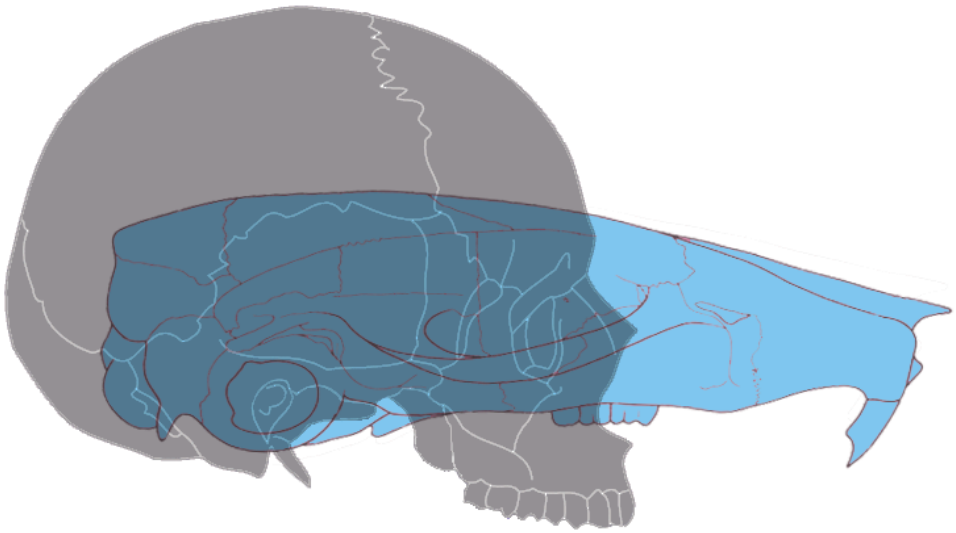
Genetics of Chronic Otitis Media
A Mouse to Man Approach

Mahmood F Bhutta

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Genetics of chronic otitis media: A mouse to man approach

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Abstract

Chronic otitis media (OM) is an archetypal complex disease, which is particularly prevalent in childhood. Epidemiological data suggest high heritability for disease susceptibility, but previous genetic association studies have had methodological flaws, and none have specifically focused on chronic OM phenotypes.

Mouse models represent one way to ascertain candidate loci for human association testing. A number of mouse models of middle ear inflammation have been reported, but many susceptibility loci remain undiscovered. I demonstrate that oto-endoscopy is a robust and scalable phenotyping platform for OM in the mouse, and discuss its value in new model discovery.

Chronic OM is also a feature of trisomy HSA21 (Down Syndrome). Through an interrogation of the mouse library of segmental trisomy models of Down Syndrome, I identify a critical trisomic region for chronic otitis media. This region may underlie OM susceptibility in Down Syndrome, but could also contribute to disease susceptibility in non-syndromic disease.

Mouse models can also be used to interrogate disease mechanisms. Our previous work has shown that the chronically inflamed middle ear is hypoxic, and that hypoxia signalling is a potential therapeutic target. Exploiting the *Junbo* mouse model, I demonstrate that surgical ventilation of the *Junbo* ear improves inflammation, and that this is associated with loss of hypoxia signalling. I present preliminary results from transcript analyses of human middle ear effusions showing marked upregulation of hypoxia signalling.

A systematic review of existing mouse models suggests that the loci *FBXO11*, *EVI1*, *SMAD2*, and *TGIF1* are good candidates genes for human association testing. I detail recruitment and collection of DNA from families in the UK where a child is undergoing grommet insertion. Association testing using a variant of the transmission disequilibrium test shows susceptibility associated with polymorphisms at *FBXO11*, and possibly also *SMAD2* and *TGIF1*.

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Abbreviations

ALSPAC	Avon Longitudinal Study of Parents and Children
AOM	Acute otitis media
cDNA	copy DNA
CEU	Utah residents with Northern and Western European ancestry in the Hapmap Project
COME	Chronic otitis media with effusion
CSOM	Chronic suppurative otitis media
DNA	Deoxyribonucleic acid
EVI	Ecotropic viral intergration site
ELISA	Enzyme linked immunosorbent assay
ENT	Ear Nose Throat
EYA	Eyes absent family of proteins
FACS	Fluorescence-activated cell sorting
FBXO11	F-box 11 gene
GWAS	Genome wide association study
HIF	Hypoxia inducible factor
IL	Interleukin
KO	Knock-out
LRTI	Lower respiratory tract infection
MAF	Minor allele frequency
M. cattarhalis	*Moraxella cattahralis*
NALT	Nasal associated lymphatic tissue
NF-κB	Nuclear factor kappa B
NHS	National Health Service
NIHR	National Institute for Health Research
NRES	National Research Ethics Service
NT*Hi*	Non-typeable *Haemophilus influenza*
OM	Otitis media
OME	Otitis media with effusion
PCR	Polymerase chain reaction
PIMO	Pimodinazole
PIS	Participant information sheets
rAOM	recurrent AOM
RNA	Ribonuleic acid
RSV	Respiratory syncitial virus
RV	Rhinovirus
S. pneumoniae	*Streptococcus pneumoniae*
SMAD	Smad gene/protein
SNP	Single nucleotide polymorphism
TGIF	Transforming growth interfering factor
TGF-ß	Transforming growth factor beta
TLR	Toll like receptor
TNF- α	Tumour necrosis factor alpha
TDT	Transmission disequilibrium test
URTI	Upper respiratory tract infection
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WT	Wild type

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Prologue

The burden of chronic otitis media

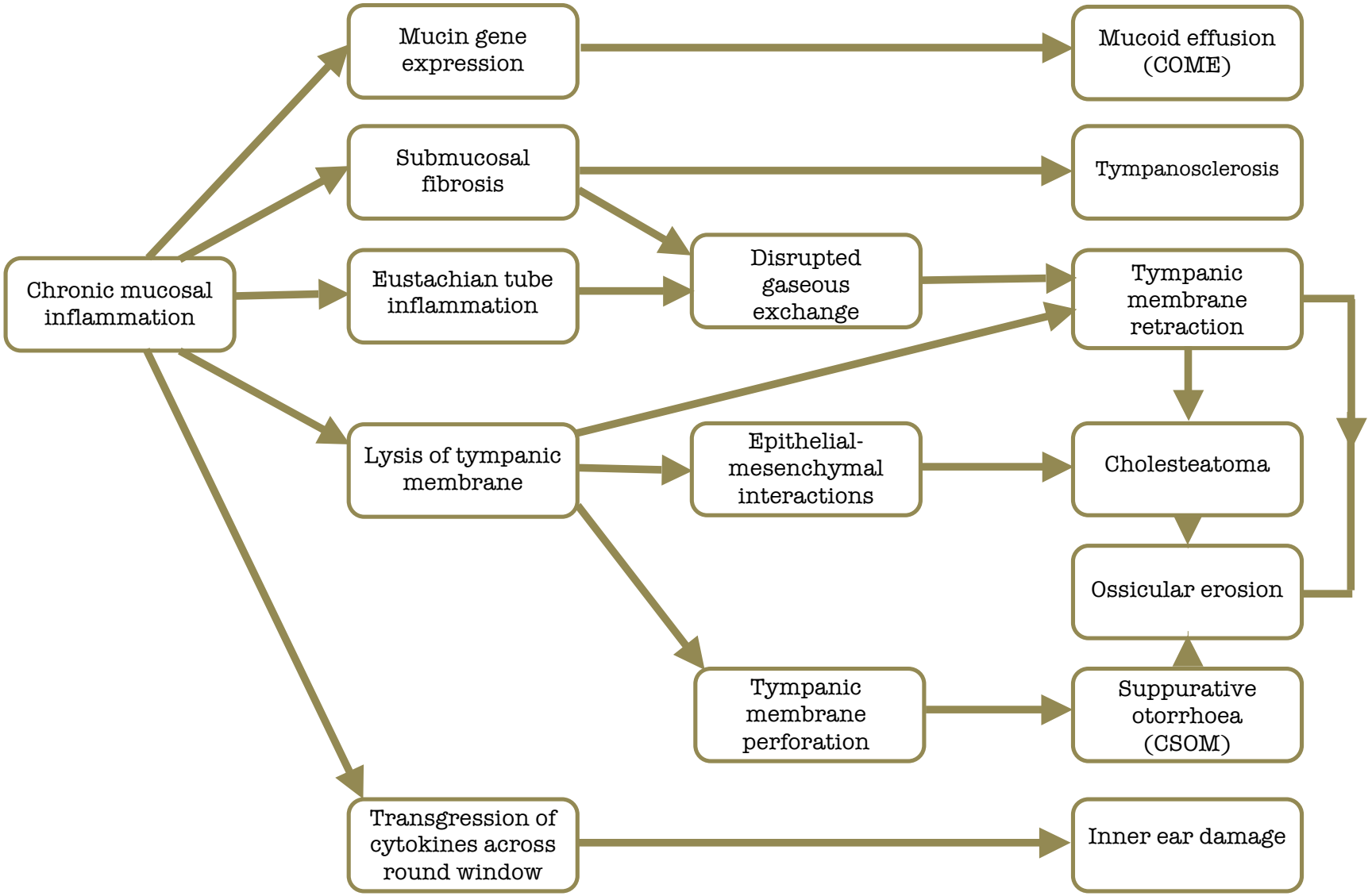
> 'In England, diseases of the ear had never met with the attention from the medical profession which their importance demanded. We have, then, great reason to complain of the little attention bestowed on the treatment of aural disease by men of science in this country.'
>
> James Yearsley (1839)
> *Deafness cured by cleaning out the passages from the throat to the ear*[1]

Inflammation of the middle ear (otitis media, OM) is a highly prevalent disease in children. Acute otitis media (AOM) is the most common bacterial infection in childhood, with two-thirds of children in the developed world having at least one episode by the age of five[2], leading to 2 million primary care consultations in the UK every year[3]. AOM rarely causes symptoms lasting more than a few days[4], and the disorder is largely confined to early childhood. By contrast, chronic middle ear inflammation causes persistent and sometimes permanent disability, due to middle ear effusion, or due to irreparable damage to middle ear tissues (figure 1).

Chronic otitis media with effusion (COME) is the prototypical form of chronic middle ear inflammation, where the middle ear becomes filled with a sero-mucoid effusion secreted by inflamed mucosa. This interferes with the conduction of air-borne sound, and COME is the most common cause of hearing loss in children in the UK[5]. Chronic suppurative otitis media (CSOM) describes when the middle ear develops a purulent effusion, leading to hearing loss and otorrhoea. CSOM is rare in the developed world, but is a major burden in the developing world, where it is estimated to affect 65-330 million people[6].

As well as causing effusion, chronic inflammation in the middle ear can also lead to tissue damage. Negative middle ear pressure can lead to tympanic membrane retraction[7], which probably occurs as a result of disruption to gaseous diffusion[8] from subepithelial fibrosis[9]. Tympanic membrane retraction affects 3-25% of all children[10-14]

Figure 1: Consequences of chronic middle ear inflammation. Consequences towards the top of the diagram are common, whereas those further down are increasingly rare. Mechanisms are somewhat speculative.



and 4-32%[15 16] of all adults, and may lead to or compound hearing loss[17 18]. Presence and duration of COME in infancy predisposes to subsequent tympanic retraction[11 19].

Chronic OM may cause erosion of the ossicles[20-23] or lead to tympanosclerosis[24], which again may compound hearing loss. There can also be effects on the neuroepithelium of the inner ear, leading to hearing loss from dysfunction of the cochlea[25-32], or imbalance due to dysfunction of the vestibular system[33].

In rare cases chronic OM precedes the development of cholesteatoma. Cholesteatoma is a self-perpetuating growth of squamous epithelium developing from the tympanic membrane. Its cause is obscure[34], but it possibly arises as a result of erosion or damage to the tympanic membrane, leading to unscheduled epithelial-mesenchymal interactions[35]. Untreated cholesteatoma is locally invasive and can lead to life-threatening meningitis or temporal lobe abscess[36 37].

The socio-economic costs of chronic otitis media have not been comprehensively evaluated, but are significant[38]. Chronic otitis media is the most common reason for minor or major middle ear surgery in the developed world. The insertion of ventilation tubes ("grommets") to treat symptomatic COME is the most common operation in children, with over 30,000 such procedures in the UK each year[39], at a cost in excess of £20 million. More than 5,000 tympanoplasty operations are performed in the UK every year[39], mostly as a treatment for the consequences of chronic otitis media, at a cost in excess of £10 million. Costs in UK primary care are not known, but it is estimated that every primary care practice will see 6-7 cases of OME per year (Ian Williamson, personal communication). Children and adults with chronic otitis media report lower disease-specific and general quality of life[40 41], and persistent hearing loss may cause

detriment to linguistic development and education[41]. Worldwide, CSOM is estimated to lead to the annual loss of 2.16 million disability-adjusted life-years[42].

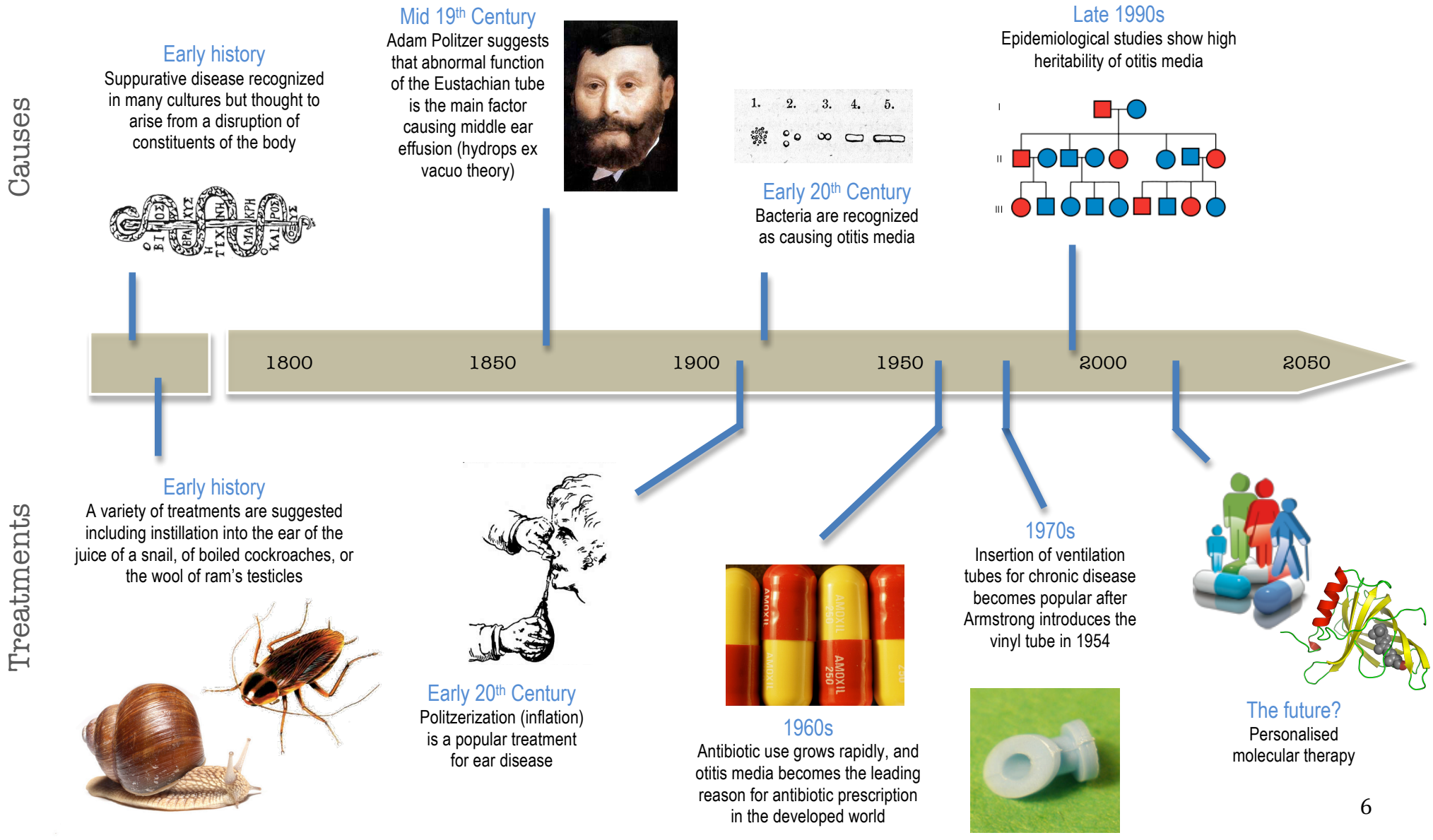
The promise of genetics

> "We are dealing with much more than a catarrhal process, we are dealing with what may probably be shown one day to be a microbic disease affecting the deeper layers of the muco-periosteum - a condition somewhat akin to " keloid." What we are in the habit of calling a catarrhal disease is no longer a catarrhal disease but a diffuse fibrosis. To keep this tendency to fibrosis in subjection is the crux of the position and has so far baffled all attempts."
>
> William Milligan (1921)
> *Chronic catarrhal otitis media: Some thoughts and suggestions*[43]

Middle ear suppuration has been recorded since the time of Hippocrates[44], but even as late as until the early 20th century, causation was still poorly understood[44-47], and therapies, of dubious value, were based around the instillation of a variety of concoctions into the ear (figure 2)[48]. Over 150 years ago Politzer promoted the theory that Eustachian tube dysfunction is the primary cause of otitis media[49], and this idea still prevails in contemporary literature[50] despite little convincing evidence to support it. In 1938 Schlanser surmised that heritability was ''the greatest single factor in the causation of the disease'[51]. Epidemiological studies in recent years have confirmed these suspicions, and demonstrate high heritability for recurrent or persistent middle ear inflammation[52-55].

Interrogation of the genetic architecture predisposing to chronic otitis media promises much. Only half of children presenting to a clinician with middle ear effusion will still have that effusion three months later[56]. A number of factors are associated with persistence of disease (upper respiratory tract infection, attendance at a day-care center, having older siblings, and a family history)[57], but current models that factor

Figure 2: A history of ideas in the causation and treatment of otitis media



patient demographics, environmental variables, or patient history to predict outcome are too inaccurate to be clinically useful[58]. With rapid advances in sequencing technology and computational capacity, it seems realistic that in the foreseeable future entering genotypes into these models will improve their accuracy and provide personalized predictions[53]. Perhaps more importantly, an understanding of genetic susceptibility willprovide new clues to the molecular architecture of inflammation in the middle ear, and so support exploration of potential targeted therapies.

Thesis structure

In this thesis I interrogate genetic susceptibility to chronic otitis media, both in mouse models, and in man. The thesis is divided into several section and chapter headings, and in each chapter I make an original contribution.

Section 1 is an analysis of otitis media phenotypes and the genetic architecture underlying them. In Chapter 1.1 I extract data from leading epidemiological studies in paediatric otitis media to construct a phenotype map and landscape for middle ear inflammation. In the field of population genetics, landscapes are a well-rehearsed method of presenting phenotypes, but this approach has not previously been applied to otitis media. In chapter 1.2 I review what is understood about host susceptibility to disease, and so provide background for potential mechanisms that could operate in genotype-phenotype correlation in OM. In particular I make an argument for the reason behind differences in otitis media prevalence in ethnic groups, again using concepts borrowed from population genetics. In chapter 1.3 I discuss methods to identify potential candidate genes for otitis media susceptibility, and use systematic review strategies to identify potential candidate loci, particularly from mouse models of

disease. A systematic approach to identify candidate genes for otitis media has not previously been undertaken. In chapter 1.4 I critically review existing human association studies, and discuss their advantages and limitations.

