelated but distinct phenotypes. OME is defined by fluid in the middle ear without acute signs of infection with conductive hearing loss as the principal symptom and may be asymptomatic in young children (61, 62). OME often occurs during the resolution of AOM once the acute inflammation has resolved but may also occur with dysfunction of the Eustachian tube in the absence of AOM (63, 64). It was therefore expected that some, but not all, signals might overlap between the two phenotypes.

Cord blood is easily accessible, which makes it ideal for large population studies such as birth cohorts. DNA methylation in cord blood may, however, not reflect DNA methylation levels in middle ear mucosa. Tissue obtained from middle ear mucosa is much more difficult to obtain, especially in population-based studies, for obvious reasons. However, AOM is an infectious disease with systemic manifestations characterized by increased inflammatory blood markers. We adjusted for estimated white blood cell counts using the Houseman method with the Reinius reference panel. No major alterations were found in our top results when comparing our complete model to one without adjustment for cell-type composition (Supplemental Table 5.3) (36). Since the analyses were completed, multiple cord blood-specific methods for white blood cell adjustment have been proposed (39, 65, 66). We compared the results of the main model adjusted for cell-type proportions estimated using the Houseman method with those adjusted using the cord blood-specific cell-type proportions within the Generation R Study, one of the largest participating studies. The results were very strongly correlated ($r = 0.97$), which is why we decided not to rerun the analyses in all cohorts. Residual confounding due to alterations in cell-type distribution cannot be ruled out completely after correction by either method. All participating cohorts used the Infinium HumanMethylation450 BeadChip, which has been shown to measure genome-wide DNA methylation reliably (67). This array nonetheless covers only 1.7% of all CpGs in the genome, leaving many CpGs unmeasured that may influence susceptibility to AOM (68).

We combined results from studies in different populations from different continents. Most subjects were of European descent; however, CBC provided two samples, one of which comprised subjects of Hispanic descent. Nevertheless, when we excluded this sample in the leave-one-out analysis, it did not significantly alter the results which increases the generalizability of our findings to non-European populations (Supplemental Figures 5.2a-j, Supplemental Table 5.4).

CONCLUSION

We did not find evidence to support an association between DNA methylation at birth and AOM in early childhood. Joint analysis of data on AOM and OME showed some potential differences in DNA methylation between cases and controls, which should be further explored using larger sample sizes.

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SUPPLEMENTARY MATERIAL

INDIVIDUAL COHORT AND METHOD DESCRIPTION

CBC

The California Department of Public Health maintains a repository of all neonatal birth bloods as blood dried on a filter paper (Guthrie card), which we term the California Birth Cohort (CBC). These are available for qualified researchers to perform specified health research as monitored by local and State level institutional review boards; all work here was performed under ethics approval at the State of California, the University of California, and the University of Southern California. Our current research project using CBC resources is a case-control study of childhood leukemia, the California Childhood Leukemia Study (CCLS) which identifies children with leukemia and matched controls (birthdate, gender, and ethnicity) from around the State of California (1). Control subjects were chosen as birthdate, gender, and ethnic matches to leukemia cases. Cases included all consecutive cases at 13 California hospitals which treat leukemia. Approximately 300-500 ng of high molecular weight DNA was extracted from a 1/4 section of a 1.5 cm^2 archived neonatal DBS (stored at -20°C from the time of birth) using Qiagen blood card extraction protocol and bisulfite treated using the EZ DNA Methylation-Direct™ Kit (Zymo). Genome-wide DNA methylation was then measured in these bisulfite-converted DNA samples using Illumina Infinium HumanMethylation450 BeadChip arrays.

Normalization and QC of DNA methylation data

CpG sites with detection p-values > 0.01 were defined as bad CpG sites and discarded. CpG sites with >15% of absence of information (i.e. >15% of total samples) were totally excluded from the analysis. A total of 540 CpGs were excluded. Samples with >15% of bad CpG sites (of the 450K loci) were also excluded from the analysis. The DNA methylation data preprocessing consisted of functional normalization according to Fortin et al. (2) to control for batch and position effects. Additional correction for probe types was accomplished with BMIQ normalization (3). Joo et al. have demonstrated that DNA methylation measured by the HM450k array on archived dried blood spots is fully correlated with DNA methylation measured by the same platform on same individuals matched frozen buffy coats (correlation coefficient = 0.99), therefore proving that this material is suitable for DNA methylation analyses (4). The DNA methylation data was normalized using functional normalization as implemented in the minfi package in R/Bioconductor. Poorly performing samples seen as those with low signal all across the array and those whose reported sex did not match that predicted were excluded. CpGs with detection p-values > 0.01 were defined as bad CpGs and discarded (replaced with "NA"). CpGs with >15% bad values (i.e. >15% of total samples) were totally excluded from the analysis. Samples with >15% bad CpGs (of the 450k

loci) were also excluded from the analysis. BMIQ normalization was then applied to adjust for the chip design type-2 bias (3).

Acute Otitis Media

Acute otitis media (AOM) was assessed by questionnaires of the mother as described in detail previously (5). Briefly, data on infectious illnesses a child had during the first year of life including ear infections, and data on covariates, were collected with an emphasis on the timing of exposure, specifically whether the child had the illness at the age of <3 months, 3–5 months, and/or 6–12 months.

Covariates

Adjustment covariates include maternal age (continuous), Parity (1, >1), maternal smoking assessed by questionnaire, family income, sex, gestational age, as well as batch correction.

Cell-types

Estimation of six different white blood cell-types (CD8+ T and CD4+ T lymphocytes, CD56+ natural killer cells, CD19+ B cells, CD14+ monocytes, and granulocytes) by Houseman method (6) was performed using the default implementation of the estimateCellCounts function in the minfi package (7).

EDEN

The EDEN (Etude des Déterminants pré et post natals du développement et de la santé de l♂Enfant) study is a prospective Birth Cohort Study (https://eden.vjf.inserm.fr/), which has been described in detail elsewhere (8). Pregnant women seen for a prenatal visit at the departments of Obstetrics and Gynecology of the University Hospital of Nancy and Poitiers before their twenty-fourth week of amenorrhea were invited to participate. Enrolment started in February 2003 in Poitiers and September 2003 in Nancy; it lasted 27 months in each centre. Among eligible women, 55% (n=2002) accepted to participate. The study has been approved by the ethical committees Comité Consultatif pour la Protection des Personnes dans la Recherche Biomédicale, Le Kremlin-Bicêtre University hospital, and Commission Nationale de l'Informatique et des Libertés. Immediately after delivery, cord blood samples were collected by research midwives from 1367 consenting cohort participants. To prevent any contamination with maternal blood, the cord was doubly clamped immediately after birth (vaginal delivery) or after extraction of the fetus through the uterine incision (elective cesarean section); repeatedly rinsed and venous cord blood serum was sampled between the 2 clamps. Samples were centrifuged within 24 hours of collection. The serum was separated, and samples were stored at −80°C.

Normalization and QC of DNA methylation data

DNA was extracted from 162 cord blood samples using the QIAamp blood kit (Qiagen or equivalent protocols), followed by precipitation-based concentration using GlycoBlue (Ambion). DNA concentration was determined by Nanodrop measurement and Picogreen quantification. 500 ng of DNA was bisulphite-converted using the EZ 96-DNA methylation kit (Zymo Research), following the manufacturer's standard protocol. After verification of the bisulphite conversion step using Sanger Sequencing, genome-wide DNA methylation was measured using the Illumina Infinium HumanMethylation450 BeadChip. After normalization of the concentration, the samples were randomized to avoid batch effects, and all paired samples were hybridized on the same chip. Standard male and female DNA samples were included in this step as control samples. In total, 439,306 CpGs are available in children with DNA measurements.

Acute otitis media

The occurrence of otitis was defined in EDEN based on the answers to parental questionnaires administered when the child was 4months, 8 months, 12 months, 24 months and 3 years of age. In each of the questionnaires, the parents were asked: "[In the last 4 months]/[Since the age of [1 or 2years]], has your children had otitis?" [yes/no]. A child was classified as a case of otitis if they had otitis during any of the questionnaires, as compared to controls, who were never reported to have had otitis in any of the questionnaires.

Covariates

Socioeconomic Status: Maternal socioeconomic class based on self-reported family income ("high" if more than 3000EUR, "middle" if between 1500 and 3000EUR, "low" if lower than 1500EUR). Ancestry: EDEN DNA methylation data were collected only on Caucasian children born to French-speaking mothers in the cities of Poitier and Nancy (France).

Cell-types

Estimation of six different white blood cell-types (CD8+ T and CD4+ T lymphocytes, natural killer cells, B cells, monocytes, and granulocytes) by Houseman method was performed using the default implementation of the estimate CellCounts function in the minfi package (6, 7).

The Generation R Study

The Generation R Study is a population-based prospective cohort study from fetal life onwards in Rotterdam, the Netherlands (9, 10). Assessments in pregnant women and children consisted of physical examinations, fetal ultrasounds, biological samples, and questionnaires. All children were born between April 2002 and January 2006. The study has been approved by the Medical Ethical Committee of the Erasmus University Medical

Center (MEC-2007-413-NL21545.078) and written consent was obtained from participating parents of their children.

Normalization and QC of DNA methylation data

DNA extracted (using the salting-out method) from cord blood from 979 Caucasian children from the Generation R focus cohort was used for this analysis. 500 ng DNA per sample underwent bisulfite conversion using the EZ-96 DNA Methylation kit (Shallow) (Zymo Research Corporation, Irvine, USA). Samples were plated onto 96-well plates in no specific order. Samples were processed with the Illumina Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, USA), which analyses DNA methylation at 485,577 CpG sites. Quality control of analyzed samples was performed using standardized criteria. Samples were excluded in case of low sample call rate (<99%, 6 samples excluded), color balance >3 (no samples excluded), low staining efficiency (no samples excluded), poor extension efficiency (no samples excluded), poor hybridization performance (no samples excluded), low stripping efficiency after extension (no samples excluded) and poor bisulfite conversion (1 sample removed). In addition, 2 samples were excluded because of a gender mismatch and 1 sample was excluded because of a retracted informed consent, leaving a total of 969 Generation R samples in the analysis. Probes with a single nucleotide polymorphism in the single base extension site with a frequency of > 1% in the GoNLv4 reference panel were excluded, as were probes with non-optimal binding (non-mapping or mapping multiple times to either the normal or the bisulphite-converted genome), resulting in the exclusion of 49,564 probes, leaving a total of 436,013 probes in the analysis. We ran DASES normalization using a pipeline adapted from that developed by Touleimat and Tost (11-13). DASES normalization includes background adjustment, between-array normalization applied to type I and type II probes separately, and dye bias correction applied to type I and type II probes separately and is based on the DASEN method described by Pidsley et al., but adds the dye bias correction, which is not included in DASEN (14). Beta-values were calculated for all CpG sites.

Acute otitis media

The following outcomes were collected through parental questionnaires: episodes of otorrhea, earache with fever, and use of eardrops prescribed by family practitioner or ear, nose and throat (ENT) surgeon. AOM was defined using these outcomes. Questionnaires were administered at the children's ages of 2, 6, 12, 24, 36, 48 and 66 months. For the current study, data was available for 773 European-ancestry mothers and their children.

Covariates

Information on maternal age, educational level as a measure of socioeconomic status, smoking during pregnancy, and parity was collected by questionnaires at enrollment. Ma-

ternal educational level was categorized as lower (none, primary or secondary education) or higher (more than secondary education). Information on maternal smoking at enrolment was combined with information on maternal smoking thereafter obtained by multiple questionnaires during pregnancy, and combined (no; yes). Parity was categorized as nulliparity or multiparity.

Cell-types

Cell-type correction was applied using the reference-based Houseman method in the minfi package in R (6, 7). This method estimates the relative proportions of six white blood cell subtypes (CD4+ T-lymphocytes, CD8+ T-lymphocytes, NK (natural killer) cells, B-lymphocytes, monocytes and granulocytes), based on a standard reference population. Recently, a cord blood-specific reference panel for cell-type correction was published, with betas that were calculated using estimated white blood cell proportions using the IDOL library selection by Gervin & Salas (15). In order to perform sensitivity analysis, we compared betas after cell-type correction using Houseman reference panel with betas after correction with the cord blood-specific reference panel (Supplemental Figure 1).

INMA

The INMA—INfancia y Medio Ambiente—(Environment and Childhood) Project is a network of birth cohorts in Spain that aim to study the role of environmental pollutants in the air, water and diet during pregnancy and early childhood in relation to child growth and development (http://www.proyectoinma.org/) (16). The Ethical Committees approved the study of each participating center, and written consent was obtained from participating parents. For this study we used data from 175 children participating in the INMA Sabadell subcohort.

Normalization and QC of DNA methylation data

Cord blood at birth was obtained in EDTA tubes and extracted using the Chemagic DNA Blood Kits (Perkin Elmer) in a Chemagen Magnetic Separation Module 1 station at the Spanish National Genotyping Center (CEGEN, http://www.usc.es/cegen/). DNA concentration was determined by NanoDrop Spectrophotometer (Thermo Scientific) measurement and Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) quantification. Some of the samples underwent a precipitation-based concentration and purification using GlycoBlue (Ambion) to uniform the concentration and purity. After normalization of the concentration, the samples were randomized to reduce batch effects. Standard male and female DNA samples were included in this step as internal controls. Samples were processed with the Infinium HumanMethylation450 BeadChip at the Genome Analysis Facility of the University Medical Center Groningen (UMCG) in Holland as part of the MeDALL project (Mechanisms of the Development of ALLergy). 500 ng of DNA of each sample were bisulfite-converted

using the EZ 96-DNA-methylation kit following the manufacturer's standard protocol. After verification of the bisulfite conversion using Sanger Sequencing, the DNA methylation was measured using the Illumina Infinium HumanMethylation450 BeadChip. IDAT files were preprocessed imported using minfi (7). The preprocessing steps were: 1) sample filtering to remove bad quality and mixed up samples, 2) 65 SNPs probes, the probes on sex chromosomes, potential crossreactive probes, and probes 6 containing SNPs at the target CpG sites with a MAF>10% (polymorphic) were excluded (crossreactive and polymorphic probes) (17). A total of 439,306 CpG probes were retained for downstream analyses, 3) Signal correction and normalization was performed using "DASEN", 4) batch correction was attained, including the significant (permutation p-value < 10^{-4}) principal components derived from the 613 negative control probes presented in 450K arrays (14). Twelve samples were excluded because they were outliers defined as more than 3 SD from the mean for PC1 or PC2, or there were sex discrepancies. The final sample size for this analysis was 175 children with information on AOM. After 10,000 permutations, five PCs were retained. The beta-values were batch corrected, incorporating these five PCs and calculating the residuals of the linear model. These residuals were used to test their association with lung function.

Acute otitis media

Mothers answered four independent questionnaires at different ages of the child: 6 months, 14 months, 2.5 years, and 4 years. Questions included the age of a child when he/she had the first episode of otitis, whether doctor assistance was required, and whether the doctor was visited in primary care or in hospital, and finally, the number of episodes of otitis. According to the answers, subjects were classified into three groups: control group – subjects with no medical history of otitis media (OM); AOM; recurrent AOM – subjects who answered >3 episodes of otitis in 12 months on questionnaires at 2.5 and 4 years of age.

Covariates

Information on maternal age, socioeconomic status, smoking during pregnancy, and parity was collected by questionnaire during pregnancy. Maternal education was based on the maternal occupation at pregnancy and categorized into three levels: low (semi-skilled/unskilled occupations), medium (skilled manual/non-manual), or high (managers/technicians). Pregnant women were asked whether they were current smokers (at week 32 of pregnancy) and, if so, how much. They were also asked if they had stopped smoking due to pregnancy and when (before pregnancy or at what month of pregnancy). Any smoking was defined as smoking any number of cigarettes at any time during pregnancy. Parity was categorized into 0 or ≥1.

Cell-types

We used the estimateCellCounts function from minfi, which estimates a constrained projection using constrained projection/quadratic programming according to Houseman et al. (6). We estimated six white blood cell-types (CD4+ T-lymphocytes, CD8+ T-lymphocytes, NK (natural killer) cells, B-lymphocytes, monocytes, and granulocytes) using the Reinius et al. reference (18).

Gene Expression

Gene expression data were collected at 4 years of age. Whole blood was collected in PAXGene tubes and extracted using the kit recommended by the company. All samples had an RNA Integrity Number (RIN) higher than 7. Gene expression data were obtained using Affymetrix HTA 2.0 at the European Institute for Systems Biology and Medicine in Lyon as part of the MeDALL project. Gene expression was normalized with the RMA algorithm using the Expression Console Software from Affymetrix, and probes were clustered to the transcript level. Expression transcripts were annotated using version 35 of Affymetrix annotation. The final sample size was 110 children for these analyses. For the DNA methylation vs. gene expression analysis, only CpGs with subthreshold association ($p<1.0x10^{-4}$) from the meta-analysis in newborns were tested. Of the 19 CpGs, only 18 passed the quality control and were analyzed. To control for technical variation in the DNA methylation dataset, a principal component analysis of 600 negative control probes using 10,000 permutations was performed, and the residuals of a linear regression model using the first five PCs were estimated. Next, the effect of sex and Houseman cell proportions estimates were controlled out in a second-stage linear regression model. Only transcripts inside a 500 kb window of selected CpG sites (250 kb downstream and 250 kb upstream) were considered in the analysis (N=5,568 transcripts). To control for technical and unwanted biological variation when estimating gene expression residuals, a model was applied in which sex and six cell estimates obtained from the DNA methylation values using the Houseman algorithm were regressed out (6). A second model was tried adjusted for surrogate variables added to sex and cell estimates, using the sva R package to estimate surrogate variables. As the dataset was too small, surrogate variables could not be estimated, and this model was dropped. Finally, linear models of residuals of gene expression vs. residuals of DNA methylation were performed. The associations between DNA methylation 4 years vs. gene expression 4 years were tested. All the analyses were performed with R.

MoBa 1 & MoBa 2

Participants in the current analysis represent two subsets of mother-offspring pairs from the Norwegian Mother, Father and Child Cohort Study (MoBa) (19-21). MoBa is a prospective population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health (19). Participants were recruited from all over Norway from 1999-2008. The women

consented to participation in 41% of the pregnancies. The cohort now includes 114.500 children, 95.200 mothers and 75.200 fathers. Blood samples were obtained from both parents during pregnancy and from mothers and children (umbilical cord) at birth (20). The establishment and data collection in MoBa was previously based on a license from the Norwegian Data protection agency and approval from The Regional Committee for Medical Research Ethics, and it is now based on regulations related to the Norwegian Health Registry Act. The current study was approved by The Regional Committee for Medical Research Ethics (REK 2017/1934-1).

Each subset in the current study is referred to here as MoBa1 and MoBa2. MoBa1 and MoBa2 study populations were part of a larger study within MoBa that was designed to evaluate the association between maternal plasma folate during pregnancy and childhood asthma status at 3 years of age (22). The current analyses include the children who had cord blood DNA methylation measurements and covariate data (N=921 MoBa1; N=416 MoBa2), and each dataset was analyzed independently. The year of birth for participants in these subsets ranged from 2000-2009, and the studies have their own approvals from the Regional Committee for Ethics in Medical Research, Norway and were approved by the Institutional Review Board of the National Institute of Environmental Health Sciences, USA.

Normalization and QC of DNA methylation data

Details of the DNA methylation measurements and quality control for the MoBa1 participants were previously described and the same protocol was implemented for the MoBa2 participants (23). Briefly, umbilical cord blood samples were collected and frozen at birth at -80°C in line with the procedures at the Biobank of MoBa (20). Bisulfite conversion was performed using the EZ-96 DNA Methylation kit (Zymo Research Corporation, Irvine, CA) and DNA methylation was measured at 485,577 CpGs in cord blood using Illumina's Infinium HumanMethylation450 BeadChip (24). Raw intensity (.idat) files were handled in R using the minfi package to calculate the DNA methylation level at each CpG as the beta-value (β=intensity of the methylated allele (M)/(intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100)) and the data was exported for quality control and processing. Probe and sample-specific quality control was performed in the MoBa1 and MoBa2 datasets separately. Similar protocols were applied to both data sets, as follows: Control probes (N=65) and probes on X (N=11,230) and Y (N=416) chromosomes were excluded in both datasets. Remaining CpGs missing > 10% of DNA methylation data were also removed (N=20 in MoBa1, none in MoBa2). Samples indicated by Illumina to have failed or have an average detection p-value across all probes < 0.05 (N=49 MoBa1, N=35 MoBa2) and samples with gender mismatch (N=13 MoBa1, N=8 MoBa2) were also removed. For MoBa1 and MoBa2, we accounted for the two different probe designs by applying the intra-array normalization strategy Beta Mixture Quantile dilation (BMIQ)(3). The Empirical Bayes method via *ComBat* was applied separately in each dataset for batch

correction using the sva package in R (25, 26). Beta-values were calculated for all CpG sites. Batch effects were corrected by surrogate variable analysis (sva).

Acute Otitis media

AOM was classified based on maternal report from questionnaires when the child was 6, 18 and 36 months. Mothers were asked whether the child had experienced an ear infection between birth and 6 months (6 months questionnaire), 6 and 18 months (18 months questionnaire), 18 and 36 months (36 months questionnaire). The outcome in the analysis was maternal report of any ear infection the first 36 months of life.

Covariates

For both datasets, information on maternal age, parity, gestational age, child sex, maternal education, and smoking was collected via questionnaires completed by the mother or from birth registry records as previously described (23). Maternal age was included as a continuous variable. Parity was categorized as 0, or ≥ 1 births. Maternal educational level was categorized into four groups based on years of education: less than high school/secondary school, high school/secondary school completion, some college or university, or 4 years of college/university or more. Maternal smoking status during pregnancy was classified into two groups: non-smoker, any smoking in pregnancy.

Cell-types

Cell-type correction was applied using the reference-based Houseman method in the minfi package in R. This method estimates the relative proportions of seven white blood cell subtypes ($CD4^+$ T cells, $CD8^+$ T cells, NK cells, B cells, monocytes, granulocytes, and nRBC), based on a standard reference population.

ALSPAC (Look-up Cohort)

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a general population pregnancy cohort study that initially recruited 14 541 pregnancies with a due date between April 1991 and December 1992 in Avon, UK (27, 28). Of these initial pregnancies, there was a total of 14,676 foetuses, resulting in 14,062 live births and 13,988 children who were alive at 1 year of age. Written informed consent has been obtained for all ALSPAC participants. Please note that the study website contains details of all the data that is available through a fully searchable data dictionary (http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/). Ethical approval was granted from the ALSPAC Law and Ethics Committee and the local Research Ethics Committees. Blood samples were collected from all consenting ALSPAC mothers and their offspring at several clinics at different time points. DNA methylation data for a sub-set of 1018 mother-offspring pairs was generated as part of the Accessible Resource for Integrated Epigenomics Studies (ARIES) project (http://www.

ariesepigenomics.org.uk/) (29). ARIES participants were selected based on availability of DNA samples at two time-points for the mother (antenatal and at follow-up when the offspring were mean age 17.1 years) and three time-points for the offspring (neonatal, childhood, mean age 7.5 and adolescence, mean age 17.1 years).

Normalization and QC of DNA methylation data

Cord or peripheral blood (whole blood or buffy coats) were collected according to standard procedures, spun and frozen at -80˚C. DNA methylation analysis and data pre-processing were performed at the University of Bristol as part of the ARIES project (ariesepigenomics.org.uk). Following extraction, DNA was bisulfite converted using the Zymo EZ DNA MethylationTM kit (Zymo, Irvine, CA). Following conversion, the genome-wide methylation status of over 485,000 CpG sites was measured using the Illumina Infinium® HumanMethylation450 BeadChip assay according to the standard protocol. The arrays were scanned using an Illumina iScan and initial quality review was assessed using GenomeStudio (version 2011.1). The level of DNA methylation is expressed as a "Beta" value (β-value), ranging from 0 (no cytosine methylation) to 1 (complete cytosine methylation). Samples from all time-points in ARIES were distributed across slides using a semi-random approach (sampling criteria were in place to ensure that all time-points were represented on each array) to minimize the possibility of confounding by batch effects. Samples failing quality control (average probe detection p-value ≥ 0.01) were repeated. As an additional quality control step genotype probes on the HumanMethylation450 were compared between samples from the same individual and against SNP-chip data to identify and remove any sample mismatches. Data were pre-processed in R (version 3.0.1) with the WateRmelon package according to the subset quantile normalization approach described by Touleimat & Tost in an attempt to reduce the non-biological differences between probes (11, 14). We removed probes that had a detection p-value >0.05 for >5% of samples (3034 probes), probes on the X or Y chromosomes and SNPs (rs probes). 471192 probes remained. For each probe, beta values were trimmed using the agreed IQR3 approach.

Otitis Media with Effusion

All the children had a measure of their middle ear function using tympanometry to determine whether otitis media with effusion (OME) ('glue ear') was present. Tympanometry was always preceded by video otoscopy. The probe of a GSI 38 tympanometer, placed at the entrance to the ear canal, measured the middle ear compliance, middle-ear pressure and ear canal volume. Staff graded the resultant tympanogram according to whether it had a normal peak, a flat shape, an abnormal shape, or whether there was a grommet or perforation present. The tympanogram was categorized after the test visit into the following groups: Type A (Normal peak, Middle ear pressure of +100 to –100 daPa. Middle ear compliance >0.00 ml. Normal middle ear function) Type B (flat trace, eardrum immobile, indicates presence

of OME), Type C1 (middle ear pressure of -101 to -200 daPa, normal peak, middle ear compliance >0.00ml, indicates slight Eustachian tube dysfunction), Type C2 (middle ear pressure <200 daPa, normal peak, middle ear compliance >0.00ml, indicates Eustachian tube dysfunction. Impossible to tell from tympanometry alone whether ear is recovering from OME or fluid is starting to build up). Grommet (grommet observed on otoscopy, flat trace, grommet in-situ. large ear canal volume indicates the grommet is patent), Perforation (perforation observed on otoscopy or reported by parents, flat trace, perforation of ear drum). These gradings were made for each ear. For the purposes of this study, I derived an "OME" variable for each time point when tympanometry was performed, up to the age of 9 (8mths, 12mths, 18mths, 25mths, 31mths, 37mths, 43mths, 49mths, 61mths, 7yrs, 9yrs). OME cases were children who had been graded Type B in one or both ears. OME controls were children who had tympanometry but had not been graded as Type B in either ear (they could be type A, type C1 or type C2). Children with grommet in-situ or perforation were excluded. OME variables for each time point were combined to give an "OME_ever" variable that defined whether a child had OME at any of the clinics. Controls were those children who had no OME at any clinic. This variable was used as the OM (combined) phenotype. We did not have data on AOM, and every child who had tympanostomy had OME at least once. There were 150 combined OM cases and 661 controls with DNA methylation data and complete data on all covariates.

Covariates

Gestational age was calculated (in days) based on the date of the mother's last menstrual period (LMP) where the mother was certain of this, but for uncertain LMPs and conflicts with clinical assessment the ultrasound assessment was used. Where maternal report and ultrasound conflicted, an experienced obstetrician reviewed the clinical records and made a best estimate. Maternal age at delivery was derived from the mother's report of her own and her baby's dates of birth. Maternal social class was classified for this study as "attended university" or "did not attend university". Parity was extracted from medical records and categorized for this study as nulliparous or parous; maternal smoking behavior was assessed during pregnancy via questionnaire and categorized for this study as 1) never smoking during pregnancy, 2) smoking before pregnancy or in the first trimester and then stopping, or 3) smoking throughout pregnancy. We excluded all multiple births (i.e. we kept only singleton pregnancies). We excluded all pregnancies in which the child was not white race (the vast majority of ARIES participants are white and we did not have large enough numbers of other races to analyze separately).

Cell-types

Cell-type proportions were estimated using the estimateCellCounts function in the minfi R package which is based on the method developed by Houseman (6, 7). This estimated the

proportion of B-cells, CD8 T-cells, CD4 T-cells, granulocytes, NK-cells and monocytes in each sample.

Adjustment for batch

During the data generation process a wide range of batch variables were recorded in a purpose-built laboratory information management system (LIMS). The LIMS also reported QC metrics from the standard control probes on the HumanMethylation450 BeadChip for each sample back to the laboratory. Of all measured batch variables, bisulfite conversion batch (96-well plate) was identified as by far the most influential on the ARIES HumanMethylation450 data. Slide level batch adjustment is less useful as each slide will only contain a small number of samples for each time point, additionally allocation to bisulfite conversion batch is more likely to contain systematic bias because samples were added to the batch according to lab priorities and convenience. However, running models with bisulfite conversion batch included as a factor variable (as is appropriate) caused non-singular fit errors (due to small batches), so we instead adjusted for batch by including in all models several surrogate variables generated using the sva() function in the sva R package (26). These variables were generated separately for each exposure. 10 SVs were generated and then those that were associated with OM were discarded. The remainder (seven SVs) were included in models as covariates. The goal of sva is to remove all unwanted sources of variation while protecting the contrasts due to traits and covariates of interest, so cell counts were included when generating SVs regardless of whether the EWAS was adjusted for cell counts. This way, the SVs will not be based on cell counts, so adjusting for cell counts should still have an effect.

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EDEN

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Generation R Study

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MoBa 1 & MoBa 2

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ALSPAC (Look-up Cohort)

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SUPPLEMENTAL TABLES & FIGURES

Supplemental Table 5.1: Characteristics of covariates per cohort.

	Male sex % (n)	Maternal age in years (SD)	Maternal SES % (n)			Maternal smoking during pregnancy % (n)	Maternal nulliparity % (n)	Gestational age in weeks (SD)
			Low	Middle	High			
CBC – Hispanic	58.1 (100)	27.4 (5.9)	52.3 (90)	27.9 (48)	19.8 (34)	8.1 (14)	40.7 (70)	39.2 (2.5)
– Caucasian	56.9 (107)	32.0 (5.6)	14.4 (27)	20.7 (39)	65.0 (122)	6.4 (12)	39.9 (75)	39.0 (2.4)
EDEN	60.8 (90)	30.2 (5.0)	21.6 (32)	13.5 (20)	47.3 (70)	21.6 (32)	41.9 (62)	39.4 (1.5)
Generation R	52.4 (773)	31.5 (4.0)	34.7 (268)	N/A	65.3 (505)	23.2 (179)	59.8 (462)	40.2 (1.4)
INMA	50.3 (88)	30.6 (4.2)	20.0 (35)	33.1 (58)	46.9 (82)	24.0 (42)	51.4 (90)	39.8 (1.3)
MoBa1	54.9 (506)	29.9 (4.3)	7.4 (68)	32.4 (298)	60.3 (555)	28.8 (265)	40.9 (377)	39.9 (1.6)
MoBa2	54.6 (227)	30.1 (4.4)	2.7 (86)	14.4 (60)	64.9 (270)	24.8 (103)	40.4 (168)	39.4 (1.6)
ALSPAC (Look-up cohort)	48.8 (396)	29.6 (4.4)	78.9 (640)	N/A	21.1 (171)	12.6 (102)	81.5 (661)	39.6 (1.5)

Supplemental Table 5.2: Look-up of FNDC1-related CpGs in meta-analysis of EWAS on AOM.

CpG	Chr	Bp position	Location in gene	Relation to UCSC CpG Island	OR	p-value	Heterogeneity (I^2)	Flagged probe[1]
cg02808397	6	159,656,576	Body	S-Shore	0.51	0.09	26.6	-
cg27118080	6	159,618,364	Body	Open Sea	0.91	0.09	0	SNP at CpG
cg18426860	6	159,592,134	Body	S-Shore	0.86	0.18	18.4	-
cg13181026	6	159,692,484	3'UTR	Open Sea	0.84	0.20	0	-
cg17975258	6	159,639,330	Body	Open Sea	1.11	0.21	0	-
cg02212846	6	159,639,257	Body	Open Sea	0.83	0.22	36.9	-
cg11944359	6	159,653,199	Body	N-Shore	1.45	0.22	0	-
cg01539510	6	159,650,954	Body	N-Shelf	0.86	0.24	0	-
cg01264126	6	159,653,488	Body	Island	0.86	0.27	0	-
cg08397344	6	159,589,874	TSS1500	Island	1.16	0.37	5.4	-
cg07917909	6	159,591,467	Body	S-Shore	1.24	0.38	0	-
cg09375620	6	159,590,155	TSS1500	Island	1.42	0.38	20.6	-
cg20951350	6	159,592,466	Body	S-Shore	0.93	0.40	54.7	-
cg21027599	6	159,639,549	Body	Open Sea	1.14	0.45	0	-
cg09107912	6	159,590,059	TSS1500	Island	0.72	0.46	0	-
cg07739841	6	159,657,657	Body	S-Shelf	0.93	0.47	0	Repeat sequence
cg01037362	6	159,660,806	Body	Open Sea	1.11	0.47	0	Repeat sequence
cg01907051	6	159,591,121	Body	Island	1.16	0.48	16.4	-
cg14500486	6	159,655,392	Body	Island	0.96	0.49	0	-
cg00219282	6	159,654,898	Body	Island	1.11	0.62	31.3	-
cg05678571	6	159,660,813	Body	Open Sea	1.08	0.62	27.3	Repeat sequence
cg00157796	6	159,590,231	TSS200	Island	0.78	0.66	0	-
cg16363238	6	159,618,265	Body	Open Sea	0.95	0.67	0	-
cg10129085	6	159,655,941	Body	S-Shore	0.89	0.68	19.9	-
cg05015938	6	159,595,260	Body	S-Shelf	1.03	0.70	49.3	-
cg08631819	6	159,589,483	TSS1500	N-Shore	1.03	0.73	0	-
cg14971597	6	159,590,578	1stExon; 5'UTR	Island	0.92	0.81	32.0	-
cg06764804	6	159,654,028	Body	Island	1.03	0.81	34.3	-
cg10617739	6	159,624,611	Body	Open Sea	1.05	0.89	0	INDEL, SNP at CpG
cg05730027	6	159,654,415	Body	Island	1.01	0.92	25.7	-

Analysis was adjusted for maternal age, maternal educational level, maternal smoking during pregnancy, parity (multiparous versus nulliparous), and gestational age at birth, sex, technical covariates and estimated white blood cell proportions. Odds ratios (OR) are presented per 10% increase in DNA methylation level. Location in gene: location of the CpG in the epigenome. N-shelf: North shelf. N-Shore: North shore. S-Shore: South shore. Flagged probe: if probe mapped to DNA containing a SNP, to repetitive sequence elements, or to DNA harboring an INDEL.