Section 2 explores novel experimental methods to identify new candidate genes in mouse models of chronic OM. In chapter 2.1 I demonstrate that otoendoscopy is a rapid, reliable, and valid method to screen for murine otitis media, which could be incorporated into phenotyping pipelines in large-scale mouse mutagenesis programs. In chapter 2.2 I mine the library of segmental trisomy mouse models of Down Syndrome to narrow a critical region underlying the chronic OM phenotype in this syndrome. This indicates not only potential pathways underlying OM susceptibility in Down syndrome, but also highlights pathways that may underlie otitis media in the non-syndromic child.

Section 3 exploits the value of genetic mouse models of chronic otitis media to interrogate potential pathobiology, and in particular the role of tissue hypoxia. In section 3.1 I find evidence that the beneficial effect of ventilation tubes (the most common treatment for symptomatic chronic otitis media) may be, at least in part, secondary to alleviation of chronic tissue hypoxia in the chronically inflamed middle ear. In section 3.2 I report preliminary data from leucocytes in human chronic middle ear effusions showing upregulation of transcripts from hypoxia responsive genes.

Section 4 details the major work of this thesis, a case-parent triad candidate gene human association study. Section 4.1 reports results of design and recruitment to the study. Section 4.2 explains the strategy to identify and genotype polymorphisms based upon loci derived from mouse models. Section 4.3 shows results of statistical association testing.

The thesis overall showcases new methods to interrogate genetic susceptibility to chronic middle ear inflammation, in particular exploiting mouse models to understand disease genetics and pathobiology in man.

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Chapter 1.1
The phenotype landscape of otitis media

Otitis media (OM) is a complex disease, and includes the phenotypes acute otitis media (AOM), recurrent AOM (rAOM), chronic suppurative otitis media (CSOM), otitis media with effusion (OME) and chronic OME (COME). These phenotypes are considered to be on a continuum of disease[1] [2], and although there are a large number of studies describing the epidemiology of OM[5] [6], the nature of this continuum has not been fully enunciated.

Recurrent or persistent middle ear inflammation is now known to be largely heritable[8], but definition of the sample space is necessary before a dissection of genotype-phenotype correlations. In this chapter I analyse relevant existing epidemiological and pathological data to justify and construct a comprehensive phenotype landscape of otitis media. I then consider the effect of important variables on phenotype prevalence, in particular the effect of age, or variation in the exposome. In the following chapter I extrapolate from a number of sources to guess at the underlying genetic structure of OM phenotypes.

Clinical Definitions

Clinical definitions of otitis media categorize disease based upon clinical symptoms and signs (table 1). A canonical pathway for the aetiology of the common forms of otitis media is well established (figure 1), whereby an upper respiratory tract infection (URTI) predisposes to suppuration of the middle ear (AOM, due to secondary bacterial infection), and a subsequent non-suppurative middle ear effusion (OME), which may become chronic (COME). This schema does not include phenotypes such as rAOM or CSOM, nor does it recognise instances of AOM, OME or COME that occur outside of this mechanism. Importantly, it is well established that all of these OM phenotypes (in the

Table 1: Clinically defined phenotypes of otitis media

Type	Abbreviation	Definition
Acute Otitis Media	AOM	History of acute onset of signs and symptoms, with signs and symptoms of middle-ear inflammation including effusion[9]
Recurrent Acute Otitis Media	rAOM	Variable. Three separate episodes of AOM in the preceding 6 months, or four or more episodes in the preceding 12 months[10]
Otitis Media with Effusion	OME	Fluid in the middle ear without signs or symptoms of ear infection[11]
Chronic Otitis Media with Effusion	COME	OME lasting greater than three months[11 12]
Chronic Suppurative Otitis Media	CSOM	Presence of discharge through a perforated tympanic membrane for longer than three months[13]

canonical pathway or not) are linked, in that the presence of one form of otitis media in an individual is associated with a coincident or subsequent risk of another form of otitis media in the same or the other ear[14 15], a condition that has been termed "otitis prone"[16]. This suggests that these middle ear inflammation phenotypes should be considered as a group, and not as having entirely separate aetiologies. The nature of this inter-relation is considered in more detail below.

Figure 1: The canonical pathway for the most common forms of otitis media



In passing, it is worth stating that here I will not consider OM in adults, unusual forms of OM, nor squamous diseases such as atelectasis or cholesteatoma. OM in adults may occur by non-canonical mechanisms[17], and is comparatively rare[18], and usually less severe[19 20]. Unusual forms of OM (table 2) probably occur by pathological mechanisms different to the common phenotypes, and may be best considered as separate entitites. Squamous middle ear disease often arises in those with a history of chronic mucosal OM, but here I focus only on mucosal disease.

Table 2: Unusual forms of otitis media

Disease Type	Disease Subtype
Atypical infection	*Mycobacterium tuberculosis*[21-25] *Mycobacterium avium intracellulare*[26-28] *Mycobacterium bovis*[29] *Mycobacterium chelonae*[30 31] *Mycobacterium fortuitum*[32] *Fusobacteria necrophorum*[33-39] *Actinomyces*[40]
Autoimmune vasculitis	Granulomatosis with polyangiitis (Wegener's)[41-67] Granulomatosis with eosinophilic polyangiitis (Churg-Strauss)[68-70] Microscopic polyangiitis (polyarteritis nodosa)[42 44 64] Giant cell arteritis[71] Behcet disease[72]
Autoimmune rheumatological	Ankylosing spondylitis[73 74] Systemic lupus erythematosis[42] Rheumatoid arthritis[75] Sjogren syndrome[76 77] Juvenile idiopathic arthritis[78]
Atopic	Eosinophilic otitis media[79 80]
Neonatal	Amniotic fluid associated[81-87] Infective (sepsis)[88-98]
Malignant	Nasopharyngeal carcinoma[99] Radiotherapy induced[100 101]

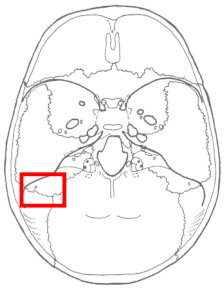
Pathological Analyses

Categorization of disease by symptoms and signs is necessary and beneficial in a clinical context, for purposes of epidemiology, prognosis or treatment, but such definitions are inaccurate, due to variations in phenotype definition[102-104], symptom reporting, or diagnostic methods[5]. AOM is defined by symptoms and signs, yet no single symptom[105-107], group of symptoms[106 108], or sign on otoscopy[109] reliably predicts the presence of contemporaneous middle ear effusion[110]. OME is defined by the presence of middle ear effusion, and again clinical tests for the presence of effusion may be inaccurate[111 112]. Pathological confirmation must be taken as the gold standard for the presence of disease. Such a definition may have limited clinical utility, but is necessary for an accurate picture of the spectrum of disease.

In general, clinically defined otitis media phenotypes do display some differing histological features (figure 2). AOM is associated with a suppurative effusion that is neutrophil rich[2 21 113 114]. In OME there is usually an effusion associated with infiltration of predominantly mononuclear cells, and hyperplasia and metaplasia of the epithelium (from squamous or cuboidal to columnar), with proliferation of goblet cells (which are the predominant source of mucins in the effusion)[113 115 116]. In CSOM, the suppurative effusion is infiltrated by lymphocytes, plasma cells and macrophages[21 113], but multinucleated giant cells and histiocytes may also be present[114]

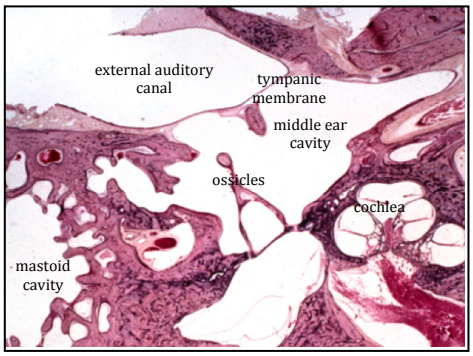
Although these histological descriptions correspond to clinical phenotypes, in reality OM occurs on a histological continuum[1 2]. OM associated with few or no clinical symptoms or signs, and no or minimal effusion has been detected in post-mortem specimens[117 118] (an entity termed chronic silent otitis media[119]). When a non-suppurative effusion is present, it varies quantitatively and qualitatively. Small volume clinically unapparent

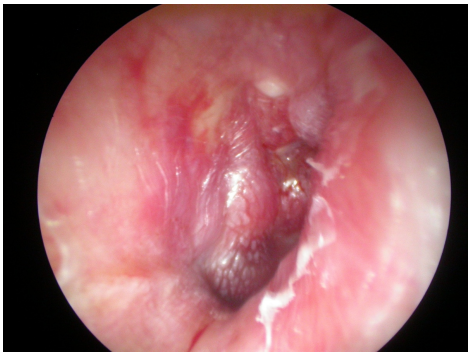
Figure 2: Otoscopic and histopathological appearances of clinically defined otitis media phenotypes. Plane of histological section shown on skull base.





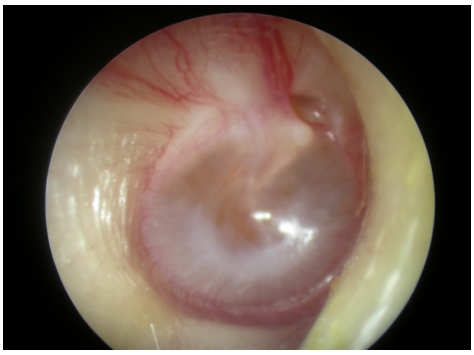
NORMAL





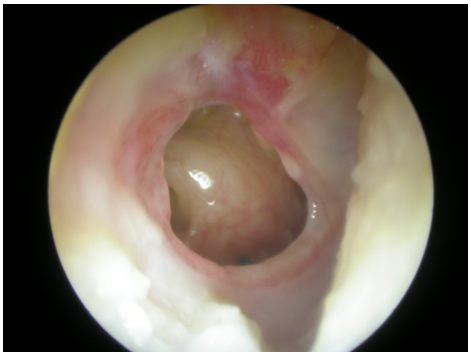
AOM



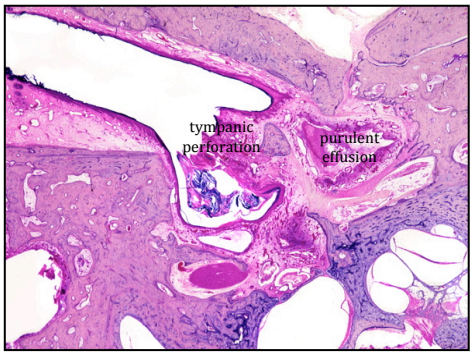


(C)OME





CSOM



Otoscopic images reproduced courtesy of David Pothier (with permission)
Histological images reproduced courtesy of Saumil Merchant (with permission)

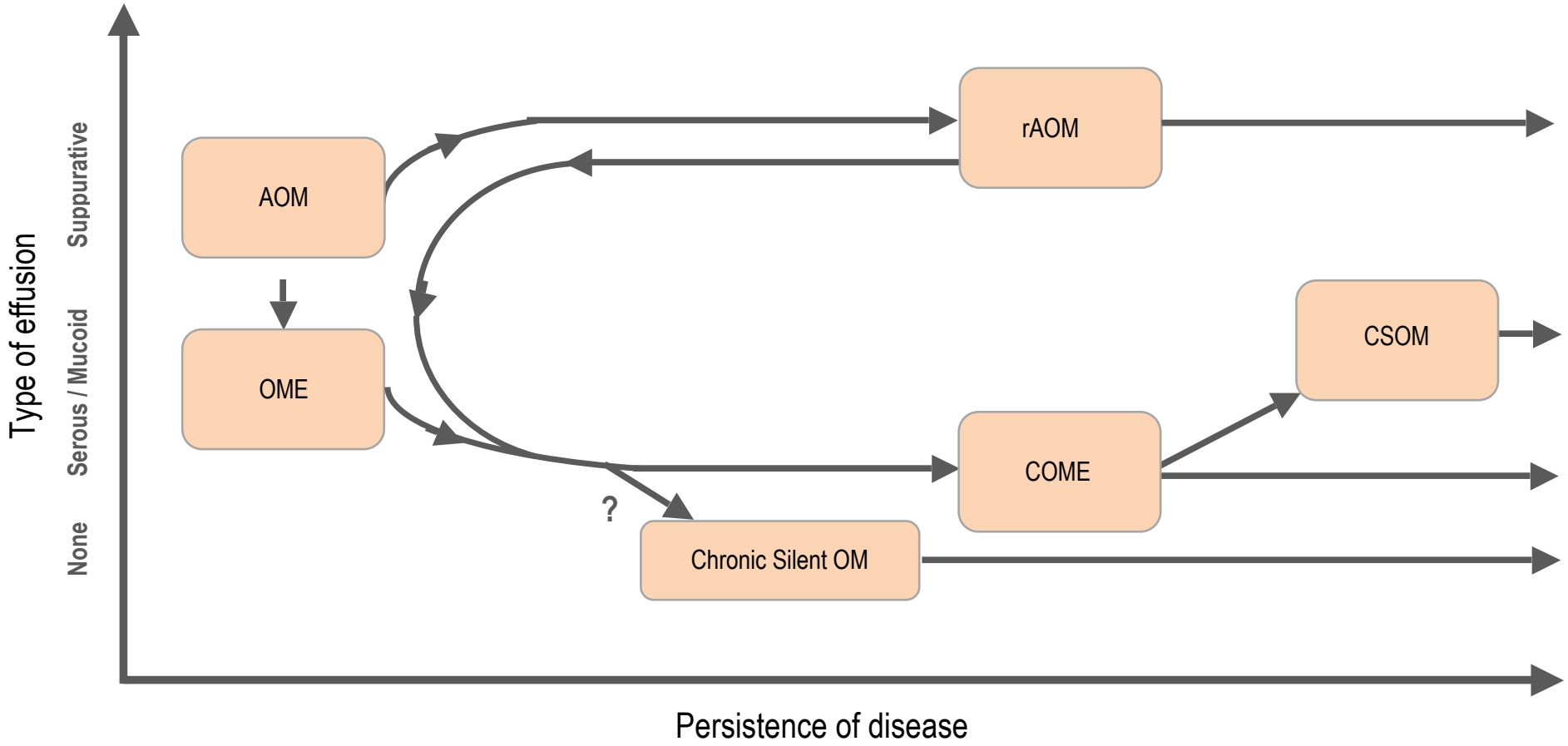
effusions may be found at tympanocentesis[109 120-129]. OME may occur with a serous (low viscosity, mucin deplete) effusion, and others with a mucoid (high viscosity, mucin rich) effusion[130]. Serous effusions are associated with less inflammation than mucoid effusions, in terms of immunoglobulin levels, inflammatory cytokines levels[131 132], and leucocyte infiltration[2 113 133] (and are more often of shorter duration[2], unilateral, and in an older age group[134]). Finally, post-mortem studies show that mucosal changes accompanying suppurative effusions have histological appearances that overlap with those accompanying non-suppurative effusions[2].

A Phenotype Map

These findings implicate that accurate phenotypic representation of middle ear inflammation should be based upon histology (as the gold standard), and must be represented as a continuum of disease, punctuated by agglomerations that correlate with clinical definitions of disease. One suggestion is that OM can be classified based upon the type of effusion[135]: serous, mucoid or suppurative. Such a schema seems to accommodate both pathological and clinical definitions, but I would extend this concept, to include silent otitis media, that without a macroscopic effusion. I would also place these effusions on a vector, whereby the type of effusion is a surrogate for the degree of inflammation. Histological analyses[2] suggest that, in general, the extent of leucocyte infiltration is of the order: purulent > mucoid > serous > silent. If we add an additional vector representing persistence of disease, a phenotype map can be created, and clinical definitions of disease placed upon this (figure 3).

Of course the map is still a simplification of reality, but it may be somewhat improved. First by adding a further vector representing prevalence of disease, and second by

Figure 3: A phenotype map of otitis media. Vectors for the degree of inflammation, and persistence of disease form axes, with clinically defined phenotypes populating this map. The canonical pathways are shown. Non-canonical pathways are discussed in the text.



considering in more detail the inter-relation of clinical phenotypes, and so creating a "phenotype landscape" for otitis media. To populate this construct requires a review of existing epidemiological data, to mathematically model phenotypes and generate landscape contours through curve fitting. Curve fitting carries the advantage of reducing the effects of noise, and potentially allowing extrapolation of missing data.

This approach may encounter difficulties. Different studies may define disease in different ways, study different populations, study disease at different time points, or vary in methodology. Indeed meta-analyses of epidemiological data suggest significant heterogeneity between OM studies[136], particularly where studies are small. A better approach is to model with single large data sets (where possible), and then impose upon these models variables that may affect outcome.

Unfortunately, and for pragmatic reasons, there is no epidemiological study that singularly contains sufficient data on all major OM phenotypes. For my analyses I combine data from what I believe are the best available sources. This is the largest single study for a particular phenotype, and that includes frequent assessment and over several years (table 3). I derive relevant studies from the systematic review of epidemiological studies of Casselbrant[5], and supplement these studies with other sources where relevant information may be inadequate. For AOM, rAOM and OME I use data from the Boston Study[3 7], supplemented with OME data from the Pittsburgh study[137]. I use data from the Finland study[138-140] in particular to examine the relationship between AOM/rAOM and OME/COME (note that the Finland study is actually larger that the Boston study, but only has data for OME in infancy). I also use data from other studies[141 142] to understand the epidemiology of URTI, a common precedent to AOM.

Table 3. Summary of major epidemiological studies used as data sources in this analysis.

Authors	Location	Year	Phenotype	Age range	Sample size	Population derivation	Frequency of Assessment	Notes
Alho et al[139 140]	Finland	1991 1995	AOM COME	3-24 months	2512	Community	3-6 months	COME defined as OME persisting for 2-4 months
Lambert et al[141]	Melbourne	2007	URTI	Birth – age 5	234	Community	2-3 weeks	Virus isolated in 74%
Revai et al[142]	Texas	2007	AOM after URTI	6-35 months	112	Community	2-3 weeks	-
Paradise et al[137]	Pittsburgh	1996	Middle ear effusion	Birth – age 2	2253	Primary care	monthly	AOM and OME
Teele et al[3 7]	Boston	1980 1989	OME after AOM AOM	Birth - age 7	877 to 498	Primary care	variable	Higher prevalence than other studies[5]. Attrition of less diseased individuals[7]

I base my analysis on clinical OM phenotypes. I define the prevalence (prev) as that of any occurrence of disease in the preceding year (i.e. a period prevalence). Within this I recognise the actual prevalence reported by studies ($prev_a$), and the predicted prevalence from modeling ($prev_p$). I represent the inter-relation of phenotypes as conditional probabilities (P(x|y)). I initially present probabilities pertaining to a two-year-old white child in a developed world environment, and subsequently consider the effect of demographic variants on disease probabilities.

Upper Respiratory Tract Infection (URTI)

Symptoms of URTI precede AOM in 94% of individuals[143], typically 3-5 days before AOM onset. The mechanism by which viral infections predispose to AOM is likely muti-faceted[143 144]. A variety of viruses are implicated, with detection of respiratory syncytial virus (RSV), influenza A or B, parainfluenza type 1, 2, or 3, adenovirus, rhinovirus, enterovirus, or coronavirus in 90% of those with AOM[143]. Because viral detection methods are not entirely sensitive (suggesting subclinical disease[145]), and because some virus species are not commonly screened for (e.g. human metapneumovirus, coronaviruses, human bocavirus[146]), it may be that URTI is an almost universal antecedent to AOM.

(1) $P(\text{URTI}|\text{AOM}) \geq 0.94$

This is the reverse probability; we can also ask the forward probability (that an URTI will lead to AOM). At age two the mean annual incidence of URTI is 6.7 episodes[141]. The probability that each episode of URTI will lead to AOM is 0.29[142], i.e.

(2) $P(\text{AOM}|\text{URTI}) = 0.29$

However multiplying the annual incidence of URTI by P(AOM|URTI) predicts 6.7 x 0.29 = 1.9 mean episodes of AOM per annum at age two. This is higher than the reported annual prevalence, which in the Boston study was 1.1[7] and in the Finland study was 1.18[139]. This implies that either, or both of the estimates for the incidence of URTI and for *P*(AOM|URTI) are too high.

Acute Otitis Media (AOM) and Recurrent AOM (rAOM)

At the age of two, 59% of children will have at least one episode of AOM[7].

(3) $\text{prev}_a(\text{AOM}) = 0.59$

Bacteria can be cultured in up to 80% of effusions, with *Streptococcus pneumoniae,* non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis* the most frequently isolated[147]. Many children will have more than one episode of AOM: at the age of two 30% will have two or more episodes, 10% three or more, and 4% four or more[7]. Definitions of rAOM vary, but if we use a definition of ≥4 episodes of AOM in a year then:

(4) $\text{prev}_a(\text{rAOM}) = 0.04$

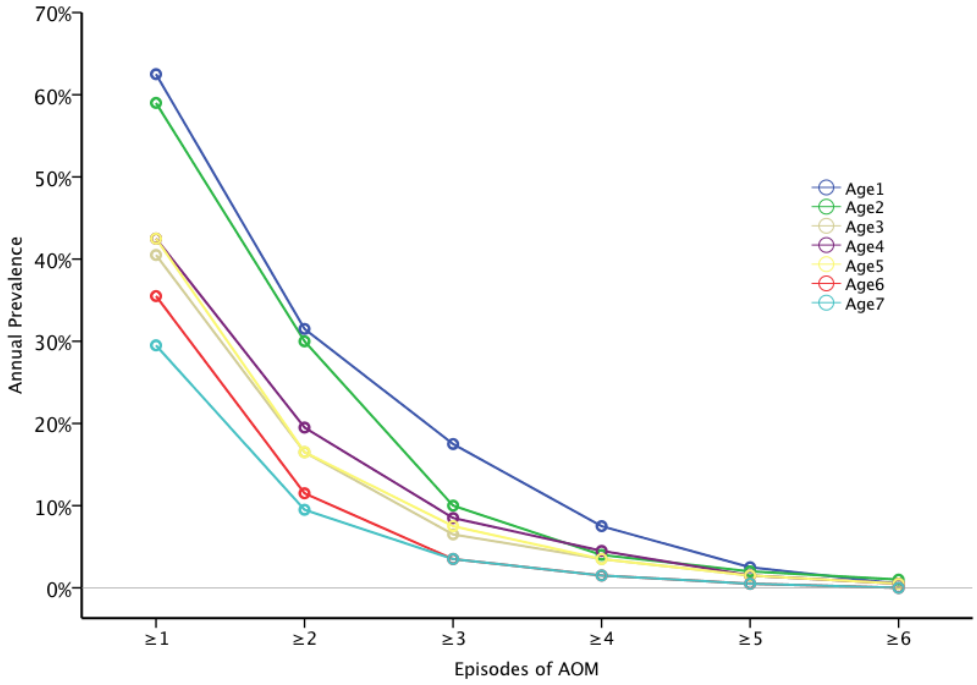
We can model population prevalence of "number of episodes of AOM" with regression. This does mean converting a discrete variable to continuous, but this is not problematic if the view is taken that the regression curve represents population susceptibility to AOM (a continuous variable), with AOM events (discrete) occurring above a liability

threshold. The data for prevalence of AOM at age two (from the Boston Study[7]) are well modeled ($r^2 = 0.994$) by the function:

(5) $prev_p(n) = 1.37\ (0.43^n)$

Where n is the minimum annual number of episodes of AOM (i.e. if n=3, that includes all those with ≥ 3 episodes of AOM). This is graphically displayed in figure 4.

Figure 4. Annual prevalence of AOM episodes, from birth until age 7 (data from Teele et al[7]). Data points are interpolated for ease of interpretation.



Otitis Media with Effusion (OME) and Chronic OME (COME)

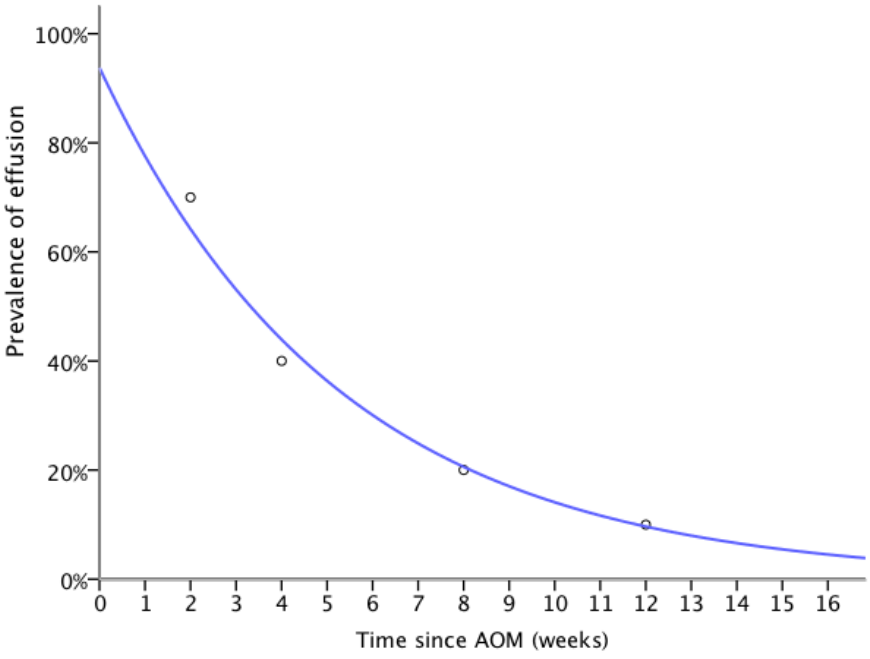
The Boston Study reported OME prevalence and duration following 612 first episodes of AOM in children aged 1-3[3] (which here I presume is representative of a two-year old

child). Duration of OME can be modeled by exponential decay[148], and here using raw data from the Boston study (figure 5) I derive the formula (r^2=0.991):

(6) $prev_p(d) = 0.938(e^{-0.19d})$

Where d is the duration of effusion in weeks. Note that this model only applies to OME of new onset. Other studies have followed OME of unknown prior duration, and report significantly lower rates of resolution[136] (because this population is biased towards individuals already with disease).

Figure 5. Prevalence of persisting effusion after first episode of AOM (data from Teele et al[3]). Data points (circles) are well fitted by an exponential decay function (see text for details).



If this formula is extrapolated towards d=0 (y-axis intercept), I predict that 94% of children with AOM will have an initial effusion:

(7) $P(OME|AOM) = 0.938$

If we extrapolate (6) forwards, then at 3 months (13 weeks), $e^{-0.19*13}$= 8.5% of those who had an effusion will still have an effusion.

(8) $P(COME|OME) = 0.085$

These extrapolations extend beyond the dataset, and so should be treated with caution.

At age two, formula (5) suggests that 58.9% of the population will have one or more episodes of AOM in a year:

(9) $Prev_p(AOM) = 1.37(0.43^1) = 0.589$

Consequently from (7), 0.589 x 0.938 = 55.4% will have an episode of OME following AOM.

However, OME also occurs without a prior history of AOM[3 149]. The Boston Study did not examine OME outside that occurring following AOM, but the Pittsburgh Study[137] included all instances of OME. Their data suggest that 78% of children are diagnosed with a middle ear effusion at some point in the year before their second birthday[137] (a finding consistent with other studies[5]). This figure encompasses both suppurative and non-suppurative effusions. From (5), 58.9% of effusions will be for AOM, but (7) 94% of these will lead to OME, so we can predict that the prevalence of OME is:

(10) $prev_p(OME) = (0.78 - 0.589) + (0.589 \times 0.938)$

$= 0.745$

This also suggests that 0.745 – 0.589 = 15.6% of all OME occurs without a prior history of AOM.

(11) $prev_p(OME_no\ prior\ AOM) = 0.156$

The Finland study reported that 16% of episodes of COME occurred with no prior history of AOM[149].

We can extrapolate these data to predict COME at age 2. Combining formulae (9), (7) and (8) gives us the predicted annual incidence of COME occurring secondary to AOM:

(12) $prev_p(COME_prior\ AOM) = prev_p(AOM) \times P(OME|AOM) \times P(COME|OME)$

$= 0.589 \times 0.938 \times 0.085$

$= 0.047$

To this we can add the prevalence of COME occurring without preceding AOM, which using (11) and (8) we can predict as

(13) $prev_p(COME_no\ prior\ AOM) = 0.156 \times 0.085$

$= 0.013$

This is on the assumption that OME is as likely to lead to COME whether there is preceding AOM or not. There are no available epidemiological data to verify this (see the next section). With this caveat, we can predict:

$$(14) \quad prev_p(COME) = prev_p(COME_prior\ AOM) + prev_p(COME_no\ prior\ AOM)$$
$$= 0.042 + 0.013$$
$$= 0.055$$

We do not have good epidemiological data with which to compare this prediction. There are many studies of point prevalence of OME[5], but no large prospective studies have reported the prevalence of COME[136 148]. A further complicating factor in epidemiological studies is potential misclassification of recurrent OME as persistent OME (COME) due to infrequent assessment. Of children aged 2-6 who have OME, a quarter will have recurrence within the same year[150]. One (unpublished) small longitudinal study with frequent assessments suggested infants could be stratified into those with persistent effusion, those with recurrent effusion, and those with occasional or rare effusion[151].

The Finland study reported a prevalence of 4.4% for middle ear effusion lasting more that two months (at any time in the first two years of life), and provides some proxy for the prevalence of COME. This estimate is lower than predicted by our model, and is also discordant with the higher estimates of COME prevalence in other studies[149].

The Relationship of AOM and rAOM to OME and COME

Although AOM and OME are strongly inter-related, not all OME follows AOM (15.6% in our model). This implies that perhaps OME is a heterogenous entity, with a complex and variable relationship to prior or coincident AOM or rAOM. Indeed there is biological evidence to support such heterogeneity. Effusions that follows a recent history of AOM are hypercellular and more often positive for bacterial culture than OME that occurs

apparently de novo[152]. In children undergoing grommet surgery, 88% of those treated for a history of COME have an effusion confirmed at operation, but only 26% of those undergoing surgery for rAOM[153].

Epidemiological studies have tried to delineate the complex relationship between COME and AOM, The Finland study[140] reported that a single episode of AOM is associated with an 11.9 odds ratio for the presence of a subsequent chronic (two month long) middle ear effusion, whereas two successive episodes of AOM increases the odds ratio for subsequent effusion to 30.0, and three successive episodes to 39.3. Other studies have also suggested that episodes of AOM[149 154 155] or URTI[156-158] contribute to persistence of effusion. These studies have not attempted to disentangle subphenotypes of OME following AOM versus OME occurring de novo.

Clinical experience suggests that, at least at the severe end of the disease spectrum, rAOM and COME may not be quite as closely partnered as these epidemiological studies suggest. The majority of children undergoing ventilation tube (grommet) surgery give a clinical history of recurrent AOM or of hearing loss suggestive of COME, but only sometimes a history of both of these symptom domains. Schilder undertook a principal component analysis of upper respiratory tract symptoms in an unselected cohort of 999 children aged 2-4, and showed that URTI was correlated to presence of AOM and OME, but that in children with more severe or persistent symptoms, occurrence of OME and AOM became more separated[159]. Consequently children with more severe disease, in particular those presenting to secondary care, are likely to differ from a cohort of children enrolled in a prospective epidemiological study, and some aspects of the analysis presented here may not be relevant to hospital populations.

Chronic Suppurative Otitis Media (CSOM)

CSOM is a relatively rare disorder, with varying definitions, and little epidemiological data is available in comparison to other mucosal OM phenotypes. The prevalence of CSOM in a two-year old white child in a developed world environment is probably less than 1%[160-162] (incidence in adults has been estimated as 0.5%[163]).

(15) $prev_a(CSOM) = 0.01$

The relationship of CSOM to other forms of OM is unclear. Those with CSOM often give a history of prior AOM, but AOM episodes are not more frequent in this group compared to the background population[164 165]. Clinical experience suggests that, whereas CSOM may occur subsequent to AOM, in the developed world often it occurs secondary to a history of COME[162 166]. In these cases it may be that non-resolving or severe inflammation in COME leads to degeneration[167 168] and eventual perforation of the tympanic membrane, which then allows periodic or persistent infection of dysfunctional mucosa by flora of the external auditory canal[169]. Chronic otorrhoea can also occur in those with grommets[170], but I do not include this in the definition of CSOM. Microbial culture from patients with CSOM often yields a mix of aerobic and anaerobic bacteria[161], notably *Pseudomonas aeruginosa* which preferentially colonises damaged respiratory epithelium[171]. CSOM may heal spontaneously[172].

Silent Otitis Media

Middle ear inflammation without symptoms and signs can occur, but because this is a pathological diagnosis there are no accurate estimates of prevalence. In clinical practice

much of OME and COME in children will be clinically silent. However subclinical inflammation can also affect adults, Meyerhoff et al reported a diverse range of histological features of middle ear inflammation in 41% of 800 temporal bones from people with clinically unexplained ear symptoms[117].

A Phenotype Landscape of Otitis Media

Our modeling can now be imposed upon the phenotype map (figure 3) to add a third dimension representing prevalence and interrelation, and so generate a phenotype landscape (figure 6). This landscape specifically relates to a population of white two-year old children in a developed world environment.

Although still an imperfect representation, the landscape does provide a more complete and heuristic concept of the complex spectrum of inter-related otitis media phenotypes. Adding additional dimensions in hyperspace may further improve this model[173], but risks making the concept unintelligible. Instead it seems better to consider in turn the significant factors that can determine and alter the contours of this landscape.

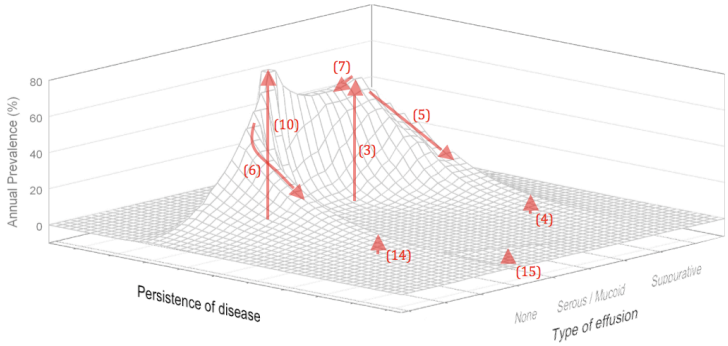
Factors Affecting the Landscape

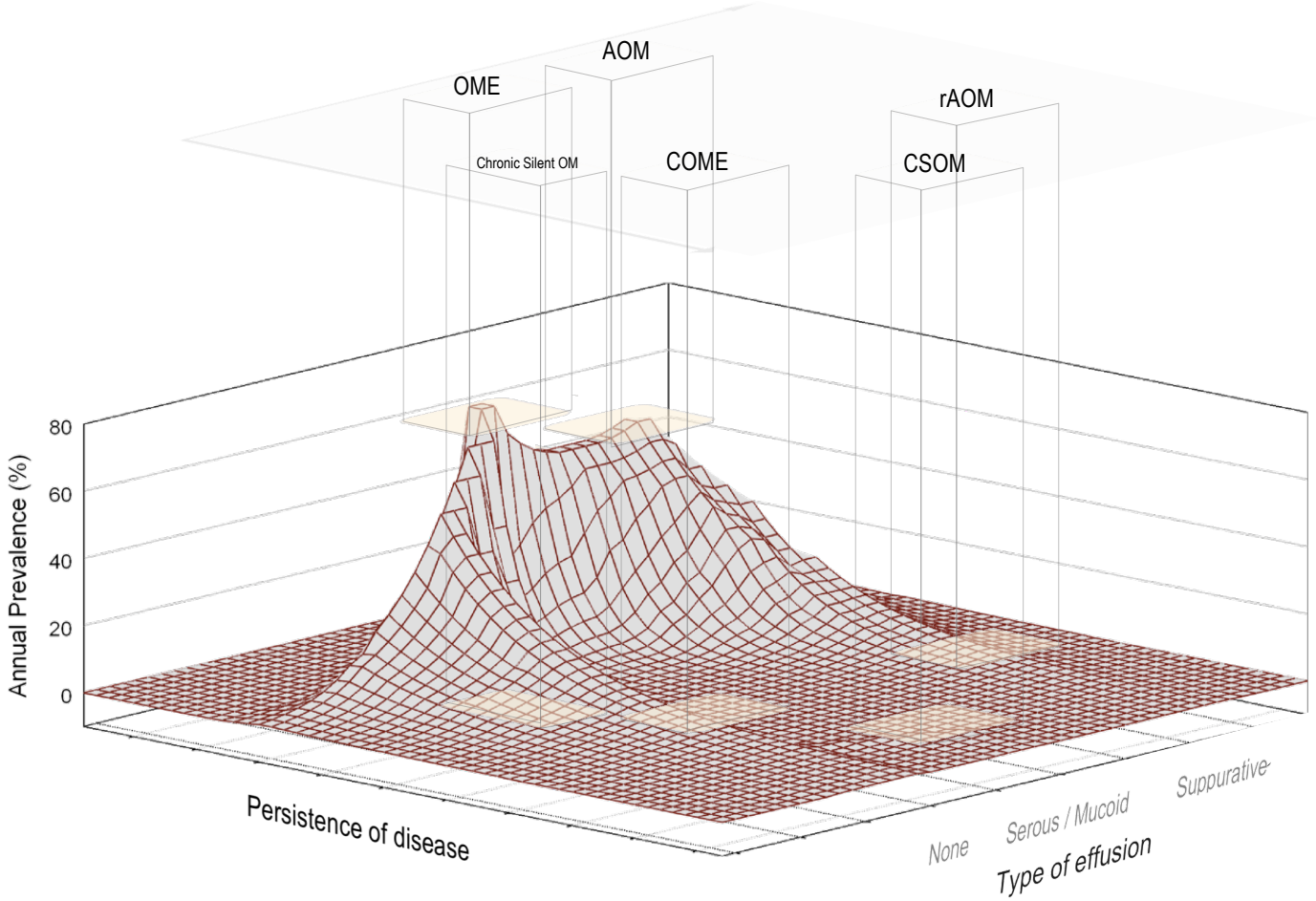
The effect of gender

Several studies (including the Boston study[7]) have suggested that males may have a slightly higher incidence of AOM or rAOM[5]. No consistent gender variation in URTI has been reported[141 174]. Most studies (including the Pittsburgh study[137]) find no gender differences in rates of OME or COME[5].

Figure 6: A hypothetical phenotype landscape for otitis media in a population of white children at age two. This is based upon the map (figure 3) but with annual prevalence of disease shown in the Z-plane. The contours represent the relationship between phenotypes, and are derived from the formulae presented and detailed in the text.