Supplemental Table 5.3: Results from meta-analysis of EWAS on AOM comparing covariate-adjusted and completely-adjusted model including cell-type composition ($p<1.0x10^{-4}$).

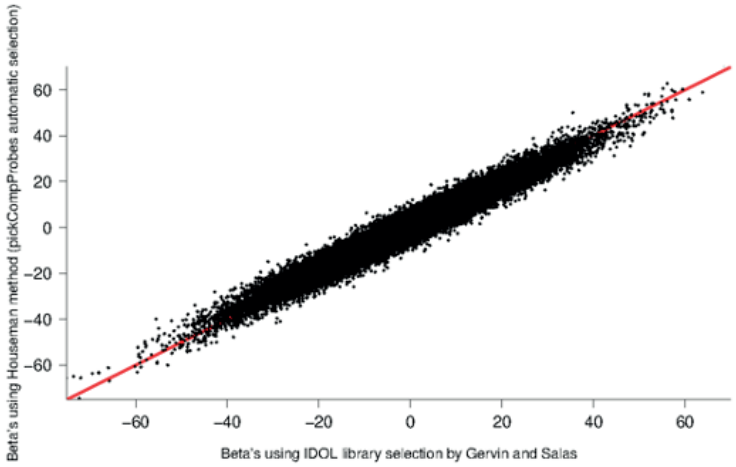
CpG	Closest gene	Adjusted model *AOM ~ CpG + cov*		Complete model *AOM ~ CpG + cov + cell*	
		OR	**p-value**	**OR**	**p-value**
cg26638266	*VIT*	1.51	1.65×10^{-7}	1.51	2.24×10^{-6}
cg04373106	*OTOP3*	2.02	1.06×10^{-6}	1.99	4.91×10^{-6}
cg20172563	*CD2AP*	1.85	1.23×10^{-4}	2.10	2.05×10^{-5}
cg27518631	-	1.62	7.30×10^{-6}	1.79	2.22×10^{-5}
cg06242243	*NCOR2*	2.03	4.16×10^{-5}	2.26	2.67×10^{-5}
cg21040575	*MIR497; MIR195*	1.48	5.39×10^{-6}	1.47	2.78×10^{-5}
cg00352106	*MACF1*	1.68	9.70×10^{-6}	1.92	3.03×10^{-5}
cg13077745	*GPR44*	2.08	1.77×10^{-6}	2.12	3.52×10^{-5}
cg02576892	-	3.25	1.13×10^{-5}	3.34	3.59×10^{-5}
cg26782169	-	2.61	3.51×10^{-6}	2.54	4.25×10^{-5}
cg10009875	*CEP104*	0.53	1.03×10^{-4}	0.44	5.03×10^{-5}
cg11201229	*PIK3C2A*	0.33	3.20×10^{-4}	0.26	5.10×10^{-5}
cg20184247	*HOXB-AS3; HOXB5*	1.63	1.30×10^{-4}	1.73	7.49×10^{-5}
cg09677945	*DNAJC15*	0.78	1.44×10^{-4}	0.77	7.90×10^{-5}
cg14157549	-	1.43	1.18×10^{-4}	1.47	7.95×10^{-5}
cg13644715	*PTGIR*	1.82	7.35×10^{-5}	1.86	8.03×10^{-5}
cg06780777	-	2.53	1.85×10^{-5}	2.45	8.07×10^{-5}
cg06838215	*SH3PXD2A*	2.26	5.29×10^{-5}	2.22	8.94×10^{-5}
cg04561120	-	0.86	0.0244	0.74	9.62×10^{-5}

Cov: covariates, knowing maternal age, maternal educational level, maternal smoking during pregnancy, parity (multiparous versus nulliparous), gestational age, plate. Cell: cell-type composition. Odds ratios (OR) are presented per 10% increase in DNA methylation level.

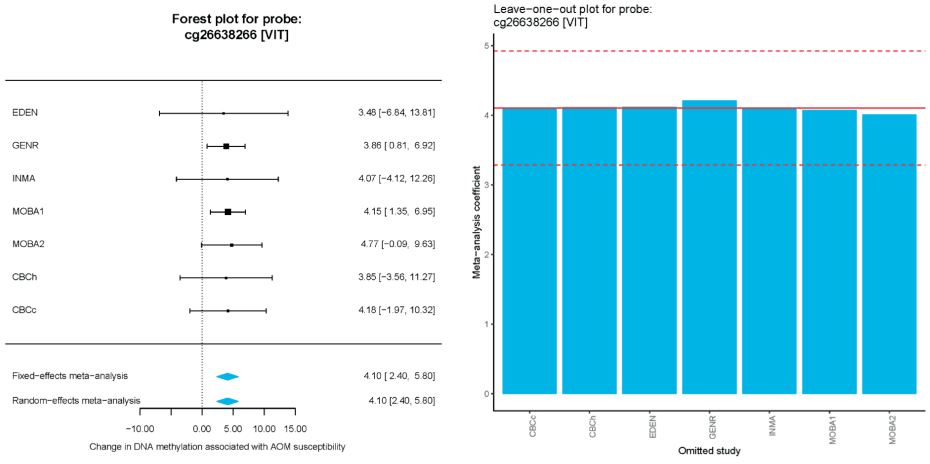
Supplemental Table 5.4: Results from meta-analysis of EWAS on AOM comparing studies with subjects of European descent (excluding CBC Hispanic) with all studies (including CBC Hispanic).

CpG	Closest gene	All studies *Including CBC Hispanic*		Studies of European Descent *Excluding CBC Hispanic*	
		OR	**p-value**	**OR**	**p-value**
cg26638266	*VIT*	1.51	2.24×10^{-6}	1.51	3.85×10^{-6}
cg04373106	*OTOP3*	1.99	4.91×10^{-6}	2.05	5.62×10^{-6}
cg20172563	*CD2AP*	2.10	2.05×10^{-5}	2.17	2.42×10^{-5}
cg27518631	-	1.79	2.22×10^{-5}	1.72	1.32×10^{-4}
cg06242243	*NCOR2*	2.26	2.67×10^{-5}	2.22	7.94×10^{-5}
cg21040575	*MIR497; MIR195*	1.47	2.78×10^{-5}	1.42	2.56×10^{-4}
cg00352106	*MACF1*	1.92	3.03×10^{-5}	1.80	3.53×10^{-4}
cg13077745	*GPR44*	2.12	3.52×10^{-5}	2.07	1.19×10^{-4}
cg02576892	-	3.34	3.59×10^{-5}	3.93	7.38×10^{-6}
cg26782169	-	2.54	4.25×10^{-5}	2.43	1.49×10^{-4}
cg10009875	*CEP104*	0.44	5.03×10^{-5}	0.42	6.20×10^{-5}
cg11201229	*PIK3C2A*	0.26	5.10×10^{-5}	0.25	7.39×10^{-5}
cg20184247	*HOXB-AS3; HOXB5*	1.73	7.49×10^{-5}	1.72	1.91×10^{-4}
cg09677945	*DNAJC15*	0.77	7.90×10^{-5}	0.78	3.09×10^{-4}
cg14157549	-	1.47	7.95×10^{-5}	1.49	8.34×10^{-5}
cg13644715	*PTGIR*	1.86	8.03×10^{-5}	1.92	7.43×10^{-5}
cg06780777	-	2.45	8.07×10^{-5}	2.23	6.69×10^{-4}
cg06838215	*SH3PXD2A*	2.22	8.94×10^{-5}	2.25	9.48×10^{-5}
cg04561120	-	0.74	9.62×10^{-5}	0.76	6.96×10^{-4}

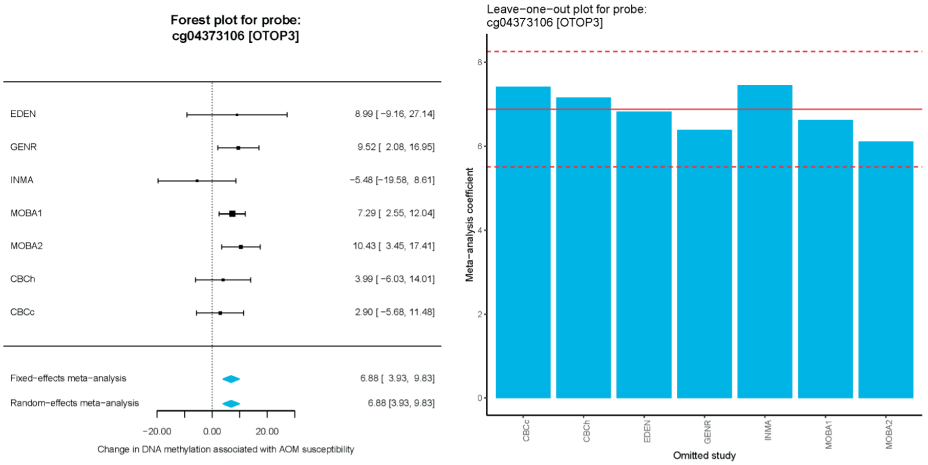
Analysis was adjusted for maternal age, maternal educational level, maternal smoking during pregnancy, parity (multiparous versus nulliparous), and gestational age at birth, sex, technical covariates and estimated white blood cell proportions. Odds ratios (OR) are presented per 10% increase in DNA methylation level.



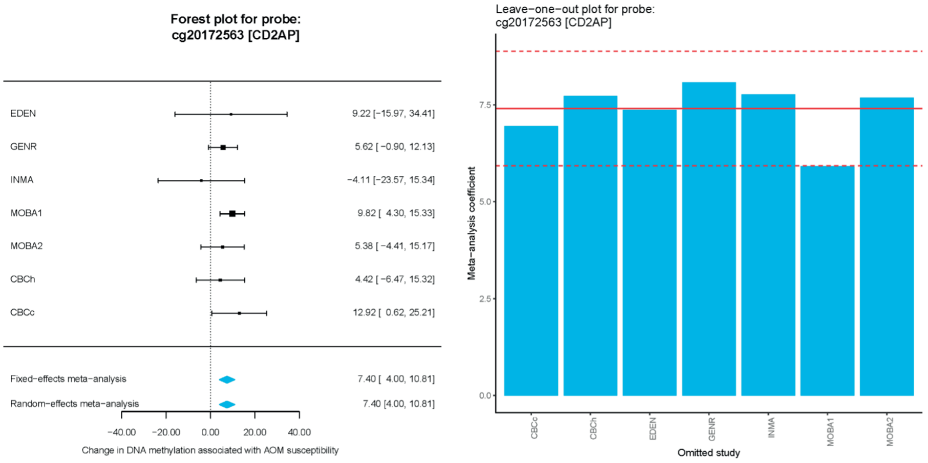
Supplemental Figure 5.1: Comparing betas in the Generation R study using two reference methods for estimating cell proportions from DNA methylation data: Houseman method versus Gervin & Salas method.



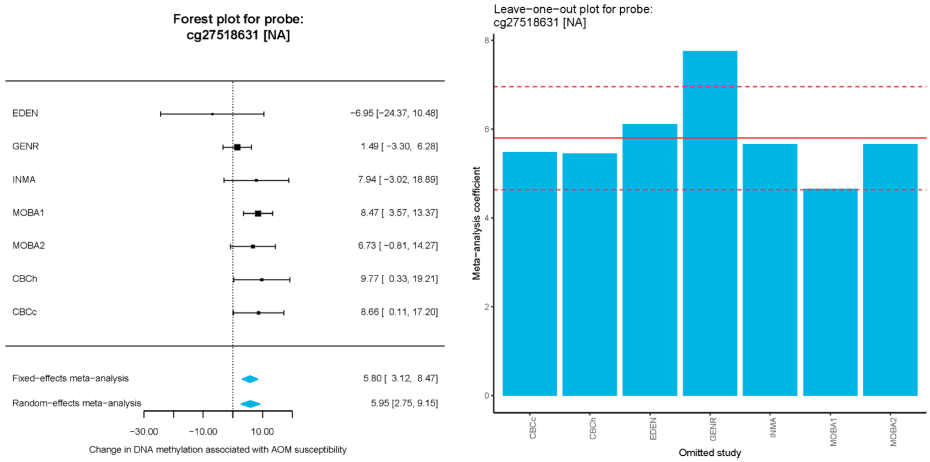
Supplemental Figure 5.2a: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg26638266 (*VIT*).



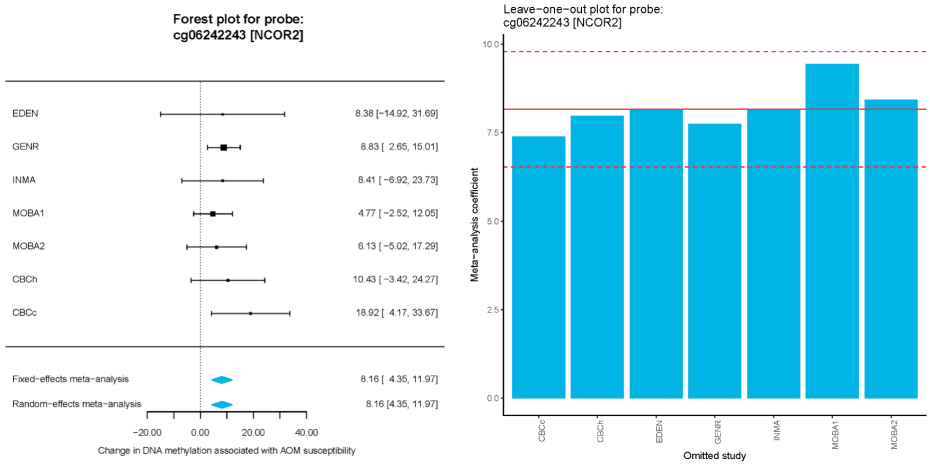
Supplemental Figure 5.2b: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg04373106 (*OTOP3*).



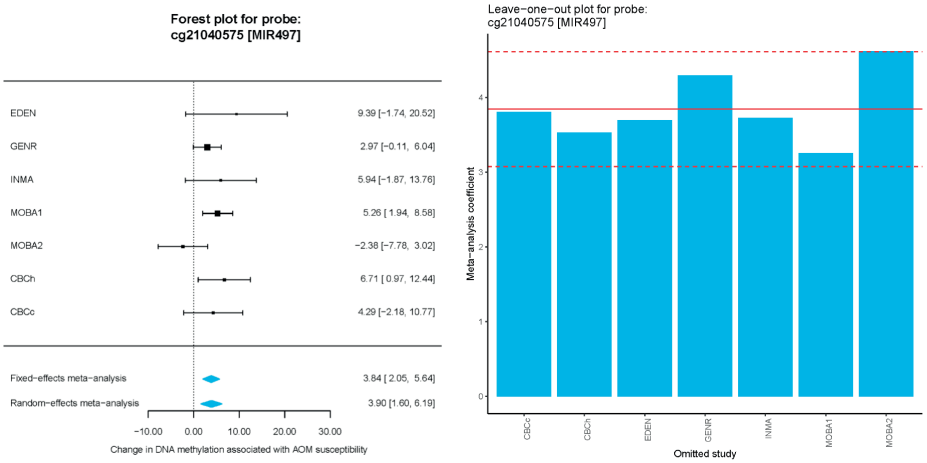
Supplemental Figure 5.2c: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg20172563 (*CD2AP*).



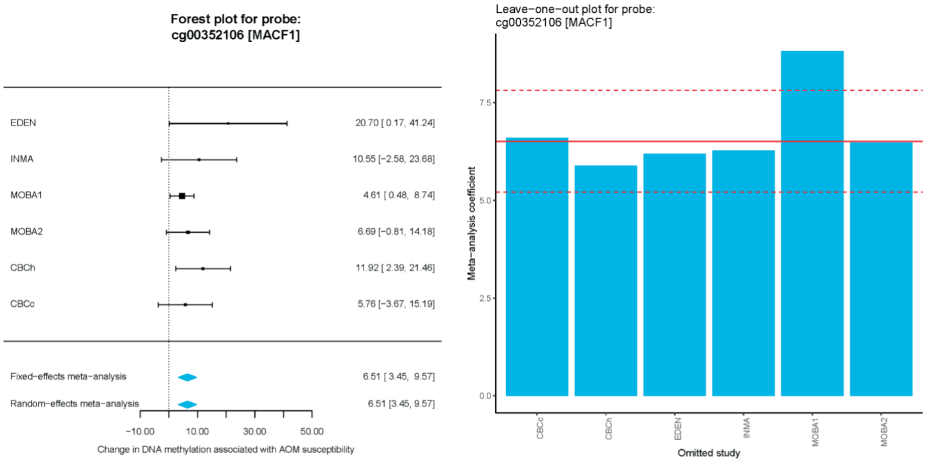
Supplemental Figure 5.2d: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg27518631.



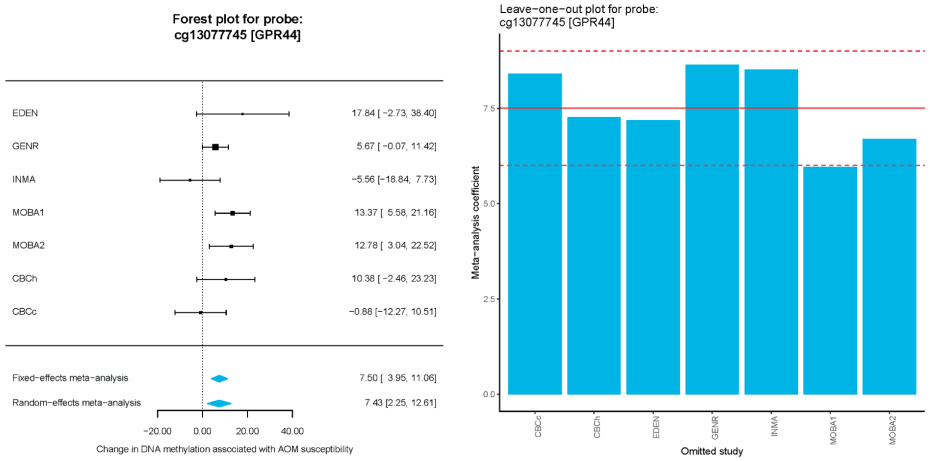
Supplemental Figure 5.2e: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg06242243 (*NCOR2*).



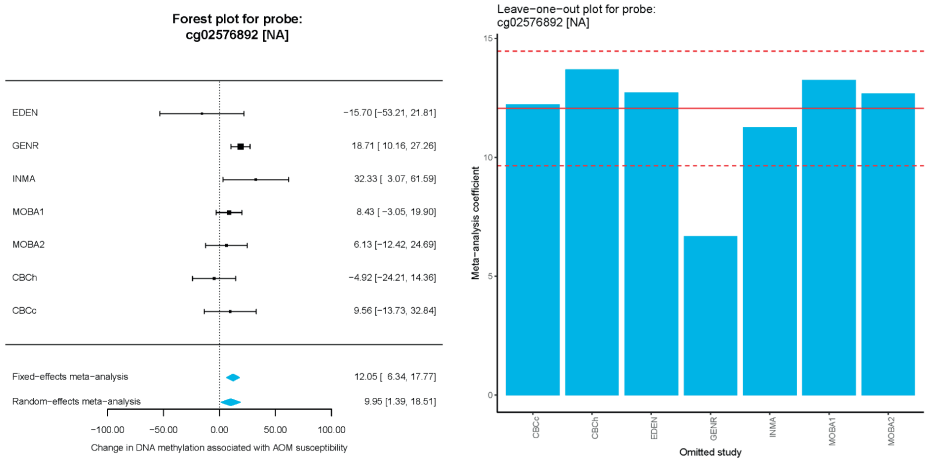
Supplemental Figure 5.2f: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg21040575 (*MIR497*).



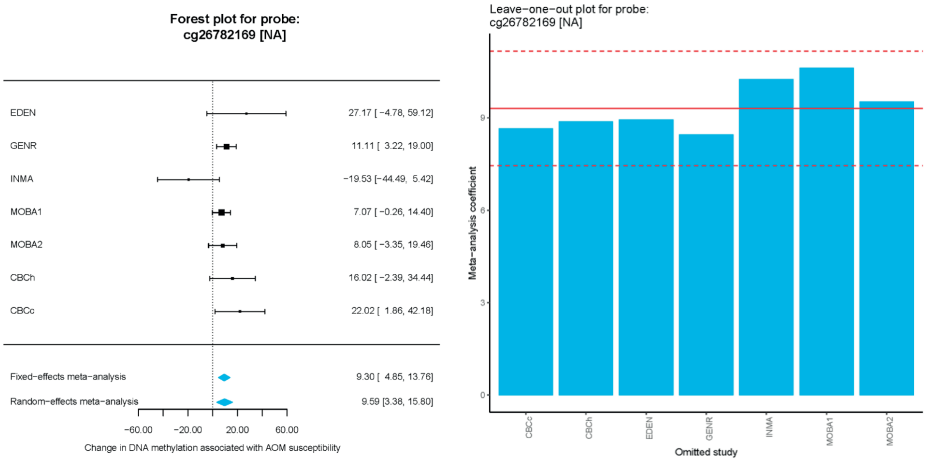
Supplemental Figure 5.2g: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg00352106 (*MACF1*).



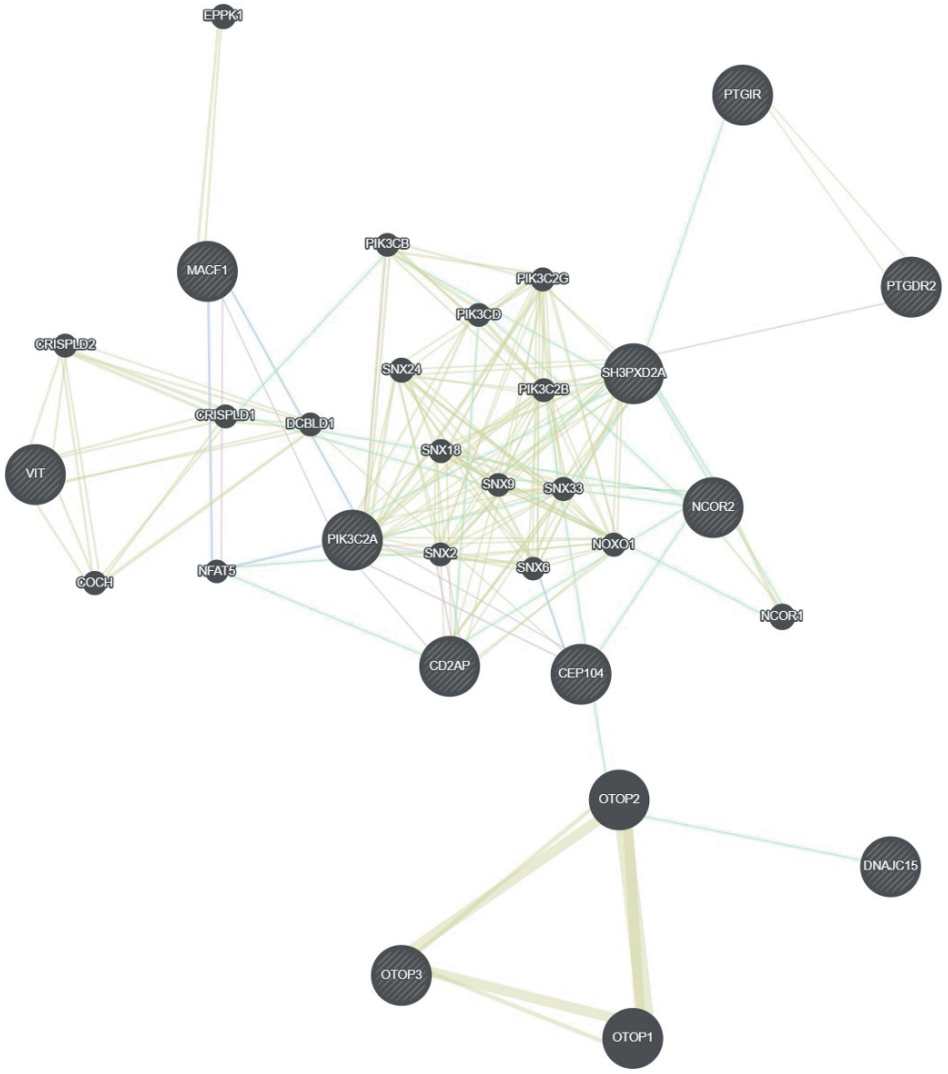
Supplemental Figure 5.2h: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg13077745 (*GPR44*).



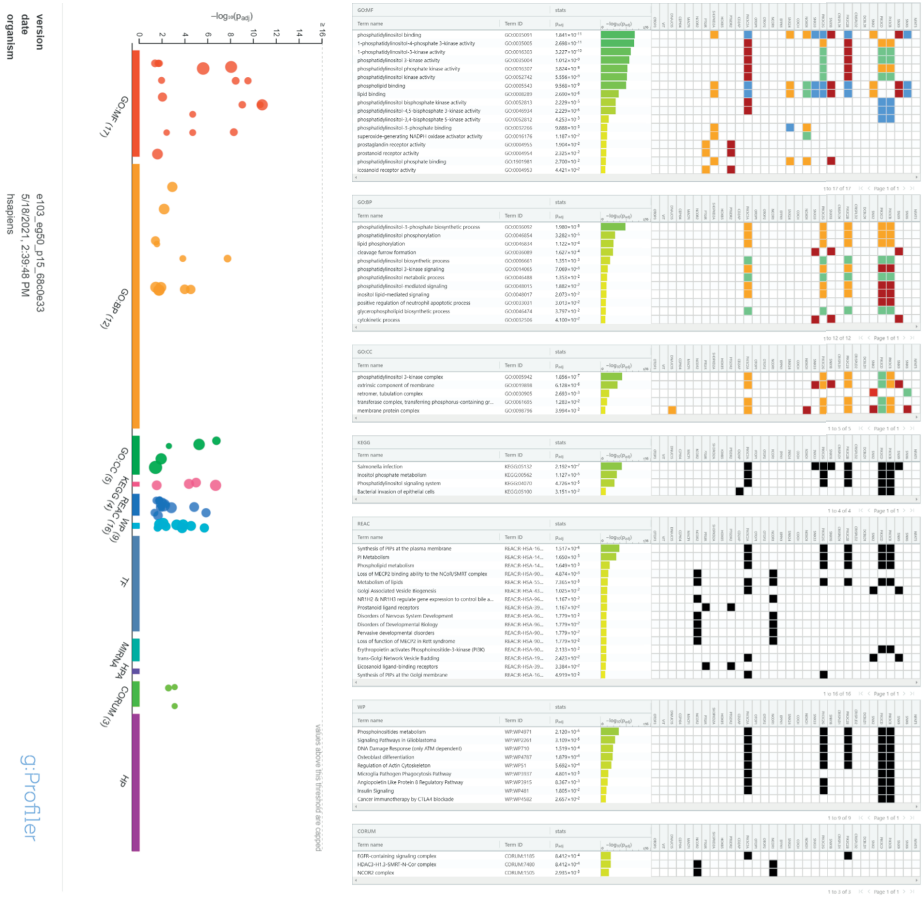
Supplemental Figure 5.2i: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg02576892.



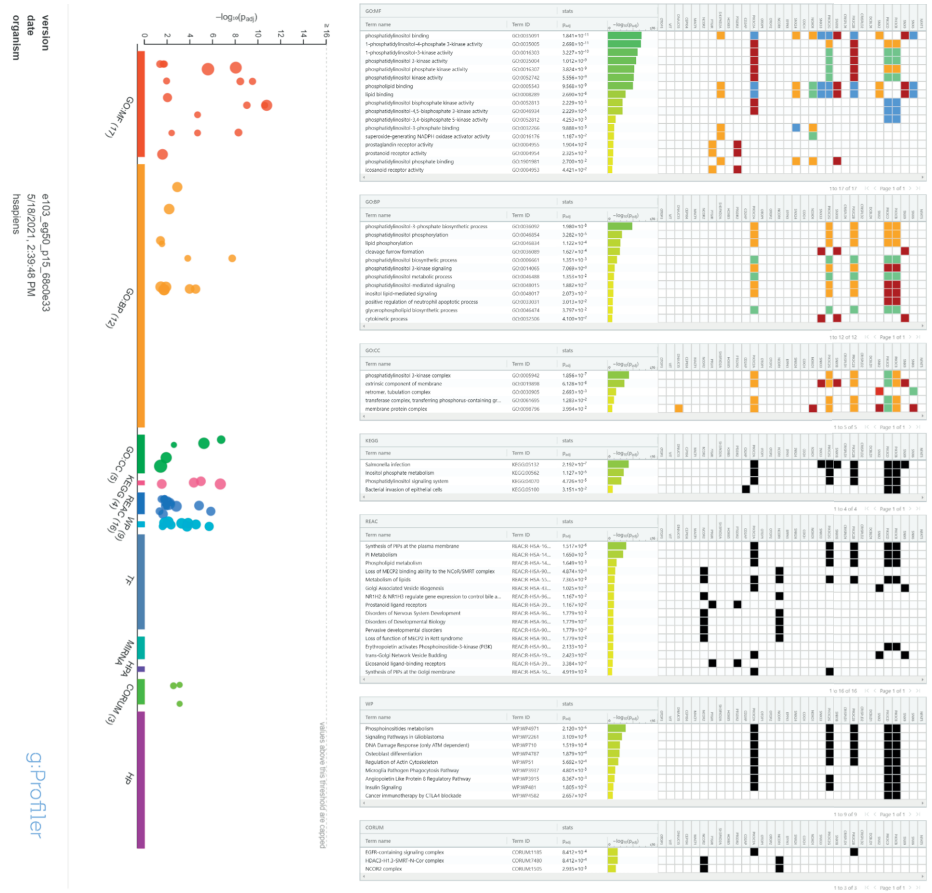
Supplemental Figure 5.2j: Forest plot and leave-one-out analysis plot showing betas on y-axis and omitted study on x-axis and for cg26782169.



Supplemental Figure 5.3: Constructed gene interaction network plot showing co-expression networks, shared protein domains, co-localizations networks and genetic interactions of genes mapped to CpG with $p<1.0x10^{-4}$ using GeneMania.



Supplemental Figure 5.4a: Functional enrichment analysis of all 32 genes of the constructed interaction network against Gene Ontology (GO) terms to find the most enriched GO terms using g:Profiler.



Supplemental Figure 5.4b: Functional enrichment analysis of all 32 genes of the constructed interaction network against Gene Ontology (GO) terms to find the most enriched GO terms using g:Profiler.

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III Hearing in children, and adverse hearing outcomes in childhood associated with acute otitis media

6 Music-induced hearing loss in children, adolescents, and young adults: a systematic review and meta-analysis

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M.P. van der Schroeff

Otol Neurotol. 2016 Oct;37(9):1208-16

ABSTRACT

Objective: Exposure to loud music has increased significantly because of the current development of personal music players and mobile phones. The aim of this study was to provide an overview of music-induced hearing loss and its symptoms in children.

Data Sources: The search was performed in the databases Embase, Medline (OvidSP), Web-of-science, Scopus, Cinahl, Cochrane, PubMed publisher, and Google Scholar. Only articles written in English were included.

Study selection: Articles describing hearing levels and music exposure in children were used, published from 1990 until April 2015.

Data extraction: The quality of the studies was assessed on reporting, validity, power, and the quality of audiometric testing.

Data synthesis: Data of each publication was extracted into spreadsheet software and analyzed using best evidence synthesis.