(3) $prev_a(AOM) = 0.59$
(4) $prev_a(rAOM) = 0.04$
(5) $prev_p(n) = 1.37\ (0.43^n)$
(6) $prev_p(d) = 0.938(e^{-0.19d})$
(7) $P(OME|AOM) = 0.938$
(10) $prev_p(OME) = 0.745$
(14) $prev_p(COME) = 0.055$
(15) $prev_a(CSOM) = 0.01$





The effect of age

Otitis media is predominantly a disease of childhood[175], and age is the single largest determinant of disease prevalence. Data from the Boston study[7] show that AOM and rAOM prevalence falls with age (table 5 and figure 4). Analysis of data from this and from other studies suggests a peak incidence between the ages of 6 and 11 months. It may be that breastfeeding confers protection against disease for a proportion of children below 6 months of age (see below).

We can re-derive the function in (5) for prevalence of AOM in each age group, with a good fit, but extrapolation to calculate the integer of each function from four to infinity, as a representation of predicted prevalence ($prev_p$(rAOM)) with age (table 4), shows poor correlation with actual prevalence ($prev_a$(rAOM)) (table 5). An alternative model for $prev_p$(rAOM) for actual values of prevalence (table 5) using logarithmic regression shows an unacceptable fit (r^2=0.855). It is likely that $prev_p$(RAOM) is poorly modeled because known data for this phenotype are sparse, causing inaccurate curve fitting.

Age	Equation	r^2	$\int_4^\infty f(n)\,dn$
1	$prev_p(n) = 2.16\ (0.39^n)$	0.963	0.053
2	$prev_p(n) = 1.37\ (0.43^n)$	0.994	0.055
3	$prev_p(n) = 0.93\ (0.43^n)$	0.995	0.038
4	$prev_p(n) = 1.13\ (0.42^n)$	0.991	0.041
5	$prev_p(n) = 1.00\ (0.42^n)$	0.997	0.035
6	$prev_p(n) = 2.46\ (0.23^n)$	0.906	0.047
7	$prev_p(n) = 1.99\ (0.24^n)$	0.895	0.046

Table 5: Regression model for episodes of AOM for each age group. $prev_p$ is the predicted prevalence of the phenotype and n is the minimum annual number of episodes of AOM (i.e. if n=3, that includes all those with ≥ 3 episodes of AOM). The final column gives the predicted prevalence of rAOM (defined as n≥4) by calculating the integral from 4 to infinity.

Table 4: The effect of age on prevalence of URTI, AOM and OME. Unless otherwise stated, prevalence is for events in the preceding year. See table 1 and text for a list of abbreviations, and further details of data source.

Age	URTI		AOM			OME		
	$prev_a$(URTI) per month[141]	P(AOM|URTI)[142]	Mean episodes AOM/yr[7]	$prev_a$ (AOM)[7]	$prev_a$ (rAOM)*[7]	$prev_a$ (OME)[137]**	P(COME|OME)	OME culture positivity[176]
1	0.50	0.36	1.2	0.62	0.11	0.87	-	60%
2	0.56	0.29	1.1	0.59	0.07	0.78	0.085**	
3	0.51	0.15	0.7	0.41	0.06	-	Age 2 -12, risk is 3.8x less than those aged<2[177]	
4	0.44	-	0.8	0.42	0.07	-		
5	0.39	-	0.7	0.42	0.06	-		
6	0.21	-	0.6	0.37	0.02	-		32%
7	-	-	0.4	0.30	0.02	-		
>8	-	-	-	-	-	-		22%

*defined as ≥4 episodes AOM
**calculated value, see formula (8)

Because we know that URTI is an almost universal precedent to AOM (1), and because we have some age-specific incidences of URTI and of P(AOM|URTI) (table 5) we can try to delineate proximate causes of the decline in AOM with age. Declining incidence of URTI will undoubtedly explain some of the decrease in AOM with age. However linear regression of the data in table 4 shows poor correlation (r^2=0.483) between prev(URTI) and annual incidence of AOM (however, I find better correlation between URTI and AOM incidence from data in a study by Henderson et al[174], r^2=0.872). If we correlate $prev_a$(URTI)*P(AOM|URTI) to mean episodes of AOM we get a much better fit (r^2=0.953). This suggests that the reduction in AOM with age factors both an age-related reduction in URTI incidence, and a reduction in P(AOM|URTI).

Unfortunately there are no data to accurately predict the annual prevalence of OME or COME with age. The Pittsburgh[137] and Finland[149] studies only looked at children up to the age of two. The Boston study[7] estimated time with middle ear effusion for children up to the age of seven by extrapolation of data from children aged under three who had preceding AOM. But to extrapolate $prev_p$(OME) from $prev_p$(AOM) is based on the assumption that neither P(OME|AOM) (7) nor P(COME|OME) (8) vary with age, and also fails to consider OME that occurs without preceding AOM. Epidemiological and pathological analyses suggest that these factors do vary with age (table 4). In older children COME is less cellular[152] and less likely to be culture positive[176 178 179], evidencing age-related variation in inflammatory response. Epidemiological studies have shown that duration of effusion, i.e. P(COME|OME), decreases with age[154 177 180], but these studies have been too small to allow this effect to be accurately quantified. Whether there is variation in P(OME|AOM) with age is not known.

Empirically, we can consider a model whereby bacterial ingress into the middle ear is a continuous variable, and this is the primary determinant of the prevalence and degree of

inflammatory response of the middle ear (the y-axis on our phenotype landscape). Bacterial ingress will reduce with age (presumably related to better adaptive immunity), and so the prevalence and the severity of otitis media will also decrease, creating a shift to less disease, a greater proportion of OME relative to AOM, and reduced probability of OME progression to COME.

The only good data we have on OME/COME prevalence are from studies of point prevalence. In general prevalence decreases with age[162], but a meta-analysis reported peaks in point prevalence at age two and at age five[112]. The later peak may be contributed to by exposure to respiratory infections when children start school, but could also include a shift towards OME rather than AOM as the pathological response, congruent with decreasing bacterial ingress.

The exposome

A number of studies have reported environmental factors that alter the risk of AOM[181], and these have been subject to meta-analysis[4 182] (table 6). Childcare outside the home and having siblings increases the risk of AOM, and this is likely related to exposure to a greater number and variety of potential viral and bacterial pathogens. Parental smoking increases risk of AOM, probably through impaired mucosal immunity[183], whereas passive immunity from secretory IgA in breastmilk[184] confers protection against AOM. Other relevant factors are season (greater prevalence of AOM in autumn and winter)[182], urbanization[137] (increased risk), and the use of a pacifier (increased risk)[4 182]. The contribution of gastro-oesophageal reflux disease[182] or socio-economic deprivation[185] to disease risk is less clear.

Potential risk factors for COME have been reported in a number of studies and appear to be similar to those for AOM, including winter season, passive smoking, day-care

Table 6. Summary of meta-analysis of exposome risk factors for AOM. Adapted from Uhari et al[4].

Risk Factor	Risk ratio (CI)	p value
Child care outside home	1.82 (1.21-1.73)	0.004
Sibling	1.92 (1.29-2.85)	0.001
Parental smoking	1.76 (1.36-2.28)	0.00002
Breastfeeding	0.48 (0.32-0.78)	0.0004
Atopy	1.23 (0.94-1.61)	0.12

attendance and having siblings[156 157], but meta-analysis of risk factors has not been undertaken. Such risk factors cannot accurately differentiate between children at risk of rAOM as opposed to those at risk of COME[186].

In the developing world the incidence of CSOM is many orders of magnitude higher than in the developed world[187], particularly in Oceania and sub-Saharan Africa[175]. Empirically this is likely due to socioeconomic deprivation leading to overcrowding, nutritional deficiency, or poor sanitation, which could lead to chronic low level bacterial ingress finto the middle ear, causing chronic inflammation, and ultimately tympanic perforation and immunological failure with consequential chronic otorrhoea. It may be that COME is the predominant form of chronic otitis media in the developed world (effective immune protection of the middle ear), whereas CSOM is prevalent in the developing world (ineffective immune protection of the middle ear). We do not have good estimates of COME prevalence in the developing world[188].

The effect of population genetics

Heritability of both frequency of AOM and time with middle ear effusion (OME, COME) has been estimated at 0.52-0.72. There are also variations in OM susceptibility by ethnic group. The genetic architecture of otitis media is the subject of the next chapter, but

clearly the genomic structure of the population being studied can significantly affect the contours of the phenotype landscape. Empirically, pathogen genetics will also have an effect, but this effect, and that of host-pathogen interactions, is poorly characterized.

The effect of interventions

Surprisingly few medical interventions significantly impact upon population prevalence of otitis media[189]. Antibiotics only marginally reduce the duration of AOM[190], but can help to prevent rAOM[191]. Consequently, policies of reduced or delayed prescribing[9] seem unlikely to significantly impact on population prevalence. By contrast, administration of the PCV-7 vaccine against *S. pneumoniae* causes a 6-7% reduction in population incidence of AOM[192]. COME is reduced by adenoidectomy[193], oral steroids[194], or ventilation tube[195] (grommet) surgery, although the effects may be short-lived. For CSOM antibiotics may reduce otorrhoea[196]. Tympanoplasty can also stop otorrhoea, but success is less likely with more severe disease (persistent otorrhoea)[197].

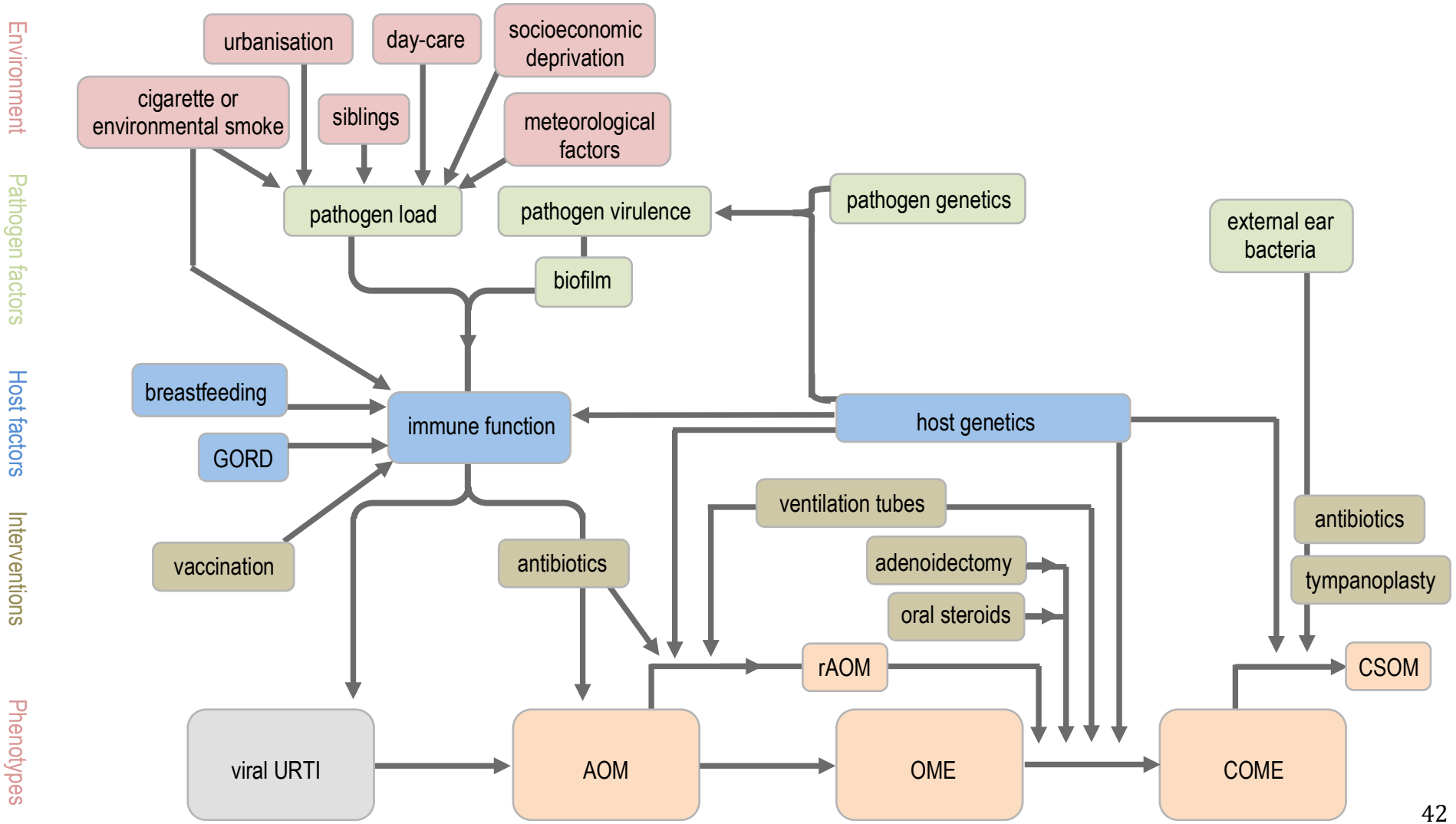
Figure 7 summarises factors known or hypothesized to impact on susceptibility to OM.

Conclusion

Epidemiological and pathological analyses suggest that middle ear inflammation is best considered as a complex spectrum of inter-related disease phenotypes. One way to map OM is based upon the type (degree) of inflammatory response, the persistence of disease, and the prevalence of disease.

The ideal epidemiological study for otitis media would follow a birth cohort of several thousand throughout childhood (and even into adulthood for those with persisting

Figure 7. A summary of factors known or hypothesized to impact on aetiology of otitis media, and how they may interact



symptoms or signs of disease), with frequent expert assessment for disease and disease risk factors. There is no single study fulfilling this ideal, and so prevalence and inter-relation of otitis media phenotypes can only be extrapolated by combining data from the best studies available.

AOM almost always follows an URTI. OME is a common sequel to AOM, but can also occur de novo. The degree and duration of inflammation in OME is variable, suggesting a somewhat heterogenous disorder. The epidemiology of CSOM is less well understood.

Age is the single largest determinant of disease prevalence, but a number of factors in the exposome also determine susceptibility. However, genetic polymorphisms likely play the greatest role in determining individual predisposition to recurrent or persistent middle ear inflammation.

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Chapter 1.2
The genetic & evolutionary landscape of otitis media

From the analysis in the preceding chapter, clearly middle ear inflammation is a complex phenotype, and an archetypal complex disease. In this review I factor heritability estimates, evolutionary forces, ethnic variations in disease incidence, and what is known about the genetic basis of extra-aural infectious or chronic inflammatory disorders, to propose a conceptual framework for genetic susceptibility to OM.

Otitis Media Heritability

Based upon twin studies, the heritability of rAOM in a white child at age two, and in a developed world environment, has been estimated by Rovers et al as 0.49[1]. This estimate is based upon a retrospective questionnaire of symptoms, and so may be subject to misreporting[2 3]. The quoted heritability estimate includes symptoms of upper airway obstruction, although these are not part of the clinical OM phenotype. If I include only the questions on symptoms of otorrhoea or otalgia from this study, I derive a revised estimate of heritability (of rAOM at age two) of 0.57.

Heritability may vary with the demographics of the population studied. Heritability at age three is 0.52, and 0.65 at age four (my revised estimate, based upon otological questions only in the Rovers et al study). These estimates are consistent with those from other questionnaires[4 5]. Rising heritability with age (if true) could reflect falling prevalence of disease, such that those with disease persisting beyond infancy are genotypically further from the norm. There is no evidence of gender difference in heritability[1 4]. There are no estimates of heritability in non-white children, nor in children outside of the developed world.

Heritability of time with middle ear effusion (OME, COME), including that following AOM or arising de novo, has been estimated by Casselbrant et al as 0.73 up to the age of two[6] (again in a predominantly white population in a developed world environment), and this is unchanged by age five (0.72)[7]. There is no difference in heritability by gender[6 7]. The effect of other demographic variables on heritability is not known.

A more recent study[8] has estimated heritability lower than prior estimates, at 0.39 for rAOM and 0.22 for COME. However this study's significant limitation is that it relied on parental reporting of their own OM history. This will likely lead to an under-estimate of parental disease prevalence, because recall of childhood medical history is likely inaccurate, and because OM (and particularly COME) was probably under-diagnosed in the past.

Because of its rarity, heritability estimation for CSOM is difficult and has not been attempted. However in a study in the Inuit population, Jensen et al reported a 2.55 odds ratio of disease in those with a parental history of CSOM (defined as perforation of the tympanic membrane)[9].

Squamous forms of OM are not part of this thesis, but I have demonstrated elsewhere that there is intrinsic susceptibility to tympanic membrane retraction[10]. Reports of familial clustering of cholesteatoma[11 12] also suggests genetic predisposition. Tympanosclerosis is associated with atherosclerosis, suggesting a genetic predisposition in common[13 14]. Empirically, the genetic basis for squamous disease is likely to significantly differ from that for mucosal disease (although predisposed to by mucosal disease).

One study suggested that chronic OM incidence is lower in people with otosclerosis[15], but otoclerosis is difficult to diagnose in the presence of chronic OM, and this finding may be spurious.

Evolutionary Considerations

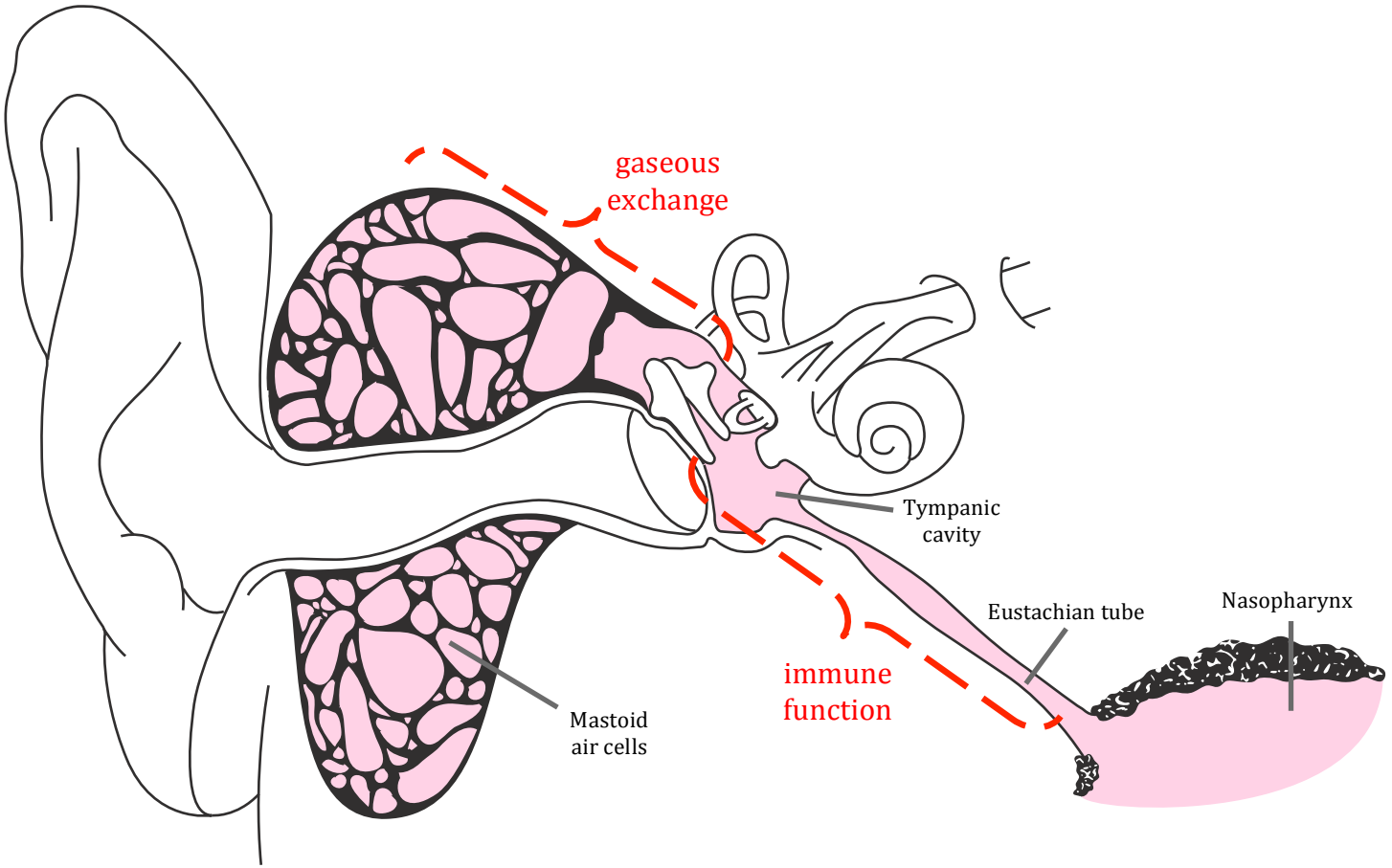
Otitis media has been and continues to be a significant cause of morbidity and mortality, with annual global deaths from OM (usually due to intracranial spread of infection) recently estimated at 28,000[16]. As a consequence, susceptibility to OM will be subject to evolutionary forces, as a medley of those maintaining middle ear function, those determining microbial colonization and pathogenicity, and those driving host immunity.