Conclusion: The prevalence of increased hearing levels (>15 dB HL) was 9.6%, and high-frequency hearing loss was found in 9.3%. The average hearing thresholds were 4.79 dB HL at low frequencies (0.5, 1, and 2 kHz) and 9.54 dB HL at high frequencies (3, 4, and 6 kHz). Most studies reported no significant association between pure-tone air thresholds and exposure to loud music. However, significant changes in hearing thresholds and otoacoustic emissions, and a high tinnitus prevalence suggest an association between music exposure and hearing loss in children.

INTRODUCTION

Exposure to noise is known to be a hazard to those working in, for example, factories and in the military. Since the 1970s, protective measures are dictated by law in many countries (1). Noise is defined as sound equal to or exceeding the level of 80 decibels, and exposure of at least 40 hours per week is considered potentially harmful (2, 3). Exposure to higher levels of sound for shorter periods of time, e.g., an hour a day, can be damaging as well. In noise-induced hearing loss, noise-trauma causes damage to hair cells and the blood supply in the cochlea (4). Although this is usually temporary, it can develop into permanent damage with increasing exposure. Hair cells that transduce high-frequency tones, located at the basal region of the cochlea, are the most sensitive to noise damage. Therefore, (early) noise-induced hearing loss is usually presented as notching or hearing loss at high frequencies in the audiogram. Noise-induced hearing loss is often noticed through hearing-related symptoms, such as tinnitus or perceived temporary hearing loss. The prevalence of tinnitus is significantly related to hearing loss in both noise-exposed workers and children (5, 6). It has been demonstrated in adolescents that these symptoms occur quite often, also when volume levels do not exceed the recommended safe listening levels (7).

Nowadays, exposure to noise is not necessarily occupational but more and more recreational. Exposure to loud sounds is available for all ages because of the widespread use of personal music players (PMPs), such as MP3- players, iPods, tablets, and mobile phones. With this development, the exposure to potentially high sound levels has risen, in particular in a younger age group. A recent systematic review has shown that up to 58.2% of the adolescents and young adults exceeded the recommended maximum daily noise dose (8). Prevention programs have been launched to raise awareness of possible risks of the use of PMPs. The topic has been addressed by governments and health departments, and media attention has risen. However, even though the number of studies and systematic reviews on this topic is mounting, evidence of the presence of music-induced hearing loss in children, adolescents, and young adults remains controversial.

The purpose of this systematic review and meta-analysis was to provide an overview of the available literature on the prevalence of music-induced hearing loss, temporary threshold shifts, and the associated symptoms in children and young adults, especially in relation to PMPs.

METHODS

Study Selection

Eight electronic databases were searched to identify studies for inclusion. The search was designed for Embase and then adapted to Medline (OvidSP), Web-of-science, Scopus,

Cinahl, Cochrane, PubMed publisher, and Google Scholar (see Supplemental Methods, or http://links.lww.com/MAO/A433, which demonstrates the full search). The last search was performed on April 7, 2015. All articles describing hearing levels in children and young adults exposed to music were eligible for assessment.

Two reviewers independently screened all articles for eligibility, using the following exclusion criteria: articles not written in English, average age of the study population above 23-year old, no original data or measurements, no exposure to music, occupational noise, or no audiometric measurements. Any disagreements were resolved by discussion.

Study Assessment

The quality of each study was assessed by two reviewers independently, on multiple aspects of study methods and outcome level: reporting, external validity, internal validity, and power as described by Downs and Black (9). In addition to the items on this checklist, quality of audiometry and its reporting were assessed because of additional relevance to the current topic. The two scores obtained were used to define a quality score (see Table 6.1, and Supplemental Table 6.1 or http://links.lww.com/MAO/A434, for the calculation of the final quality score). Studies assessed to be of low quality were excluded from further analyses. The studies of medium and high quality were reviewed, and audiometric data from high-quality articles were additionally included in the meta-analysis.

Table 6.1: Criteria of calculated quality score.

Risk-of-bias score \ Quality of audiometry	0	1	2	3	4
0	Low	Low	Low	Low	Medium
1	Low	Low	Low	Medium	High
2	Low	Low	Low	Medium	High

Data Extraction and Analysis

Primary outcome measures were hearing thresholds and the presence of acquired (music-induced) hearing loss. The secondary outcome measure was the prevalence of hearing-related symptoms, such as tinnitus. Data was extracted independently by two reviewers in a data extraction sheet in spreadsheet software. The extracted data comprised the study setting, characteristics of the study population, presence of confounders and risk factors, interventions (if applicable), type of audiological measurements, and the obtained results, including hearing thresholds, prevalence of (temporary) hearing loss, and prevalence of hearing-related symptoms.

Outcome measures were evaluated in three separate categories: hearing levels in children and young adults exposed to music during leisure-time, temporary threshold shifts directly

after exposure to music, and subjective hearing-related symptoms. A meta-analysis was performed to calculate average hearing thresholds in the included population. Hearing thresholds were additionally compared between users and nonusers of PMPs, with the use of two-sided *t* tests.

RESULTS

Study Selection

The search identified 3,154 citations. One citation was added from the reference lists. After removing duplicates, 1,865 citations were reviewed by two independent reviewers. Another 1,550 citations were excluded based on title and abstract. A total of 315 full-text citations were assessed, which resulted in 52 studies deemed eligible for inclusion by agreement of the two reviewers (Fig. 6.1).

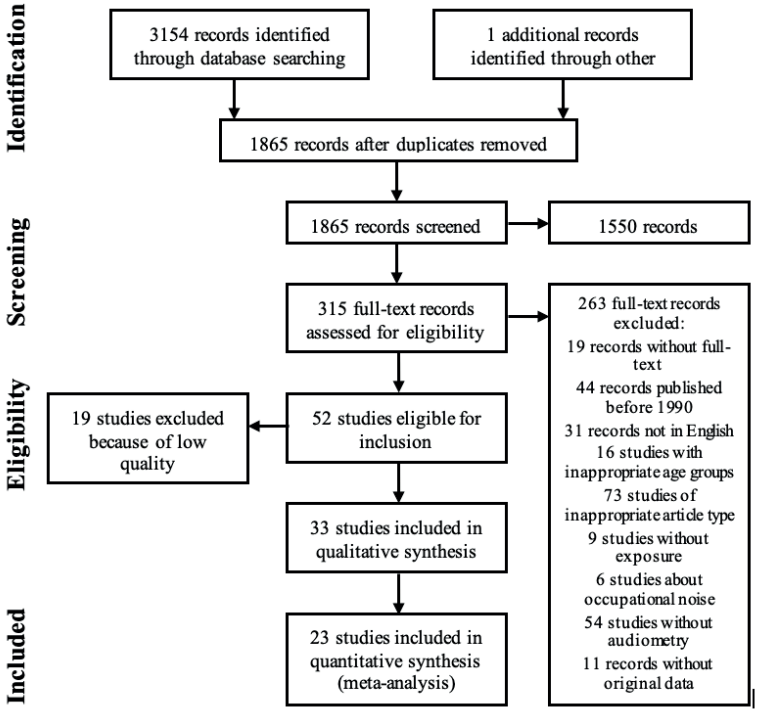

Figure 6.1: Flow diagram of the inclusion process

Study Assessment

The risk-of-bias assessment using the Downs and Black checklist (9) resulted in scores between 6 and 27, with a median score of 19. The quality of the audiometry was assessed as "good" in over half of the studies. After combining both scores, 19 studies were assessed to be of low quality, 10 studies of medium quality and 23 studies of high quality (Table 6.2). Studies of low quality were excluded from further analyses.

Table 6.2: Risk-of-bias and quality scores of the 52 included studies

Study	Reporting	External validity	Internal validity -bias	Internal validity - confounding	Power	**Total score**	**Risk-of-bias score**	**Quality of audiometry**	**Overall Quality**
Barlow, 2011.	6	3	5	3	0	**17**	**1**	**1**	**Low**
Berg, 2011.	9	3	5	3	5	**25**	**2**	**4**	**High**
Bhagat, 2008.	7	2	5	3	0	**17**	**1**	**4**	**High**
Biassoni, 2005.	8	3	5	3	2	**21**	**1**	**4**	**High**
Chen, 2011.	9	3	5	2	5	**24**	**2**	**1**	**Low**
Cone, 2010.	10	3	5	3	5	**26**	**2**	**4**	**High**
da Silva, 2012.	9	3	4	2	2	**20**	**1**	**4**	**High**
de Beer, 2003.	6	2	5	3	3	**19**	**1**	**4**	**High**
Derebery, 2012.	10	2	4	4	0	**20**	**1**	**4**	**High**
Emmerich, 2002.	4	1	3	0	0	**8**	**0**	**4**	**Medium**
Emmerich, 2008.	4	2	5	1	2	**14**	**0**	**1**	**Low**
Feder, 2013.	7	3	4	2	3	**19**	**1**	**1**	**Low**
Figueiredo, 2011.	9	3	5	4	1	**22**	**1**	**4**	**High**
Gierek, 2009.	8	3	5	2	2	**20**	**1**	**1**	**Low**
Helleman, 2015.	8	1	4	3	0	**16**	**1**	**4**	**High**
Holgers, 2006.	7	2	6	1	3	**19**	**1**	**4**	**High**
Jaffer, 2004.	3	2	4	2	0	**11**	**0**	**4**	**Medium**
Job, 2000.	7	3	5	2	5	**22**	**1**	**4**	**High**
Keppler, 2010.	6	2	5	1	0	**14**	**0**	**4**	**Medium**
Kim, 2009.	6	3	5	2	3	**19**	**1**	**3**	**Medium**
Kuchar, 2010.	6	2	5	2	0	**15**	**0**	**1**	**Low**
Kumar, 2009.	9	2	4	2	1	**18**	**1**	**4**	**High**
Le Prell, 2013.	9	2	4	2	1	**18**	**1**	**4**	**High**
Lee, 2014.	11	3	3	5	5	**27**	**2**	**4**	**High**

Table 6.2: Risk-of-bias and quality scores of the 52 included studies (continued)

Study	Reporting	External validity	Internal validity -bias	Internal validity - confounding	Power	**Total score**	**Risk-of-bias score**	**Quality of audiometry**	**Overall Quality**
Mansfield, 1999.	10	2	4	3	0	**19**	**1**	**2**	**Low**
Martinez-Wbaldo, 2009.	7	2	4	3	3	**19**	**1**	**1**	**Low**
McBride, 2013.	3	1	2	0	0	**6**	**0**	**1**	**Low**
Mercier, 2002.	8	2	3	2	4	**19**	**1**	**1**	**Low**
Mostafapour, 1998.	9	2	4	2	0	**17**	**1**	**4**	**High**
Naik, 2014.	3	2	2	1	4	**12**	**0**	**1**	**Low**
Park, 2014.	7	3	4	2	5	**21**	**1**	**1**	**Low**
Peng, 2007.	8	2	5	2	2	**19**	**1**	**4**	**High**
Phillips, 2010.	8	2	4	2	3	**19**	**1**	**4**	**High**
Poissant, 2012.	9	2	4	3	0	**18**	**1**	**4**	**High**
Rao, 2014.	8	1	4	3	2	**18**	**1**	**3**	**Medium**
Rosanowski, 2006.	5	1	5	3	1	**15**	**0**	**4**	**Medium**
Schlauch, 2011.	4	2	2	1	5	**14**	**0**	**4**	**Medium**
Schmuziger, 2006.	7	3	3	0	0	**13**	**0**	**4**	**Medium**
Sekhar, 2011.	8	3	5	3	3	**22**	**1**	**1**	**Low**
Serra, 2014.	8	2	4	2	2	**18**	**1**	**4**	**High**
Shah, 2009.	7	2	5	2	1	**17**	**1**	**1**	**Low**
Sulaiman, 2013.	8	3	4	2	2	**19**	**1**	**1**	**Low**
Tin, 2000.	10	2	6	4	0	**22**	**1**	**3**	**Medium**
Toh, 2002.	9	3	3	4	4	**23**	**2**	**1**	**Low**
Torre III, 2014.	9	2	4	3	2	**20**	**1**	**4**	**High**
Torre III, 2007.	7	2	5	2	3	**19**	**1**	**4**	**High**
Tung, 2013.	8	3	5	2	3	**21**	**1**	**1**	**Low**
Weichbold, 2012.	8	2	5	4	5	**24**	**2**	**1**	**Low**
West, 1990.	9	2	4	3	1	**19**	**1**	**3**	**Medium**
Widen, 2009.	9	3	4	2	3	**21**	**1**	**1**	**Low**
Wong, 1990.	8	2	4	4	2	**20**	**1**	**4**	**High**
Zocoli, 2009.	10	2	4	4	0	**20**	**1**	**4**	**High**

Study Characteristics

Twenty-five of the 33 analyzed studies were observational studies, describing hearing levels in general young populations. The remaining eight studies were experimental studies, which assessed the occurrence of (temporary) threshold shifts shortly after music exposure. Participants were exposed to music through headphones, attended a discotheque, or performed a musical instrument practice session. The 33 studies comprised a total of 26,379 participants, ranging between 15 and 8,710 participants per study. The median age was 20-year old with an interquartile range of 16;9 to 21;6 years. Characteristics of all studies in the analyses are available in the table of Supplemental Table 6.3, or http://links.lww.com/MAO/A435.

Hearing Levels in Children, Adolescents, and Young Adults

There was a large variation in audiometric results between studies. Average pure-tone hearing thresholds in all included studies were reported between 0 and 16.4 dB HL, with a median of 9.2 dB HL (10-12). The prevalence of hearing loss varied significantly, possibly because of differences in definitions (see Table 6.3). When evaluating average thresholds, the prevalence of hearing loss was found between 0 and 12.6% (10, 13-17). An increased threshold in at least one frequency was reported in 14.2 to 34.9% (18, 19). The weighted average of the prevalence of hearing loss was 9.6%. High-frequency hearing loss was both absent in some studies (12, 20), as well as present in other studies (10, 21), with a weighted average of 9.3% (Table 6.3).

Notches in audiograms as a sign of noise-damage were often assessed using the criteria described by Niskar et al. (22) in 2001. These notches were found in 8.3 up to 46% of the participants (14, 16, 23-25).

When focusing on PMP users, average hearing thresholds were between 6 and 13 dB HL (12, 16). The prevalence of hearing loss was 0.9 to 14.2% (14, 16, 19). The prevalence of high-frequency hearing loss again showed a wide range from 0 to 23.6% (20, 21). In general, the number of notched audiograms seemed to increase among participants with increased exposure to music and among older participants. In a selection of habitual PMP users and music students, 45% and 46% of the participants showed a notched audiogram respectively (14, 23).

Otoacoustic emissions were compared between participants who were exposed to recreational noise on a regular basis and participants with less exposure. Distortion-product otoacoustic emission (DPOAE) amplitudes at frequencies around 4 and 6 kHz were decreased in participants with more exposure to music (20, 26-28). This group also showed a decrease in amplitude of transient-evoked otoacoustic emissions (TEOAE) at frequencies between 2 and 4 kHz (17, 27, 29). Failure on both DPOAE and TEOAE in at least one ear was observed in 79.9% of the participants with regular music exposure, with more failed tests in higher frequencies (30).

Table 6.3: Prevalence rates of hearing loss, high frequency hearing loss and tinnitus for the 34 analyzed studies.

Hearing loss	
Definition of hearing loss (study)[a]	*Range (no. of participants)*
PTA > 15 dB HL	0.9-9.7% (7,777)
PTA > 20 dB HL (17)	0% (103)
PTA > 25 dB HL	12.6% (103)
Threshold > 18 dB HL at ≥ 1 frequency	34.9% (172)
Threshold > 25 dB HL at ≥ 1 frequency	14.1% (150)
Weighted average*	9.6% (8,305)
High-frequency hearing loss	
Definition of high-frequency hearing loss (study)[b]	*Range (no. of participants)*
HFPTA > 15 dB HL	0-19.2% (9,951)
HFPTA > 25 dB HL	0% (100)
Weighted average*	9.3% (10,051)
Tinnitus	
Definition of tinnitus (study)	*Range (no. of participants)*
Self-reported transient or noise-induced tinnitus	8-69% (10,927)
Weighted average*	38.8% (10,927)

PTA – pure-tone average; HFPTA – high-frequency pure-tone average

* Weighted average was calculated taking study quality into account.

a Hearing loss included both unilateral and bilateral hearing loss, based on an average of thresholds between 0.5 and 8 kHz.

b HFPTA: Average hearing threshold at frequencies between 3 and 6 or 8 kHz.

Temporary Threshold Shifts After Exposure to Music

Eight studies examined the occurrence of temporary threshold shifts after exposure to music. The admitted exposure varied from 57.8 to 108 dBA between the studies, with occasional peak sound levels up to 130 dBA, and lasted from 30 minutes up to 4 hours.

In seven studies, pure-tone air conduction thresholds were compared before and after exposure (15, 31-36). Changes in hearing thresholds were reported as nonsignificant in one study (31), small but significant in three studies (15, 34, 35), and significant at all frequencies in all participants in another study (33). Overall, the observed significant threshold shifts occurred more often in case of increased volume levels and at high frequencies, with threshold shifts between 4.3 and 17 dB at 2 to 8 kHz (32, 36).

Studying change in OAE amplitudes after exposure to music, the change in DPOAE amplitudes was nonsignificant in two studies (35, 37). Other studies found significant decreases in DPOAE amplitudes of 1.4 to 1.8 dB SPL at high frequencies (15, 31, 32). TEOAE amplitudes showed significant decreases at 2 and 2.8 kHz (35). These significantly decreased OAE amplitudes occurred more often in participants exposed to higher volume levels (33, 35).

Both pure-tone hearing thresholds and OAE amplitudes returned to baseline levels after a maximum of 2 days (15, 33-36). Recovery was often observed within hours after ending the exposure, which supports that the observed threshold shifts were temporar

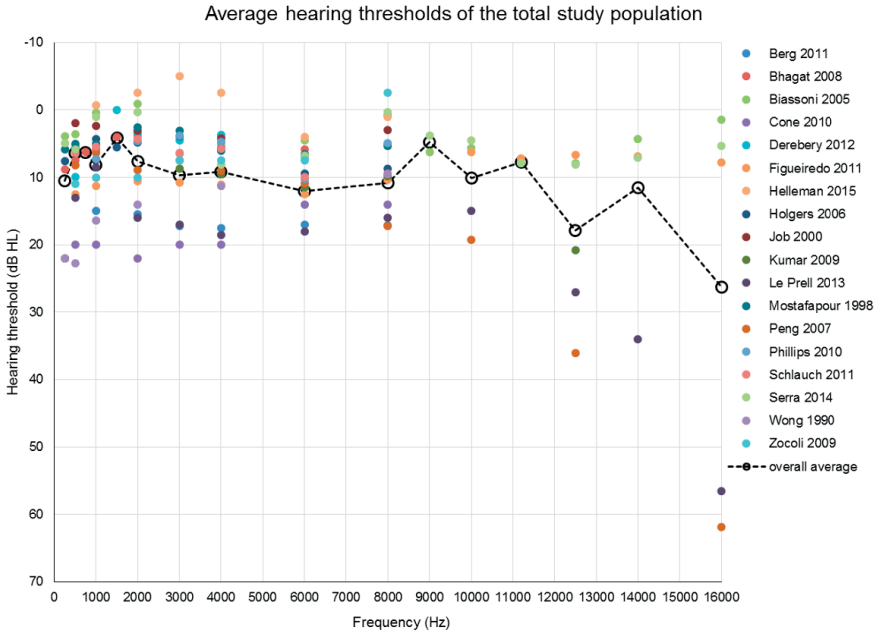

Figure 6.2A: Average hearing thresholds (in dB HL) of the total study population for all available frequencies (in Hz). The overall average was calculated for all included studies, weighted on sample size.

Hearing-related Symptoms

Based on self-reporting, up to 69% of the analyzed participants had experienced hearing-related symptoms after exposure to music, which most often was tinnitus, but also the feeling of pain or pressure in the ears and perceived hearing difficulties were regularly reported. Tinnitus was experienced by a wide range of 8 to 69% of participants, with a weighted average of 38.8% (Table 6.3) (10, 12, 14, 21, 24, 25, 29, 32, 33, 36, 38, 39). Permanent tinnitus was reported by 0 to 2.2% of the participants (14, 25, 38).

Meta-analysis of Hearing Levels

Eighteen of the 23 high-quality studies reported pure-tone hearing thresholds, offering the opportunity to obtain average pure-tone hearing thresholds, as is visualized in Figure 6.2A (10, 13, 14, 18-21, 23, 25, 29, 31, 32, 34, 38-43). Weighted average hearing thresholds at standard audiometric frequencies (0.125–8 kHz) did not exceed 15 dB HL, being between 4.18 and 12.04 dB HL (Table 6.4). Increased thresholds were found at two extended high

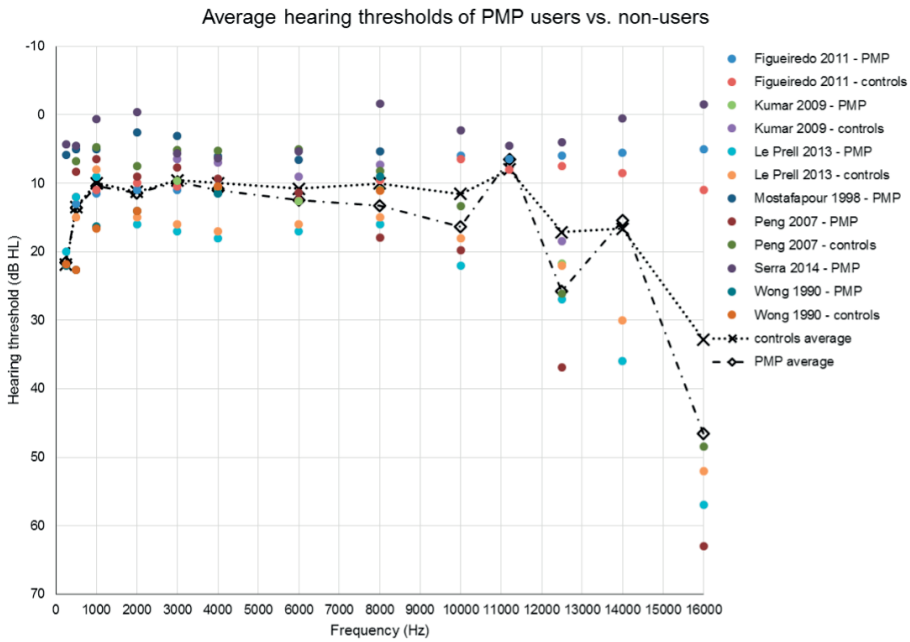

Figure 6.2B: Average hearing thresholds (in dB HL) stratified for PMP users versus non-users for all available frequencies (Hz). Average thresholds per frequency were calculated using only studies who reported hearing levels for both users and non-users, weighted on sample size.

frequencies, namely 12.5 and 16 kHz, with average hearing thresholds of 17.85 and 26.26 dB HL, respectively. The weighted average low-frequency PTA (0.5, 1, and 2 kHz) was found at 5.50 dB HL, the high-frequency PTA (3, 4, and 6 kHz) at 10.43 dB HL. When applying uniform criteria, 18.8% of the studies (or 31.2% of the participants) had mean hearing thresholds exceeding 15 dB HL at high frequencies 3, 4, and 6 kHz.

Figure 6.2B shows average hearing levels when stratified for the use of PMPs. Hearing threshold levels in both groups were comparable in lower frequencies yet diverted at higher frequencies. Hearing thresholds at high frequencies 4, 6, 8, 10, 12.5, and 16 kHz were significantly poorer in PMP users compared with control subjects (Table 6.4).

Only three of the high-quality studies examined change in DPOAE amplitudes after exposure to music. We therefore concluded that there was insufficient information to perform meta-analysis on TTS in DPOAE after exposure to music.

DISCUSSION

Over the last decade, attention for hearing and hearing loss in teenagers has increased. A relationship between hearing loss and exposure to loud sounds and music is suggested,

Table 6.4: Average pure-tone hearing thresholds for all available frequencies for the total population and for samples consisting of PMP users versus non-users.

	Average threshold (dB HL)					
	Overall average		PMP users		Non-users	
Frequency (Hz)	*n*	Threshold	*n*	Threshold	*n*	Threshold
250	629	10.52	104	21.54	53	21.91*
500	6777	6.42	278	13.62	129	13.50
1000	9794	8.11	278	10.46	129	9.99
1500	144	4.18	-	-	-	-
2000	9794	7.65	278	11.44	129	11.28
3000	9108	9.68	270	9.75	134	9.56
4000	9894	9.22	348	11.08	159	9.99**
6000	9108	12.04	270	12.45	134	10.79**
8000	9894	10.85	348	13.31	159	10.05**
9000	274	4.73	-	-	-	-
10000	611	10.12	200	16.37	104	11.57**
11200	374	7.71	54	6.50	46	8.00†
12500	711	17.85	270	25.82	134	17.15**
14000	461	11.51	80	15.41	74	16.64
16000	611	26.26	200	46.54	104	32.86**

PMP – Personal Music Player

* Significantly better hearing threshold for PMP users compared to the hearing threshold of non-users, $p < 0.01$.

** Significantly worse hearing threshold for PMP users compared to the hearing threshold of non-users, $p < 0.01$.

† One study, insufficient information to perform t-test.

especially because of the simultaneous development and increased use of PMPs. We must however acknowledge that the association between music and hearing loss in children is mainly based on hypotheses instead of evidence. With the present systematic review and meta-analysis, we aimed to examine hearing levels and the prevalence of acquired hearing loss in children, and so try to elucidate a possible association with exposure to music.

The 52 eligible studies varied in methodological quality, often depending on whether observed hearing levels were described for individual frequencies. Studies of low quality were subsequently excluded from the analyses to avoid the inclusion of unreliable outcomes. However, large variations in the hearing levels and prevalence rates of predefined hearing loss persisted. With the analysis of hearing thresholds instead of prevalence rates, we tried to eliminate the bias caused by the use of different cut-off values for the diagnosis of hearing loss. Nevertheless, the obtained results should be interpreted with caution.

Over half of the primarily included studies (29 of 52) achieved the maximum score on quality of audiometry and its reporting, which we considered an encouraging finding. However, definitions and outcome measures still differed widely between the included studies. The use of more consequent definitions of normal and abnormal hearing, like clas-

sified by Niskar et al. 1998 (44), would contribute to better comparable studies. Also, study populations, sample sizes, and the assessment of other risk factors and exposures varied considerably, introducing heterogeneity in the analyses.

An important issue in the evaluation of the effect of music exposure on hearing is the extent of the exposure. Studies that administrated music for the evaluation of TTS had well-measured and well-reported exposures. However, in most studies on hearing in general, exposure was based on self- or parent-reporting. Although the general use of PMPs is likely to be reported truthfully, reports on for instance listening volume can suffer from social desirability. This further complicates drawing reliable conclusions on dose-effects. To study these dose-effects, objective measurements of music exposure are required.

Besides the exposure being self-reported, tinnitus as one of the outcome measures was self-reported as well. As this diagnosis is based on subjective symptoms, this is currently the best available option. It is helpful to distinguish between temporary noise-induced tinnitus, which was experienced by most subjects at some point, and permanent tinnitus, which is considered a more severe condition. Fortunately, the latter was rare.

Both pure-tone audiometry and otoacoustic emissions were used to measure hearing. Pure-tone audiometry is considered the golden standard for evaluating hearing levels. Alternatively, OAEs seem more sensitive in detecting early sensorineural hearing loss (45). The current results support this statement, as alterations in hearing after music exposure were more often found in OAE than in pure-tone audiometry.

Implications of the Obtained Results

Various studies conclude that exposure to music and the use of PMPs are hazards to hearing. The current safety standards for noise were based on the assumption of 40 years of exposure to occupational noise (2). It might be that the current generation will accumulate more than 40 years of noise-exposure. It has to be acknowledged that many children, adolescents, and young adults are at risk of developing hearing loss (8). However, we must not interpret this risk of hearing loss as the presence thereof. Although the results of the current systematic review suggest an association between music and hearing loss, the evidence is not uniform.

The aim of this systematic review was to provide an overview of hearing levels in children with and without music exposure. Although equally clinically relevant, our conclusive study population was of slightly older age than originally intended. Less research is done on the effect of music exposure in elementary school children than in, for example, university students. Young adults may be more exposed to loud music by attending discotheques and pop rock concerts than younger children. However, we think that younger children can be highly exposed as well through the use of PMPs. Hence, more studies are required in, for instance, elementary school children to explore consequences of PMP use at young ages. Like all acquired diseases, knowledge of acquired music-induced hearing loss and the possible age of onset would benefit greatly from longitudinal studies with repeated measurements.

CONCLUSION

The association between exposure to loud music and hearing levels in children, adolescents, and young adults remains difficult to prove. Changes in hearing were frequently reported. However, there were no significant differences in the prevalence of hearing loss and the prevalence of high-frequency hearing loss between children, adolescents, and young adults who are exposed to loud music and those who are not. Deviations were found when there were increasing amounts of exposure, but not all correlations were reliable, and the large spread of results hindered drawing reliable conclusions. Nevertheless, the significant poorer hearing thresholds in PMP users in the meta-analysis, the significant decreases in DPOAE amplitudes after exposure, and the large number of participants experiencing tinnitus suggest that there might be an association between music exposure and hearing loss at a young age already.

Detailed acknowledgements and online resources can be found in the published article online: https://journals.lww.com/otolog-neurotology/Abstract/2016/10000/Music_induced_Hearing_Loss_in_Children,.4.aspx

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Search strategy for Embase: ('MP3 player'/de OR 'tape recorder'/de OR music/de OR 'compact disk'/de OR (mp3 OR recorder OR stereo OR music OR ((listen* OR audio) NEAR/3 (device* OR equipment*)) OR earphone* OR headphone* OR ((ear OR head) NEXT/1 phone*) OR walkman* OR disco OR discos OR discothe* OR nightclub* OR concert* OR popconcert* OR rockconcert* OR club OR clubs OR ipod* OR (I next/1 pod*) OR 'compact disk' OR discman* OR ((cd OR cassette) NEXT/1 player*)):ab,ti) AND ('hearing disorder'/exp OR 'noise injury'/de OR 'otoacoustic emission'/exp OR (((hear* OR auditor*) NEAR/3 (loss OR impair* OR defect* OR damage* OR harm* OR effect* OR level* OR disabilit* OR ability* OR screen* OR problem* OR health OR risk*)) OR deaf* OR (noise NEAR/3 injur*) OR tinnit* OR (ear NEAR/3 (buzz* OR ring* OR beep* OR sizzl* OR whistl* OR sound*)) OR (otoacoust* NEXT/1 emission*) OR OAE):ab,ti) AND (child/exp OR adolescent/exp OR adolescence/de OR (kid OR kids OR teen* OR boy* OR girl* OR minors* OR underag* OR (under NEXT/2 ag*) OR juvenil* OR youth* OR puber* OR pubescen* OR prepubescen* OR prepuberty* OR school* OR preschool* OR highschool* OR adolescen*):ab,ti OR (child* OR pediatric* OR paediatric*))

Supplemental Table 6.2: Risk-of-bias and quality score calculation.

Score 1: Quality of audiometry		**Score 2: Downs & Black score**			**Overall quality**	
4	Adequate audiometry with reporting of separate measured frequencies	2	> 70%	> 22	High quality (low risk of bias)	4 in quality of audiometry and minimum of 1 in Downs & Black score
3	Description of adequate audiometry with some measured frequencies reported	1	50-70%	16-22	Medium quality (medium risk of bias)	Minimum of 3 in quality of audiometry and minimum of 1 in Downs & Black score
2	Adequate audiometry reporting mean hearing thresholds/index of hearing threshold	0	< 50%	< 16	Low quality (high risk of bias)	All others
1	Reporting a prevalence of hearing loss					
0	Hearing loss unknown or experienced symptoms via questionnaire					

Supplemental Table 6.3: Characteristics of the included studies.