Evolution of the middle ear

As I have detailed elsewhere[17], the middle ear evolved as an air-filled sound transformer to overcome the air-fluid impedance mismatch that occurred on transition from an aquatic to a land-based existence. In man the middle ear provides up to a 27 decibel gain in auditory thresholds for air-borne sound[18]. Between the Carboniferous and Triassic periods the middle ear independently evolved in five lineages (the anurans, turtles, lepidosaurs, archosaurs and mammals[19]), and such convergent evolution clearly evidences the survival advantage of tympanic hearing to terrestrial existence.

Thus, the primary function of the middle ear is to provide and maintain a gas pocket at atmospheric pressure, and even relatively minor alterations to the gaseous composition can affect hearing[20-23]. Exchange of oxygen, carbon dioxide, and nitrogen between the middle ear space and venous blood[24-26] occurs across the flat or cuboidal mucosal epithelium of the postero-superior middle ear cleft[27] (figure 1). The Eustachian tube plays little role in normal passive middle ear ventilation[28], but can equilibrate gross pressure alterations through active opening (such alterations are rarely encountered in the natural environment). Many mammals have a posterior extension of the middle ear known as the bulla, but in hominids this extension

Figure 1: The function of the middle ear cleft (shaded pink) is partitioned into immune defence in the antero-inferior portion, and trans-mucosal gas exchange in the postero-superior portion. Image created with assistance of Steve Thomas.



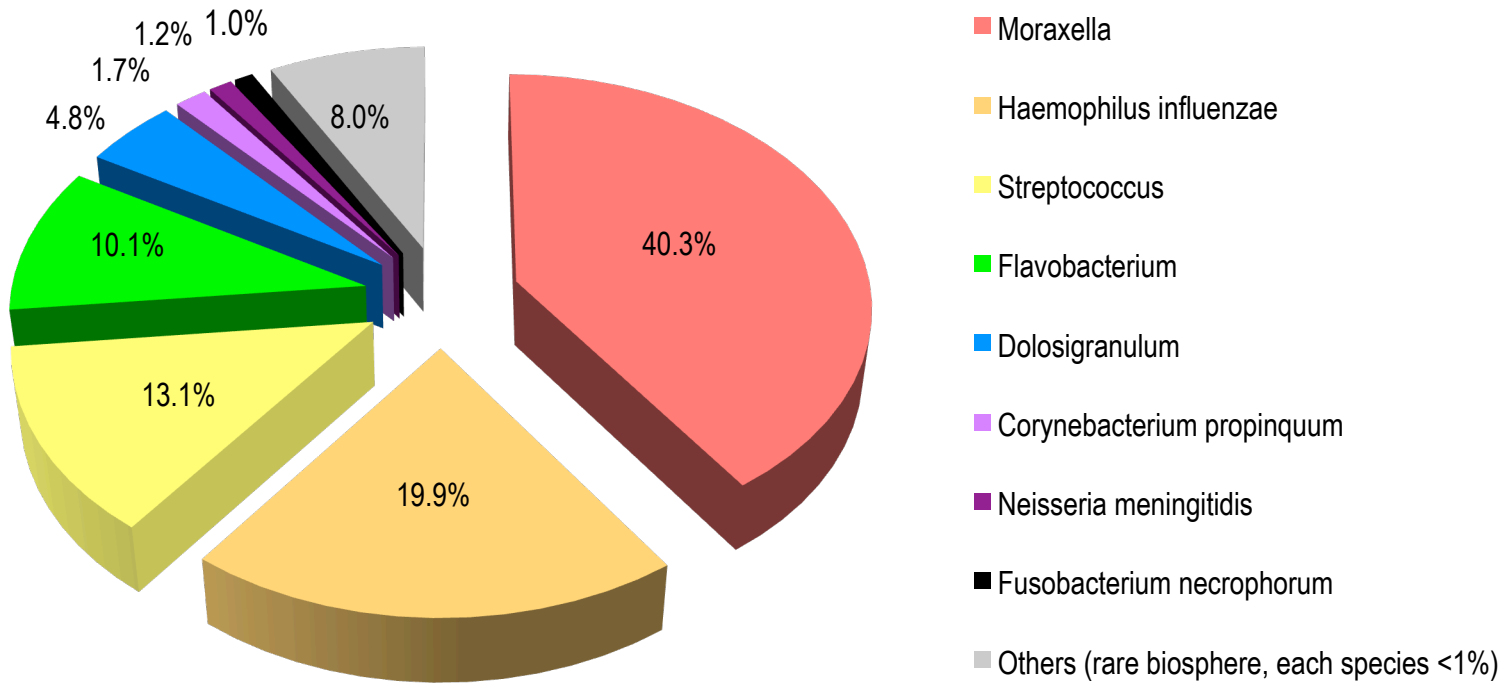
forms a series of interconnected air cells in the mastoid process of the temporal bone. It may be that the presence of these air cells increases the area for mucosal gas exchange[29], but why this should be evolutionarily advantageous in hominids is not clear[30]. In humans compared to other hominids the mastoid process projects inferiorly and is large[31], which may reflect the altered pull of the sternocleidomastoid muscle on the skull as a result of human adoption of an upright stance[32].

To maintain a surface for gaseous exchange[33] the middle ear is kept free of pathogens[34] and particulate matter (and in this regard resembles the lung). Hence the antero-inferior region of the middle ear (including the Eustachian tube) is tasked with host defence[35] (figure 1) and is lined with a ciliated columnar epithelium that secretes and propels a mucociliary escalator to defend against bacterial ingress[36]. The normal middle ear has a paucity of immunocytes[36] suggesting that mucociliary clearance alone is usually effective, despite the infant middle ear being positioned only 2cm[37] from the microbiome of the nasopharynx.

Evolution of the nasopharyngeal microbiome

Nasopharyngeal colonisation is the source of bacteria that cause AOM[38 39], and therefore the initiating site for almost all OM phenotypes. Colonisation occurs rapidly after birth, and the nasopharyngeal flora becomes established in the first year of life[40]. By infancy the nasopharyngeal microbiome is complex and formed of an average of six million bacteria (colony forming units)[41] and 20-87 different species encompassing 13 taxonomic phyla. The most common genera are *Moraxella, Haemophilus, Streptococcus,* and *Flavobacterium* (figure 2)[42]. The flora is dynamic and individualised[42]: bacterial species may be acquired, lost and re-acquired[43], including through inter-species competition[44 45], or subject to seasonal variations[42].

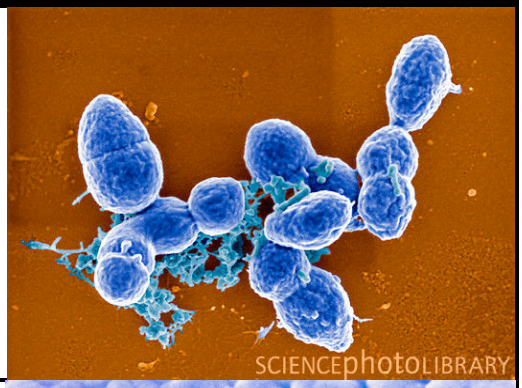
Figure 2: Genera found on metagenomic analysis of the nasopharynx microbiome of 18-month old infants. Adapted from Bogaert et al, 2011[42].



Nasopharyngeal commensals are ecotropic, site-specific, and have co-evolved with their host. The majority of commensals exist in a symbiotic, or at least tolerated relationship: they rarely cause disease, may prevent colonization with potential bacterial pathogens, and can reside in the nasopharynx of their host throughout life. In contrast some species, notably *S. pneumoniae*, NT*Hi*, or *M. catarrhalis*, do have pathogenic potential (table 1). Exposure to new strains of these bacteria can result in clearance, asymptomatic colonization[46 47], or respiratory tract infection (including AOM[48 49], but also rhinosinusitis[50] or pneumonia[51]). However disease usually occurs following viral URTI (see chapter 1.1), suggesting virulent bacteria and upper respiratory tract viruses have likely co-evolved. Mechanisms of viral-bacterial co-evolution have not been explored. No particular virus appears to synergise with any bacterial strain[52], and there appears to be convergent evolution in that viruses use species-specific mechanisms for synergistic bacterial infection[53]. I propose that perhaps synergism allows viral and bacterial species to together seize an opportunity to overwhelm the host immune system, and so expand their populations. Synergism could also be to capitalise on symptoms such as cough (which can be invoked by viruses[54] and possibly also bacteria[55]) or sneezing, which generate bioaerosols and so enhance pathogen spread[56].

Bacteria that colonise the human nasopharynx seem to have adopted strategies of either virulence or avirulence, and mathematical models[57 58] predict both of these two outcomes as co-evolutionary stable states. Avirulence will likely lead to host tolerance and long-term carriage, thus favouring microbe spread between hosts over prolonged periods. Virulence enables massive bacterial population expansion during the pathogenic phase, thus promoting spread between susceptible hosts (which in the case of AOM will largely be immunodeficient infants), but with the disadvantage that colonization may be only short-term if (when) the host generates an immune response sufficient for eradication. The major otopathogens *S. pneumoniae*, NT*Hi*, or *M. catarrhalis* can often be cultured from the nasopharynx of infants, but are rarely found in

Table 1: Major pathogens causing AOM. Culture isolation is based upon children in North America, post pneumococcal vaccination (Block et al, 2004[59]). For a review of culture isolation in other geographic regions see Vergison, 2008[60]. Images from science photo library[61].

Species	Culture isolation in AOM	Classification	Electron microscope image
Streptococcus pneumoniae	31%	Gram positive cocci. Alpha-haemolytic anaerobe	
Non-typeable *Haemophilus influenzae*	56%	Gram negative rod. Aerobe or facultative anaerobe	
Moraxella cattarhalis	11%	Gram negative diplococci. Aerobe	

adults[40 62], and I suggest this is because immune response in the host leads to their eventual clearance (although biofilm formation[44] may afford some protection).

Advances in genetic sequencing have provided further insight into the transition between evolutionary strategies for the nasopharyngeal microbiome. For example *M. cattarhalis* probably attained virulence coincident with, and as a consequence of, human population expansion[63] (although the molecular mechanisms are unclear[64]). Conversely, sequencing data suggests that the nasopharyngeal commensals *Streptococcus mitis* and *Streptococcus parapneumoniae* have recently evolved as avirulent derivatives of *S. pneumoniae*[65]. The evolutionary history of *Haemophilus influenzae* is not well understood[66]. Of course, these strategies are not mutually exclusive. Not all colonization with *S. pneumoniae*, NT*Hi*, or *M. cattarhalis* leads to disease, and commensals like *Fusobacterium necrophorum* or group A streptococci that are normally avirulent can occasionally be pathogenic[67 68]. Indeed the evolutionary dynamics will be affected by many factors, including pathogen transmission rates and risk of mortality[69] but also, and importantly, host population ecology[70]. In particular, expanding host populations favour pathogen virulence, whereas stable or declining populations favour avirulence.

Evolution of host immune response

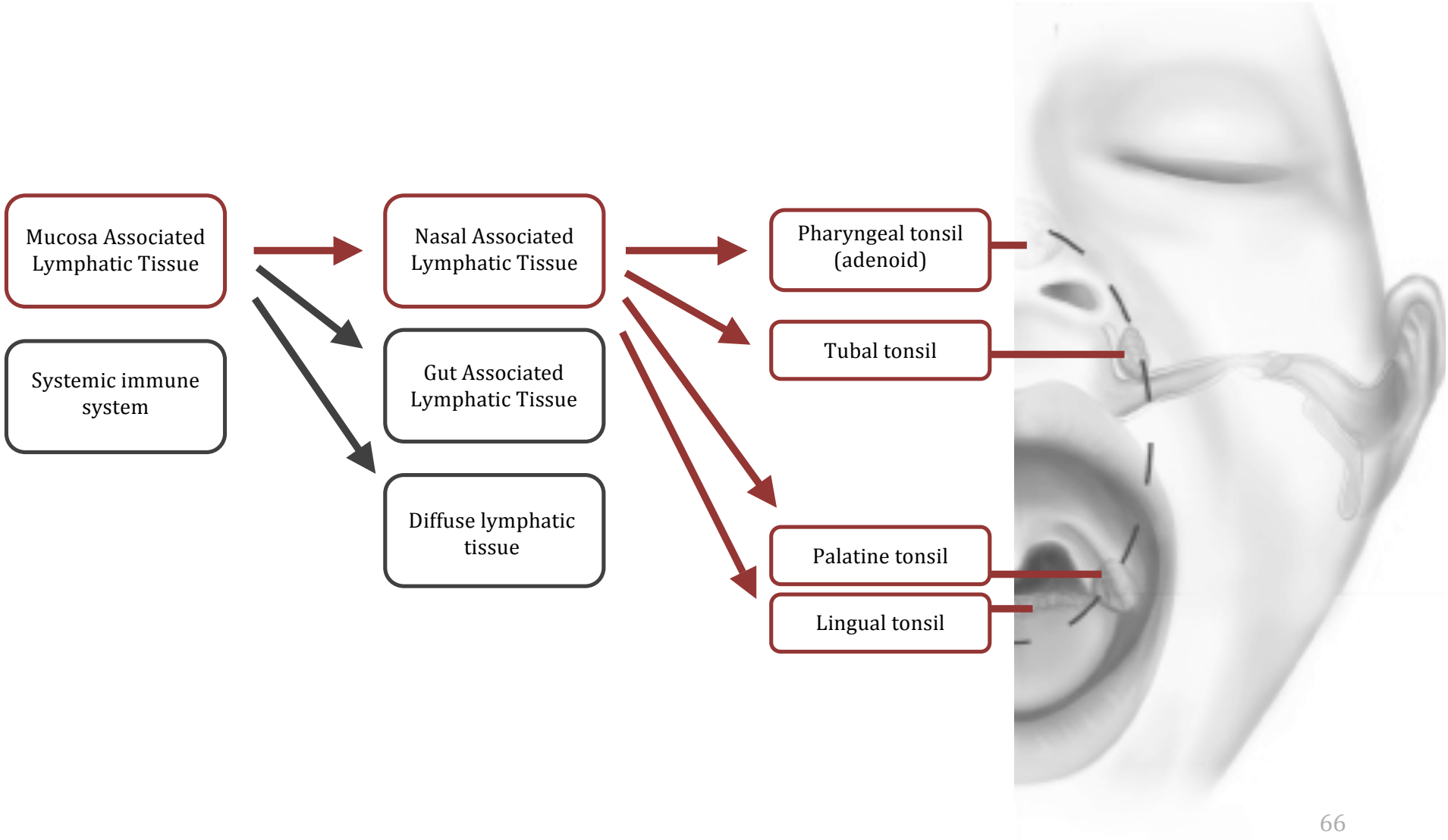
The ontogeny and phylogeny of the immune system is from mucosal epithelium[71], reflecting that this is the entry point for most infectious agents[72]. The mucosa associated lymphatic tissue (MALT) contains 80% of all immunocytes, is separate in structure and function from the systemic immune system, and is compartmentalised into gut-associated lymphatic tissue (GALT) and nasal associated lymphatic tissue (NALT)[73].

Infectious disease is a major driver of evolution[74], and given the morbidity and mortality of respiratory infections[75], function of NALT will have been, and continue to be, subject to major evolutionary pressure. However, mucosal immune regulation remains poorly understood, and in particular the mechanisms that determine tolerance versus an active immune response. (although secretory IgA appears to play an important role here[76]).

NALT in man is particularly well developed when compared to other mammals such as rodents, and is arranged as lymphoid aggregates that form a ring (Waldeyer's ring) at the pharyngeal inlet (figure 3)[77]. This development perhaps reflects the fact that man is a societal species, with high exposure to air-borne microorganisms through social contact. Importantly, NALT is embryologically and functionally distinct from GALT[78 79], evidencing that it has been subject to site-specific evolutionary forces[80]. Empirically, this suggests that upstream regulators of the immune response in NALT have also evolved to be site-specific, although downstream (phylogenetically ancient) signaling may utilise pathways in common with the rest of the immune system. Evidence from other contexts reveals wide variation in immune function in different tissue types[81]. The components of different tissues that determine this variation in immune function are not well understood, but seem important.

A priori it may appear that the host population should be constantly striving to win the arms race against potentially pathogenic microorganisms, but this is not necessarily true. The immune system consumes significant energy capital[82], and so the usage costs (and risks of morbidity and mortality) of fighting an infection are balanced by the resource investment required to maintain an immune system[83]. In addition, because pathogens usually have a shorter generation time and larger population size than their hosts, they are more adaptable, and so the host may never completely win this battle[84]. Population models suggest that for species such as man, that are long-lived and utilise acquired immunity, optimum evolutionary

Figure 3: Nasal Associated Lymphatic Tissue (NALT) as a component of the immune system. Artwork adapted from *Toronto Notes 2011*[85].



strategy may be to allow the occasional infection[86]. If the host survives the infection, acquired immunity will prevent similar infection in the future, but the pre-programmed lower basal investment in immune maintenance may increase fecundity over the lifetime of the individual. The optimum strategy will vary with host and population dynamics, thus engendering intra-species diversification, and genotypic variation with regard to host immune response[87].

Prolonged (chronic) inflammatory response may occur as a result of persistent antigenic stimulation, but may also be pre-programmed, for example as a strategy to enhance clearance of antigens[88]. There is evidence that man has evolved a more pro-inflammatory genotype compared to other primates, perhaps to enhance pathogen clearance, and thus engender longevity of our species[89] and the unique system of multigenerational support in child nurture that this affords[90]. However, chronic inflammation can be maladaptive[91], and risks causing irreparable tissue damage[92]. Pro-inflammatory genotypes exemplify antagonistic pleoitropy[93], whereby a polymorphism may serve a useful function in the short term but have negative consequences in the longer term.

Evolution of otitis media

Otitis media in man is not a recent phenomenon. In contrast to most mucosal infections[94], AOM can erode bone, and so leave traces in ancient remains[95 96], and probably the earliest evidence of OM is in a 40,000 year old human skull[97 98].

It has been suggested that OM is particularly prevalent in man when compared to other species[99], but this is difficult to verify without a systematic assessment of other species in their natural environment. One hypothesis advanced to explain human propensity to disease is in craniofacial morphology[100], and in particular the angulation and reduced calibre of the Eustachian tube[99 101], suggested to occur as a consequence of our species' adoption of an upright

stance. Such ideas arise from the notion that function of the Eustachian tube is an important determinant of susceptibility to OM[102]. However, the primacy of Eustachian tube function as a determinant of disease susceptibility is now questioned[26 103], and it has been suggested that the main driver for evolution of the Eustachian tube was a need to reduce the volume of the middle ear to attune hearing to higher frequencies[104].

The evidence that Eustachian tube function plays a significant role in initial susceptibility to OM is not convincing, and the idea that this is the main evolutionary reason for OM susceptibility in man seems insensitive to the complexities of population genetics. Rather, the higher propensity to OM in our species is probably a consequence of human population dynamics, whereby our large and increasing demes favour evolution of virulence in respiratory microorganisms. If OM becomes chronic, dysfunction of the Eustachian tube may then occur, and contribute to ongoing chronic inflammation, but such dysfunction seems unlikely to be a primary mechanism of disease.

The Genetic Landscape of Otitis Media

We can apply some of the broad principles of population genetics to understand the genetic landscape of otitis media.

Susceptibility to rAOM

Importantly, the phenome of susceptibility to rAOM includes susceptibility to other respiratory tract infections, including tonsillitis[105 106], rhinosinusitis[106], and bronchopulmonary infections[107], and, to a lesser extent, susceptibility to extra-respiratory mucosal infections[107]. The association with bronchopulmonary infections occurs because AOM and most lower respiratory tract

infections (LRTI) are alternate (or common) outcomes of nasopharyngeal colonization by the same species of respiratory pathogens. Because mortality due to LRTI[75] is more prevalent than that due to intracranial spread of middle ear infection[16], prevention of LRTI will likely be the main driver of host investment in NALT immune function. The lower respiratory tract is also likely to be the preferred site for infection by pathogenic bacteria; LRTI aids bioaerosol dispersion, but there is relatively little to be gained for the pathogen through infection of the middle ear space. Thus, the middle ear may be an unfortunate bystander in the battle of host versus respiratory pathogen. The middle ear is susceptible to infection, yet exerts comparatively little evolutionary pressure to prevent such infection.

The high heritability of rAOM implies that host response is the main determinant of disease susceptibility, specifically NALT immune response to potentially virulent infectious agents[40 108 109]. Some evidence supports this notion. First the pathogens causing rAOM are the same as those causing AOM[110], but children prone to rAOM experience nasopharyngeal colonisation more frequently[47 111] and with higher density[112] (although this may also be found in children with COME[113]). Quantitative or qualitative defects in plasma immunoglobulins have been found in some, but not all, of those with rAOM[114-120].

Extrapolation from other infectious diseases suggests that susceptibility to rAOM will likely factor a very large number of genes[121]. The arms race between pathogen and host has engendered a complex immunological network of pathogen recognition and signaling molecules in the host, and with considerable redundancy[122] (see also chapter 1.3). Susceptibility loci may encompass those determining bacterial adhesion, mucociliary clearance, innate immune response, or adaptive immune response, and may be those regulating such functions throughout the mucosa, or more specifically only in nasopharyngeal mucosa.

Susceptibility to COME

For chronic otitis media, we may hypothesise from evolutionary theory[91] that because persistent middle ear inflammation is common it must have at least some beneficial effect[123]. Empirically, this inflammation could aim to enhance clearance of, or prevent proliferation of, microorganisms[103], and there is evidence to support this idea. Bacterial mRNA can be detected in 37-94% of effusions in COME[124-131], suggesting that metabolically active bacteria are present, but are constrained in their proliferation. Indeed, the effusion in OME has been shown to be bacteriostatic[132] and bacteriocidal[133], presumably due to the mucins, immunoglobulins, and cytokines within the effusion. The viability of bacteria in effusions negatively correlates to the levels of such constituents[129 134 135]. There is a (small) increase in AOM following insertion of ventilation tubes for COME (which removes the effusion), which further evidences potential anti-bacterial effects of the effusion[136] (although very few studies of ventilation tubes have specifically looked at this outcome[137 138]).

The reverse hypothesis has also been advanced, that the effusion in OME provides the milieu for increased bacterial colonization, and so contributes to AOM. It is true that AOM can be immediately preceded by OME, but this may reflect the imperfection of a sero-mucoid effusion in its attempt to prevent suppuration, rather than implying that it contributes to that suppuration. With rAOM, the common observation that OME is also present may represent recurrent rather than chronic OME.

Whereas protection against microorganisms may possibly be the advantage, and possibly the evolutionary purpose of chronic otitis media, clearly prolonged inflammation can also be detrimental. Prolonged effusion in COME creates an air-fluid interface that prevents the transmission of sound, and thus counteracts the entire purpose of the middle ear. The consequential hearing loss could reduce audition of danger but the handicap will be somewhat

ameliorated, at least during infancy, by the long parent-child bond in our species which affords protection from danger. Reduced hearing can also interfere with language acquisition, although the long-term consequences of COME on linguistic abilities are debated[139]. Consequently chronic inflammation of the ear in infancy may have relatively little effect on fecundity, and there may be little evolutionary pressure to select against it. In the longer-term middle ear fibrosis, tympanic retraction, chronic otorrhoea, or ossicular erosion can lead to irreversible hearing loss (see prologue), but these consequences are relatively rare and may be offset by any potential advantage of chronic inflammation in earlier life.

Pathways driving chronicity of inflammation are not well understood. It is not known to what extent chronic inflammation results from a failure of catabolism of pro-inflammatory mediators, and to what extent it may be a failure of induction of pro-resolution programs[140]. Nor do we know the relative roles of leucocytes, stromal cells or mucosal cells in this process. Whereas the acute inflammatory response invokes a very large number of pathways, and with redundancy, empirically it is plausible that a smaller number of pathways are involved in driving chronicity of inflammation. Indeed homozygous polymorphisms in acute inflammatory response genes rarely cause a significant phenotype, whereas similar polymorphisms in key regulators of inflammation can lead to chronic inflammation[141].

Chronic OM is not knowingly associated with chronic inflammation in extra-tympanic sites, in childhood nor in later life (but this question has never been adequately investigated). Given the development of site-specificity of immune response in latter phylogeny, and the lack of evidence of extra-tympanic inflammation, it may be that susceptibility to chronic middle ear inflammation is determined by only a handful of genes that function as locoregional regulators of inflammation.

A summary of evolutionary forces in otitis media

In the preceding chapter (chapter 1.1) I presented a phenotype landscape for otitis media, albeit a simplification. Because evolution works on phenotypes, there is an argument that the phenotype landscape is akin to an adaptive landscape, whereby a prevalent phenotype is seen to represent a "fitness peak".

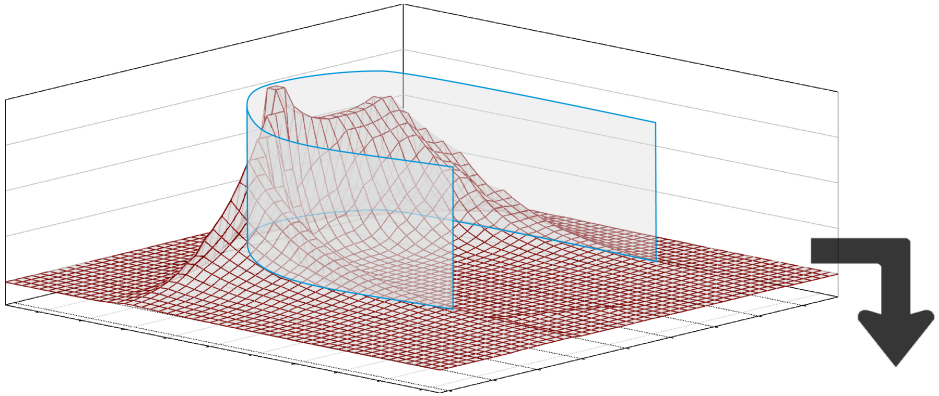
The landscape approach to representing evolutionary forces has its proponents, who argue that it can at least be useful to conceptualise ideas, although even proponents do not suggest using it as an analytical tool[142 143]. But the metaphor can be illusory[144 145]. Phenotypes represent a balance of evolutionary trade-offs and constraints, with the "fitness peak" determined by the context in which the phenotype is expressed.

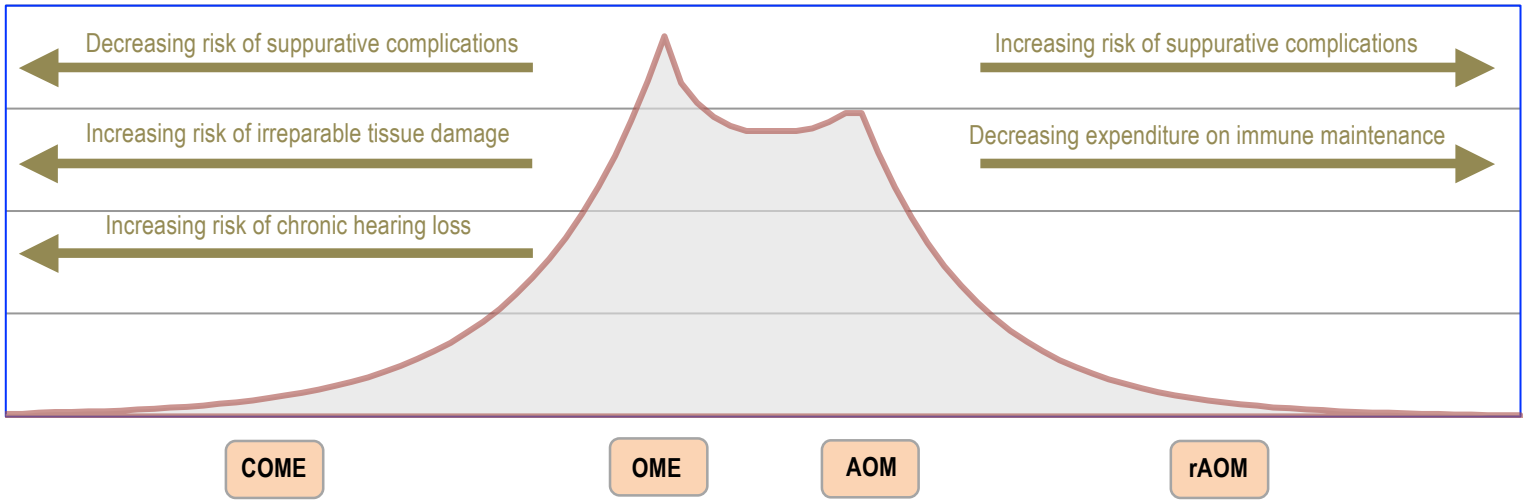
Figure 4 summarises evolutionary forces I consider to be at play on middle ear phenotypes.

Ethnic variations in disease incidence

A number of studies have reported differences in prevalence of otitis media by ethnicity. These differences could be due to ethnic variations in socio-economic factors[146] or in illness-seeking behaviour[147], as well as genetics, but because many studies have been retrospective they have been unable to control for such confounders. Furthermore studies have been contradictory, and the conclusions of systematic[148] and non-systematic[102 149 150] reviews do not agree on differences in OM prevalence between white, black, Hispanic or Asian children in the developed world. In the developing world only CSOM has been studied in any detail, but methodological differences

Figure 4: Opposing evolutionary forces that may impact upon the common otitis media phenotypes. I have placed the phenotypes onto a single vector, with rAOM and COME at opposite spectrums, but the relationship between these phenotypes is complex (chapter 1.1).





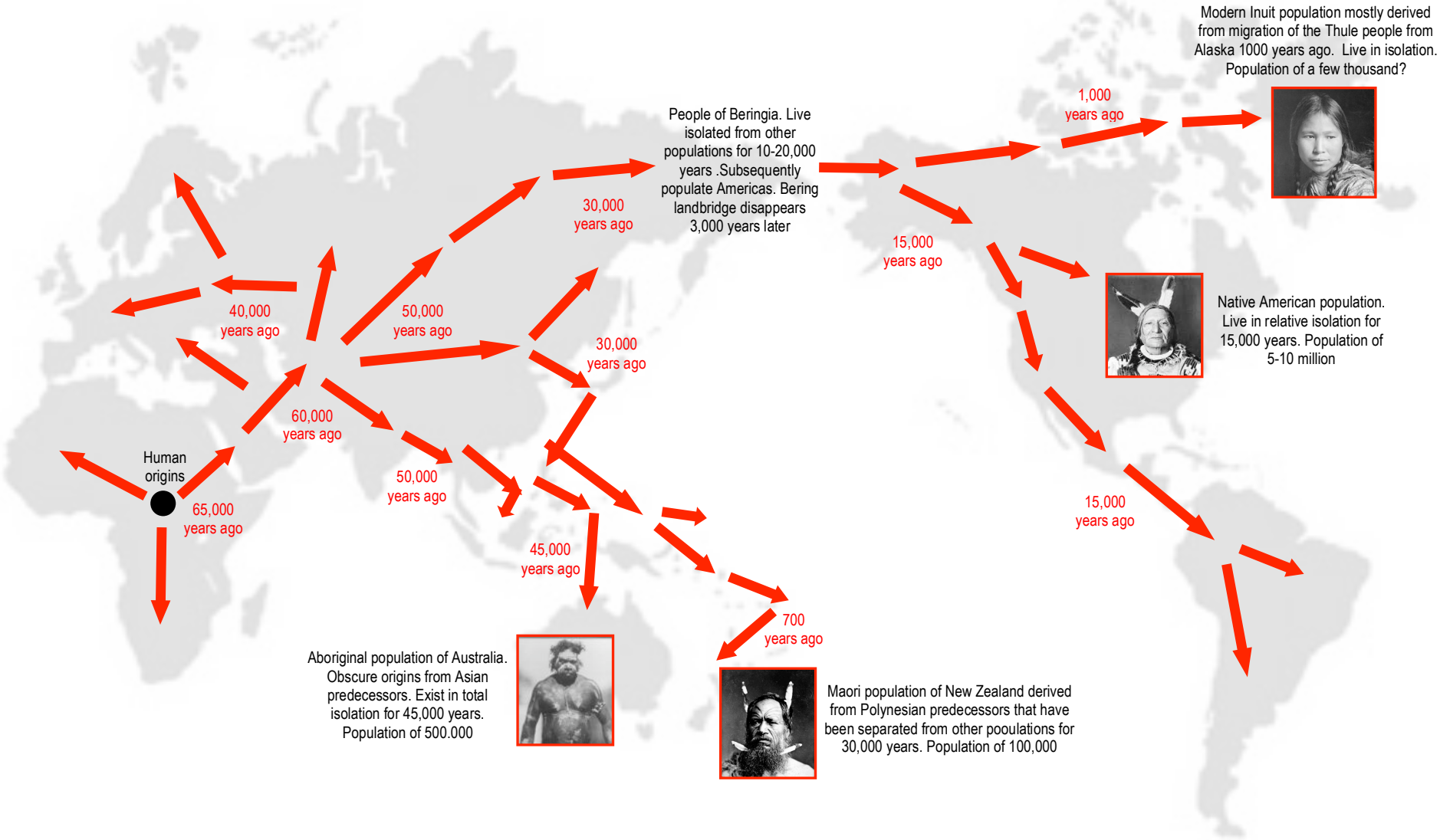
and inconsistent disease definition make it difficult to compare reported geographical variations in prevalence[16].

However, there are reliable data that show a high prevalence of otitis media in children of the indigenous Inuit[151-154], native American[154-156], Maori[157-159], or Australian Aborigine[160-163] populations when compared to white conspecifics. These indigenous groups seem more susceptible to the full spectrum of middle ear mucosal inflammation: AOM, rAOM, COME and CSOM. Socioeconomic disparities may contribute, but the incidence of OM in indigenous groups is exceptionally high, suggesting genetic differences play a significant role.

It has been mooted that anatomical factors in Eustachian tube morphology or in middle ear anatomy may underlie ethnic variation in OM incidence, but data to support this theory are weak[102 164-166]. Craniofacial differences in human populations appear to arise largely as a consequence of genetic drift[167], rather than evolutionary pressures. Because of the current lack of good evidence that a particular craniofacial form is responsible for ethnic variations in OM susceptibility, theories based on this premise appear reductionist, and could be construed as racist.

I present an alternative hypothesis, based upon population genetics and phlyogeography. Consider human populations prior to European exploration and colonization in the late 15th and 16th Centuries (figure 5). At this time the Eurasian and African populations numbered in the hundreds of millions, and were sympatric (they probably interbred until 20-40,000 years ago[168]). It seems likely that microorganisms would have spread across the African and Eurasian populations, aided by regional and inter-continental trade, and (for respiratory microorganisms) by wind. In comparison, the Inuit, native American, Maori, and Australian Aboriginal populations at this time had relatively small total and demic population sizes, and

Figure 5. A simplified map of human population migrations prior to onset of European world colonization (c.500 years ago). Annotations are focused on populations known to have a high contemporary incidence of OM. Data derived from a number of sources[169-173].



each of these populations lived in relative geographic and social isolation. Hence, for the large part they were exposed only to local or regional microorganisms. In addition, indigenous populations were derived from population bottlenecks[174], and such canalization has, in general, lead to less genetic diversity in these isolated groups[173] when compared to Eurasian or African conspecifics[175].

Given that the Inuit, north American, Maori or Australian Aborigine populations were separated from Eurasian and African populations for a period of 30-45,000 years, we would expect these groups to have diverged through genetic drift. Similarly we would expect drift to lead to geographic divergence in the nasopharyngeal microbiome. Hence empirically, due to co-evolution between man and microorganism, evolutionary stable strategies will invoke different loci (in host and pathogen) in geographically isolated populations, and the nature of the strategy likely will vary with local host population size and dynamics. In particular, large and expanding populations, such as the European population, favour virulence in microorganisms, whereas smaller populations, such as the Inuit, north American, Maori, or Australian Aborigine populations favour avirulence[176]. With the advent of European world exploration in the 15th Century, indigenous populations became exposed to virulent respiratory microorganisms from Europe, to which they had not evolved specific immunity. This seems to me a likely explanation for the high prevalence of otitis media in these groups.

This model would predict that indigenous Mesoamerican or South American populations would also have suffered OM following European colonisation, but this is difficult to verify. Contemporary populations in these regions comprise a mixture of native American, European, and African ancestry[177], and (perhaps as a consequence) OM is not particularly prevalent[148].

There is (indirect) evidence in support of this hypothesis. Population genetics modeling confirms predicted divergence of host-pathogen co-evolutionary strategy in allopatric populations[178]. Indeed sampling of flora in sites such as the oral cavity[179] or stomach[180 181] confirms geographic variations in bacterial genotype[182]. Similarly, allele frequencies in immunomodulatory loci have been found to differ between ethnic groups[183] (although the functional effect of these variations is not well understood). Furthermore, population genetics predicts potential catastrophic infectious disease from the coalescence of groups that historically have been spatially segregated[178]. History documents this effect, whereby Old World diseases such as smallpox, influenza, bubonic plague and pneumonic plague were introduced by colonization of the New World and were responsible for the decimation of biologically naïve native populations. Importantly, accounts from the time suggest that a similar effect occurred even with pathogens to which these populations were not entirely naïve: native Americans suffered from tuberculosis prior to European colonisation, yet mortality from tuberculosis increased significantly following colonisation[184], implying the introduction of a more virulent variant of *Mycobacterium tuberculosis*.