Source (author, year)	Study design, sample size	Study population characteristics	Measurements	Findings	Additional comments
Barrenas, 1996.	Nonrandomized trial, n = 12	Caucasian volunteers, average age 13.6 years (range 12-16 years old)	Pure-tone audiometry	Baseline hearing thresholds were 10.5 ± 3.4 dB HL in the blue-eyed group and 8.0 ± 2.8 dB HL in the brown-eyed group.	Influence of eye color
Berg, 2011.	Retrospective cohort study, n = 8710	Latino with low SES, average age 15.8 years (range 12-20 years old)	Pure-tone audiometry and questionnaire	Prevalence of HFHL was 19.2% at the end of the study. Of PMP users 19.8% has tinnitus and 23.6% shows HFHL. Of tinnitus, 99.7% uses PMP. Of HFHL, 84% uses PMP.	
Bhagat, 2008.	Pre-post experimental study, n = 20	Young adults, age range 18-38 years old	DPOAE, SSOAE and pure-tone audiometry	No significant alteration of the group hearing sensitivity of a sample of normal-hearing adults. Significant reductions in DPOAE half-octave band levels centered at frequencies from 1.4-6.0 kHz were observed following the music exposure in this study.	Exposure (rock music song) in half of the participants: mean sound pressure 85 dBC ± 3dB for 30 minutes via earphones.
Biassoni, 2005.	Long-term study, n = 102	Students in two middle class schools, age 14 years old	Pure-tone audiometry	35% of the boys and 24% of the girls had hearing thresholds >20 dB HL in at least one frequency. The significant threshold shifts over a four year period, mostly in participants with noisy recreational activities.	
Cone, 2010.	Cross-sectional cluster sample survey, n = 6581	Formal school students, average ages 7 and 11 years old	Pure-tone audiometry and speech perception tests	Prevalence of 0.88% of slight-mild bilateral SNHL. Significantly higher PMP use in the SNHL group, adjusted OR 1.7.	
da Silva, 2012.	Cross-sectional descriptive study, n = 134	Middle school students without otologic problems, age range 14-19 years old	TEOAE and DPOAE	79.9% failed on both TEOAE and DPOAE in at least one ear. 94.0% of the total study wears earphones and 82.8% frequently visits places with loud music.	
de Beer, 2003.	Longitudinal cohort study, n = 238 with known PMP exposure	Sample of a birth cohort, age 18 years old	Pure-tone audiometry	No differences between PMP users and non-users, and between extensive PMP users and non-users.	

Supplemental Table 6.3: Characteristics of the included studies. (continued)

Source (author, year)	Study design, sample size	Study population characteristics	Measurements	Findings	Additional comments
Derebery, 2012.	Repeated measures pre- and post-concert assessment of hearing, n = 29 (27 analyzed)	Normally hearing teenagers and young adults, average age 17.7 years (range 13-20 years old)	DPOAE and pure-tone audiometry	59.3% of the subjects had threshold increases at 3 or more frequencies, with an average TTS of 6.3 dB (right ears) and 6.5 dB (left ears). After the concert, 25% experienced tinnitus, and 53.6% felt pressure in their ears.	Exposure (concert): average sound level 98.5 dBA (range 82-110 dBA), with encouragement of earplug use.
Emmerich, 2002.	Repeated measures pre- and post-concert assessment of hearing, n = 36	Subjects, age range 16-18 years old	OAE, pure-tone audiometry and questionnaire	A TTS up to 20-25 dB SPL over all frequencies in all subjects. This TTS almost disappeared within 2 hours. Nearly 65% of the participants reported tinnitus after a discotheque visit.	Exposure (discotheque): average sound level 95 dB, with peaks increasing up to 130 dB
Figueiredo, 2011.	Cross-sectional study, n = 100 (54 regular PMP users)	School students, teachers and staff, average ages 17.5 and 21.3 years (range 15-30 years old)	TOAE, high frequency pure-tone audiometry and questionnaire	The regular use of PMPs has a relationship with the incidence of tinnitus, elevated audiometric thresholds at 8 kHz, and decreased otoacoustic emission amplitudes at 2 kHz.	
Helleman, 2015.	Cross-over intervention study, n = 18	Normally hearing young adults, average age 21.4 years old	Pure-tone audiometry	An observed average TTS of 1.7 dB for right ears and 3.4 dB for left ears. After a pause of an hour the TTS doesn't fully recover to baseline condition. There is no difference in TTS after two hours of music exposure with or without a break.	Exposure (dance music via headphones): 2 hours of music presented at 91 dB
Holgers, 2006.	Cross-sectional study, n = 274	School students, age range 9-16 years old	Pure-tone audiometry and questionnaire	95 tinnitus-patients had correlations between HFPTA and TSQ scores (Spearman's r=0.29, p=0.013) and stronger correlations between HFPTA and visual analog scales on both tinnitus loudness and tinnitus annoyance during the day or week. No such correlations were found for the LFPTA. 53% of all participants had experience of noise-induced tinnitus, which was found in significantly older children and more in girls than in boys.	

Supplemental Table 6.3: Characteristics of the included studies. (continued)

Source (author, year)	Study design, sample size	Study population characteristics	Measurements	Findings	Additional comments
Jaffer, 2004.	Case-control study, n = 30	Students, age range 19-25 years old (mean age 22 years in the study group, and 21.5 years in the control group)	DPOAE and pure-tone audiometry	DPOAE amplitudes in controls are more robust than in Walkman users, at all the frequencies, with larger differences for increased Walkman use. They were statistical significant at and above 2kHz ($p<0.05$). This suggests that Walkman noise has affected the cochlear hair cells of its users. The damage was not manifested in their pure-tone audiogram, yet, since all participants had normal hearing.	
Job, 2000.	Cross-sectional study, n = 1208	Young men undergoing selection for the army, age range 18-24 years old	Pure-tone audiometry and questionnaire	9.7% had hearing loss at medium frequencies and 15% had HFHL. Hearing thresholds were the highest at 4-8 kHz (4.13 dBHL ±15.5). Hearing thresholds were significantly higher in the exposed group versus the non-exposed group ($p=0.04$ for disco and concert attendants and $p<0.001$ for personal stereo users).	
Keppler, 2010.	Pre-post experimental study, n = 49	Volunteers, age range 19-28 years old	TEOAE, DPOAE and pure-tone audiometry	Significant 1.12 dB and 1.17 dB deterioration in hearing thresholds between pre-exposure and post-exposure measurements at 0.25 kHz ($p<0.05$) and 8.0 kHz ($p<0.05$), respectively. TEOAE amplitudes showed significant decreases of –0.47 dB and –0.70 dB at 2.0 kHz ($p<0.05$) and 2.8 kHz ($p<0.001$), respectively. The odds for significant threshold shifts were 2.18 up to 4.40 times greater in the noise exposure group with earbuds or headphones at 50% or higher compared with the control group.	Intervention exposure (pop rock music songs) in half of the participants: sound level 76.87 to 102.56 dBA for earbuds and from 71.69 to 97.36 dBA for supra-aural headphones, presented for approximately 1 hour and 2 minutes.

Supplemental Table 6.3: Characteristics of the included studies. (continued)

Source (author, year)	Study design, sample size	Study population characteristics	Measurements	Findings	Additional comments
Kim, 2009.	Cross-sectional study, n = 490	Middle and high school students, age range 13-18 years old	Pure-tone audiometry	Hearing thresholds were significantly higher in male than in female students, and higher in right ears than left ears (16.4 and 10.8 dB for males and 14.7 and 9.2 dB for females). There was no significant association between hearing threshold and daily PMP use, but there were significant elevated hearing thresholds at 4 kHz in students who had > 5 years of usage.	
Kumar, 2009.	Case-control study, n = 70 and 30, respectively	Graduate students, mean age of experimental group 20.5 years (range 17-24 years old)	DPOAE and pure-tone audiometry	No significant difference in mean pure-tone thresholds in PMP users and non-users. None of the PMP users had thresholds > 25 dB HL in 3 to 8 kHz in both ears. There was a significant positive correlation between hearing thresholds at 6000 Hz and exposed music levels, in both ears. All subjects had normal DPOAE in frequencies 1031 to 7277 Hz, without significant differences between the PMP use groups. There was a significant negative correlation between DPOAE and exposure at high frequencies, indicating lesser DPOAE amplitudes and SNRs in individuals listening to music at higher output levels.	
Le Prell, 2013.	Retrospective analysis of a laboratory database, n = 87	Normally hearing adult college students, average age 21.6 years (range 18-29 years old)	Extended high-frequency pure-tone audiometry and questionnaire	Male subjects had higher (worse) thresholds than female subjects. Differences in PTA thresholds were statistically reliable for the HFPTA comparison ($p \leq 0.05$), but not LFPTA or EHFPTA comparisons ($p > 0.05$). There was no statistically reliable relationship between hearing thresholds and PMP use. However, at and above 10 kHz there were small but statistically reliable differences for increased PMP use. Listening in noisy backgrounds associated with worse EHFPTA thresholds. 50% reported tinnitus in the absence of noise after noise exposure.	

Supplemental Table 6.3: Characteristics of the included studies. (continued)

Source (author, year)	Study design, sample size	Study population characteristics	Measurements	Findings	Additional comments
Lee, 2014.	Cross-sectional study, n = 1724	Freshman students of school of engineering, average age 18.4 years (range 15-22 years old)	DPOAE, TEOAE and questionnaire	DPOAE levels were lower for the high risk group at all frequencies, except 8 kHz. TPOAE levels were significantly lower for the high risk group at all frequencies. TEOAE amplitudes at 1.5 and 4 kHz, and DPOAE amplitudes at 4 kHz were significantly lower for males than for female subjects.	Occupational vs. control
Mostafapour, 1998.	Cross-sectional study, n = 50	College student volunteers with chronic PMP use, average age 22.1 years (range 18-30 years old)	Pure-tone audiometry and questionnaire	22% showed a 10 dB notch at 3 to 6 kHz (18% unilateral, 4% bilateral) and 28% had 15 to 25 dB notches (18% unilateral, 10% bilateral). 14% could be diagnosed with early NIHL based on bilateral notching. 46% of the PMP users showed a notch in either ear. There was no difference in pure-tone thresholds, speech reception thresholds, speech discrimination, and word recognition between PMP users and non-users. There was no correlation between PMP use and tinnitus and between length of exposure and notched audiograms.	
Peng, 2007.	Cross-sectional study, n = 150	Student volunteers, average age 20,6 years (range 19-23 years old)	Pure-tone audiometry	There were significant differences between PMP users and controls in hearing thresholds of conventional frequencies ($p < 0.01$), especially when PMPs were used > 5 years. At extended high frequencies, thresholds were higher for the PMP users ($p < 0.01$) as well. 14.1% of the ears showed hearing loss (> 25 dB HL) following long-term PMP use.	

Supplemental Table 6.3: Characteristics of the included studies. (continued)

Source (author, year)	Study design, sample size	Study population characteristics	Measurements	Findings	Additional comments
Phillips, 2010.	Cross-sectional study, n = 329	Classical music students, age range 18-32 years old	Pure-tone audiometry	0.9% of the music student population had substantial hearing loss. 45% had a notch at 4000 or 6000 Hz in at least one ear with a depth of at least 15 dB. Unilateral notches were more prevalent in the left ear (63%) than in the right ear (37%). 26% of the notches were bilateral. Bilateral NIHL was seen in 11.5% of the subjects. The prevalence of tinnitus was found at 17.1-18% and did not differ between students with or without notches. PMP use was not significantly related to the presence of a notch.	
Poissant, 2012.	Pre-post experimental study, n = 15	College-age trumpet players, age range 18-24 years old	DPOAE and pure-tone audiometry	Pure-tone thresholds 20 minutes post exposure were significantly poorer than pre-exposure thresholds (mean difference of 1.07 dB). DPOAE amplitudes significantly decreased in OAE SPLs at 2 minutes post exposure compared with pre-exposure levels. The largest decrease in DPOAE amplitude occurred in the 3000 Hz composite frequency band. Recovery of DPOAE amplitudes to baseline level was complete at approximately 40 minutes post exposure.	Music exposure: trumpet practice session following their normal routine for 1 hour, average L_{eq} unweighted 96.0 to 96.6 dB SPL, A-weighted L_{eq} 94.8 to 96.1 dBA.
Rao, 2014.	Cross-sectional study, n = 103	Students using PMPs, average age 20.14 years (range 18-25 years old)	Pure-tone audiometry	Median hearing thresholds were 13 dB for the right ear and 12 dB for the left ear, which was higher in males than in females. 12.62% had SNHL, mostly males (69.2%). There were significant relations between SNHL and listening music at loud volume and increased listening times. 4.8% showed a bilateral dip at 4 kHz. 6.8% showed a unilateral dip at either 1, 4 or 8 kHz.	

Supplemental Table 6.3: Characteristics of the included studies. (continued)

Source (author, year)	Study design, sample size	Study population characteristics	Measurements	Findings	Additional comments
Schlauch, 2011.	Cross-sectional study NHANES III, n = 5089	NHANES participants, age range 6-19 years old	Pure-tone audiometry	Almost all thresholds exceeded 0 dB HL at each frequency. Across all age groups, thresholds for 6.0 kHz were elevated relative to those for other frequencies, even among children with the best hearing.	
Schmuziger, 2006.	Retrospective assessment of medical records, longitudinal follow-up study, n = 33	Previous clinical patients exposed to continuous noise, average age 21.5 years shortly after exposure (range 12-34 years old), and 28.5 years at follow-up (range 15-45 years old)	Pure-tone audiometry and questionnaire	For the average thresholds of 3, 4, 6, and 8 kHz, thresholds shortly after exposure were 4 to 9 dB HL and 0 dB HL at follow-up. Extended high-frequency audiometry showed symmetrical median thresholds, without hearing loss. Uncomfortable loudness levels were significantly lower for the continuous noise group than for controls. All subjects reported tinnitus after the noise exposure. At follow-up, tinnitus was present in 67%, and mostly in both ears. HFPTA was not correlated with subjective auditory symptoms, such as hearing difficulties, hypersensitivity to sound and tinnitus.	
Serra, 2014.	Cross-sectional study, n = 172	Technical school students, age range 14-15 years old	TEOAE and pure-tone audiometry	34.9% had a slight or significant shift of hearing thresholds in at least 1 frequency. Differences between slight and significant shifts were significant in the extended high frequency range ($p < 0.05$). 16% had low-level or absent TEOAE amplitudes, with significant different amplitudes from ears with present amplitudes in all frequencies ($p < 0.01$). Subjects with hearing threshold shifts had significantly lower TEOAE amplitudes. There were no differences between the groups with slight or significant shifts in hearing threshold levels. Groups with increased hearing threshold levels had higher (but non-significant) participation rates of high music exposure.	

Supplemental Table 6.3: Characteristics of the included studies. (continued)

Source (author, year)	Study design, sample size	Study population characteristics	Measurements	Findings	Additional comments
Tin, 2000.	Pre-post experimental study, n = 48	Normally hearing tertiary institution students, average age 22 years (range 19-31 years old)	Pure-tone audiometry and questionnaire	Threshold shifts occurred in 72.7 up to 75.6% of the subjects, with changes of 7.7-10.6, 4.8-4.3, and 16.9-17 dB at 4, 6 and 8 kHz, respectively. 75.7 to 81.8% perceived their hearing to be worse after exposure and had significant threshold shifts in their audiograms. Tinnitus was experienced by 63.6% of those exposed to 108 dBA. Tinnitus was significantly related to the perception that music volume was too loud. Hearing thresholds and tinnitus all recovered in two days.	Music exposure (single discotheque visit): average sound level 101.3 dBA or 108 dBA for four hours.
Torre III, 2014.	Pre-post experimental study, n = 101	Normally hearing students, average age 21.4 years	DPOAE	Very little change in DPOAE after listening to music, with no significant effect. Males had significantly larger decreases than females at 3, 4, and 6 kHz.	Music exposure (at preferred listening volume): average sound level 57.8 dBA for 1 hour via earbuds.
Torre III, 2007.	Cross-sectional study, n = 436	Normally hearing marine recruit men, average age 19.2 years (range 17-29 years old)	DPOAE, pure-tone audiometry and questionnaire	Recruits who reported > 3 times loud live music exposure in the past year, had lower DPOAEs for frequencies 4.5 kHz, 5.0, 5.6, and 8.0 kHz ($p < 0.05$). Those who reported tinnitus after loud noise exposure, had significantly lower DPOAEs at 8 kHz ($p < 0.05$). In all groups with lower DPOAEs, the mean pure-tone thresholds were at most 2 dB poorer, which may account for the difference in DPOAEs. Risk factors smoking, loud music exposure and tinnitus had the lowest DPOAEs compared to those groups with fewer or no risk factors.	

Supplemental Table 6.3: Characteristics of the included studies. (continued)

Source (author, year)	Study design, sample size	Study population characteristics	Measurements	Findings	Additional comments
West, 1990.	Cross-sectional study, n = 49	Comprehensive school and university students, average age controls 15;6 years (range 15;2-15;11 years old), average age young exposed 15;9 years (range 15;3-16;2 years old) and average age older exposed 21;11 years (range 19;10-23;10 years old)	Pure-tone audiometry and questionnaire	21.4% of all ears showed notches. This was similar in the young exposed and the controls (15.6 and 14.7%, respectively), and increased in the older exposed (34.3%, p = 0.03). 14% of all notched subjects had bilateral notches. Unilateral notches were equally divided between left and right ears. Spontaneous tinnitus and subjective hearing difficulties of the exposed were reported by 6% and 18%, respectively. After exposure, this was 69.7% in the exposed, compared to 6% in the controls. There was no significant correlation between exposure and spontaneous tinnitus or hearing difficulties.	
Wong, 1990.	Cross-sectional study, n = 103	Youths from youth centers, age range 15-24 years old	Pure-tone audiometry	There was no significant difference in mean hearing thresholds between PMP users and non PMP users.	
Zocoli, 2009.	Cross-sectional study, n = 24	Private school students, average age 15.7 years (range 14-18 years old)	Pure-tone audiometry and questionnaire	8.3% had bilateral notches of at least 25 dB at 6 kHz. Subjects showing a 6 kHz notch used PMPs for 6-10 hours per day. There was no significant correlation between a mean attitude towards noise and hearing thresholds. Tinnitus was reported more by women (41%) than by men (28%), and only 0.4% reported permanent tinnitus.	

7 Prevalence of hearing loss among children 9 to 11 years old: the Generation R Study

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ABSTRACT

Importance: Hearing loss (HL), a major cause of disability globally, negatively affects both personal and professional life.

Objective: To describe the prevalence of sensorineural hearing loss (SNHL) among a population-based cohort of 9- to 11-year-old children, and to examine potential associations between purported risk factors and SNHL in early childhood.

Design, setting, and participants: The study was among the general, nonclinical, pediatric community within the city of Rotterdam, the Netherlands, and was conducted between 2012 and 2015 as a cross-sectional assessment within the Generation R Study, a population-based longitudinal cohort study from fetal life until adulthood. Participants are children of included pregnant women in the Generation R Study with an expected delivery date between April 2002 and January 2006. They form a prenatally recruited birth cohort.

Main outcomes and measures: Pure-tone air-conduction hearing thresholds were obtained at 0.5, 1, 2, 3, 4, 6, and 8 kHz, and tympanometry was performed in both ears. Demographic factors and parent-reported questionnaire data, including history of otitis media, were also measured.

Results: A total of 5368 participants with a mean age of 9 years 9 months (interquartile range, 9 years 7 months-9 years 11 months) completed audiometry and were included in the analyses. A total of 2720 were girls (50.7%), and 3627 (67.6%) were white. Most of the participants (4426 children [82.5%]) showed normal hearing thresholds 15 dB HL or less in both ears. Within the cohort, 418 children (7.8%) were estimated to have SNHL (≥16 dB HL at low-frequency pure-tone average; average at 0.5, 1, and 2 kHz or high-frequency pure-tone average; average at 3, 4, and 6 kHz in combination with a type A tympanogram) in at least 1 ear, most often at higher frequencies. In multivariable analyses, a history of recurrent acute otitis media and lower maternal education were associated with the estimated SNHL at ages 9 to 11 years (odds ratio, 2.0 [95% CI. 1.5-2.8] and 1.4 [95% CI, 1.1-1.7], respectively).

Conclusions and relevance: Within this cohort study in the Netherlands, 7.8% of the children ages 9 to 11 years had low-frequency or high-frequency HL of at least 16 dB HL in 1 or both ears. A history of recurrent acute otitis media and lower maternal education seem to be independent risk factors for presumed SNHL in early childhood.

INTRODUCTION

Hearing loss (HL) can have an impact on communication and relationships on a personal level and can negatively affect education and occupation (1-3). Globally, HL is the second leading cause of years lived with disability (4), making HL an important issue for society as well (5, 6). Although most apparent in later stages of life, it is probable that hearing acuity gradually declines with age and should therefore be studied at young ages as well (6). The prevalence of low-frequency or high-frequency HL in at least 1 ear (≥16 dB HL) was 14.9% among children aged 12 to 19 years in the cross-sectional National Health and Nutrition Examination Survey III (NHANES III) in 1988 to 1994 (7). The prevalence rose to 19.5% in NHANES 2005-2006 and the prevalence of bilateral HL (≥16 dB HL) was 5.5%. A cross-sectional cluster sample survey (8) of 6240 7- to 11-year-old Australian children published in 2010 showed 0.9% of the children having bilateral slight to mild sensorineural HL (SNHL) of 16 to 40 dB HL in low and high frequencies. There is a large spread in prevalence among the published literature, which consists mostly of cross-sectional studies (9, 10). The prospective cohort study Avon Longitudinal Study of Parents and Children (ALSPAC) found a prevalence of mild and high frequency bilateral HL of 0.5% at the age of 11 years (11).

To determine whether an increase of the prevalence of acquired HL in children is present, and if so, which factors are associated, there is a need for more longitudinal population-based studies with accurate outcome measures and covering numerous potential risk factors. Previous cross-sectional studies have observed associations between a history of otitis media and permanent HL among young adults (12-14). Other associations have been found for sex, socioeconomic status, and the use of personal music players (PMPs) (7, 8). A recent study among 9-year-olds showed that most seldom or never listen to music with headphones (15). Exposure to loud music, such as through PMPs and concert and party attendance, rises significantly around the age of 11 to 13 years (16, 17).

Our purpose is to perform a reliable longitudinal hearing evaluation, with repeated measurements, that has the ability to study associations with the development of permanent HL. To do this accurately, it is necessary to determine the baseline situation. With the current study, we aim to assess the prevalence of SNHL among 9- to 11-year-old children in a large cohort study, preceding the exposure to risk factors for acquired HL, such as smoking, alcohol use, and exposure to loud music. Furthermore, we aimed to examine potential associations with purported risk factors of early childhood, predominantly from socioeconomic background and medical history.

METHODS

Design and Sample

The current study was performed as part of the Generation R Study, a longitudinal cohort study that enrolled nearly 10,000 children born in Rotterdam, the Netherlands (18). Pregnant women with an expected delivery date between April 2002 and January 2006 were eligible for participation. The children born from these pregnancies form a prenatally included birth cohort that will be followed at least until young adulthood (19). The study is designed to identify early environmental and (epi)genetic causes and causal pathways leading to normal and abnormal growth, development, and health during fetal life, childhood, and adulthood. The research aims, follow-up rates, and measurements are described in more detail by Jaddoe et al (20). All children who were not withdrawn or lost to follow-up from the cohort at the start of the examination phase at the age of 9 to 11 years were eligible for participation, resulting in the invitation of 8548 children (Supplemental Figure 7.1). Participants were invited to the research center to undergo various physical examinations and measurements and received extensive questionnaires on medical history, family history, environmental factors, and lifestyle. A total of 5862 children underwent testing at the research center (68.6% of those invited). These children did not differ in year of birth from the children who did not participate or who participated solely by questionnaires (Supplemental Table 7.1). The participating children more often had higher educated mothers (59.3% [2953 children] vs 46.0% [586 children]; absolute difference of 13.3% [95% CI, 10.3%-16.4%]), were more often white (68.2% [3898 children] vs 52.6% [1777 children]; absolute difference of 15.6% [95% CI, 13.6%-17.7%]), and had more equal boy-girl distribution (49.7% [2913] boys among participating children vs 52.1% [2024] boys among nonparticipating children; absolute difference of 2.4% [95% CI, 0.37%-4.42%]) than children who did not visit the research center. Audiometric measurements included pure-tone audiometry and tympanometry. Of the 5862 children visiting the research center, 5434 (92.7%) participated in pure-tone audiometry. Data were collected between April 2012 and October 2015.

Oral and written informed consent of the parents was collected for all measurements. Participants received a small incentive for participation (a simple backpack), but there was no financial compensation. The general design, all research aims, and the specific measurements within the Generation R Study have been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam.

Pure-tone Audiometry

Hearing levels were obtained in a sound-proofed booth that met the maximum permissible ambient sound pressure levels of ISO 8253-1. In all participants, hearing was measured with a clinical audiometer (Decos audiology workstation; version 210.2.6 with AudioNigma interface) and TDH-39P earphones with MX-41/AR ear cushions. Calibration met the ref-

erence equivalent sound pressure levels specified by ISO 389-1. The calibration of all equipment was checked every 6 months. Pure-tone air-conduction thresholds were obtained at frequencies 0.5, 1, 2, 3, 4, 6, and 8 kHz by dedicated research assistants who were trained by a member of the Speech and Hearing Center. All thresholds were measured according to the shortened ascending method based on ISO-standard 8253-1, which means that thresholds were defined by the intensity level at which the tone was heard in 2 out of 3 ascents, resulting in a true clinical audiogram. The right or left ear was alternately tested first, and the measurement variance was 5 dB HL. When no response could be obtained, even at maximum stimulation level, the threshold was set to 5 dB above the maximum stimulation level. Owing to time constraints within the tight schedule of the examination day, masking was not applied, and bone conduction thresholds were not measured.

The air conduction pure-tone thresholds were averaged at low frequencies (0.5, 1 and 2 kHz) for a low-frequency pure-tone average (LPTA), and at high frequencies (3, 4 and 6 kHz) for a high-frequency pure-tone average (HPTA), as described by Niskar et al (21). Normal hearing was defined as both LPTA and HPTA ≤15 dB in both ears, regardless of tympanometry results. Increased hearing levels included slight (16 to 25 dB HL), mild (26 to 40 dB HL), moderate (41 to 55 dB HL), moderately severe (56 to 70 dB HL), severe (71 to 90 dB HL), or profound (≥ 91 dB HL), in accordance with the American Speech-Language-Hearing Association guidelines and in line with other prevalence studies (8, 21, 22).

Tympanometry

After air-conduction testing was completed, tympanometry (Interacoustics AT235h; stimulus frequency, 226 Hz) was performed for both ears, unless a contraindication, such as otorrhea or recent ear surgery, was present. Ear-canal volume, static compliance, middle ear pressure, and gradient were automatically calculated during the pressure sweep. Ears with an ear canal volume smaller than 0.3 mL were excluded to avoid ear canal collapse or occlusive cerumen influencing the results. Tympanograms were categorized as described by Jerger (23), assessing a value of at least 0.25 mL as normal compliance and a value between −100 and 100 daPa as normal middle ear pressure. Middle ear function was judged by the tympanograms to distinguish between conductive hearing loss (CHL) and SNHL in the absence of bone conduction threshold measurements. Any HL in combination with a type A tympanogram (suggesting normal middle ear function) was considered SNHL, since it is unlikely, although possible, that conductive hearing impairment would be present in ears with normal tympanograms (24). Loss of middle ear function, presented via type B and type C tympanograms, in combination with HL, was categorized as CHL, although mixed or underlying SNHL could not be excluded.

Demographic Covariates and Otitis Media

Demographic information of the participants and information on maternal education were collected via questionnaires at different time points, as part of the general study. Sex, age, race/ethnicity, gestational age at time of birth, and maternal education were selected to analyze their possible association with HL based on literature (7). Because of the large variety in races/ethnicity in the Generation R Study, race/ethnicity was grouped as white and nonwhite (20). Maternal education was used as a marker of socioeconomic status, categorized as lower (did not follow or finish higher education) or higher (finished higher education, namely higher vocational education or university). A history of acute otitis media was determined based on parent-reported otitis media at the ages of 2 and 6 months and 1, 2, 3, 4 and 5 years. Otitis media was classified as having no history of otitis media, acute otitis media or recurrent acute otitis media (Supplemental Figure 7.2).

Statistical Analysis

Pure-tone averages (mean [SD]) were evaluated in the context of the accompanying tympanogram, and between-group differences were analyzed using independent samples *t*-tests. We calculated prevalence estimates and 95% CIs for unilateral and bilateral HL at low and high frequencies (LFHL and HFHL, respectively). SNHL was the main interest in this study, and therefore only children with presumed SNHL and children with normal hearing were included in further analyses. Statistical analyses using independent samples *t*-tests compared the proportions of demographic and exposure variables between children with presumed SNHL and those with normal hearing. Univariable and multivariable logistic regression analyses were carried out to assess associations between the demographic and exposure variables (sex, age, gestational age at birth, maternal education, and otitis media) and the presence of presumed SNHL by calculating odds ratios (OR) with 95% CIs. IBM SPSS Statistics (version 21.0 for Windows) was used for data management and analyses, and an $\alpha = 0.05$ was used for statistical significance.

RESULTS

A total of 5368 participants with a mean age of 9 years 9 months (interquartile range, 9 years 7 months–9 years 11 months) completed audiometry and were included in the analyses. A total of 2720 (50.7%) were girls, and 67.6% (3627) were white. Children who completed audiometry at all frequencies in both ears were included in our analyses (n = 5368). Nearly 4800 of these children completed tympanometry (88.2%), of which 4344 right ears (80.9%) and 4298 left ears (80.1%) could be classified using the Jerger classification (23). The included 5368 children were grouped based on their audiological and tympanometry results,

shown in Figure 7.1. The demographics of the children with normal hearing, SNHL, and CHL are presented in Table 7.1.

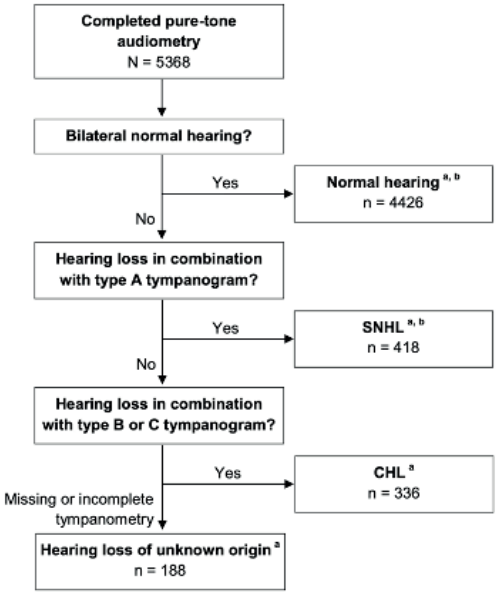

Figure 7.1: Flow chart of classification in groups based on audiological and tympanometry results.
Abbreviations: CHL, conductive hearing loss; SNHL, sensorineural hearing loss
[a] Included in prevalence estimate calculations.
[b] Included in regression analyses to assess associations between demographic and exposure variables and SNHL.

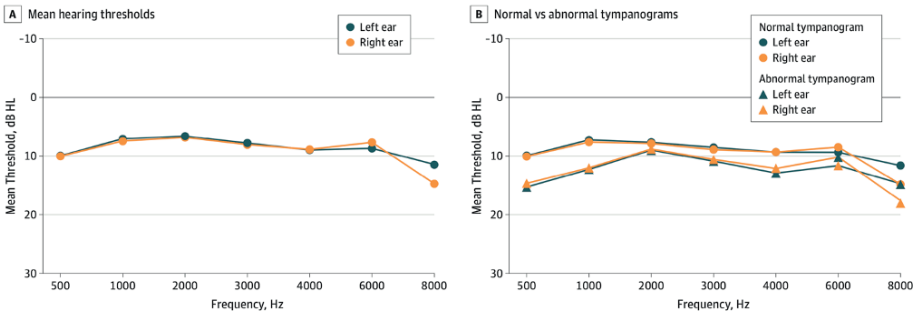

Figure 7.2: Mean hearing thresholds of the right and left ears.
A, Total study cohort of 5368 participants. B, Normal vs abnormal tympanograms.

Audiological Results

The mean (SD) LPTA of the total cohort was 8.10 (5.60) dB HL for right ears and 7.91 (5.78) dB HL for left ears (Figure 7.2, and Supplemental Table 7.2). The mean HPTAs were 8.22 (6.16) dB HL and 8.51 (6.18) dB HL for right and left ears, respectively. Tympanograms were most often of type A (3289 [75.7%] for right ears and 3301 [76.8%] for left ears), and 66.6% of the participants had bilateral type A tympanograms. Of the total studied cohort, 4426 children (82.5%) had normal hearing. Presumed SNHL (≥16 dB HL with a type A tympanogram) in at least 1 ear was present in 418 children (7.8%) at LPTA and/or HPTA, namely 7.7% (203) in boys and 7.9% (215) in girls. In 47 of these children (0.9% of the cohort) HL was of mild degree or worse (≥26 dB HL). More specifically, 33 children (0.6% of the cohort) had HL in the mild category (26-40 dB HL), 7 children (0.1%) had moderate SNHL, 5 children (0.1%) had moderately severe SNHL, and 2 children (0.0%) had profound SNHL. Sensorineural hearing loss was unilateral in 350 children (6.5%), and 68 children (1.3% of the cohort) had bilateral HL of at least 16 dB HL. In 103 of those children (1.9% of the cohort), HL was present at low and high frequencies in the same ear (all unilateral).

Table 7.1: Characteristics of the participants[a].

Characteristic	No. (%)		NH vs SNHL, Difference (95% CI)[d]	CHL, No. (%)[e]	NH vs CHL, Difference (95% CI)[d]
	NH[b]	SNHL[c]			
No.	4426	418		336	
Age, median (IQR)	9 y 9 mo (9 y 7 mo to 9 y 11 mo)	9 y 9 mo (9 y 7mo to 9 y 11 mo)	0.01 (−0.03 to 0.04)	9 y 9 mo (9 y 7 mo to 9 y 11 mo)	0.04 (−0.01 to 0.07)
Sex					
Male	2199 (49.7)	203 (48.6)	1.1% (−3.9% to 6.1%)	154 (45.8)	3.9% (−1.7% to 9.4%)
Female	2227 (50.3)	215 (51.4)	NA	182 (54.2)	NA
Gestational age at birth, mean (SD), wk	39.8 (1.9)	39.6 (2.0)	0.15 (−0.04 to 0.34)	39.8 (2.1)	0.02 (−0.23 to 0.19)
Race/ethnicity					
White	2996 (67.7)	272 (65.1)	2.6% (−2.2% to 7.4%)	226 (67.3)	0.1% (−5.1% to 5.3%)
Nonwhite	1332 (30.1)	136 (32.5)	NA	100 (29.8)	NA
Unknown	98 (2.2)	10 (2.4)	NA	10 (3.0)	NA
Maternal education					
Higher	2351 (51.8)	183 (43.8)	7.0% (1.5% to 12.5%)	174 (51.8)	1.0% (−4.8% to 6.8%)
Lower	1501 (34.3)	161 (38.5)	NA	120 (35.7)	NA
Unknown	574 (13.9)	74 (17.7)	NA	42 (12.5)	NA
History of otitis media[f]					
No	1827 (41.3)	144 (34.4)	6.4% (1.3% to 11.5%)	96 (28.6)	12.4% (7.0% to 17.8%)
AOM	1902 (43.0)	174 (41.6)	NA	162 (48.2)	NA
rAOM	444 (10.0)	67 (16.0)	NA	48 (14.3)	NA
Unknown	253 (5.7)	33 (7.9)	NA	30 (8.9)	NA

Abbreviations: AOM, acute otitis media; CHL, conductive hearing loss; HPTA, high-frequency pure-tone average; HL, hearing level; IQR, interquartile range; LPTA, low-frequency pure-tone average; NA, not applicable; rAOM, recurrent acute otitis media; SNHL, sensorineural hearing loss.

[a] The participants with hearing loss of unknown origin, because of incomplete or missing tympanometry (n = 188) are not shown.

[b] Hearing thresholds ≤15 dB HL at both LPTA and HPTA in both ears.

[c] Children with hearing thresholds ≥16 dB HL at LPTA or HPTA in either ear with a normal tympanogram, indicating SNHL.

[d] Differences were calculated based on participants with complete data regarding the variable.

[e] Children with hearing thresholds ≥16 dB HL at LPTA or HPTA in either ear with a type B or type C tympanogram, indicating CHL.

[f] Based on parent reports up to the age of 5 years.

Within ears with normal middle ear function (type A tympanograms) the average LPTA was 7.09 (4.79) dB HL in right ears and 6.84 (4.92) dB HL in left ears (Supplemental Table 7.2). LFHL was present in 3.9% of the right ears and 3.7% of the left ears. Table 7.2 shows hearing levels of children with bilateral normal tympanograms. HPTA was 7.49 (5.80) dB HL for right ears and 7.67 (5.47) dB HL for left ears, with a prevalence of 5.4% HFHL in right ears and 5.3% HFHL in left ears (Table 7.2).

Abnormal tympanograms were found in 1055 (24.3%) of the right ears and 997 (23.2%) of the left ears. Type B and type C tympanograms were bilaterally present in, respectively, 139 (3.6%) and 287 (7.3%) children. Children with tympanograms of types B and C had worse average hearing thresholds than children with type A tympanograms (mean difference in right ear, 2.9 dB HL [95% CI, 2.5-3.3]; mean difference in left ear, 3.5 dB HL [95% CI, 3.0-3.9]; Supplemental Table 7.2). Increased hearing thresholds were more prevalent among these ears (153 [29.8%] at low frequencies and 149 [29.0%] at high frequencies in either ear), resulting in 336 children (6.3% of the cohort) fulfilling the criteria of CHL.

Table 7.2: Hearing levels based on low-frequency pure-tone average in 2631 children with bilateral normal tympanograms[a].

	Right Ear, % (95% CI)		
Left Ear	**Normal (≤15 dB HL)**	**Slight (16-25 dB HL)**	**≥Mild (≥26 dB HL)**
Low Frequency[b]			
Normal (≤15 dB HL)	94.4 (93.5-95.3)	2.2 (1.6-2.8)	0.1 (0.0-0.3)
Slight (16-25 dB HL)	2.0 (1.5-2.6)	1.0 (0.6-1.4)	0.0 (0.0-0.1)
≥Mild (≥26 dB HL)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.2 (0.0-0.3)
High Frequency[c]			
Normal (≤15 dB HL)	92.5 (91.5-93.5)	2.7 (2.0-3.3)	0.2 (0.0-0.4)
Slight (16-25 dB HL)	2.8 (2.2-3.5)	1.1 (0.7-1.5)	0.2 (0.1-0.4)
≥Mild (≥26 dB HL)	0.2 (0.0-0.3)	0.2 (0.1-0.4)	0.2 (0.0-0.3)

Abbreviation: HL, hearing level.

[a] Hearing levels presented as prevalence (95% CI) per category.

[b] Low-frequency pure-tone average = (500 + 1000 + 2000) Hz / 3.

[c] High-frequency pure-tone average = (3000 + 4000 + 6000) Hz / 3.

Purported Risk Factors in Young Children (Ages 9-11 Years) with SNHL

There were no significant differences in sex (1.1% [95% CI, −3.9% to 6.1%]), age (0.01 [95% CI, −0.03 to 0.04]), gestational age at birth (0.15 weeks [95% CI, −0.04 to 0.34 weeks]), and race/ethnicity (2.6% [95% CI, −2.2% to 7.4%]) between children with SNHL and those with normal hearing (Table 7.1). Higher maternal education was less prevalent in the group with SNHL than in the group with normal hearing (mean difference, 7.0%; 95% CI, 1.5%-12.5%). A history of otitis media was present more often in children with SNHL than in children with normal hearing (mean difference, 6.4%; 95% CI, 1.3%-11.5%). Associations between several available risk indicators and presumed SNHL were statistically evaluated. Sex, age, gestational age at birth, and race/ethnicity were not associated. Recurrent acute

otitis media was significantly associated with presumed SNHL (OR, 1.9; 95% CI, 1.4-2.6), whereas merely a history of otitis media (without being of recurrent origin) was not (OR, 1.2; 95% CI, 0.9-1.5). Another significant association was found between the educational status of the mother and the presence of presumed SNHL, with OR, 1.3 (95% CI, 1.1-1.7) for maternal education of a lower level. These positive associations remained present in a multivariable analysis, while adjusting for sex, age, gestational age, and race/ethnicity, indicating independence of the associations (OR, 1.4 [95% CI, 1.1-1.7], for lower maternal education, and OR, 2.0 [95% CI, 1.5-2.8], for recurrent otitis media).

DISCUSSION

To our knowledge, this study is the first large population-based study to examine hearing acuity among school-aged children born in the Netherlands. It demonstrates that 7.8% of the children were estimated to have SNHL in low or high frequencies in at least 1 ear, which was similar in boys and girls. Most of the HLs were unilateral and of slight degree (16-25 dB HL). The prevalence of mild or more severe HL of our study was 0.9%, and bilateral SNHL was present in 1.3%. The latter is slightly higher than the prevalence of bilateral SNHL that was reported in previous large studies among children of similar age, namely 0.5% (11) and 0.9% (25). Altogether, 17.5% of the cohort had hearing thresholds exceeding 15 dB HL (7.8% SNHL, 6.3% CHL, and 3.5% HL of unknown origin). This is roughly comparable with the prevalence of 16.0% HL among 12- to 13-year-old children in the NHANES studies, which did not distinguish between SNHL and CHL (7).

To achieve optimal comparability between our study and most of the previously published literature, we chose to use the most frequently used HL categorization of low and high frequencies as described by Niskar et al (21). This criterion includes even small HLs in either low- or high-frequency regions. Most of the HLs in the current study were of slight degree and are probably subclinical. Although the clinical relevance of these slight losses might be uncertain (2, 25, 26), they are indicative of mild cochlear damage, which in turn might be relevant regarding the young age of the participants and their future. Moreover, cochlear synaptopathy (hidden HL) does not, or does slightly, elevate hearing thresholds in pure-tone audiometry, while significantly contributing to hearing-in-noise difficulties and tinnitus (27). Therefore, even though HL is only of slight degree in pure-tone audiometry, possible effects should not be underestimated.

We found that recurrent acute otitis media was associated with the presumed SNHL, which is in line with some (12-14), but not all (8), previous studies among children and young adults. Limited evidence exists that inflammatory mediators present in otitis media can cause changes in the auditory structures, including causing cochlear damage and sensorineural hearing loss over and above CHL (28). Maternal education was associated with

worse hearing acuity as well, showing that increased odds were present for lower maternal educational status. This could be related to the more general socioeconomic status, which is partly determined by educational level among other factors (29). Also, maternal education has been found to be a prognostic indicator for a child's success with a cochlear implant (30, 31). Together with associations between socioeconomic variables and HL that have been found before (7), maternal education and socioeconomic status seem important factors in relation to hearing. This could be topic of further studies, while including other household variables and preferably modifiable factors in search of possible targets for prevention.

The current study was conducted as part of the Generation R Study, which, besides a large sample size, has the benefits of being closely related to clinical practice and comprises a large number of determinants. Testing was performed by dedicated personnel with a small variance, and accordingly we do not expect this variance to explain the presented results. Although this is a longitudinal study, the audiometric assessments described herein were cross-sectional. As a result, we cannot be certain whether the found HLs are acquired or congenital. Most of the included children probably participated in the universal newborn hearing screening that is available in the Netherlands (32), but these measurements are less sensitive for slight HL compared with pure-tone audiometry and are unfortunately not available within the Generation R Study. Hearing evaluation within the Generation R Study will be repeatedly measured in future assessments, and longitudinal data will become available in the future. This will provide information on the permanence and the course of the detected hearing losses. In addition, it gives the possibility to assess the test-retest variability and reliability, for instance, due to attentive factors within the children.

Limitations

Our audiometry was limited by the lack of bone-conduction hearing thresholds owing to strict time constraints in conducting audiological measurements among several other assessments within the Generation R Study. Therefore, tympanometry served as a measure to estimate middle ear function to differentiate between CHL and SNHL. Our main interest was to focus on permanent HL, without possible interference of middle ear pathology. To achieve this, we chose to include only those children with type A tympanograms in the main analyses on presumed SNHL and to accept the decrease in sample size. Also, there was a small but significant difference between the participants of the study and those who chose not to participate, and maternal educational level was missing in a noteworthy proportion of the participants. This might influence the generalizability of the results. However, because an association was found between lower maternal education and presumed SNHL, and those who participated in the study seemed higher educated than those who did not, the current presented prevalence of SNHL is probably rather an underestimation than an overestimation.

Sensorineural hearing loss, and likely acquired SNHL, is not only a problem of the older adolescents, but it also occurs at younger ages as well (6, 7). It is possible that the acquired HL that becomes apparent at older age actually starts to develop at younger age. More importantly, identifying HL at an early stage could prevent communicational and educational difficulties. The association of recurrent acute otitis media with presumed SNHL suggests a possible effect thereof. However, it would be too strong of a claim within this study to state that acquired HL was caused by ear infections earlier in life. Further studies must evaluate the association between otitis media and HL, while taking other confounding factors into account. More detailed information on possible risk factors is required, which would ideally be collected in an objective and not a self- or parent-reported manner.

CONCLUSION

In this cross-sectional assessment of a population-based prospective birth cohort study, 7.8% of children were estimated to have low- or high-frequency SNHL in either ear, with a prevalence of 0.9% of mild or more severe SNHL (≥26 dB HL). A history of recurrent acute otitis media and lower maternal educational status were associated with the presence of presumed SNHL.

Detailed acknowledgements and supplemental content can be found in the published article online: https://jamanetwork.com/journals/jamaotolaryngology/fullarticle/10.1001/jamaoto.2017.1068

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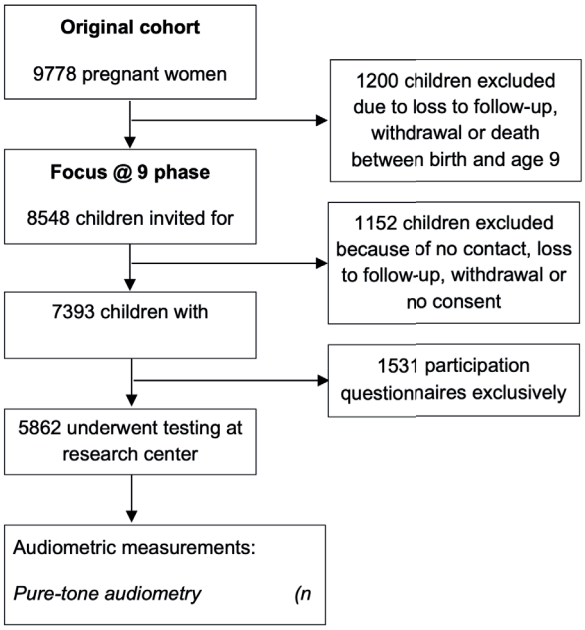
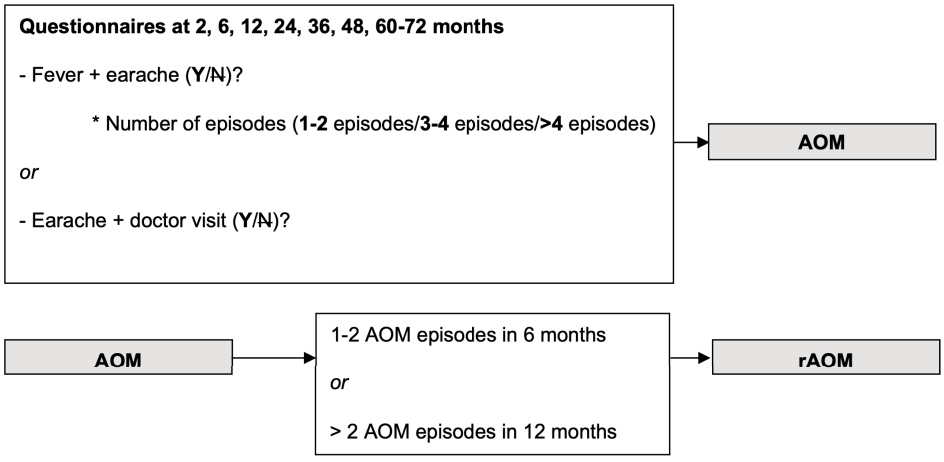
SUPPLEMENTARY MATERIAL



Supplemental Figure 7.1: Flow chart of the Generation R Study participation.



Supplemental Figure 7.2: Flow chart of questionnaire data used to define otitis media phenotypes.

Supplemental Table 7.1: Characteristics and comparison of study participants versus non-participants.

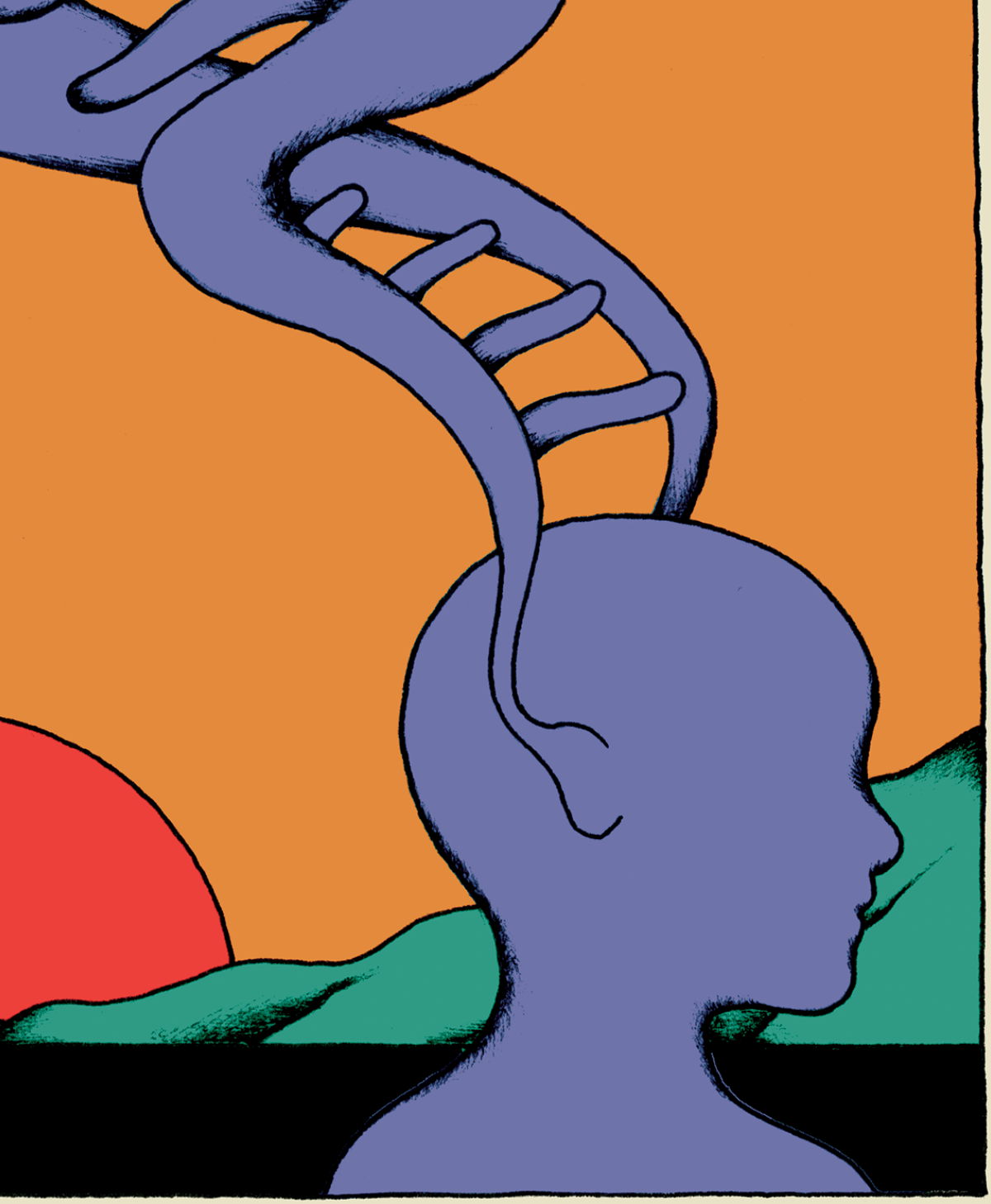
Characteristic		Tested at research center		Difference	95% CI
		Yes, n (%)	No, n (%)	%	
Year of birth	2002	578 (9.9)	374 (9.6)	0.24	-0.97 - 1.44
	2003	1936 (33.0)	1315 (33.8)	-0.80	-2.72 - 1.11
	2004	1700 (29.0)	1174 (30.2)	-1.20	-3.06 - 0.65
	2005	1626 (27.7)	1018 (26.2)	1.55	-0.25 - 3.34
	2006	22 (0.4)	6 (0.2)	0.22	0.02 - 0.42
Sex	Male	2913 (49.7)	2024 (52.1)	2.40	0.37 - 4.42
	Female	2948 (50.3)	1861 (47.9)		
Ethnicity	Caucasian	3898 (68.2)	1777 (52.6)	15.64	13.57 - 17.71
	Non Caucasian	1815 (31.8)	1602 (47.4)		
Maternal education	Higher	2953 (59.3)	586 (46.0)	13.34	10.28 - 16.40
	Not higher	2027 (40.7)	689 (54.0)		

Supplemental Table 7.2: Hearing thresholds for the total study cohort, and for ears with normal and abnormal tympanograms.

Ear	Frequency (Hz)	Hearing Threshold (dB HL)[a]			Absolute difference (dB HL)	95% CI
		Total Cohort	Normal Tympanogram	Abnormal Tympanogram		
Right		*N = 5368*	*n = 3289*	*n = 1055*		
	500	10.04 (7.06)	8.73 (6.14)	13.63 (7.59)	4.90	4.40-5.41
	1000	7.44 (6.58)	6.14 (5.66)	10.82 (7.24)	4.68	4.20-5.16
	2000	6.82 (6.34)	6.40 (5.91)	7.39 (6.78)	0.99	0.56-1.44
	3000	8.09 (6.37)	7.50 (5.99)	9.30 (6.50)	1.80	1.36-2.25
	4000	8.86 (7.34)	7.94 (6.81)	10.93 (7.98)	2.99	2.46-3.53
	6000	7.70 (7.87)	7.04 (8.73)	8.88 (9.22)	1.84	1.21-2.48
	8000	14.72 (9.86)	13.83 (9.17)	16.79 (10.65)	2.96	2.24-3.67
Left		*N = 5368*	*n = 3301*	*n = 997*		
	500	10.00 (7.21)	8.60 (6.18)	14.29 (8.26)	5.69	5.13-6.24
	1000	7.10 (6.78)	5.73 (5.72)	11.10 (7.86)	5.67	4.84-5.89
	2000	6.63 (6.61)	6.19 (6.23)	7.58 (7.47)	1.39	0.88-1.90
	3000	7.82 (6.39)	7.09 (5.83)	9.61 (7.26)	2.52	2.03-3.02
	4000	8.99 (7.36)	7.95 (6.49)	11.76 (8.68)	3.81	3.23-4.40
	6000	8.71 (9.04)	7.98 (8.48)	10.41 (10.19)	2.43	1.73-3.13
	8000	11.47 (10.01)	10.42 (9.27)	13.70 (11.19)	3.29	2.52-4.05

[a] Hearing thresholds are presented as mean (SD).

IV New models of otitis media



8 Biotechnology, biomedical engineering and new models of otitis media

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ABSTRACT

Objective: To summarize recently published key articles on the topics of biomedical engineering, biotechnology and new models in relation to otitis media (OM).

Data Sources: Electronic databases: PubMed, Ovid Medline, Cochrane Library and Clinical Evidence (BMJ Publishing).

Review methods: Articles on biomedical engineering, biotechnology, material science, mechanical and animal models in OM published between May 2015 and May 2019 were identified and subjected to review. A total of 132 articles were ultimately included.

Results: New imaging technologies for the tympanic membrane (TM) and the middle ear cavity are being developed to assess TM thickness, identify biofilms and differentiate types of middle ear effusions. Artificial intelligence (AI) has been applied to train software programs to diagnose OM with a high degree of certainty. Genetically modified mice models for OM have further investigated what predisposes some individuals to OM and consequent hearing loss. New vaccine candidates protecting against major otopathogens are being explored and developed, especially combined vaccines, targeting more than one pathogen. Transcutaneous vaccination against non-typeable Haemophilus influenzae has been successfully tried in a chinchilla model. In terms of treatment, novel technologies for trans-tympanic drug delivery are entering the clinical domain. Various growth factors and grafting materials aimed at improving healing of TM perforations show promising results in animal models.

Conclusions: New technologies and AI applications to improve the diagnosis of OM have shown promise in pre-clinical models and are gradually entering the clinical domain. So are novel vaccines and drug delivery approaches that may allow local treatment of OM.

Implications for practice: New diagnostic methods, potential vaccine candidates and the novel trans-tympanic drug delivery show promising results, but are not yet adapted to clinical use.

INTRODUCTION

Otitis media (OM) is a major cause of health care visits, antibiotic prescriptions and surgery, especially in the pediatric population (1, 2). Its complications and sequelae are important causes of preventable hearing loss as well as serious infections, particularly in developing countries. This report from our 'Biotechnology, Biomedical Engineering and New Models of Otitis Media' panel as drafted by 12 clinicians and scientist who convened during the 20th International Symposium on Recent Advances in Otitis Media, Hollywood, CA, USA in June 2019. We focused on articles on the above topics which were published since the last report in 2015 (3).

METHODS

Panel members were assigned to review the following topics: imaging of the tympanic membrane (TM) and the middle ear, bioengineering, mechanical models, animal models (advances in genetics, microbiology and vaccines), innovative treatment approaches, material science and tissue engineering. Each panel member designed a topic-specific key-word search strategy for the various electronic databases [PubMed, Ovid Medline, the Cochrane Library and Clinical Evidence (BMJ Publishing)]. Databases were searched for publications with an English language abstract published from 5/31/2015 to 5/31/2019. Additional relevant articles identified during the meeting were added. In total, 132 articles were included in this manuscript.

RESULTS

1: IMAGING TECHNOLOGIES

Visible light (pneumatic) otomicroscopy is considered the best diagnostic tool for OM currently available. Specific features of the TM, such as bulging and redness, are vital for making a correct diagnosis (4). However, otomicroscopes are not available everywhere, and require training. The second-best option is pneumatic otoscopy; however, it has multiple and well documented limitations regarding diagnostic certainty (5-8). To overcome current diagnostic challenges in otoscopy, several new technologies are being developed, aimed at mapping the TM and assessing and quantifying the presence of middle ear effusion (MEE). Table 8.1 summarizes these recent advances in imaging technologies.

1.1: Tympanic Membrane

Optical coherence tomography (OCT) has been proposed to evaluate structural changes of the TM. OCT is an optical imaging technique analogous to ultrasound, enabling a cross-sectional view of the TM and the identification of thickness changes and biofilm attachment to the TM (9-12). OCT coupled with pneumatic otoscopy enables quantification of TM mobility (compliance) and middle ear pressure (13, 14). Optical methods to delineate the three-dimensional (3D) contour of the TM have also been explored. One modality utilizes structured light to form a 3D image (15). Another 3D imaging method is light field where a plenoptic camera records numerous measurements corresponding to rays passing through different locations in the aperture. Plenoptic data can be demultiplexed to a set of multi-view images forming a 4-dimensional structure called the light field. From this, a 3D picture can be computed (16). Another technology is terahertz otoscopy, based on the high affinity of terahertz waves for water. This technique has been explored *ex vivo*, but there is no clinical data to this date (17).

1.2: Middle Ear

Evaluation and diagnosis of the middle ear status using conventional oto(micro)scopy and tympanometry have remained dependent on the physician's experience and interpretation. Superior resolution and assessment of the middle ear cavity and its content can potentially reduce over-prescription of antibiotics for AOM, ventilating tube (VT) insertion if prolonged MEE is detected, and diagnostic exploratory surgery in challenging otologic pathology. High frequency ultrasound (HFUS) has been successfully utilized *ex vivo* and *in vitro* to visualize middle ear pathologies, like ossicular pathologies, with high resolution (18-20). Spectral gradient acoustic reflectometry (SGAR) has been tested to predict the presence of MEE, with high sensitivity following short training, but specificity is relatively poor compared to tympanometry (21). Transmastoid ultrasound has been used to detect MEE with high accuracy, yet improved design of the probe for clinical use is needed (22). Visual light techniques include multicolor reflectance imaging and narrow band imaging (NBI). Multicolor reflectance imaging produces high-definition images with demarcation of morphological structures to detect MEE (23). The technique provides superior imagery of middle ear pathology compared to current methods, but patient movement and image distortion remain substantial challenges. NBI has been used to investigate middle ear anatomy using specific blue and green light wavelengths that interact with hemoglobin to enhance illustration of TM vascularity (24). Near infrared light techniques include anti-confocal middle ear assessment and OCT. Anti-confocal spectroscopic measurements to assess middle ear inflammation by analyzing blood content have been successful *in vitro*, but *in vivo* assessment has yet to be conducted (25). OCT enables non-invasive characterization of middle ear pathology *in vivo*, in addition to TM imaging (Figure 8.1). OCT has the largest number of publications over the specified time period and, most notably, has been

shown to facilitate non-invasive differentiation of the type of MEE (9, 10, 20, 26-34). An otoscope sensitive to shortwave infrared (SWIR) wavelengths of light provides two primary advantages over conventional visible light-based pneumatic otoscopy: it can help identify MEE based on the strong light absorption by fluid in the SWIR spectral region, and can penetrate deeper through tissue, enabling a view of middle ear anatomy behind the TM (35, 36) (Figure 8.2). Raman spectroscopy is able to distinguish serous from mucoid MEEs *ex vivo* (37). Scanning laser doppler vibrometry can detect changes in chinchilla TM motion evoked by sound during OM, with reduced amplitudes and a shift towards lower frequencies (38). Synchrotron radiation phase-contrast imaging has shown improved contrast and thereby visualization and finite-element modeling of middle ear structures (39).

Table 8.1: Recent advances in middle ear imaging.

Technique	Basic Principles	Purpose	Results	Benefits / Limitations	Ref.
Acoustic Techniques					
High frequency ultrasound (HFUS)	HFUS waves are used to visualize behind the eardrum. High frequency waves allow for higher resolution at smaller depths.	Visualization of middle ear anatomy and pathology.	HFUS allows for visualization of the middle ear past an intact TM in real time.	HFUS enables visualization of middle ear and contents, however no *in vivo* studies have been done and HFUS may not translate to imaging through thicker soft tissue.	(18-20)
Spectral gradient acoustic reflectometry (SGAR)	Inaudible sonar waves are reflected off the middle ear wall to assess the presence / absence of effusion.	MEE detection.	SGAR has high sensitivity, but suffers poor specificity.	SGAR has a high negative predictive value, and is relatively easy technology to use clinically, even on less than compliant patients. SGAR however cannot detect effusion progression/clearance or differentiate from AOM. Also, SGAR is not superior to nor synergistic with tympanometry.	(21, 50)
Transmastoid ultrasound	Detection of effusion via the mastoid air system.	MEE detection.	Transmastoid ultrasound has a high accuracy rate (81%).	Transmastoid ultrasound has a high accuracy but overcoming states when probes cannot be inserted into the external ear canal is a drawback.	(22)

Table 8.1: Recent advances in middle ear imaging. (continued)

Technique	Basic Principles	Purpose	Results	Benefits / Limitations	Ref.
Visible Light Techniques					
Multicolor reflectance imaging	By incorporating RGB (multicolor) narrow-band reflectance imaging to a standard video otoscope, differences in tissue properties can be assessed.	Detection of AOM, MEE, and cholesteatoma.	The high-definition images depict middle ear mucosal structures to a better extent, combined with better demarcation of morphological structures.	Multicolor reflectance imaging provides high quality imagery of middle ear pathology. However, this technology is particularly susceptible to patient movement and image distortion.	(23)
Narrow band imaging (NBI)	Narrow band light penetrates the tissue at different depths and indicate areas of hypervascularity.	Improved visualization of tissue based on varying degrees of vascularity.	NBI is a feasible technology for measuring the extent of a disease, and small residual disease is not ignored.	NBI enables differentiation of healthy and diseased tissue, and the extent of the disease.	(24)
Near Infrared Techniques					
Anti-confocal middle ear assessment	Spectroscopic measurements to assess inflammatory states of middle ear, by analyzing blood content.	MEE detection.	On phantom ear models, human MEE inserted was detected.	While this *in vitro* study shows promising results for detection of MEE, extrapolation into use in humans may prove less capable due to patient movement.	(25)
Optical coherence tomography (OCT)	Low coherence (broadband) interferometry (light interference) penetrates the tissue to obtain depth visualization in high resolution. Scanning the near infrared beam laterally enables 2/3D imaging.	MEE detection and differentiating acute or chronic OM states.	Using the TM thickness combined with the presence of MEE enabled differentiation of fluid type. Additionally, OCT uniquely enables visualization of middle ear biofilm structure *in vivo*.	OCT enables structural and dynamic visualization of TM thickness, middle ear contents (as well as MEE differentiation), and biofilm structure, in high resolution. While OCT can be used to visualize the ossicles, it cannot image through them. Yet multiple clinical studies have been conducted using OCT, and this modality has the largest body of evidence of the imaging techniques discussed.	(26-34)

Table 8.1: Recent advances in middle ear imaging. (continued)

Technique	Basic Principles	Purpose	Results	Benefits / Limitations	Ref.
OCT: quantitative pneumatic otoscopy	Measure TM micro-displacement differences using a pneumatic OCT-otoscope. TM compliance and middle ear pressure are calculated via displacement amplitude ratio.	MEE detection and differentiating acute or chronic OM states.	Proof of technical capability shown, wherein decreased compliance in MEE cases, decreased amplitude ratio in AOM cases, due to positive middle ear pressure.	Pneumatic OCT-otoscopy enables objective quantification of both middle ear mobility (compliance) and middle ear pressure. However, this technique still suffers the requirement of an ear canal seal to collect data.	(14)
OCT: TM thickness mapping	TM thickness mapping using a 1-D OCT-otoscope and sampling TM thickness measurements at 500 different TM locations.	Visualization of middle ear anatomy and pathology.	Proof of concept shown, wherein TM thickness was seen to increase 100–200% when MEE and/or AOM are present.	While this new application for a low-cost OCT device shows promise, this approach struggles with differentiation of MEE type and suffers a relatively long image processing time.	(11, 38)
Radiology Techniques					
Synchrotron radiation phase-contrast imaging (SR-PCI)	A phase-shifted beam interferes with the original beam to produce measurable fringes that correspond to the surfaces and structural boundaries of the sample.	Visualization of middle ear anatomy and pathology.	SR-PCI edge-enhancement provides clearer visualization of bone structure and brighter borders for higher density structures, as well as soft tissues like TM, ligaments, and joints.	R-PCI provides improved contrast and detectability of soft tissue in intensity profile compared to absorption contrast micro-CT. Images provide a more accurate 3D reconstruction of the ossicles. However, this radiative imaging modality poses standard health risks.	(39)

2: BIOENGINEERING

2.1: Computerized Software

Artificial intelligence has been used to train computer software to diagnose OM from otoscopic images of the TM with a diagnostic accuracy of over 90% (40-42). A computer vision system was able to automatically detect VTs in otoscopic images of high as well as low quality. The offline training process constructed a 3-layer cascaded classifier, with each layer reflecting specific characteristics of the VT. When trained using 215 images, it could achieve a 90% accuracy in terms of classifying otoscopic images with and without VTs (43).