To find evidence of this effect for otitis media is more difficult. Middle ear disease is not documented in historical records from the time of European colonisation. However, suppurative OM can arrest pneumatisation of the mastoid bone, and archaeological evidence in ancient fossilized skulls of both the native American[185] and the Greenland Inuit[186] does suggest an increase in OM subsequent to European colonisation. Genetic variation in the nasopharyngeal flora by ethnic groups has not been studied, either in contemporary or bioarchaeological remains, but the nasopharynx of Australian Aborigine children is known to be colonized by potential otopathogens at an earlier age[187] and in higher numbers[188] (and this is predictive of subsequent middle ear suppuration[189]). This supports an idea that Aborigine populations are colonized by variants of nasopharyngeal commensals with which they have not

co-evolved, and to which they may not have the genetic repertoire to mount a directed and specific immune response. More frequent and more dense nasopharyngeal colonization by otopathogens has also been documented in the Inuit[190].

If nasopharyngeal host-pathogen co-evolution is responsible for ethnic variations in OM, we would predict variation in susceptibility to other respiratory infections. Indeed childhood LRTI is highly prevalent in the Inuit[191-194], native American[195-199], Maori[200-202], and Aborigine populations[203-208], and this is not fully explainable by socio-economic factors.

Implications for OM Treatment

From the discussion presented in this chapter, the common phenotypes of otitis media appear to be evolutionarily stable strategies for the human population, factoring potential negative consequences of infection or inflammation against investment and usage costs of the immune system. Indigenous populations with high incidence of OM may be the exception, where previously evolutionarily stable strategies could have been disrupted by fairly recent European colonisation.

If current phenotypes and their prevalence are indeed an evolutionary stable strategy, this raises the question of whether it is wise to interfere with this process. I would argue that contemporary prevalence of OM phenotypes is not the stable or desirable strategy, particularly so for a developed world environment.

The developed world has altered dramatically in the past few centuries, with advances in living conditions and public health measures thwarting virulent microorganisms[209]. AOM and rAOM have become less common, and data from health consultations in UK primary care suggest that this decline may still be occurring[210] although now reaching a plateau (Ian Williamson, personal communication). Incidence could also be reduced further with advances in mucosal vaccination. However, the trend could be reversed in the future, as a result of an increase in the risk factors for AOM: use of child-care facilities, and increasing population urbanization[211]. In terms of evolution, the reduced prevalence of suppurative otitis media (and infectious disease generally) may mean that genotypes programming lower immune investment become fitter. But, this effect could be counterbalanced by the abundance of food in the developed world, which makes efficient use of energy capital less related to fecundity.

There has also been a decline in health consultations in UK primary care for COME (Ian Williamson, personal communication), but whether this reflects falling prevalence of chronic middle ear inflammation, or a change in health seeking behaviour is unknown. If chronic inflammation is a genetically programmed strategy, perhaps to prevent middle ear infection, then any advantage in the short term is counterbalanced as the inflammation persists, causing damage to surrounding tissues and hearing loss. Any advantage of chronic OM becomes less in contemporary developed world environments, where there is a reduced prevalence, and therefore risk of infectious disease, and increasing importance assigned to hearing (for educational outcomes). Genotypes programming chronic OM become increasingly maladaptive.

Modeling suggests that evolutionary adaptation to new environments is a long-term phenomenon[212]. In this context, anthropogenic efforts to understand or modulate chronic inflammatory response in the middle ear should be welcomed.

Conclusion

There is high heritability to recurrent or chronic otitis media. A number of evolutionary forces and constraints have operated upon middle ear inflammation, which are likely to have engendered a complex genetic architecture conveying susceptibility to disease, but may also account for ethnic variations in disease.

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Chapter 1.3
Identifying candidate association loci

Two methodological approaches can be taken to identify loci conferring susceptibility to chronic otitis media: a discovery orientated genome-wide association study (GWAS), or a hypothesis orientated candidate gene association study.

GWAS has become a popular method in genetic epidemiology, but carries a high risk of false positive findings, particularly if sample sizes are small or phenotypes are poorly defined[2]. I believe GWAS to be an important research strategy in the genetic epidemiology of chronic otitis media, but disease cohorts are probably as yet insufficient in size to reliably enable such analyses (see chapter 4.1).

The candidate gene approach tests a restricted and biologically plausible hypothesis based upon only a few loci. The approach has been criticized because of frequent non-replication of findings from this method, or because of the failure of the method to undertake comprehensive testing of candidate loci[3]. Non-replication is an issue for candidate gene approaches, but this is a problem also found in genome-wide approaches[4]. In many cases non-replication may be due to variation in study design, particularly if phenotypes are poorly matched[3]. Older candidate-gene studies did fail to comprehensively evaluate candidate loci, but this was largely due to a lack of any useful data on human haplotype blocks. This limitation has now been overcome by the International HapMap project[5].

I describe a candidate gene association study in section 4 of this thesis. The value of this approach is maximised if appropriate candidate loci are selected, which is the subject of this chapter. To minimize risk of false discovery, a good and cohesive biologically plausible hypothesis is the first step[3 6]. The ideal is to find persuasive (multi-faceted) evidence that a particular biological pathway is responsible for signaling persistent middle ear inflammation. In principle several approaches can be used to find such evidence, including gene expression

profiling, comparison of variations in disease incidence in different ethnic groups, analysis of disease associated with genetic syndromes, or the use of animal (mouse) models.

Gene expression profiling

Many studies have looked at mRNA or protein levels of cytokines, chemokines, and arachidonic acid metabolites in the inflamed middle ear, in an attempt to identify key regulatory pathways[7]. Microarrays have hugely expanded capacity for transcriptome analysis, and there are reports of altered transcripts of over 16,000 genes in middle ear mucosa following injection of *S.pneumo* or NT*Hi*[1] (figure 1). It is improbable that so many genes play a role in middle ear inflammation, but this does evidence complexity and redundancy in inflammatory signalling, and the difficulty inherent in post-hoc analysis of the inflamed microenvironment. An additional limitation is that transcriptome analysis is only a partial model of gene expression[8], and does not account for protein translation or enzyme kinetics.

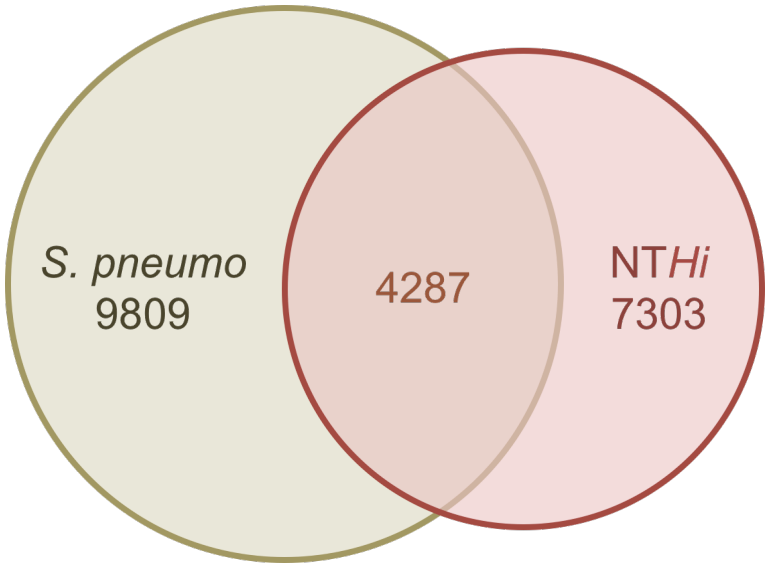


Figure 1: Microarray analysis of upregulated genes in murine middle ear mucosa after injection of the pathogens *S.pneumo* and NT*Hi.* Adapted from Lim et al, 2009[1].

Nevertheless, review of data from human and animal models[7 9] do suggest that certain pathways are prominent in the acute inflammatory response in OM, and others in the chronic phase of inflammation[10]. In particular, persistent inflammation is associated with upregulation of IL-10, TNF-α, and/or TGF-ß.

Ethnic variations in disease

A comparison between different ethnic groups could suggest potential candidate loci for disease (but see chapter 1.2). In particular isolated populations may be valuable as they may be enriched in unique variants[11]. However, at present we have limited understanding of genotypic variation between ethnic groups, and of how this may impact OM susceptibility.

Syndromic disease

OM is more prevalent in certain congenital syndromes, and increasingly the genetic polymorphisms underlying such syndromes are known. Where pathology of syndromic OM mimics that of non-syndromic OM, it may suggest candidate loci or biological pathways underlying non-syndromic disease. I have undertaken a systematic (narrative) review of syndromic OM, with results summarized in table 1.

Using the terms "otitis" and "syndrome" I searched Pubmed (1141 articles screened) and Web of Science (1760 articles screened) for case series and pathological reports. Single case reports were excluded. Where a syndrome associated with OM was confirmed I undertook further

relevant searches to identify the loci affected, evidence of underlying biological mechanisms, and mouse models of that syndrome.

Immunodeficiency

Primary immunodeficiency presents with frequent infections and often in several organs. Some immunodeficiencies seem to be often associated with rAOM (table 1), but in others this does not feature. In particular susceptibility to rAOM is associated with null mutation in *TACI*, *ICOS*, *BTK*, or *CD40L*, genes that seem to play a role in B-lymphocyte function, including secretion of immunoglobulin A. Defects in T-lymphocyte or granulocyte function can also lead to rAOM, but here infections tend to be multifocal and caused by multiple pathogens.

There is accumulating evidence that less severe immunodeficiency phenotypes may be due to mutations in the same loci underlying primary immunodeficiency, albeit mutations with a lesser effect on gene function[12]. Consequently polymorphisms in *TACI*, *ICOS*, *BTK*, or *CD40L* could confer susceptibility to disease in some children who develop rAOM but without infection in extra-aural organs.

My focus in this thesis is on chronic otitis media phenotypes, and so I have not explored this further here.

Craniofacial syndromes

Several syndromes with craniofacial deformation are associated with susceptibility to COME, notably cleft palate and the craniosynostoses. Much of the existing literature suggests that this association is due to dysmorphism and consequent dysfunctional opening of the Eustachian tube[13].

Table 1: Otitis media in association with genetic syndromes

Syndrome	Affected Locus/Protein	Population Incidence	General Phenotype	Otitis Media Phenotype	OM in Mouse Model
Immunodeficiency					
Common Variable Immune Deficiency	*TACI*/ *ICOS*/ others	1 in 40,000	Recurrent bacterial infections	rAOM 60%[14 15]	-
X-linked agammaglobulinaemia	*BTK*	1 in 100,000 males	Recurrent bacterial infections	rAOM >70%[16 17]	Not known[18-21]
Hyper-IgM syndrome	*CD40L*	1 in 1,000,000	Viral, bacterial, fungal and parasitic infections	rAOM 40%[22]	-
Wiskott-Aldrich syndrome	*WASP*	1 in 1,000,000 males	Eczema, microthrombocytopaenia and immune dysfunction	rAOM >30%[23]	-
Hyper-IgE syndrome	*STAT3*	very rare	Staphylococcal skin abscesses, recurrent pneumonia	rAOM 50%[24]	Not known[25]
Severe Congenital Neutropenia	*ELA2*	rare	Recurrent bacterial infections	rAOM[26]	No known phenotype[27]
Cyclic neutropenia	*ELA2*	rare	Episodic infections	rAOM[28]	No known phenotype[27]
Leucocyte Adhesion Deficiency	Integrins, selectins	rare	Peripheral leucocytosis, bacterial infections, defective pus formation	rAOM 50%[29]	-
Schwachman-Diamond Syndrome	*SBDS*	1 in 50,000	Exocrine pancreatic insufficiency, skeletal and bone marrow defects	rAOM 30%[30]	-
Severe Combined Immunodeficiency	various	1 in 300,000	Multiple infections	rAOM 25%[31]	-
Bare Lymphocyte Syndrome	*RFX5*/ *RFXAP*/ *CIITA*/ *RFXANK*	rare	Multiple infections	AOM 50%[32]	-

Craniofacial					
Cleft palate	multiple	1 in 1000	Cleft palate, 30% have other defects	rAOM 45%,[33-35] COME 66%[34-50]	Not known
Velopharyngeal incompetence	multiple	unknown	Velopharyngeal incompetence	rAOM 40%[51], COME 30%[51 52]	-
Apert syndrome	*FGFR2*	1 in 60,000	Craniosynostosis, bony syndactyly	OME 90%[53-55]	Not known[56-58]
Crouzon syndrome	*FGFR2*	1 in 50,000	Craniosynostosis, overtly normal limbs	OME 29-50%[55 59 60]	-
Crouzon syndrome with aganthosis nigricans	*FGFR3*	rare	Craniosynostosis, overtly normal limbs, acanthosis nigricans	OME 14%[61]	-
Pfeiffer syndrome	*FGFR2/ FGFR3*	1 in 100,000	Craniosynostosis, broad thumbs and broad great toes	COME 44%[62]	-
Achondroplasia	*FGFR3*	1 in 30,000	Short stature, short proximal limbs, midface hypoplasia,	rAOM 25%, COME 25%[63 64]	Not known or not present[65-72]
Choanal Atresia	multiple	1 in 6,000	Choanal atresia, 75% have other defects	COME 30%[72]	-
CHARGE syndrome	*CHD7*	1 in 25,000	Choanal atresia, coloboma, genital hypoplasia, retarded growth, inner ear and cardiac malformation	rAOM[73] , COME 81-97%[73-76]	Chronic OM[77]
Kabuki syndrome	unknown	1 in 50,000	Abnormal facial features, skeletal and dermatoglyphic abnormalities, short stature, mental retardation	rAOM 60%[78-81]	-
Nager syndrome	unknown	very rare	Complex mandibulofacial and upper limb dysplasia	OME > 90%[82]	-
Cornelia de Lange syndrome	*NIBPL/ SMC1A/ SMC3*	1 in 100,000	Craniofacial malformation, mental retardation, hirsutism, growth retardation, limb anomalies, sensorineural hearing loss	OME > 90%[83]	-

Chromosomal					
Down syndrome	47,XX,+21	1 in 750	Skull/midface malformation, cardiac anomalies, hypotonia, delayed growth, thyroid disease, obesity	OME > 66%[84-89]	Chronic OM (Ts65Dn mouse) [90]
22q11.2 deletion syndrome	46,XY,del(22)(q11.2)	1 in 6000	Craniofacial and conotruncal defects	rAOM 50%[91-98]	Chronic OM (BAC 316 mouse) [99 100]
Turner syndrome	45,X	1 in 2,500 females	Short stature, gonadal dysgenesis, webbing of neck, malformed aorta	rAOM ? 66%[101-106]	Not known[107]
Emmanuel syndrome	45,XY,t(11;22)	rare	Craniofacial defects, global developmental delay, cardiac, urogenital anomalies	rAOM >90%[108]	-
17q21.31 microdeletion syndrome	46,XY del(17q21.31)	very rare	Global developmental delay, epilepsy, cardiac, renal, urogenital anomalies	? OM in 27%[109]	-
Tetrasomy 18p	tetrasomy 18p	very rare	Global developmental delay, cardiac, skeletal anomalies	rAOM 33%[110]	-

Mucopolysaccharidoses					
MPS type I	α-L-iduronidase	1 in 100,000	Widespread lysosome deposition	Effusion 50%[111-113]	Chronic OM[114]
MPS type II	L-sulfoiduronate sulfatase	1 in 250,000	Widespread lysosome deposition	Effusion 72-91%[115-117]	Chronic OM[118]
MPS type IIIB	N-acetyl- α -D-glucosaminidase	1 in 50,000	Widespread lysosome deposition	Animal models only	Chronic OM[119]
MPS type IVA	N-acetylgalactosamine-6-sulfatase	1 in 100,000	Widespread lysosome deposition	Effusion >33%[120 121]	-
MPS type VII	ß-glucuronidase	very rare	Widespread lysosome deposition	Animal models only	Chronic OM[122 123]

Other					
Primary Ciliary Dyskinesia	*DNAI1/ DNAH5/ DNAH11/ DNAI2/ KTU/ RSPH9/ RSPH4A/ TXNDC3*	1 in 15,000	Situs invertus, infertility, sinusitis, bronchiectasis	Effusion 60%[124-130]	Not known[131]
Ligneous conjunctivitis	*PLG*	very rare	Widespread deposition of fibrin-rich material	Fibrinous chronic OM[132-135]	Chronic OM[136]
Ectodermal dysplasia	multiple	very rare	Teeth, skin and hair anomalies	rAOM 28-49%[137-140]	-

It is likely that Eustachian tube dysmorphology is important in aetiology, but it may not be the only or main factor, and susceptibility is more likely complex, heterogeneous, and to some extent specific to each syndrome. There are very few studies of Eustachian tube dysfunction in syndromic OM, and the oft-quoted evidence that this contributes to susceptibility to OM in non-syndromic children is also questionable[141 142]. Gross craniofacial malformation can occur with no increased susceptibility to OM[143]. Craniofacial maldevelopment is a result of disrupted pharyngeal arch embryology, and the pharyngeal arches also function in lymphocyte maturation[144 145]. A factor not often recognized is the co-existence of additional overt or subtle immunodeficiency in craniofacial malformation, which could play a role in the high prevalence of otitis media. For example immunodeficiency has been demonstrated in Chromosome 22q.11.2 deletion[146 147], Kabuki syndrome[148], CHARGE syndrome[149] and Down syndrome[150 151].

Cleft palate and velopharyngeal incompetence

The association of OM with cleft palate occurs in syndromic and non-syndromic cleft palate[35], and any anatomical variant of cleft[34 35 42 45 152]. Disease is usually bilateral even if the malformation is unilateral[153]. The pathology of OM in cleft palate is similar in terms of histology[154 155] and microbiology[156] to that in non-syndromic cases, but the inflammation may be more severe[154]. Anatomical variations in the Eustachian tube[157], skull base[158 159] or attachment of the tensor-veli-palatini muscle are inconsistent[160-162], or of uncertain functional consequence[163]. Studies of Eustachian tube dynamics have been contradictory, with some reports suggesting failure to open[164], others suggesting failure to close[165], some suggesting improved opening following palatal repair[166], but others suggesting no change[167].

Cleft palate leads to velopharyngeal incompetence, and OM occurs with equal frequency in velopharyngeal incompetence without overt cleft palate[51 52], yet palatal defects without velopharyngeal incompetence are not associated with OM[168] (e.g. bifid uvula[169-171], very small

submucous clefts[172], or isolated cleft lip[35 173]). The association of OM may therefore be with velopharyngeal incompetence of whatever cause, rather than cleft palate *per se*. I suggest that this is because velopharyngeal incompetence leads to repeated aspiration of oral contents (chemical irritants or microbes) into the nose or nasopharynx, causing inflammation of respiratory mucosa and local immunological dysfunction. Those with cleft palate or velopharyngeal incompetence are known to have impaired mucociliary clearance[174] and a higher prevalence of sinusitis[174 175] as well as OM. An additional factor may be that many cleft palate patients have other, sometimes subclinical abnormalities[176 177], including a higher incidence of leukaemia and lymphoma[178], suggesting uncharacterised immune dysfunction.

Animal models of cleft palate have been of limited help in understanding pathophysiology. Cleft palate is often lethal in non-human mammals due to defective suckling, although survival is possible for animals in captivity or kept as pets[179]. Dogs and cats with palatal defects develop OM in most cases[180 181], and surgical clefting of the palate in primates[182 183], rat[184 185 186], or chinchilla[187] induces OM. A mechanism involving dysfunction of the Eustachian tube has been suggested[188], but the evidence is contradictory[182 183 189]. Hundreds of mutated mice with cleft palate have been identified[179], mostly syndromic, although non-syndromic models with null-mutations in *Tgf- β3*[190] and *Tbx22*[191] have also been described. The occurrence of OM in almost all of these mouse models has not been reported, but investigations may be compounded by early lethality.