2.2: 3D Models

3D reconstructions of cadaver ears from patients with chronic OM or cholesteatoma has shown reduced volumes of the bony portion of the Eustachian tube (ET) compared to normal controls (44). Computerized tomography (CT) scans have been used to measure ET diameter in healthy adults and young children <10 years old who exhibit reduced ET opening efficiency, and thus were considered to be OM prone. A smaller diameter was detected in the latter (45). CT scans can reliably measure the length of the ET cartilaginous portion, but this measurement seems to be of limited prognostic value for surgical treatment, i.e. successful ET opening dilatation (46). Multi-scaled modeling has been employed to study if the ET is more affected by mucosal adhesion in children than in adults, potentially contributing to their increased susceptibility to OM (47). A 3D model of the chinchilla ear, based on Xray micro-CT images, has helped characterizing middle ear functions (48). A conventional 2D monocular endoscope coupled with a computer-based 3D imaging system has been tested during otologic surgery for chronic otitis media (COM), cholesteatoma, otosclerosis and cochlear implant, thereby giving 3D vision endoscopic procedures (49). The presence of MEE has also been explored by a machine-learning software algorithm able to assess TM mobility using speakers and microphones within a normal smartphone (50).

3: ANIMAL MODELS

Several animal models of OM have been used in recent years. The mouse has become one of the favored models because of the mouse life span, its easy breeding and the well-established methods for introducing genetic modifications. The similarities in auditory structure between mouse and human and the similarity of the genomes make the mouse a valuable model to study the genetics of hearing.

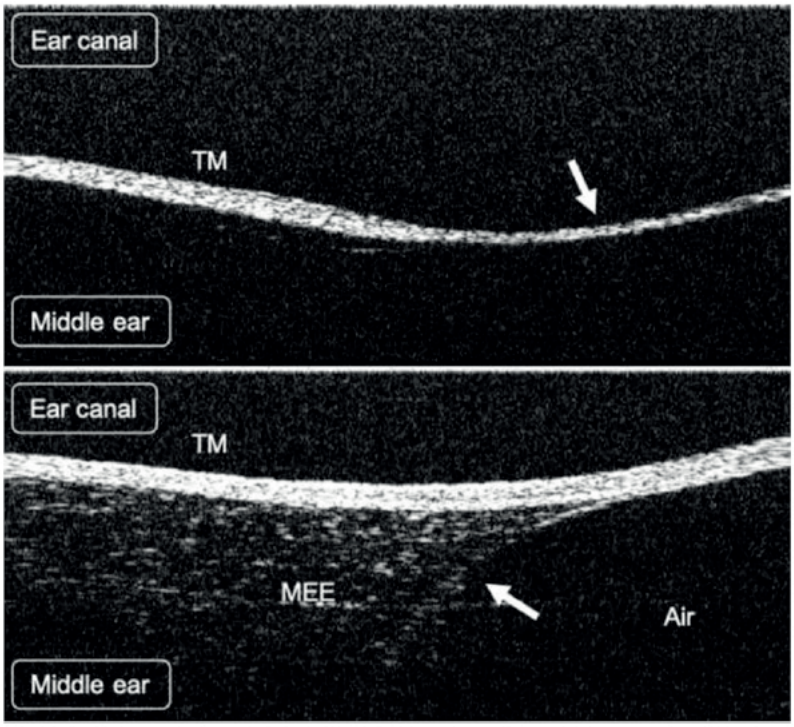

Figure 8.1: Optical coherence tomography (OCT) images of the tympanic mem- brane (TM) and underlying middle ear space. Top: Both normal thickness and monomer (thinned) portions (arrow) of the TM can be assessed in OCT's cross- sectional views. Bottom: Middle ear effusion (MEE) and air-fluid boundary (arrow) can be directly visualized as well using OCT.

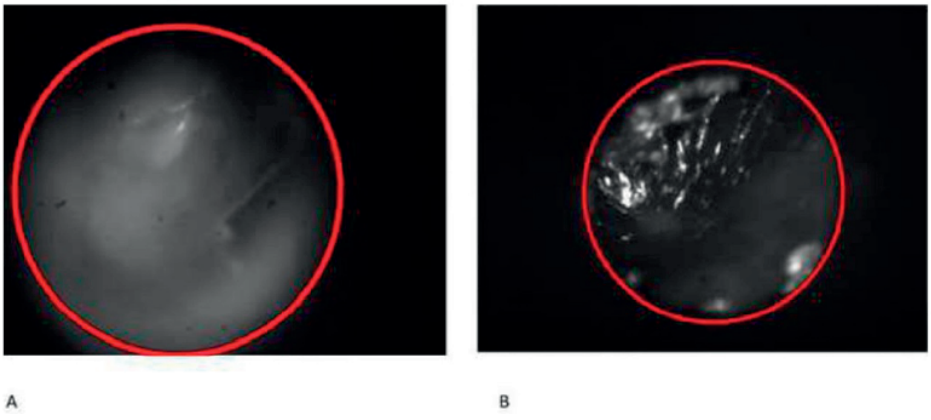

Figure 8.2: Otoscopy Images on the SWIR: (A) with evidence of the promontory and incus seen through the tympanic membrane; (B) blackout effect in otitis media with effusion due to water absorption in the SWIR.

3.1: Genetics

In the past four years, new genetic models to study the predisposition to OME have been developed:

1.) FLI1 and ETS1 are transcription factors known to be homozygous in Jacobsen syndrome. They play a role in the development of the nose, middle ear cavity and ossicles. The Fli1 ± and Ets1 ± Fli1 ± mice exhibit hearing impairment associated with chronic OM, a small middle ear cavity, and fusions deformities of the ossicles (51).
2.) Ages-with-stiffened-joints mutant mice, which have a point mutation in the Enpp1 gene develop conductive hearing loss, MEE and ME deformities at an early age (52).
3.) Mutations in the EDA, EDAR and EDARADD genes are associated with the development of OM, rhinitis and nasopharyngitis (53).
4.) A point mutation in the Nischarin (NISCH) gene results in the development of conductive hearing loss due to chronic OM. Homozygous mice spontaneously develop chronic OM at three weeks of age, and sometimes an inflamed TM (54).
5.) BPIFA1, a bacterial permeability-increasing fold innate defense protein, is one of the most abundant secretory proteins in the upper respiratory tract. Bpifa1 knock out mice do not develop spontaneous OM up to six months, although BPIFA1 is highly expressed in the middle ear epithelium. However, deletion of Bpifa1 in Junbo mice resulted in significant exacerbation of the phenotype including thickening of the middle ear mucosa and increased collagen deposition. This finding indicates a role of BPIFA1 in mucosal protection (55).

3.2: New Animal Models

A novel *in vitro* model of mouse middle ear epithelial cells, incorporating an air-liquid interface (ALI) has been developed (56) and recapitulated the characteristics of the native murine middle ear epithelium. After bacterial infection, it mimicked features of the epithelium in OM.

3.3: Microbiology and New Vaccines

Animal models and cell cultures have been used in vaccine research, including studies of basic immunologic mechanisms and those aimed at finding vaccine candidate molecules and protein carriers (57). *Streptococcus pneumoniae* (Spn), non-typeable *Haemophilus influenza* (NTHi) and *Moraxella catarrhalis* (Mcat) are the major AOM bacteria. With viral-bacterial interactions playing an important role in the pathophysiology of AOM, vaccines against respiratory syncytial virus (RSV) and influenza virus are being developed (58, 59).

3.3.1: Streptococcus Pneumoniae (Spn)

Vaccines against a larger number of pneumococcal serotypes than in the current 10- and 13-valent pneumococcal conjugated vaccines are being developed, but no new trials have

been conducted with them (60). The main findings on research about pneumococcal OM are summarized in Table 8.2. Most research has focused on the identification of virulence peptides in both encapsulated and non-encapsulated strains and the investigation of quorum sensing peptides in bacterial persistence (61). Recent studies have shown that a secreted metabolite byproduct of the LuxS/AI-2 quorum sensing system enables Spn to utilize galactose as a carbon source and increases the production of capsular polysaccharide and the development of a hypervirulent OM phenotype in a mouse model (62). Single nucleotide polymorphisms in the raffinose uptake and utilization genes *rafR* or *rafK* dictate the nature of pneumococcal disease (63) and genes involved in the metabolism of sugars influence the virulence of bacteria during OM in chinchillas (64).

Table 8.2: Recent advances in research on Spn induced OM.

Field of Research	Model	Key Results	Ref.
Identification of novel virulence peptides and quorum sensing pathways	Chinchilla middle ear epithelial cells	A novel virulence peptide 1 (vp1), under the Rgg family of transcription regulators, was characterized. Infection with mutant Vp1 pneumococcal strain produced biofilms with reduced biomass and thickness which was restored by addition exogenous synthetic VP1.	(129)
	Mouse model	briC (Biofilm regulating peptide induced by Competence) gene was identified as a novel peptide involved in pneumococcal competence and virulence and induced ComE, the master regulator of quorum sensing competence signaling pathway. Mice challenged with briC-deleted mutant of the D39Δ pneumococci show reduced bacterial counts in nasal lavages.	(130)
	Rat model	Transbullar infection of rats using Spn strain with mutation in the quorum sensing component, LuxS, let to no biofilm formation in the middle ears and resulted in lower bacterial titers in the middle ear effusions.	(62)
Targeting the pneumococcal polysaccharide capsule	Mouse model	Uncapsulated pneumococci were more susceptible to *in vitro* phagocytosis assays and exhibited reduced ability to colonize the nasal passages in an *in vivo* murine model in comparison to capsulated variants.	(65)
		Chitosan nanoparticles were used as vectors for pneumococcal surface adhesin protein A (PsaA), a lipoprotein common to all serotypes of pneumococcus. Intranasal inoculation of mice with these particles induced protection against pneumococcus (serotype 14) induced OM.	(66)
		Mice immunized with a conjugate recombinant PSA vaccine with the Hib polysaccharide (Hib-PSA) improved elimination pneumococcus and reduced inflammation of the middle ear.	(67)
Targeting non-encapsulated strains (NESp) of pneumococci	Mouse and chinchilla models	NESp expressing AliC and AliD were found to be more virulent compared to mutants and enhanced murine nasopharyngeal colonization. The OM phenotype was significantly attenuated in absence of AliC and AliD in the chinchilla model.	(68)

3.3.1.1: New Vaccines

The pneumococcal polysaccharide capsule has been extensively studied because of its role in AOM development (65). Attempts at developing peptide vaccines have involved chitosan nanoparticles, used as vectors for pneumococcal surface adhesion protein A (PsaA) (66) and the development of a conjugate recombinant PsaA vaccine with the *Haemophilus influenzae* type b (Hib) polysaccharide (67). Non-encapsulated Spn strains lack genes involved in capsule synthesis. Loss of these genes have been shown to dampen the OM phenotype in mouse and chinchilla models (68-70) (Figure. 8.3). Mice vaccinated with trivalent vaccine including some of these genes have shown reduced inflammatory responses in experimentally induced Spn-AOM (70). Hib-PsaA conjugate vaccine can induce both anti-Hib and anti-PsaA immune responses in young mice and elicits effective protection against Spn-induced AOM (67). These studies emphasize the importance of developing combined vaccines that can target multiple virulence factors.

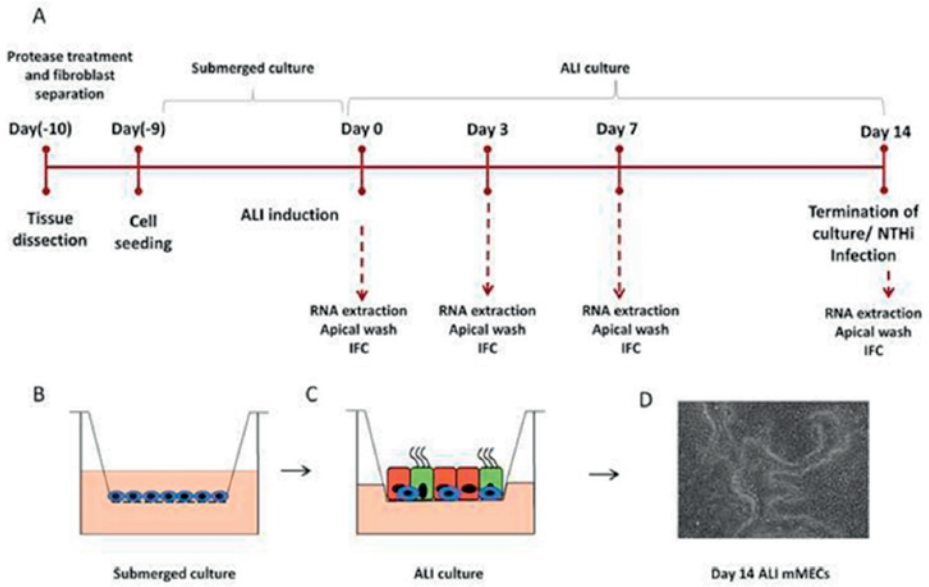

Figure 8.3: A novel in vitro model of the primary mouse middle ear epithelial cells. Timeline for culture of mouse middle ear epithelial cells (mMECs). Bullae were dissected, treated with pronase for dissociation of the epithelial cells, and fibroblasts were excluded from culture by differential adherence to plastic (A). Epithelial cells were grown in submerged culture until confluence (B), before ALI was induced (C). Cultures were terminated at ALI day 14, at which point the cultures were composed of flat polygonal and compactly clustered pseudostratified cells with active cilia (D). Scale bar: 200 μm. This figure has been adapted with permission from Disease Models & Mechanisms 2016 9: 1405–1417; https://doi.org/10.1242/dmm.026658.

Table 8.3: Recent advances in research on NTHi induced OM.

Pathophysiology of NTHi induced OM			
Novel Emerging Concepts	**Animal Model Utilized**	**Supporting observations**	**Ref.**
Pre-existing middle ear inflammation in genetic models of OM can provide a niche in which micro-aerophilic bacteria such as NTHi can successfully establish an infection and persist following transfer through the ET	Evi1Jbo/+ (Junbo mouse)- spontaneous chronic OM	Single intranasal dose of NTHi in a model of spontaneous OM development induced robust infection rates of up to 90%. Pre-existing middle ear fluid was a pre-requisite for successful NTHi infection.	(71)
	Bpifa1−/−Evi1Jbo/+-spontaneous chronic OM	Loss of the putative innate immunity protein BPIFA1 alone did not cause spontaneous or NTHi-induced OM development in young mice, but deletion of BPIFA1 in the pre-existing inflammatory Junbo middle ears led to significant exacerbation of OM severity.	(55)
Clinical features such as the type and viscosity of middle ear fluid pathology can influence bacterial load during OM development	Evi1Jbo/+ (Junbo mouse)- spontaneous chronic OM	NTHi was absent in the serous ear fluids, but almost 100% ears containing highly viscous fluid were culture positive. Viscous fluids were accompanied by higher neutrophil infiltration and percentage of necrotic and apoptotic cells and reduced number of monocytes.	(72)
Suppressor T-cells (Treg cells) confer infectious tolerance to NTHi in the middle ear during chronic OM, contributing to persistence of infection and inflammation	Trans-bullar NTHi inoculation of murine bullae followed by persistently blocking the ET for 2 months with the introduction of a gelatin plug post infection	Culture positive ears showed mucosal inflammation and elevated of Treg cells. Depletion of Treg cells caused a 99.9% reduction in NTHi bacterial counts in the middle ear fluids and in levels of proinflammatory cytokines.	(74)
Intracellular entry of NTHi contributes to bacterial persistence during OM	Chinchilla model *In vitro* NTHi infection of chinchilla middle ear epithelial cell line	Heme-iron restriction of NTHI led to bacterial entry into epithelial cells and formation of intracellular bacterial communities (IBCs). IBCs survive the hostile microenvironment by escaping the endolysosomal pathway for degradation. Prevention of macropinocytosis in cultured middle ear epithelial cells reduced the number of IBCs	(75, 76)

Table 8.3: Recent advances in research on NTHi induced OM. (continued)

Pathophysiology of NTHi induced OM			
NTHi variable regulons contribute to increased disease severity	*In vivo* chinchilla infection with NTHi ModA2 variants	NTHi variants in which ModA2 was OFF at inoculation but shifted ON in the middle ear showed a more severe disease phenotype than those with challenged with NTHi variants, that were either inoculated OFF and remained OFF or inoculated ON and remained ON	(77, 78)
A novel model of the murine middle ear epithelium	NTHi infection of primary cultures of mouse middle ear epithelial cells at the Air-liquid interface	This new *in vitro* model recapitulates the differentiated in situ murine middle ear epithelium as indicated by expression of various epithelial markers at a transcriptional and proteomic level. The model demonstrated effectiveness for studying longitudinal NTHi infections	(56, 79)
Development of Therapeutic Strategies			
Strategy	**Animal Model Utilized**	**Key Findings**	**Ref.**
Targeting intracellular bacterial persistence by blocking invasion of NTHi into host cells	Chinchilla model *In vitro* chinchilla middle ear epithelial cell line	Pharmacological blockade of the actinremodeling complex, Arp2/3 which is involved in invasion of NTHi by the host cells prevented the formation of IBCs. Inhibition of macropinocytis and re-direction of internalized bacteria towards the endolysosomal pathway of degradation can serve as a therapeutic intervention.	(76)

Table 8.3: Recent advances in research on NTHi induced OM. (continued)

Pathophysiology of NTHi induced OM			
Identification of surface-exposed proteins (SEPs)	Infant rat model of AOM	Antisera against five of the most highly SEPs offered protection to NTHi invasive infection.	(80)
	Rabbit immunization	Identification of antibody accessible moieties on the intracellular elongation factor thermo-unstable (EF-Tu) protein that are capable of mounting a host immune response.	(81)
	Evi1Jbo/+ (Junbo mouse)	*Haemophilus* outer member lipoprotein e (P4) acts as a receptor for host extracellular matrix proteins lamin, fibronectin and vitronectin. P4 deficient NTHi showed reduced binding to extracellular matrix and reduced middle ear infection.	(82)
	Chinchilla model	Antisera against NTHi outer membrane vesicles resulted in an opsonophagocytic killing of homologous and heterologous NTHi strains. Immunization with prototype OMVs provided protection to infection by homologous strains of NTHi.	(83)
	Chinchilla model	Macrophage survival factor (Msf) was identified as a new NTHi virulence gene family. Deletion of Msf1-4 displayed decreased in phagocytosis and survival in macrophages which was restored by a single copy of msfA1 gene.	(84)
Broad spectrum approach for biofilm disruption	Chinchilla model	Monoclonal antibodies (MAbs) against DNAbinding tip regions of the alpha- and beta subunits of the DNAIIB protein, integration host factor (IHF) disrupted biofilm formation.	(131)
Combinatorial immunization	Chinchilla model – novel transcutaneous route	combining the majority subunit of Type IV pili (Tfp) of NTHI called rsPilA in a vaccine formulation with the DBAIIB protein, IHF significantly resolved planktonic and adherent populations of NTHi.	(85)

3.3.2: Non-typeable Haemophilus Influenzae (NTHi)

The major findings of studies on the pathophysiology of NTHi-induced OM have been summarized in Table 8.3. Several studies suggest that pre-existing effusion in the middle ear of mice that spontaneously develop chronic OM promotes infection and persistence of NTHi in the middle ear (55, 71). A direct correlation between fluid viscosity and bacterial load has been observed in Junbo mice (72). This is supported by a study on children with OME, which identified NTHi as the predominant pathogen in MEEs with a higher content of mucins (73). Other studies have focused on understanding the factors involved in the persistence of NTHi during OM (74, 75). A chinchilla study has revealed that early disease is accompanied by host immunosuppression and actin morphogenesis, along with bacterial aerobic respiration (76). Phase variable alleles in NTHi are involved in epigenetic regulation of several virulence genes, and the impact of one such allele being turned 'ON' or 'OFF' has been evaluated. Chinchillas infected with bacteria where the allele had been turned OFF at inoculation but shifted ON in the middle ear has shown a more severe disease compared to those where it was either inoculated OFF and remained OFF or inoculated ON and remained ON (77, 78). An *in vitro* model of middle ear epithelial cells cultured at the air-liquid interface has been developed and used to study the effect of NTHi infection on middle ear epithelium (56, 79). Given the genetic diversity of NTHi, there was an increasing interest in "microbiome-sparing", i.e. developing approaches targeted towards genes specific to the disease-causing strains of NTHi, such identifying surface-exposed proteins (SEPs) in different strains of NTHi which can act as receptors, secretory systems and sensors, and function to establish host-pathogen interactions (80-84). Another strategy has been to develop new approaches to target bacterial biofilms (85).

3.3.2.1: New Vaccines

Several outer membrane proteins including outer membrane protein 1, 2, 4 and 6, Tbp1, Tbp2, protein D, Haemophilus adhesin protein, high-molecular-weight protein 1 and 2, and *H. influenzae* adhesin (Hia) have been reported as possible vaccine candidates (86, 87).

3.3.3: Moraxella Catarrhalis (MCat)

Key observations from studies on MCat studies have been summarized in Table 8.4.

Table 8.4: Recent advances in research on MCat induced OM.

Field of Research	Model	Key Results	Ref.
Study of mechanisms by which MCat adheres to host tissue	Smoke-induced COPD mouse model	Clinical isolates of MCat adhered to network-forming collagens IV and VI and fibrillar collagen types I, II and III through the trimeric autotransporter adhesins ubiquitous surface protein A2 (UspA2) and UspA2H receptors. UspA2 and UspA2H deletion mutants showed reduced adherence to the respiratory tract in the COPD mouse model, compared to wild-type bacteria.	(92)

3.3.3.1: New Vaccines

MCat obtained from patients with OM has been examined with genome mining, showing that AfeA, a substrate binding protein, is an excellent candidate vaccine antigen. It was present in all examined strains, it is highly conserved among clinical isolates, it expresses abundant epitopes on the bacterial surface, it is highly immunogenic and induces protective immune responses in the mouse following aerosol challenge with MCat. It is expressed during human infection (88, 89). Substrate binding protein 2 and sulfate binding protein are other promising vaccine antigens candidates (90, 91). MCat components involved in adherence to host tissue have been studied (92). Lactoferrin binding protein A and oligopeptide permease A were identified as potential candidates for vaccine development (93, 94).

3.4: Polymicrobial Infections

Table 8.5 summarizes recent findings on polymicrobial infections in OM. Neuraminidase NanA works synergistically with influenza A neuraminidase to exacerbate colonization by Spn (58). Intranasal inoculation with live attenuated influenza virus before or after intranasal pneumococcal inoculation has been shown to increase the transfer of bacteria into the middle ear cavity in mice (95). There has been increasing interest in methicillin-resistant *Staphylococcus aureus* (MRSA) as a cause of OM in combination with other otopathogens in pre-clinical and clinical studies (96-98).

Table 8.5: Recent advances in research on polymicrobial OM.

Field of Research	Model	Key Results	Ref.
Polymicrobial infection of viruses and Spn	Mouse and Chinchilla models	Mice inoculated by pneumococci deficient in the primary neuraminidase, NanA exhibited reduced nasal colonization which was only partially restored upon co-infection with IAV, which also expresses neuraminidase. IAV potentiated middle ear colonization by NanA-deficient pneumococci to a lesser extent that the Wt strain. Intranasal vaccination of mice with live attenuated Influenza virus before or after pneumococcal increased the transfer of bacteria into the middle ear.	(58, 95)
		Precedent intranasal infection of chinchillas with Wt type 5 adenovirus increased the incidence of middle ear infection upon challenge with Spn.	(132)
Polymicrobial infection of MRSA with other otopathogens	Rat model	RNA-sequencing of total transcriptome after co-infection of rat middle ears by MRSA and after *Pseudomonas aeruginosa* showed that exclusive differential induction of a number of host response genes that were not expressed with single species infection.	(96)
	Rat and guinea pig models	Eugenol, a naturally occurring phytogen and the KR-12 peptide of human cathelicidin LL-37 were shown to have an antimicrobial effect against MRSA.	(98)

3.5: Viral Infections

Apart from influenza virus, there are currently no available vaccines against respiratory viruses; no significant advances have been reported in the last 4 years. Yet, it has been reported that the administration of RSV fusion protein can induce potent neutralizing antibody responses against RSV, which may facilitate the development of an effective vaccine (99-101).

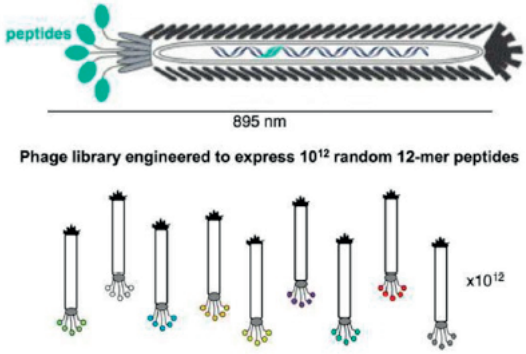

Figure 8.4: A phage display library was used to investigate trans-tympanic delivery. In this method, bacteriophages are genetically engineered to express one of 1010 random peptides on surface filaments. Peptides were selected by application of successively selected libraries to the surface of the TM, with sampling from the middle ear, which resulted in library collapse to several very rare sequences that could actively cross the membrane carrying phage as cargo.

4: INNOVATIVE TREATMENT APPROACHES

4.1: Trans-tympanic Drug Delivery

Trans-tympanic drug delivery approaches allow antimicrobials to enter the middle ear without systemic side effects. A drug delivery system that forms a strong hydrogel on the TM surface has been developed, thus allowing the antibiotic to flux into the middle ear (102). All chinchillas (n = 10) treated with this 1% hydrogel-ciprofloxacin combination cleared NTHi-induced OM, in contrast to 63% of animals who received 1% ciprofloxacin ear drops alone. In another chinchilla study, trans-tympanic delivery of ciprofloxacin also cleared Spn-induced OM (103). Trans-tympanic delivery minimized systemic side effects. Peptides have also been studied for trans-tympanic drug delivery: peptides that can actively cross an intact TM into the middle ear were identified in rats with OM (104, 105) (Figure. 8.4). The addition of six specific amino acids further enhanced the transport capacity of the peptides (106). In an *in vitro* study done on human TM fragments discarded during otologic surgery, human trans-tympanic transport capacity was found to be as effective as that of rats, rabbits or guinea pigs (107) (Figure 8.5). Trans-tympanic delivery of analgesics has also been achieved in chinchillas with experimentally-induced AOM (108).

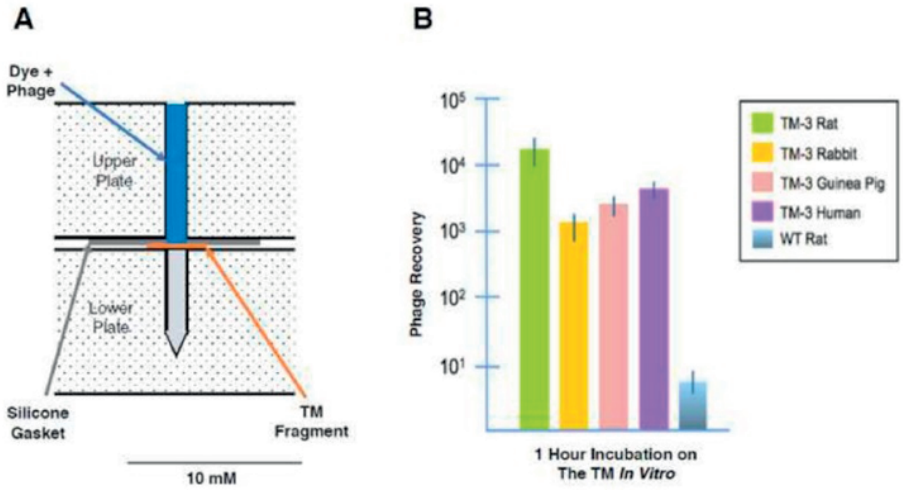

Figure 8.5: (A) In vitro assay developed to evaluate transport of a phage bearing a trans-tympanic peptide across fragments of the TM, from the upper to the lower chamber. (B) Transport of a trans-tympanic peptide phage TM-3 across the tympanic membranes of various species, including the human membrane.

4.2: Transcutaneous Immunizations

Transcutaneous immunization against NTHi using band-aids placed post-auricularly has been tested in chinchillas. The use of antibodies against the major subunit of type IV Pili resulted in eradication of NTHi, disruption of mucosal biofilms and rapid resolution of AOM (109). One band-aid vaccine resolved experimental NTHi-induced AOM in chinchillas significantly faster than saline alone (110). Monoclonal antibodies directed against specific epitopes of bacterial DNA binding proteins common in biofilm matrixes have been shown to be highly effective in disrupting biofilms *in vitro* and resolve experimental OM in chinchilla and murine models (85).

4.3: Other Treatment Options

The effect of caffeic acid phenethyl ester and thymoquinone on OME treatment has been studied in a rat model. Submucosal neutrophil leukocyte count was significantly lower among rats receiving the intraperitoneal treatment (10 mg/kg), as compared to rats receiving thymoquinone, methylprednisolone, or saline (111). The use of Hypericum perforatum (St. John's Wort) on prevention of myringosclerosis after myringotomy has also been evaluated in rats (112). Oral or topical administration of this extract suppressed inflammation and fibroblastic activity, thus reducing the severity of myringosclerosis. In another study, clarithromycin showed similar effects in an animal model (113). Mucosal biofilms play a significant role in OME and many strategies to remove biofilms have been investigated. Middle ear irrigation with saline or 1% baby shampoo was effective in reducing biofilm

formation in chinchilla middle ears. Irrigation treatment did not affect hearing, vestibular or facial nerve functions (114).

5: MATERIALS SCIENCE

It has been shown in rats that human adipose-derived stromal cells could regenerate temporal bone defects following mastoidectomy; rats that received human adipose cells had significantly higher bone formation compared to controls (115). Tissue-engineered autologous middle ear cell sheets have been examined to regenerate middle ear mucosa lost during surgery in a rabbit model. While granulation tissue formation and bone hyperplasia were inhibited, increased mucosal regeneration was observed in the cell sheet-grafted group compared to controls (116). Ossicular prostheses in which silver nanoparticles have been embedded have been tested *in vitro* and demonstrated that the released silver had an antimicrobial effect (117). Several studies have been promising for improved TM regeneration. Heparin binding epidermal growth factor-like growth factor enhances regeneration of TM perforations in mice (118). In another study in rats, epidermal growth factor-releasing nanofibrous patches showed improved TM healing (119). Similarly, improved TM regeneration has been achieved using chitosan patch scaffolds incorporated with insulin-like growth factor-binding protein 2 in a rat model (120). Bioprinted acellular grafts have been developed to treat TM perforations with the use of endoscopic imaging to create customized grafts, showing improved healing in chinchillas (121). In humans with small to moderately sized perforations, bacterial cellulose graft myringoplasty as an alternative to fat graft/temporal fascia myringoplasty have shown similar post-operative healing and hearing results (122). The use of gelatin sponge soaked with fibroblast growth factor during myringoplasty may also improve TM regeneration (123, 124). A novel VT made from Nitinol with titanium dioxide coating has been shown to inhibit biofilm formation *in vitro* when inoculated with a carbenicillin-resistant *Pseudomonas aeruginosa* strain (125). ET balloon dilation has been introduced to overcome ET dysfunction and its sequala, chronic OM, yet evidence is only available from case series showing short-term improvement of symptoms (126, 127). To prolong the effect of ET dilation, the application of a stent has been proposed. A cobalt-chrome coronary stent introduced from the nasopharynx into the ET has been shown to enhance middle ear ventilation in sheep (128). Whether this intervention has an effect on the disease course of OM remains to be seen.

DISCUSSION

New ways of preventing, diagnosing and treating OM have continued to be investigated, though most new findings have not yet been translated to testing in humans. Prevention with polymicrobial vaccines and the targeting of multiple antigens is promising in the

laboratory, but it remains to be seen to what extent OM could be prevented in a clinical setting, and whether the effect would be lasting. Improving the diagnostic accuracy of OM to avoid unnecessary antibiotic overuse without compromising the outcome for those actually suffering from the disease is still of high priority. Easy-to-learn, reliable diagnostic methods could improve the management, especially of pediatric patients, with OM, but the techniques have to be widely used and taught, and affordable for doctors in primary care if they are to make a difference. Trans-tympanic drug delivery has the potential to reduce not only systemic side effects for the individual, but also the selective pressure of antibiotics, thus giving a scope for a slow-down of antimicrobial resistance development.

IMPLICATIONS FOR CLINICAL PRACTICE AND FUTURE RESEARCH GOALS

Further development of new techniques may provide better ways of diagnosing, preventing and treating OM and its sequelae. The use of experimental models to further elucidate the basic properties of disease mechanisms shows promising results and warrants further exploring. Further development of these novel modalities may provide an enhanced ability to diagnose middle ear disease in conjunction with, or as replacement of, current technologies in the future. They might also make it easier to evaluate new means of prevention, pharmaceutical delivery or surgical intervention studied clinically or in animal models. The use of experimental models to further elucidate the basic properties of the disease mechanisms to be able to target new treatment models shows promising results and new fields to explore further.

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V General discussion and summary

9 General Discussion

INTRODUCTION

Approximately 35 years ago it was first suggested that early life factors could play an important role in the development of diseases in adulthood (1, 2). This hypothesis called "Developmental Origins of Health and Disease", proposed that development of an organism is dependent on its environment, and that early adverse exposures may lead to diseases later in life (3). These principles form the basis of this thesis.

The major aims of this thesis were: 1) to identify which environmental determinants are associated with childhood acute otitis media, and if such relationships exist at specific ages; 2) to identify genetic and epigenetic variants associated with acute otitis media in children; and 3) to investigate the prevalence of sensorineural hearing loss in 9- to 11-year-old children and assess whether it may be attributable to recurrent acute otitis media in the first years of life.

This chapter offers a general overview and interpretations of the main results presented in the previous chapters. It presents relevant methodological considerations, clinical implications, and proposes future directions for further studies in the field of childhood AOM.

INTERPRETATON OF MAIN FINDINGS

Environmental exposures

While nearly all children go through AOM during childhood, some children may be more susceptible to frequent episodes of AOM than are others. The odds of acquiring AOM are influenced by many factors. Certain factors in a child's environment are related to an increase or decrease in viral pathogens, which in turn is associated with altered incidence of URTIs – and thus of ear infections. These factors include having older siblings, day-care attendance, season-of-birth, pet keeping, breastfeeding, and socioeconomic status (SES) in general (4-6). Maternal factors with a proposed impact on childhood AOM include prenatal and postnatal (household) smoking and maternal age (7). In part 1 of this thesis, we examined associations between environmental factors and AOM in a large cohort (N= 7863) of children. Data on outcome and possible determinants was measured repeatedly until 10 years of age. We used two different statistical approaches: one using repeated measurements to assess associations with AOM with longitudinal odds at different ages, considering correlations between the repeated measurements; the other using latent-class-analyses to discover if distinct AOM-trajectories in our population existed, and if so, if determinants were associated with membership of a specific trajectory. Three distinct trajectories were identified; knowing, non-otitis prone children, children that were otitis-prone throughout childhood (persistent-AOM), and children who suffered AOM episodes until 3 years of age but not beyond (early-AOM). Half of all AOM-prone children recovered after the age

of 3 years. Several determinants were associated with AOM, including gender, day-care attendance, (lack of) breastfeeding, season-of-birth, and postnatal household smoking.

Male gender increased odds of AOM in children aged 2 to 4 years and was associated with membership of the early-AOM trajectory. Previously, male gender had been generally associated with increased odds of AOM (8-11). The association among younger children is in line with literature, as several studies among older subjects described no significant difference between boys and girls aged from 3 to 16 years (12, 13). These findings confirm that gender could perhaps serve as a predictor for AOM in the first years of life, but not beyond the age of 3 or 4 years.

It is well established that day-care attendance exposes a child to a higher amount of viral pathogens which in turn can lead to more frequent URTI's and ear infections. Day-care attendance has indeed often been related to increased risk of AOM, with risk further increasing for children who attend day-care for a longer period (>12 months) (8, 10, 11, 14). A Danish study found that children who had started day-care attendance before the age of 12 months had an increased probability risk of experiencing more than three episodes of AOM at 18 months, and to a lesser extent at 7 years of age (15). These findings may explain in part why in our study the percentage of day-care attendance was highest among children prone to early-AOM, and that only this subgroup showed increased odds of acquiring AOM.

In both chapter 2 and 3, we showed the beneficial, protective effect of breastfeeding regarding lowering odds of AOM through childhood, with the strongest association in the first months of life. The positive effect of breastfeeding over formula on AOM susceptibility is widely accepted (8, 10, 16, 17). Possible explanations of this positive effect include a lower rate of URTI's through the effects of secretory IgA, cytokines, and long-chain fatty acids in breastmilk, which are paramount to development of the infant's immune system (18). The greatest protection of breastfeeding on AOM has been described in children breastfed for at least 6 months (8, 17). Shorter duration of breastfeeding induced higher AOM rates, both below 2 years of age and later in childhood (13-15). In the Netherlands, data from the national registry (www.volksgezondheidenzorg.info) showed that in 2018 approximately 70% of all mothers started breastfeeding at childbirth, however, prevalence of breastfeeding decreased to 31% and 19% at 3 and 6 months postpartum respectively. Cumulative evidence from literature and this thesis show that longer duration of breastfeeding, at least until 6 months of age, can lower odds of AOM in all children. This view is supported by the Ministry of Health, Welfare and Sport in the Netherlands, who published a systematic review on many health outcomes for child and mother (https://www.rivm.nl/bibliotheek/rapporten/2015-0043.pdf) including AOM. It showed that breastfeeding has a beneficial health effect on both the child and the mother compared to formula feeding. There was convincing evidence that breastfed infants for example, run a lower risk of contracting gastrointestinal infections, respiratory tract infections, and indeed AOM. The beneficial effect was maintained after breastfeeding was stopped. It further showed that breastfeeding

likely reduced the risk of developing obesity, asthma and wheezing in children and diabetes, rheumatoid arthritis, and hypertension in their mothers.

Season-of-birth may be associated with either an increase or decrease in the rate of URTIs, depending on the season. We found a beneficial effect of being born in the summer and autumn as compared to being born in the spring in the persistent-AOM trajectory, yet interpreting this result is difficult. Literature on the possible relationship between AOM and season-of-birth is contradictory; some studies report that birth in the autumn would carry higher odds of AOM, whereas other studies report lower AOM rates among subjects born in the autumn (5, 6, 19). Moreover, definitions of seasons vary across countries, which limits generalizability of these results.

Public health in general is influenced by health behaviors associated with socioeconomic disparities. Literature on the association between SES and AOM has been contradictory; some studies reported absence of an association and others reported a negative effect of lower SES on AOM (9, 20). The most important unhealthy behavior is smoking. We studied both prenatal maternal smoking and household smoking. We found that household smoking raised the odds of AOM which was also reported in recent literature including two meta-analyses (7, 11, 21-23). Still, literature is ambiguous as several other studies found no convincing association (8, 9, 13, 24).

This study was limited by the availability of covariates. Several determinants have been proposed in other studies, yet we could not test or correct for as no data was available in our dataset. Examples include – but are not limited to – pacifier use, low compliance to the national vaccination program, family history of AOM, adenoid hypertrophy, pathogenic bacterial colonization of the nose/nasopharynx, and craniofacial abnormalities (8, 13, 15, 21).

Genetic and epigenetic susceptibility

Heritability of AOM is well-established in family studies, sibling, and twin studies, with the fraction of phenotype variability attributed to genetic variation (h2) estimated between 0.22 and 0.74, but genetic susceptibility of AOM is yet not well understood (25-28). Genome-wide association studies (GWASs) have been the main approach in genetic research to associate specific genetic variations with common diseases (29). In general, the (A)OM field has lagged relative to other respiratory and infectious disease arenas in terms of large-scale agnostic discovery approaches such as GWAS. It is an approach which involves scanning the genomes from many different people and looking for genetic markers (Single-nucleotide polymorphisms (SNPs)) that can be used to predict the presence of disease. Once such genetic markers are identified, they can be used to understand how genes contribute to the disease.

In chapter 4, we have performed the first GWAS of AOM and the largest genetic study of OM to date. We discovered genome-wide significance at a locus on 6q25.3 that contains

the *FNDC1* gene and replicated the association in an independent pediatric cohort. We further showed that the mouse homologue of this gene is expressed in the middle ear and is upregulated upon lipopolysaccharide treatment which we used to induce AOM in the mice. In addition to the tissue expression patterns, the eQTL and methylation data also implicate *FNDC1* as the causal gene underlying the association. While the biological function of *FNDC1* has not been well studied, it has been shown that *FNDC1* has a role in inflammation. *FNDC1* was identified as a differentially expressed mRNA from human dermal fibroblasts. Its expression level increased following treatment with TGF-β, IL-1 and TNF-α (30). Partially unfolded type III fibronectin module has been shown to induce the expression of IL-8 and TNF-α via activation of the NF-κB signalling pathway, suggesting that fibronectin matrix remodeling may impact cytokine expression (31, 32). Both tissue remodeling and increased levels of cytokines are involved in AOM (33, 34). Consistent with the association of *FNDC1* with AOM and its expression being modulated by lipopolysaccharide, several important players involved in these signaling pathways of inflammatory responses also showed at least nominal association with AOM in our GWAS. The cumulative evidence from literature and the results from our GWAS and mouse experiments suggest that *FNDC1* has a role in the pathogenesis of AOM, likely through altered immune or inflammatory response.

We scanned our GWAS results for genes that have been previously proposed to be involved in OM pathogenesis in literature. We found more than half of them exhibited nominally significant association, suggesting consistency between genetic studies and/or with results from model organism studies. It is not surprising that some of the candidate genes did not show significant association, considering the following possible reasons. In our GWAS, we focused on a more defined phenotype of early-onset AOM, which is different from the phenotypes examined in many of the previous OM genetic studies including OM in general, chronic OM or OM with effusion. In addition, different ethnicity of the study population is another influential factor to consider. Furthermore, polymorphisms of candidate genes, proposed based on evidence from differential gene expression, molecular, cellular functions and rodent model studies, may not always present an effect large enough to be captured by GWAS.

To date, 21 significant genetic loci have been identified in 5 genome-wide association studies (GWAS) on otitis media (OM), including our study – the first on AOM – from which only 5 genes of variants were replicated in an independent OM dataset including our result (35-37).

Epigenetic mechanisms such as DNA methylation could link environmental exposures with the occurrence of childhood AOM. DNA methylation is an epigenetic mechanism that is dynamic and may affect gene expression. It is associated with health-related outcomes and is influenced by environmental, genetic, and stochastic factors (38-42). Epigenome-wide association studies (EWASs) are large-scale studies of human disease-associated epigenetic variation, specifically variation in DNA methylation. We hypothesized that differential DNA

methylation measured in cord blood would be associated with AOM in early childhood. The study presented in chapter 5 was the first EWAS in the field of (A)OM. Our EWAS meta-analysis did not yield epigenome-wide significant CpGs associated with AOM in children. We found 19 CpGs, mapping to 15 genes, with a suggestive association $p<1.0x10^{-4}$ and used these in several exploratory follow-up analyses. Although this study comprised all available studies to date on AOM with epigenome-wide data and its sample size was thus as large as it could be, it was still limited in size. Joint analysis of data from AOM and OME showed some signals which could be further explored in larger sample sizes.

Hearing and acute otitis media

Hearing is essential in today's high demanding communication-based society. The negative effects of early SNHL can be profound on both the short-term and the long-term. Children with mild to severe hearing loss (HL) have worse language development and school performance than those without (43-45). In children with only slight SNHL, more behavioral problems are observed as compared with normal hearing children (46). The adverse effects of HL can add-up to severe disability later in life. In literature mild HL has been associated with poor academic performance (P<0.001) in primary school students (47). Several studies have shown that uncorrected hearing loss then often gave rise to poorer quality of life, related to isolation, reduced social activity, and a feeling of being excluded later, leading to an increased prevalence of symptoms of depression (48, 49). Patients with hearing loss were more likely to be unemployed than those without hearing loss (adjusted OR 2.2; p<0.001), and were less likely to have any wage income (adjusted OR 2.5; p<0.001) (50). These studies illustrate that early SNHL that goes untreated may have profound impact years later, on interpersonal communication, psychosocial well-being, employment opportunities, and quality of life (48-51). Although AOM is associated with short-term conductive hearing loss, there is evidence suggesting that sensorineural hearing loss (SNHL) could be a rare complication of AOM (52). Several studies investigated sensorineural hearing loss (SNHL) as a sign of permanent damage to the cochlea after chronic or recurrent AOM episodes (53-55). A history of AOM was previously associated with an increased risk of sudden sensorineural hearing loss later in life (56). The pathogenesis of chronic sensory hearing impairment may be related to inflammatory noxious substances that cross the round window membrane, which leads to serous labyrinthitis, or to fluid in the middle ear that impedes oxygen transport to the inner ear, or to an adverse effect of ototoxic drugs (57).

In part 3, we first provided an overview of all existing data on SNHL in children and adolescents, also considering the possible negative effect of noise-exposure. We discovered a large spread in prevalence of early SNHL. In two large cohort studies in Australia and England, it was shown that among 7- to 11-year-old children, an estimated prevalence of 0.5% to 1% of slight to mild, bilateral SNHL existed (58, 59). Previous cross-sectional studies have observed associations between a history of otitis media and permanent hearing

loss (HL) among young adults (60-62). We assessed prevalence of SNHL and the possible associations to a history of AOM, in a large population-based cohort of 9- to 11-year-old children. We demonstrated that 7.8% of the children were estimated to have SNHL in low or high frequencies in at least 1 ear, which was similar for boys and girls. Most of the HLs were unilateral and of slight degree (16-25 dB HL). The prevalence of mild or more severe HL of our study was 0.9%, and bilateral SNHL was present in 1.3%. The latter is slightly higher than the prevalence of bilateral SNHL that was reported in previous large studies among children of similar age, namely 0.5% (59) and 0.9% (63). Altogether, 17.5% of the cohort had hearing thresholds exceeding 15 dB HL (7.8% SNHL, 6.3% conductive hearing loss (CHL), and 3.5% HL of unknown origin). This is roughly comparable with the prevalence of 16.0% HL among 12- to 13-year-old children in the NHANES studies, which did not distinguish between SNHL and CHL (64). We found that a history of recurrent AOM was associated with the presumed SNHL, which is in line with some (60-62), but not all (58), previous studies among children and young adults. As previously mentioned, evidence exists that inflammatory mediators present in otitis media can cause changes in the auditory structures, including causing cochlear damage and sensorineural hearing loss over and above CHL (52). This was in keeping with several other studies that investigated sensorineural hearing loss (SNHL) as a sign of permanent damage to the cochlea after chronic or recurrent AOM episodes (53-56). A second factor that was associated with worse hearing acuity in chapter 7 was maternal education, showing that increased odds were present for lower maternal educational status. This could be related to the more general socioeconomic status, which is partly determined by educational level among other factors (65). Also, maternal education has been found to be a prognostic indicator for a child's success with a cochlear implant (66, 67). Together with associations between socioeconomic variables and HL that have been found before (64), maternal education and socioeconomic status seem important factors in relation to hearing.

Phenotype definition

The studies presented in this thesis were embedded in the Generation R Study, a large population-based prospective cohort study. Outcomes were parent-reported using frequent questionnaires on episodes of otorrhea, earache with fever, and use of eardrops prescribed by family practitioner or ear, nose and throat (ENT) surgeon. Parent-reported outcomes hold the risk of introducing recall bias (see Methodological Issues below). There are some opposing views with regard to parent-reported AOM symptoms and their reliability in diagnosing AOM. An episode of AOM is often a painful experience for a young child, one that parents may not easily forget. The diagnostic value of parent-reported earache, fever, and otorrhea in AOM was shown previously with a sensitivity and specificity of 71 and 80%, respectively (68-70). Yet, in other studies specific symptoms such as ear tugging/rubbing and restless sleep were not significantly associated with occurrence of AOM (71). A possible

positive effect of using parent-reported outcome is, that incidence of AOM based on medical records instead of parent-reports may underestimate the community incidence of AOM as parents may not visit a doctor every time acute ear symptoms occur (70). Added to that is that the study design with its frequent questionnaires permitted repeated measurement analyses.

In the genetic and epigenetic analyses, we focused on a more defined phenotype of early-onset AOM, which is different from the phenotypes examined in many of the previous OM genetic studies including OM in general, chronic OM or OM with effusion (37, 72-75). In chapter 4, we meta-analyzed data with data form the Children's Hospital of Philadelphia (CHOP). Case-status for AOM was defined using ICD-9 codes from the medical history of subjects. Subjects in the database with no history of middle ear disease were labelled as controls. Limitation herein is severity of disease, as in the CHOP population cases consisted of children who were specifically diagnosed with AOM, likely the more severe episodes, whereas the Generation R population we used extensive questionnaires, discovering even the mildest of episodes. These differences were resolved by the increased power of combining the studies, underlined by results showing the same direction of effect and similar effect size between both. Furthermore, the p-value of homogeneity test in our meta-analysis is >0.9 for the genome-wide significant SNP rs2932989, which indicates that there is no suggestion of between-study heterogeneity.

METHODOLOGICAL CONSIDERATIONS

The Generation R Study aims to identify early environmental and (epi)genetic factors affecting children's growth, development, and health. For the genome-wide association study (GWAS) we meta-analyzed individual participant data or effect estimate with two independent cohort studies from Philadelphia, the United States. For the epigenome-wide association study (EWAS) we combined effect estimate data from six European birth cohorts and one birth cohort from California, the United States.

Cohort studies are longitudinal, observational studies that observe a group of people over an extended period to detect disease occurrence and differences in exposure or genes between diseased and non-diseased. This study design works well if an outcome is common, and if there is a plausible hypothesis linking exposure to an outcome. Yet, as outcomes may develop a long time after an exposure, cohort studies can take years and be costly. There are several methodological issues associated with this study design with respect to internal and external validity, and specific methodological issues concerning (epi)genetic analyses.

Internal Validity

Internal validity defines the extent to which an observed outcome is causally related to a determinant and is not due to methodological errors such as selection bias, information bias, or confounders. Internal validity is determined by how well a study rules out such errors. The specific threats to internal validity are each discussed in the paragraphs below.

Selection bias

Selection bias can occur when an association between exposure and outcome differs between subjects that participate in the study, and subjects who do not participate (76). A source of potential selection bias in a cohort study is low overall response rate at baseline. In total, 9778 mothers who resided in the study area during pregnancy, enrolled in the Generation R Study during the inclusion period with an estimated response rate at baseline of 61% (77). The non-response at baseline was likely not random. It was established that non-participating mothers were more often from ethnic minorities, of lower socioeconomic status, and suffered more adverse birth outcomes, as compared with the Generation R participants (78). This suggests a selection towards a slightly healthier population. A second source of potential selection bias cohort studies are prone to, is selective loss to follow-up (76). Of all known live births (N=9749) of the originally included mothers, 85.2% (N=8305) of all children participated in the follow-up studies at 6 years, and 75.8% (N=7393) participated at 9 years (77). General characteristics of the mothers who remained in the study were compared with all mothers who were enrolled in the study at baseline. Compared to the baseline characteristics, the mothers who still participated in the study at follow up were older, more frequently of Western ethnicity and higher educated, and their children of higher birth weight (77).

We performed a similar analysis for the studies that form the basis of this thesis. General characteristics of mothers and children who were excluded from analyses due to missing questionnaires on AOM-related outcome, were compared with the studied population. It confirmed that mothers in the excluded sample were younger at enrolment and more frequently of non-Western ethnicity. It further showed a lower prevalence of breastfeeding at 6 months (19.9% vs. 33.2%) and higher rates of smoking both during and after pregnancy. Selection towards a healthier population could lead to an underestimation of the prevalence of AOM with regards to the excluded sample specifically considering the protective effect of breastfeeding (chapter 2 and 3). The potential bias is difficult to quantify. To limit risk of selection bias due to missing covariate values, we performed multiple imputation.

Information bias

Information bias is a systematic error which arises when a key variable (e.g., exposure, confounder, or outcome) is inaccurately measured or misclassified. Information bias can be differential or non-differential. Non-differential misclassification occurs when misclas-

sification is equal (random) between exposed and non-exposed and will mostly result in an underestimation of the true effect estimate. Misclassification is differential when the misclassification is different for those with and without the exposure or outcome of interest. It may lead to over- or underestimated effect estimates and to invalid conclusions to be drawn. Some adverse lifestyle variables, such as smoking during pregnancy, are known to be underreported in epidemiologic studies. Similarly, overreporting of lifestyle factors with potential beneficial effects such as duration of breastfeeding, may give rise to differential misclassification of the exposure, leading to underestimation of effect sizes. Still, the chance of differential misclassification in our studies was limited, as participants were unaware of the specific research question under study, and most determinants, including birth weight, breastfeeding, smoking, genetic and epigenetic samples, were measured before the outcome occurred. Recall bias is another potential form of information bias due to differences in accuracy in recalling past exposures between subjects with or without a specific outcome. This thesis used parent-reported outcome which holds the risk of introducing recall bias. There are some opposing views with regard to parent-reported AOM symptoms and their reliability in diagnosing AOM, as mentioned in the previous paragraph on phenotype definition. Yet, an episode of AOM is often a painful experience for a young child, one that parents may not easily forget. The diagnostic value of parent-reported earache, fever, and otorrhea in AOM was shown previously with a sensitivity and specificity of 71 and 80%, respectively (68-70).

Confounding

A confounder is a third variable that is associated with both the exposure and the outcome and is not in the causal pathway. It biases the measure of association we calculate between exposure and outcome and can lead to over- and underestimation of effect estimates. Confounders in the studies of this thesis were based on literature. We further examined the confounding effect of potential confounders in each statistical model and adjusted for potential confounders in all studies. Still, residual confounding may be an issue due to unmeasured variables of variables not known to be confounders, such as interactions between environment and (epi)genetic susceptibility.

Methodological issues in genetic and epigenetic studies

GWAS is an agnostic discovery approach which involves scanning the genomes from many different people and looking for genetic markers (SNPs) that can be used to predict the presence of disease. Once such genetic markers are identified, they can be used to understand how genes contribute to the disease. Despite the potential of GWAS to examine the associations of millions of genetic variants at once, there are some methodological issues to be considered. The currently available genotyping platforms and imputation techniques include chiefly SNPs with minor allele frequencies of 1% or more. The identified risk alleles

associated with AOM only explain a very small proportion of the genetic susceptibility of this disease. Current studies are underpowered to identify more rare variants of the complex, multigenetic disease that is AOM. The combined, yet unidentified, rare variants may potentially have larger effects than the discovered common variants on disease risk (79). A second consideration in GWAS, pertains population stratification. This problem arises when the population studied contains subpopulations that differ both in allele frequency and in the prevalence of the disease under study, or when two different populations are meta-analyzed (80). In our analysis in chapter 4, we balanced power and optimal quality control. Compared with previous genetic studies on (A)OM, this study applied more stringent quality control and more robust correction for population substructure. Indeed, by performing principal-component analysis (PCA) to identify participants of European ancestry, followed by a second PCA on this selected population, we were able to correct for population stratification more thoroughly. The last methodological issue related to GWAS, concerns correction for multiple testing. In GWAS, determining the correct p-value threshold for statistical significance is critical to control the number of false-positive associations. The common statistical threshold for significance in GWASs has been set to $p<5.0x10^{-8}$, reflecting a Bonferroni correction of testing one million variants (0.05/1,000,000). One the one hand, false-positive findings may still occur due to insufficient statistical power for common genetic variants with small effects. On the other hand, the Bonferroni correction provides a slightly conservative bound even if the associations tested are independent from one another. If the tests are correlated, as is often the case in GWAS data due to linkage disequilibrium, then the bound becomes more conservative. Thus, applying Bonferroni correction may be too conservative. Meta-analyzing data from multiple cohort studies has increased power for identification of common variants. By a well-defined phenotype in both populations, we were able to combine data from two studies despite different designs. Limitation herein is severity of disease, as in one population cases consisted of children who were specifically diagnosed with AOM by a doctor, likely the more severe episodes, whereas the other population used extensive questionnaires, including even the mildest of episodes. These differences were resolved by the increased power of combining the studies, underlined by results showing the same direction of effect and similar effect size between both. We found no suggestion of between-study heterogeneity for our top results.

EWASs are large-scale studies of human disease-associated epigenetic variation, specifically variation in DNA methylation. They present novel opportunities but there are some methodological issues to address. Firstly, the BeadChip array which all participating cohorts used in chapter 5, and is generally used in EWAS globally, is the Infinium HumanMethylation450 BeadChip. This array has been shown to measure genome-wide DNA methylation reliably (81). This array nonetheless covers only 1.7% of all CpGs in the human genome, leaving many CpGs unmeasured that may influence susceptibility to AOM (82). This Illumina BeadChip array works with hybridization of genomic fragments to probes on the chip.

There are genomic factors, however, that may compromise the ability to measure methylation, such as SNPs, small insertions or deletions (INDELSs), or regions with repetitive DNA (83). In fact, in probes that mapped to DNA containing a SNP, the methylation levels detected could simply be the reflection of underlying genetic polymorphisms and could be misinterpreted as true signals (84). It is not yet known to what extend these genomic factors do impact association analyses. In chapter 5, probes that mapped to DNA containing a SNP, repetitive sequence elements, or DNA harboring an INDEL were therefore not excluded, but flagged in the results. EWASs are subject to confounding through environmental, genetic, and technical factors. All participating cohorts in chapter 5 corrected for these in the main model. In our analyses, we used cord blood to measure DNA methylation at birth. Cord blood is easily accessible, which makes it ideal for large population studies such as birth cohorts. DNA methylation in cord blood may, however, not reflect DNA methylation levels in middle ear mucosa. Tissue obtained from middle ear mucosa is much more difficult to obtain, especially in population-based studies, for obvious reasons. As AOM is an infectious disease, it has systemic manifestations characterized by increased inflammatory blood markers. We adjusted for estimated white blood cell counts using the Houseman method with the Reinius reference panel. No major alterations were found in our top results when comparing our complete model to one without adjustment for cell-type composition (85). Multiple cord blood-specific methods for white blood cell adjustment have more recently been proposed (86-88). We compared the results of the main model adjusted for cell-type proportions estimated using the Houseman method with those adjusted using the cord blood-specific cell-type proportions. The results were very strongly correlated ($r = 0.97$). Residual confounding due to alterations in cell-type distribution cannot be ruled out completely after correction by either method. Finally, multiple testing correction and the benefits and limitations of meta-analysis are similar to those of GWASs, and, thus, the same methodological considerations apply.

External Validity

External validity reflects how well results from this thesis can be generalized to other populations. The Generation R Study is a population-based prospective cohort study in the Rotterdam metropolitan area. It is a multi-ethnic cohort, comparable to the general ethnic distribution of the population of the region. The results of this thesis could presumably be applied to western populations with mixed ethnicities. The genetic and epigenetic studies were performed in populations mostly of Caucasian origin. The GWAS examining genetic susceptibility to AOM comprised two cohort studies from the United States of Caucasian origin. The EWAS however combined data from 7 birth cohorts throughout Europe and the United States, with one subset comprising children of Hispanic descent. When we excluded this sample in the leave-one-out analysis, it did not alter the results which increases the generalizability of our findings to non-European populations.

CAUSALITY

The studies that form the basis of this thesis are observational by design, and, thus, assessed associations between exposures and outcome, but not causality. The Bradford Hill criteria are widely used in epidemiology as a framework to determine the causality of observed association (89). The criteria are strength of the association, consistency with other data/populations, specificity, and temporality – exposure should precede outcome – of observed associations. Moreover, if biological plausibility or gradient exists, if experimental evidence is available, if the relationship agrees with current knowledge of natural history, or is in line with other established cause-effect relationships, these can further strengthen causality (89). The results presented in chapter 2, 3 and 7 were in line with previously reported associations. Exposure often preceded outcome as, for instance, hearing loss occurred after AOM episodes, and AOM episodes were suffered after (lack of) breastfeeding or attributable to gender or season-of-birth. Generally, there were plausible underlying mechanisms which were discussed in each chapter, and several factors were discovered in other previous studies in literature as well. GWASs do not directly identify causal genetic variants. These studies rely on linkage disequilibrium between SNPs and imputed causal variants. Prove of gene expression of the identified variant supports that the variant is within a causal gene. In chapter 4 we showed that, in addition to the tissue expression patterns, the eQTL and methylation data also implicated that the *FNDC1* gene was indeed the causal gene underlying the association with AOM susceptibility. To further strengthen this claim, we performed an animal experiment. We observed that the mouse homologue of this gene was expressed in the middle ear and was upregulated upon lipopolysaccharide treatment by which AOM is induced. In chapter 5, we used cord blood to measure DNA methylation at birth. As our outcome was measured years later, the temporality criterium is well addressed. Still, DNA methylation is a dynamic biologic process, with altering patterns across the human life course, which can make interpretation from results form an observational EWAS challenging (90).

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The studies presented in this thesis were based on epidemiological studies using observational data from a healthy population. As discussed, identification of causality and translation to clinical practice is challenging. We can draw several conclusions from these studies leading to future research perspectives.

1.) Some exposures are only associated with altered odds at specific ages. We further observed that children prone to AOM until 3 years of age but not beyond, and children who remain prone to AOM throughout, are associations with different exposures. In

the future, we hope that these observations could be used in clinical models to predict probability of the development of AOM in childhood. If we could predict which child will become prone to AOM at presentation of the first episode, we could tailer treatment to the individual patient, and perhaps take preventative measures at an earlier age.

2.) We discovered FNDC1 as a disease contributing gene to AOM in children. We further showed that the mouse homologue of this gene is expressed in the middle ear and is up-regulated upon lipopolysaccharide treatment by which AOM is induced. In addition to the tissue expression patterns, the eQTL and methylation data also implicated FNDC1 as the causal gene underlying the association. The cumulative evidence from literature and the results from our GWAS and mouse experiments suggest that FNDC1 has a role in the pathogenesis of AOM, likely through altered immune or inflammatory response. More research is needed to reveal the exact role of FNDC1 in humans. On a broader scope, we expect that soon human GWASs using next-generation sequence data will further illuminate how coding and non-coding variants with a wider MAF spectrum, i.e., both common and rare variants, play a role in AOM susceptibility. Further studies in non-European populations, however, are needed to elucidate AOM susceptibility variants that are important in various human ethnic groups. In addition, there continue to be many advances in the identification of genes that play a role in OM from animal studies, virtually all in the mouse. These include natural and ENU-induced mutations, as well as studies of knockout and other gene-modified mice. The categories that influence AOM include genes related to immunity, inflammation, secretory activity, morphology, and tissue growth. This diversity of AOM-related pathways suggests that many more such OM-related genes will be discovered in human and mouse studies, which are important to understanding mechanisms of disease in AOM.

3.) We did not find convincing evidence that epigenetic changes in fetal life are associated with the risk of AOM in the first years of life. Although we used all available studies to date on AOM with epigenome-wide data and its sample size was thus as large as it could be, it was still limited. Therefore, it is possible that actual signals may have gone unnoticed due to insufficient statistical power. Joint analysis of data from AOM and OME showed some signals which could be further explored in larger sample sizes. More studies in non-European populations are required to increase the generalizability of findings to non-European populations.

4.) The observation that recurrent episodes of AOM were associated with the SNHL, highlights the prevention of AOM as a possible target for prevention of early SNHL. Future studies are required to evaluate if the association between AOM and SNHL can be discovered in other populations. Further research is needed to unravel if recurrent exposure to inflammatory mediators present in AOM, can cause changes in the auditory structures, including causing cochlear damage and SNHL.

5.) We reviewed new ways of preventing, diagnosing, and treating AOM. Most new findings have not yet been translated to testing in humans. Prevention with polymicrobial vaccines and the targeting of multiple antigens is promising in the laboratory, but it remains to be seen to what extent OM could be prevented in a clinical setting, and whether the effect would be lasting. Trans-tympanic drug delivery has the potential to reduce not only systemic side effects for the individual, but also the selective pressure of antibiotics, thus giving a scope for a slow-down of antimicrobial resistance development. The use of experimental models to further elucidate the basic properties of the disease mechanisms to be able to target new treatment models shows promising results and new fields to explore further.

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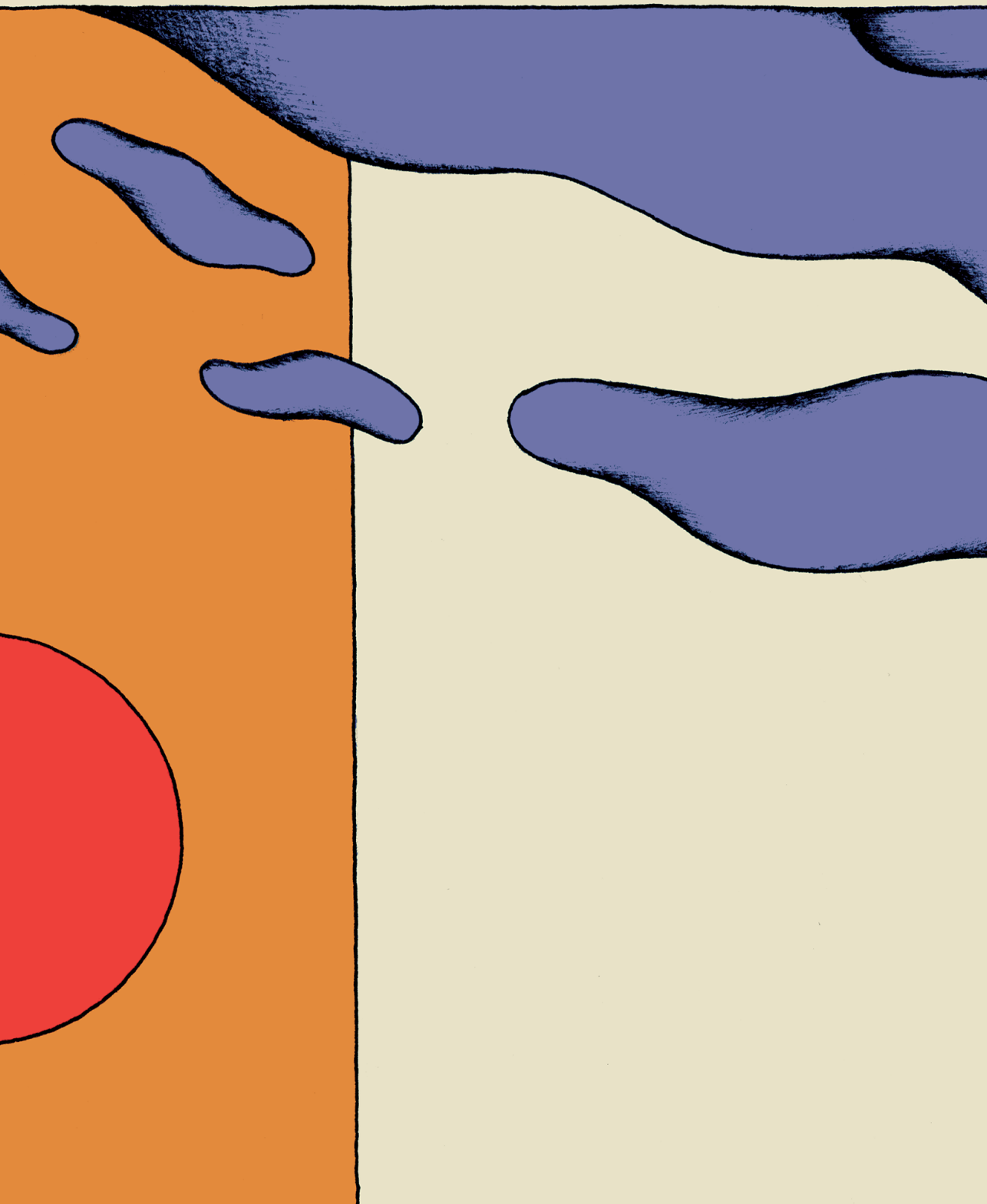

10 Summary Samenvatting

SUMMARY

In this thesis, we examined the hypothesis that adverse environmental exposures in fetal life and early childhood, along with genetic susceptibility, could lead to an increase or decrease of acute otitis media (AOM) in early childhood. Further, we examined the hypothesis that epigenetic mechanisms exist, which may be influenced during fetal life and could explain altered susceptibility to AOM in the first years of a child's life. Finally, we examine if early sensorineural hearing loss (SNHL) could be a long-term adverse outcome of endured acute otitis media (AOM) episodes in childhood. By gaining better understanding of the complex pathogenesis of AOM, we hope to learn which children are most susceptible to this disease, where earlier clinical intervention may have the most impact tailored to the individual patient and help prevent AOM altogether.

In **chapter 1**, background and rationale of our thesis are given. We expand on aims and general outlines of the subsequent individual studies that form the basis of this thesis.

Part I: Environmental determinants associated with acute otitis media in children

Chapter 2 describes associations between environmental factors and AOM in childhood for 7863 children embedded in the Generation R Study. Data on outcome and possible determinants is measured repeatedly until 6 years of age. We examine associations with AOM with longitudinal odds at different ages, considering correlations between repeated measurements. We report that male gender increases odds of AOM in children from 2 to 4 years but not at other ages. Postnatal household smoking, presence of siblings, and pet birds increase odds of AOM. Breastfeeding decreases AOM odds, most notably in the first months of life. This study shows that several determinants are associated with altered odds of acquiring AOM in childhood, and that several are associated with AOM at specific ages.

In **chapter 3**, we take a novel approach to assess AOM among 7863 children in the Generation R Study. The extensive outcome data measured at repeated intervals until 10 years of age, is used as input in latent-class-analyses. With this technique, one can detect if distinct AOM trajectories in childhood exist in population, and if so, whether determinants are associated with a specific trajectory. Three distinct trajectories are identified; that is, non-otitis prone children, children that are otitis-prone throughout childhood (persistent-AOM), and children who suffered AOM episodes until 3 years of age but not beyond (early-AOM). Male gender and day-care attendance are associated with increased odds of early-AOM. Breastfeeding is beneficial for both early-AOM and persistent-AOM trajectories. Season-of-birth is associated with altered odds only in the persistent-AOM trajectory. Half of all AOM-prone children recovered after the age of 3 years, which corresponds with our clinical experience at the department of otolaryngology. This supports the hypothesis that children that are otitis-prone are not one homogeneous population. There appears to be a distinct difference between children who are prone to AOM until three years, but not beyond, and children that remain otitis-prone throughout childhood.

Part II: Genetic and epigenetic susceptibility to acute otitis media in children

Chapter 4 describes the first GWAS of AOM and the largest genetic study of OM to date (N=11047). We discover genome-wide significance at a locus on 6q25.3 that contains the *FNDC1* gene and replicate the association in an independent pediatric cohort (N=2012). We further show that the mouse homologue of this gene is expressed in the middle ear and is upregulated upon lipopolysaccharide treatment by which AOM is induced. In addition to the tissue expression patterns, the eQTL and methylation data also implicate *FNDC1* as the causal gene underlying the association. Thus, this chapter provides evidence of a genetic component influencing susceptibility to AOM in children.

In **chapter 5**, we examine whether differences in DNA methylation a birth are associated with susceptibility to AOM in the first years of life. We perform meta-analysis of all available epigenetic data comprising 6 international cohort studies (N=2793). We find no clear evidence to support the hypothesis that DNA methylation at birth has impact on gene expression and subsequent susceptibility to AOM in early childhood.

Part III: Hearing in children, and adverse hearing outcomes in childhood associated with acute otitis media

Chapter 6 provides an overview of hearing levels from early childhood until young adulthood. Exposure to loud noise at an early age, for instance through exposure to loud music, can be hazardous for a child's hearing. The aim of this systematic review was to provide an overview of hearing levels, and music-induced hearing loss and its symptoms in children. The prevalence of increased hearing levels (>15dB) was 9.6%, and high-frequency hearing loss was 9.3%. The average hearing thresholds were 4.8dB hearing loss at low frequencies, and 9.5dB hearing loss at high frequencies. No significant association between pure-tone air thresholds and exposure to loud music was reported. However, significant changes in hearing thresholds and otoacoustic emissions, and a high tinnitus prevalence suggested an association between music exposure and hearing loss in children.

In **chapter 7**, we describe the prevalence of sensorineural hearing loss (SNHL) among 5368 children from the Generation R Study, aged 9 to 11 years. Next, we examine whether there are factors that could explain early SNHL. In this population, 7.8% of the children have low-frequency of high-frequency hearing loss of at least 16dB in one or both ears. A history of recurrent AOM and lower socioeconomic class is associated with the early SNHL in these children. Reduction of AOM episodes in early life could lead to better hearing outcomes in this young population.

Part IV: New models of otitis media

In **chapter 8**, we review key articles on the topics of biomedical engineering and biotechnology in relation to AOM. We highlight new diagnostic methods, potential vaccine candidates and the novel trans-tympanic drug delivery.

Part V: General discussion

Chapter 9 provides the general overview and interpretation of the results of this thesis. We highlight methodological issues and discuss causality of the observed associations. Finally, ideas and directions for future research in the field of AOM are discussed.

SAMENVATTING

In dit proefschrift bestuderen wij de hypothese dat vroege omgevingsfactoren tijdens de zwangerschap en in de eerste jaren van het leven van een kind, in combinatie met genetische aanleg, een toename of afname van het aantal doorgemaakte oorontstekingen op kinderleeftijd teweeg kunnen brengen. Vervolgens bestuderen wij of er epigenetische mechanismen bestaan die tijdens de zwangerschap kunnen worden beïnvloed, en welke in de jaren na de geboorte van invloed zijn op de gevoeligheid voor het ontwikkelen van oorontstekingen van het kind. Tenslotte onderzoeken wij of perceptief gehoorverlies een langetermijneffect zou kunnen zijn van het hebben doorgemaakt van otitis media acuta episoden. Door de complexe pathogenese van oorontsteking verder te ontrafelen, hopen wij in de toekomst beter te begrijpen waarom het ene kind gevoelig wordt voor het ontwikkelen van oorontsteking, waar het andere er zelden last van heeft. Zo hopen wij in de toekomst vroegere behandeling beter toe te spitsen op de individuele patiënt, en het ontwikkelen van oorontstekingen te helpen voorkomen.

In **hoofdstuk 1** wordt de achtergrond en rationale van onze hypothese uiteengezet. Van de verschillende studies die samen dit proefschrift vormen worden doelen en de grote lijnen beschreven.

Deel I: Omgevingsfactoren geassocieerd met otitis media acuta op kinderleeftijd

In **hoofdstuk 2** beschrijven wij verschillende associaties tussen omgevingsfactoren en AOM tot 6-jarige leeftijd in een groep van 7863 kinderen in de Generation R Study. Door gebruik te maken van de vele meetmomenten, en te corrigeren voor mogelijke correlaties tussen deze tijdmomenten, is het ook mogelijk om te kijken naar mogelijke associaties op specifieke leeftijden. We laten zien dat jongens een grotere kans hebben op het ontwikkelen van AOM tussen 2- en 4-jarige leeftijd, maar niet op andere leeftijden. Blootstelling aan sigarettenrook in de thuissituatie, het hebben van broers of zussen, en het bezitten van bepaalde huisdieren (vogels) vergroten de kans op het ontwikkelen van AOM tot 6 jaar. Borstvoeding verkleint deze kans juist, met het grootste effect in de eerst maanden van het leven. Hiermee tonen wij aan dat verschillende determinanten bestaan die van invloed zijn op de gevoeligheid voor het ontwikkelen van AOM op kinderleeftijd, en dat enkele determinanten die invloed alleen kennen in bepaalde fasen van de vroege jeugd.

In **hoofdstuk 3** kijken we op een nieuwe manier naar het Generation R cohort van 7863 kinderen betrekking tot onze uitkomst AOM. Door gebruik te maken van latent-class-analyses is het mogelijk te zoeken naar verschillende banen (trajectories) die een kind kan volgen vanaf de geboorte tot aan 10-jarige leeftijd. Deze banen zijn volledig gebaseerd op het al dan niet doormaken van (enkele of meerdere) AOM-infecties. We ontdekken dat er 3 verschillende banen bestaan: kinderen die hun gehele jeugd gevoelig zijn en blijven voor AOM, kinderen die dat niet zijn, en kinderen die gevoelig zijn voor AOM tot 3-jarige leeftijd maar daarna niet meer. Mannelijk geslacht en dagverblijfbezoek zijn geassocieerd

met deze laatste groep. Borstvoeding is beschermend in zowel de AOM-gevoelige kinderen tot 3 jaar, als in de groep die AOM-gevoelig blijven. In welk seizoen een kind wordt geboren is geassocieerd met kinderen die AOM-gevoelig blijven. De helft van alle kinderen die gevoelig zijn voor AOM, zal volledig herstellen vanaf 3-jarige leeftijd. Dit beeld herkennen wij in onze KNO-kliniek. Dit onderzoek ondersteunt de hypothese dat kinderen die gevoelig zijn voor AOM-infecties niet toebehoren aan één homogene groep. Er lijkt daadwerkelijk een verschil te bestaan tussen kinderen die enkele jaren last hebben van AOM en nadien herstellen, en kinderen die kwetsbaar blijven voor AOM.

Deel II: Genetische en epigenetische gevoeligheid voor otitis media acuta op kinderleeftijd

Hoofdstuk 4 beschrijft de eerste GWAS over AOM en de grootste genetisch OM studie tot nu (N=11047). We ontdekken genoombrede significantie op locus 6q25.3 wat ligt in het *FNDC1* gen en we repliceren de associatie in een onafhankelijk pediatrisch cohort (N=2012). Daarnaast laten we zien dat het muis-analoge Fndc1 gen tot expressie komt in het middenoor, en dat expressie toeneemt wanneer AOM wordt geïnduceerd bij deze muizen na inspuiten van lipopolysaccharide in het middenoor. Verder ondersteunt de eQTL en methylatiedata de associatie met het *FNDC1* gen. Aldus levert dit hoofdstuk bewijs voor een genetisch component die gevoeligheid voor AOM op kinderleeftijd mede bepaald.

In **hoofdstuk 5** onderzoeken wij of verschillen in DNA methylatie bij de geboorte geassocieerd zijn met gevoeligheid voor AOM in de eerste jaren van het leven. We verrichten een meta-analyse van alle beschikbare epigenetische data bestaande uit 6 internationale cohortstudies (N=2793). We vinden geen overtuigend bewijs om de hypothese te ondersteunen dat DNA methylatie patronen bij de geboorte invloed hebben op genexpressie en daaropvolgende AOM-gevoeligheid.

Deel III: Gehoorniveau van kinderen, en gehoorverlies in relatie tot otitis media acuta op kinderleeftijd

In **hoofdstuk 6** bestuderen wij eerst wat het gehoorniveau is op kinderleeftijd tot aan jonge volwassenheid. Een belangrijke factor die invloed kan hebben op vroeg perceptief gehoorverlies is (vroege) blootstelling aan lawaai, zoals via luide muziek. We voeren een systematic review uit waarmee wij een overzicht geven van alle beschikbare literatuur met betrekking tot gehoorniveaus en lawaaiexpositie op jonge leeftijd. De prevalentie van licht tot matig perceptief gehoorverlies (>15dB) was 9,6% en van hoge frequentie gehoorverlies 9,3%. De gemiddelde gehoordrempel in de lagere frequenties was 4,8dB en in de hogere frequenties 9,5dB. Er werd geen associatie bewezen tussen de gemeten gehoorverliezen en blootstelling aan lawaai. Echter, significante veranderingen in gehoordrempels en in otoakoestische emissies, gecombineerd met de hoge tinnitus prevalentie na lawaaiexpositie, maken een associatie tussen gehoorverlies op kinderleeftijd en de toegenomen lawaaiexpositie wel waarschijnlijk.

Hoofdstuk 7 toont de gehoordrempels op 9 tot 11-jarige leeftijd van een groep van 5368 deelnemende kinderen in de Generation R Studie. We beschrijven welke factoren een vroeg perceptief gehoorverlies zouden kunnen verklaren. Het blijkt dat 7,8% van de kinderen laag- of hoogfrequent perceptief gehoorverlies heeft van minimaal 16dB in één of beide oren. Een voorgeschiedenis met meerdere AOM-episodes en lagere sociaaleconomische klasse zijn geassocieerd met vroeg perceptief gehoorverlies op kinderleeftijd. Mogelijk zou reductie van het aantal AOM-episodes kunnen leiden tot betere gehooruitkomsten in deze jonge populatie.

Deel IV: Nieuwe modellen

In **hoofdstuk 8** bespreken we belangrijke nieuwe ontwikkelingen op het vlak van biotechnologie en biomedische technologie in relatie tot AOM. We geven een overzicht over nieuwe diagnostische methoden, bespreken potentiele kandidaat vaccins en transtympanale medicatie toediening.

Deel V: Algemene discussie

Hoofdstuk 9 geeft een samenvatting en interpretatie van de resultaten van dit proefschrift. We belichten methodologische vraagstukken en causale verbanden van de gevonden associaties. Tenslotte geven wij suggesties voor toekomstig onderzoek op het vlak van AOM.

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United States of America

Center for Applied Genomics, Children's Hospital of Philadelphia, Philadelphia, United States. J. Li, R., Y.R. Li, M.E. March, M. Bakay, F.D. Mentch, K. Thomas, X. Chang, H.S. Hain, P.M. Sleiman, H. Hakonarson.

Department of Computer Science, New Jersey Institute of Technology, Newark, United States. Z. Wei.

Department of Epidemiology, Geisel School of Medicine at Dartmouth, Hanover, United States. L.A. Salas.

Department of Otolaryngology Head & Neck Surgery, Stanford University, Palo Alto, United States. T.A. Valdez.

Department of Pediatrics, The Perelman School of Medicine, University of Pennsylvania, Philadelphia, United States. H. Hakonarson.

Division of Human Genetics, Children's Hospital of Philadelphia, Philadelphia, United States. H. Hakonarson.

Division of Otolaryngology, Department of Surgery, University of California, San Diego, La Jolla, United States. A.F. Ryan.

Los Angeles Children's Hospital, Los Angeles, United States. E. Nickels.

PhotoniCare, Inc., Campaign, United States. R.M. Nolan.

San Diego Veterans Affairs Healthcare System, Research Department, San Diego, United States. A.F. Ryan.

The Stripp Lab, Pulmonary Department, Cedars Sinai Medical Center, Los Angeles, United States. A. Mulay.

University of California San Francisco, San Francisco, United States of America. R. Roy, J.L. Wiemels.

University of Southern California, Los Angeles, United States. S. Li, E. Nickels, J.L. Wiemels.

LIST OF PUBLICATIONS

F.V.W.J. van Zijl, **G. van Ingen**, R.J. Baatenburg de Jong, F.R. Datema. Systematic and prospective outcome evaluation of septoplasty without concurrent turbinate surgery. Submitted

G. van Ingen, R. Roy, L.A. Salas, S.E. Reese, C.M. Page, G. Pesce, G.C. Sharp, R.J. Baatenburg de Jong, S. Li, M. Casas, S.E. Håberg, M.C. Magnus, N. Baïz, E. Nickels, M. Gascon, J.F. Felix, C.L. Parr, L.K. Tanno, C.L. Relton, I. Annesi-Maesano, W. Nystad, S.J. London, J.Sunyer, J.L. Wiemels, M.P. van der Schroeff. Newborn DNA methylation and risk of acute otitis media in childhood: Meta-analysis of epigenome-wide association studies. Submitted

G. van Ingen, C.M.P. le Clercq, V.W.V. Jaddoe, H.A. Moll, L. Duyts, H. Raat, R.J. Baatenburg de Jong, M.P. van der Schroeff. Identifying distinct trajectories of acute otitis media in children: a prospective cohort study. Clin Otolaryn. 2021 July

S.P.C. Koenraads, M.P. van der Schroeff, **G. van Ingen**, S. Lamballais, H. Tiemeier, R.J. Baatenburg de Jong, T. White, M.C. Franken, R.L. Muetzel. Structural brain differences in pre-adolescents who persist in and recover from stuttering. Neuroimage Clin. 2020 June

M. Gisselson-Solén, P.A. Tähtinen, A.F. Ryan, A. Mulay, S. Kariya. A.G.M. Schilder, T.A. Valdez, S. Brown, R.M. Nolan, A. Hermansson, **G. van Ingen**, T. Marom. Panel 1: Biotechnology, biomedical engineering and new models of otitis media. Int J Pediatr Otorhinolaryngol. 2020 March

G. van Ingen, C.M.P. le Clercq, C.E. Touw, L. Duijts, H.A. Moll, V.W.V. Jaddoe, H. Raat, R.J. Baatenburg de Jong, M.P. van der Schroeff. Environmental determinants associated with acute otitis media in children: a longitudinal study. Pediatr Res. 2020 January

B.C. Oosterloo, **G. van Ingen**, M.P. van der Schroeff, S.J.C. Verhaegh. Otitis media met effusie: wat is de rol van reflux in de pathogenese. NTvKNO. 2018 January

C.M.P. le Clercq, **G. van Ingen**, L. Ruytjens, A. Goedegebure, H.A. Moll, H. Raat, V.W.V. Jaddoe, R.J. Baatenburg de Jong, M.P. van der Schroeff. Prevalence of hearing loss among children 9 to 11 years old: the Generation R Study. JAMA Otolaryngol Head Neck Surg. 2017 September

C.M.P. le Clercq, M.P. van der Schroeff, J.E. Rispens, L. Ruytjens, A. Goedegebure, **G. van Ingen**, M.C. Franken. Shortened nonword repetition task (NWR-S): a simple, quick, and less expensive outcome to identify children with combined specific language and reading impairment. J Speech Lang Hear Res. 2017 August

C.M.P. le Clercq, **G. van Ingen**, L. Ruytjens, M.P. van der Schroeff. Music-induced hearing loss in children, adolescents, and young adults: a systematic review and meta-analysis. Otol Neurotol. 2016 October

G. van Ingen*, J. Li*, A. Goedegebure, R. Pandey, Y.R. Li, M.E. March, V.W.V. Jaddoe, M. Bakay, F.D. Mentch, K. Thomas, Z. Wei, X. Chang, H.S. Hain, A.G. Uitterlinden, H.A. Moll, C.M. van Duijn, F. Rivadeneira, H. Raat, R.J. Baatenburg de Jong, P.M. Sleiman, M.P. van der Schroeff, H. Hakonarson. Genome-wide association study for acute otitis media in children

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S.L. den Boer, M.L. van Osch-Gevers, **G. van Ingen**, G.J. du Marchie Sarvaas, G.G. van Iperen, R.B. Tanke, A.P.C.M. Backx, A.D.J. Ten Harkel, W.A. Helbing, T. Delhaas, A.J.J.C. Bogers, L.A.J. Rammeloo, M. Dalinghaus. Management of children with dilated cardiomyopathy in the Netherlands: implications of a low early transplantation rate. J Heart Lung Transplant. 2015 July

PHD PORTFOLIO

Name:	Gijs van Ingen
Department:	Otolaryngology and Head & Neck Surgery
Research School:	Netherlands Institute for Health Sciences (NIHES)
PhD period:	2013 – 2021
Promotor:	Prof. Dr. R.J. Baatenburg de Jong
Co-promotor:	Dr. M.P. van der Schroeff

PhD training	Year	Workload (ECTS)
Master of Science in Clinical Epidemiology (NIHES)	2013 – 2015	70
General courses - Erasmus Summer Program		
Principles of Research in Medicine	2013	0.7
Clinical Decision Analysis	2013	0.7
Methods of Public Health Research	2013	0.7
Topics in Meta-analysis	2014	0.7
Health Economics	2013	0.7
Genome-wide Association Analysis	2014	1.4
Principles of Genetic Epidemiology	2014	0.7
Genomics in Molecular Medicine	2014	1.4
Markers and Prognostic Research	2013	0.7
The Practice of Epidemiologic Analysis	2013	0.7
Core Curriculum		
Study Design	2014	4.3
Biostatistical Methods 1: Basic Principles	2013	5.7
Clinical Epidemiology	2013	5.7
Methodologic Topics in Epidemiologic Research	2014	1.4
Biostatistical Methods 2: Classical Regression Models	2014	4.3
English Language	2013	1.4
Introduction to Medical Writing	2015	1.1
Courses for the Quantitative Researcher	2014	1.4
Advanced Courses		
Repeated Measurements in Clinical Studies	2015	1.4
Advances in Genome-wide Association Studies	2015	1.4
A First Encounter with Next-generation Sequencing Data	2015	1.4
General Academic Courses		
MRI safety training	2014	0.3
Radiation safety training 5R	2013	0.3
Endnote course	2013	0.3
'Zoeken in Pubmed' course	2013	0.3
'Zoeken in andere databases' course	2013	0.3

Seminars and Workshops

Research meetings Generation R Study (1 oral presentation)	2013 – 2016	4.0
Research meetings at Center for Applied Genomics, Philadelphia (1 oral pres.)	2013	1.0
Maternal and Child meetings Generation R Study (3 oral presentation)	2013 – 2015	3.0
Otolaryngology Annual Research Day Erasmus MC (2 oral presentations)	2013 – 2018	3.0
Biannual National ENT meeting of NVvKNO (5 oral pres., 2 posters)	2013 – 2018	6.0

International conferences and research visits

ENT/SAAA/SASLHA Congress, Cape Town (1 oral presentation)	2014	1.4
European Society Pediatric Otolaryngology 2016, Lisbon (1 oral presentation)	2016	1.4
18th Symposium on Recent Advances in Otitis Media, Gold Coast (1 oral pres.)	2017	1.4
Research visit to Child Health CheckPoint, Melbourne (1 oral presentation)	2017	0.7
European Society Pediatric Otolaryngology 2018, Stockholm (1 oral pres.)	2018	1.4
19th Symposium on Recent Advances in Otitis Media, Los Angeles (1 oral pres.)	2019	1.4

Teaching Activities

Tutor for 1st year medical students, supervision and lectures	2013	1.0
Supervising various workgroups for 3rd and 5th year medical students	2015 – 2018	1.0
Supervising various workgroups for ER, OR, oncology nurses in training	2016 – 2018	1.0
C.E. Touw, MSc Student, "Environmental Determinants of AOM"	2017	3.0
B.C. Oosterloo, MSc Student, "OME en de rol van reflux in de pathogenese"	2017	3.0

Awards

NVvKNO Poster Award 1st Prize	2014
Junior Award ESPO 2016 - 1st Prize	2016
Nominee Science and Innovation Award of Federatie Medisch Specialisten	2017
Junior Award ESPO 2018 - 2nd Prize	2018

Grants

European Union 7th Framework Program; grant no. 247642, GEoCoDE	2013
Erasmus Trustfonds Conference Participation Grant – ENT/SAAA/SASLHA	2014
Erasmus Trustfonds Conference Participation Grant – ESPO 2016	2016
Erasmus Trustfonds Conference Participation Grant – ESPO 2018	2018

Other

Review papers for Scientific Reports (1) and Frontiers in Genetics (1)	2017, 2019	0.4

1 ECTS (European Credit Transfer System) is equal to a workload of 28 hours

ABOUT THE AUTHOR

Gijs van Ingen was born on the 24th of December 1985 in Nieuwegein, the Netherlands. After graduating from secondary school (Atheneum, Maerlant Lyceum, The Hague), he studied Medicine at the Erasmus University in Rotterdam, and graduated cum laude in 2012. In the last two years of his study, he became interested in otolaryngology during his clinical internships at Amphia Ziekenhuis in Breda. The final six months of his study were completed as a surgical intern at Malamulo Hospital in Makwasa, Malawi.

In 2013, Gijs started his PhD-project on acute otitis media, which was embedded in The Generation R Study (promotor: Prof. R.J. Baatenburg de Jong; co-promotor: dr. M.P. van der Schroeff). He combined his PhD-project with a Master of Science in Clinical Epidemiology at the Netherlands Institute of Health Sciences and spent 3 months at the Center for Applied Genomics in Philadelphia, the United States, focusing on Genetic Epidemiology.

From August 2015 until March 2020, Gijs combined his PhD-project with otolaryngology residency under Prof. R.J. Baatenburg de Jong and dr. R.M. Metselaar at Erasmus MC, dr. P.G.J. Ten Koppel at Maasstad Ziekenhuis, and dr. G.K.A. van Wermeskerken at Amphia Ziekenhuis. After completing his training as an otolaryngologist, he completed a 1-year fellowship in Facial Plastic and Reconstructive Surgery supervised by dr. W.M. Boek (Ziekenhuis Gelderse Vallei, Ede) and dr. K.J.A.O. Ingels (Radboud UMC, Nijmegen). Currently, he works as an otolaryngologist at Franciscus Gasthuis & Vlietland in Rotterdam, the Netherlands.

Gijs and his girlfriend Freija Stokmans, live in Amsterdam with their daughter Phileine and newborn son Julius.

DANKWOORD

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Prof. dr. R.J. Baatenburg de Jong, beste Rob, als enthousiaste coassistent zat ik eind 2012 voor je. Ik had gesolliciteerd voor de opleiding tot KNO-arts en sprak de wens uit mij wetenschappelijk verder te ontwikkelen. Jij bood mij die kans, en koppelde mij aan Marc en de Generation R Studie waarvoor ik je ontzettend dankbaar ben. Je gaf me de ruimte om binnen Generation R te ontdekken welke kansen er lagen. Je herkende de potentie van GWAS en EWAS, en steunde mijn uitstapje naar Philadelphia wat leidde tot onze belangrijkste paper. Mede dankzij jouw visie, input en kritische blik is dit proefschrift geworden tot wat het is.

Dr. M.P. van der Schroeff, beste Marc, vanaf het begin ben je de rustige steun op de achtergrond die ik nodig had. Je liet me freewheelen in de eerste maanden toen bleek dat audiologische data nog op zich liet wachten. Hierdoor had ik de tijd om de enorme schat aan data binnen Generation R te ontdekken. Na enige tijd kwamen wij bij elkaar om te brainstormen over potentiële onderzoeksvragen. Het kladblaadje dat we die middag gebruikten voor losse aantekeningen is de basis gebleken voor drie promotietrajecten waarvan één leidde tot dit boekje. Dank voor je geduld, je betrokkenheid en het meedenken over de vaak technisch complexe statistiek. Ook buiten de wetenschap zie ik je als voorbeeld voor hoe ik mij in de kliniek wil presenteren als KNO-arts, en als klinisch supervisor van arts-assistenten. Het oppassen op jouw huis tijdens je vakantie zal altijd een belangrijke herinnering blijven voor Freija en mij. Dank voor alles.

De overige leden van de **kleine** en **grote promotiecommissie** wil ik danken voor hun tijd en interesse in dit proefschrift.

Mijn paranimfen, **drs. C.M.P. le Clercq** en **drs. M.L. Gerdes**; beste Carlijn, Generation R was ons terrein, waar we een schat aan interessante data ontdekten. Jij dook vol in de audiologie, en ik in de oorontsteking. Hier zijn een paar prachtige gezamenlijke projecten en publicaties uit voortgekomen. Heel veel dank voor je statistische en wetenschappelijke input. Ik kijk met veel plezier terug naar het werkbezoek dat Marc en ik mochten brengen aan het Murdoch Children's Researche Institute in Melbourne waar jij enkele maanden zat. Het was wetenschappelijk gezien een belangrijke reis. De Great Barrier Reef was prachtig.

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STELLINGEN BEHORENDE BIJ HET PROEFSCHRIFT

Early Environmental, Genetic and Epigenetic Determinants of Acute Otitis Media in Children

1) Borstvoeding verlaagt de kans op het ontwikkelen van otitis media acuta op kinderleeftijd. (Dit proefschrift)

2) Er is een verschil tussen een kind dat gevoelig is voor het ontwikkelen van otitis media acuta tot 3 jaar, en een kind dat hier zijn gehele jeugd gevoelig voor blijft. (Dit proefschrift).

3) Genetische locus *FNDC1* is een ziekte-bijdragend gen van otitis media acuta op kinderleeftijd. (Dit proefschrift)

4) Veranderde DNA methylatie tijdens de foetale fase heeft geen invloed op de gevoeligheid van een kind om otitis media acuta te ontwikkelen. (Dit proefschrift)

5) Het herhaaldelijk doormaken van otitis media acuta in de vroege jeugd is geassocieerd met een verhoogd risico op perceptief gehoorverlies op 9- tot 11-jarige leeftijd. (Dit proefschrift)

6) Toekomstige studies naar otitis media zouden baat hebben bij beter gedefinieerde fenotypes.

7) Een reductie van het aantal otitis media acuta episoden van een kind is meetbaar in het aantal gewonnen uren slaap van de ouders.

8) Grote genetische studies ontdekken in vlot tempo associaties tussen veelvoorkomende ziekten en genetische aanleg, maar begrip van onderliggende mechanismen blijft achter.

9) Bekwaamheid in statistiek wordt in onze maatschappij net zo belangrijk als lezen en schrijven.

10) Artificiële intelligentie kan de menselijke interactie tussen zorgverlener en patiënt niet vervangen.

11) (Epi)genetica leert ons dat het goede rapport van mijn dochter kan worden verklaard door haar genen en haar omgeving; is het rapport niet goed, dan moet zij beter haar best doen.