Cleft palate represents the phenotypic end-point of a number of potential genetic and environmental defects during embryogenesis. It is syndromic in 30% of cases, with over 300 syndromes recognised. Similar pathways may be involved in syndromic and non-syndromic cases[192], particularly in *IRF6*, *MSX1*, and *FGF* pathways. Given heterogeneity in aetiological and

pathological mechanisms, loci leading to cleft palate seem unlikely candidate genes for non-syndromic OM.

Craniosynostoses

Apert, Crouzon and Pfeiffer syndrome are the more common craniosysnostoses, and are due to hypermorph mutations in fibroblast growth factor receptors (FGFR). The high prevalence of COME may be associated with Eustachian tube dysmorphology[13], which obstructs mucociliary clearance. There is some evidence to support this[53]. However other mechanisms may be at play, including associated velopharyngeal incompetence[193-196]. One other alternative explanation is that hypermorphic mutations in FGF receptors have pleoitropic effects, leading to both premature fusion of skull calvaria and chronic pro-inflammatory signaling in the middle ear (FGF-1 and FGF-2 are expressed in the subepithelium in chronic OM[197]). Interestingly, the *Hush Puppy* mouse has a mutation in *fgfr1* and develops spontaneous chronic OM[198] (table 2), but this mutation is a hypomorph[199]. Further analysis of mechanisms, perhaps using mouse models may aid our understanding.

Chromosomal disorders

Chromosomal disorder phenotypes are presumed to occur due to altered gene dosage, but mechanisms are not well understood. However, copy number variation may mimic gain or loss of function mutations, and thus chromosomal disorders may present interesting candidates regions for susceptibility in non-syndromic populations.

Down syndrome (partial or total trisomy HSA21) is the most common chromosomal disorder associated with OM, and the evolving library of partial of total mouse models of Down syndrome[200] presents a great opportunity to narrow the region responsible for OM in this syndrome. Such an analysis is the subject of chapter 2.2.

Chromosome 22q11.2 deletion syndrome is due to a 3Mb microdeletion, with the main phenotypic effect thought to be due to haploinsufficiency of *TBX1*[201], but with phenotypic modification through epistasis[147]. Overt or subclinical anatomical or functional palatal anomalies are common[202]. The syndrome is also frequently associated with thymic hypoplasia and diminished circulating T-lymphocytes[146], and an 80% prevalence of immunodeficiency[147]. Temporal bone histology of a boy with probable 22q11.2 deletion and chronic OM revealed a paucity of T-lymphocytes in the inflammatory response[203]. It may be that COME here is due to diverse defects in T-cell function, and/or due to palatal anomalies. The function of most genes in the deleted segment of 22q is not well understood.

Turner syndrome is due to a variable loss of X chromosome material, but why this lead to increased susceptibility to OM in not clear. The syndrome is associated with autoimmune disease[204] suggesting immunological dysfunction, but no consistent immune abnormality has been demonstrated[205-210].

The pathophysiological basis of other (rare) aneuploidies is even less well understood.

Other syndromes

In some syndromes the pathology of chronic OM appears to be unique. Ligneous conjunctivitis is due to mutations in *PLG*, which leads to deficiency of plasminogen, a serine protease involved in the breakdown of fibrin in wound healing. Accumulation of fibrin leads to chronic inflammation in the ear, as well as at multiple other sites. The mucopolysaccharidoses[211] are due to defects in enzymes required for the breakdown of polysaccharide glycosaminoglycans (GAGs). Accumulation of GAGs in the middle ear triggers chronic inflammation[211]. In Primary Ciliary Dyskinesia there is a chronic middle ear effusion, but it is not clear if this is due to a

simple failure of clearance of mucus or due to another mechanism. The evidence of disrupted ciliary function in non-syndromic COME is contradictory[212-217], and where disruption has been observed, it is not clear whether this is cause or effect.

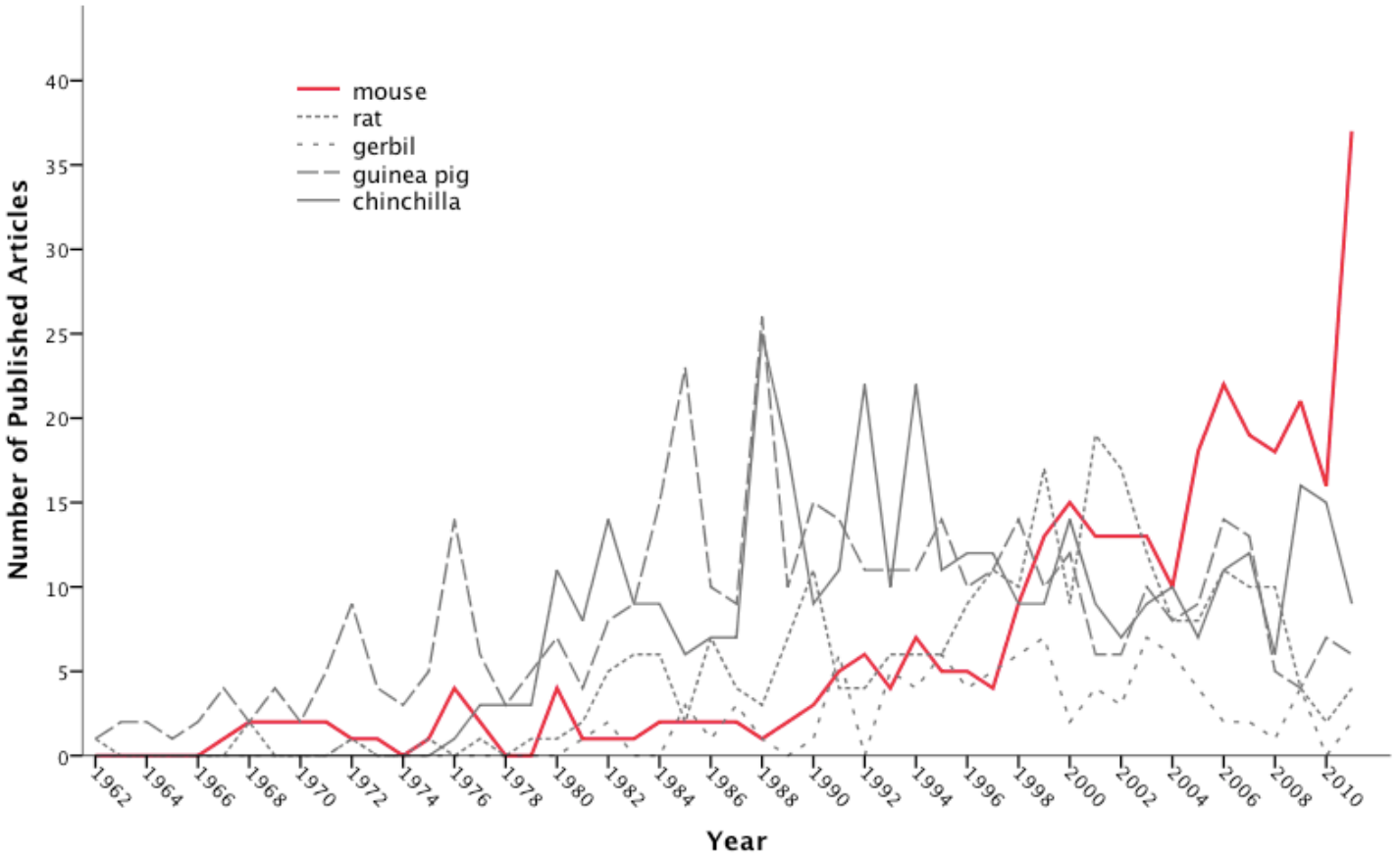
OM is also more prevalent in CHARGE syndrome, Kabuki syndrome, Nager syndrome, Cornelia de Lange syndrome, and Ectodermal dysplasia. These syndromes are rare and are associated with multiple anomalies, evidencing widespread disruption of embryology. The genetic basis for these syndromes is not well understood, but seems unlikely to be relevant to the aetiology of non-syndromic OM.

In summary, current knowledge on mechanisms of syndromic otitis media do not provide convincing loci for non-syndromic disease. Disease in cleft palate, craniosynostoses, ligneous conjunctivitis, mucopolysaccharidoses, or primary ciliary dyskinesia probably invoke mechanisms of disease that are unique to these syndromes. Analysis of OM associated with chromosomal disorders does seem a more promising strategy, whereby mapping a region or locus with altered copy number may suggest a locus underlying non-syndromic disease. As knowledge of gene function improves it may be possible to narrow candidate genes for the OM phenotype from these chromosomal disorders, and mouse models may also help. I explore this approach for mouse models of Down Syndrome in chapter 2.2.

Mouse models

Over the decades a variety of species have been exploited for otitis media research, but in particular rodents. Enthusiasm in the 1960s and 1970s was for the guinea pig (figure 2),

Figure 2. Rodents have long been used in otitis media research, but in recent years the mouse has become the preferred model. Data shows the number of published articles listed in the Pubmed database between 1962 and 2011 (search terms “mouse” or “rat” or “gerbil” or “guinea pig” or “chinchilla” and “otitis”).



reflecting the popularity of this species in other branches of medical research. In the late 1970s the chinchilla was adopted from its role in inner ear research to investigation of the middle ear, not least because the large bulla in the chinchilla affords easy access to the middle ear[218]. By the 1980s the gerbil was popular, in particular due to the propensity of this model to develop cholesteatoma[219]. However, in more recent years the mouse has become the preferred model organism for OM research[220-222], because of its utility in exploring the genetic basis of human disease[223].

The mouse is the current preferred genetic model organism for several reasons: first, determination of the mouse genome sequence indicates that 99% of mouse genes have human homologues[224]; second, the mouse shares many developmental, biochemical and physiological processes with human; and finally there are a large repertoire of techniques available to manipulate it's genome[225].

Mice have been used to model acute OM by inoculation of the middle ear with human otopathogens via trans-bullar injection[221] or pressure induced translocation from the nasopharynx[226]. Interest is developing in the mouse as a model of mucosal vaccination against OM[227]. Models of chronic OM[220 222] have been recovered from small-scale, as well as international collaborative mouse mutagenesis programs, including gene-driven knockout approaches[228] and phenotype-driven ENU mutagenesis[229].

I have reviewed the mouse-to-man approach as it pertain to otitis media elsewhere (see Appendix)[222]. Here I present an update to this review (table 2) based upon a systematic search strategy.

Table 2: Current mouse models of acute and chronic OM. KO = knock-out, SNP = single nucleotide polymorphism. Updated and modified from Rye, Bhutta, Cheeseman et al, 2011[222].

* currently being characterized at MRC Harwell (H. Tateossian)
**currently being characterized at Kings College London (A. Tucker)
***currently being mapped and characterized at MRC Harwell. Early results suggest the mutated locus may be *Nisch* (M. Crompton)

Pathway	Locus	Chr	Model type	Background Strain	General Phenotype	OM Phenotype	Reference
Acute (induced) otitis media							
Complement	*Bf*	17	KO	C57BL/6	Mild immune defects	Delayed clearance of acute infection	Tong et al, 2010[230]
	Bf/C2	17	Double KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Tong et al, 2010[230]
	C1qa	4	KO	C57BL/6	Autoimmune disease, increased mortality, glomerulonephritis	Delayed clearance of acute infection	Tong et al, 2010[230]
Toll-like Receptors	*MyD88*	9	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Hernandez et al, 2008[231]
	Tlr2	3	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Leichtle et al, 2009[232]
	Tlr4	4	SNP or KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Hirano et al, 2007[233]; Macarthur et al, 2006[234]
	Tlr9	9	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Leichtle et al, 2011[235]
	Ticam1 (Trif)	17	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Leichtle et al, 2011[236]

TNF	*lpr*	19	SNP	MRL/MpJ	Autoimmune disease	Delayed clearance of acute infection	Rivkin et al, 2005[237]
	TNFA	17	KO	C57BL/6;129S6	Immune defects	Spontaneous mild mucosal hyperplasia. Delayed clearance of acute infection	Leichtle et al, 2010[238]; Ebmeyer et al, 2011[239]
Miscellaneous	*Cyld*	8	KO	C57BL/6	lymphoid hyperplasia, susceptibility to colitis and colonic adenocarcinoma	Enhanced inflammation	Lim et al, 2007[240]
	Il-10	1	KO	C57BL/6.129P2	Reduced size, anaemia, chronic enterocolitis	Reduced mucins in acute infection	Tsuchiya et al, 2008[241]
	Pycard (*Asc*)	7	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Wasserman et al, 2011[242]
	Nlrp3 (*Nalp3*)	11	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Ryan et al, 2011[10]
	Nod1	6					
	Nod2	8					
	Rip2	13					

Chronic otitis media							
TGF-β	*Fbxo11*	17	SNP	C3H/HeN	Mild sensorineural hearing loss	Spontaneous chronic in heterozygote	Hardisty et al 2003[243]; 2006[244]
	*Tgif1**	17	KO	C57BL/6J	Smaller size, craniofacial defect	Spontaneous chronic	Not yet reported
	Evi1 (Mecom)	3	SNP	C3H/HeN	Extra digits	Spontaneous chronic in heterozygote	Parkinson et al, 2006[245]
NF-κB	*IκBαΔN*	9	Knock-in	C57BL/6	Defective epithelial appendages, lymph node formation, and macrophage function	Spontaneous chronic	Schmidt-Ullrich et al, 2001[246]

EYA	*Eya1***	1	KO	129/SvJ, C57BL/6 or Balb/C	Ossicular and inner ear defects, renal anomalies	Spontaneous chronic in heterozygote	Xu et al, 1999[247]
	Eya4	10	KO	CBA/J	Sensorineural hearing loss	Spontaneous chronic in heterozygote	Depreux et al, 2008[248]
Ciliary Defects	*Cby1*	15	KO	C57BL/6	Absence of motile cilia	Spontaneous chronic	Voronina et al, 2009[249]
	Dnahc5	15	Transgenic	C57BL/6;CBA/J	Immotile cilia, respiratory infection, left-right defect, hydrocephalus,	Spontaneous acute/chronic	Ibañez-Tallon et al, 2002[250]
	Dnahc11	12	SNP	C3H / HeH or mixed	Immotile cilia, sperm dysmotitility, rhinitis, sinusitis	Spontaneous chronic	Lucas et al, 2011[251]
Lysosomal Disorders	*Gus*	5	Spontaneous transposition	C3H/HeOuJ	Widespread lysosomal storage, dysostosis	Spontaneous chronic, lysosomal granules	Vogler et al, 2001[123]
	Ids	X	KO	unknown	Widespread lysosomal storage.	Spontaneous chronic, lysosomal granules	Kim et al. 2010[118]
	Idua	5	KO	C57BL/6	Widespread lysosomal storage, dysostosis	Spontaneous chronic, lysosomal granules	Schachern et al, 2007[252]
	Naglu	11	KO	C57BL/6	Widespread lysosomal storage, neurological defects.	Spontaneous chronic, lysosomal granules	Heldermon et al. 2007[119]
Miscellaneous	*Chd7*	4	Spontaneous deletion	BALB/cByJ	Keratoconjunctivitis, circling behavior, growth retardation	Spontaneous chronic	Tian et al, 2012[77]
	Dbh	2	KO	C57BL/6; 129/ SvCPJ	altered metabolism, thermoregulation, motor function, learning	Spontaneous chronic	Maison et al, 2010[253]
	E2f4	10	KO	C57BL/6	Erythropoeitic defects, chronic rhinitis	Spontaneous chronic	Humbert et al, 2000[254]
	Fgfr1	8	SNP	C3HeB/FeJ	Pinna and ossicle malformation, cochlear defects, shortened skull	Spontaneous chronic in heterozygote	Pau et al, 2005[198]; Calvert et al 2011[255]

	Isl1	13	SNP	C3HeB/FeJ	Ossicular fusion	Spontaneous chronic in heterozygote	Hilton et al, 2011[256]
	Lmna	3	KO	-	enlarged nuclear morphology of macrophages, hyperphosphatemia	Spontaneous chronic	Zhang et al, 2012[257]
Miscellaneous	*Mcph1*	8	KO	C57BL/6	Eye defects, erythrocyte defects, infertile	Spontaneous chronic	Chen et al, 2011[258]
	Nf2	11	Conditional KO	FVB/N C57Bl/6	Schwannomas, osteosarcomas, other tumours, craniofacial defects	Spontaneous chronic	Giovannini et al, 2000[259]
	p73	4	KO	B57BL/6 or BALB/c	Lethal gastrointestinal and intracranial haemmorhage, neurological malformation	Spontaneous chronic	Yang et al, 2000[260]
	Phex	X	Spontaneous deletion	BALB/cAnBomUrd	Craniofacial, cochlear, and bone defects, endolymphatic hydrops	Not well characterized	Megerian et al, 2008[261]
	Plg	17	KO	C57BL/6 DBA/1J	Widespread fibrin deposition in organs	Spontaneous chronic. Fibrin deposition.	Eriksson et al, 2006[136]
	Rpl38	11	Spontaneous deletion	TsJ/LeJ.BlackSwiss	Shortened, kinked tail, reduced body weight, anaemia, skeletal anomalies	Spontaneous chronic in heterozygote, with crystal deposition	Noben-Trauth and Latoche, 2011[262]
	Sall4	2	KO	C57BL/6JTyrC-Brd;129/SvEvBrd	Exencephaly, renal hypoplasia, anogenital defects	Not well characterized	Warren et al, 2007[263]
	Sh3pxd2b	11	Spontaneous deletion	B10.A-H2h4/ (4R)SgDvEg	Reduced growth, craniofacial and ocular defects, infertility	Not well characterized	Mao et al, 2009[264]
	Tbx1	16	Transgenic	C57BL/6.129	Cardiovascular defects, craniofacial and pharyngeal arch anomalies	Spontaneous chronic	Moraes et al, 2005[265]
	MMU16 (Ts65Dn)	16	Partial trisomy	C57BL/6EiC3Sn	Neurological deficits, craniofacial malformation	Spontaneous chronic	Han et al, 2009[90]
Not mapped	Unknown *nmf391*	?	ENU mutant	C57BL/6J	Mild muscular dystrophy, tremor, episodic spasms	Spontaneous chronic	JAX Neuroscience Mutagenesis Facility, 2004[266]
	Unknown *Edison*[***]	14	ENU mutant	C3H/HeH	Reduced size, facial defect	Spontaneous chronic in homozygote	Not yet reported

I searched the MGI phenotype database (term "otitis"), PubMed (terms "mouse" and "otitis", 410 articles screened) and Web of Science (terms "mouse" and "otitis", 461 articles screened) to identify reported mouse models of OM. Additional unpublished models were identified from conference presentations, and from personal communications.

Limitations of Mouse Models

As I have published elsewhere[267], it is important to recognise the inherent difficulties with the mouse-to-man approach. Some of these limitations are generic[268 269] and some are specific to otitis media (table 3). These are worth expounding before determining which, if any, of the mouse models in table 2 present compelling candidate loci for otitis media in man.

First, although man and mouse are largely symplesiomorphic, important apomorphies exist. Mouse and man evolved from a common ancestor 65-75 million years ago, and so have been subject to divergent evolutionary pressures, amplified by species differences in ecology and lifespan. This is pronounced for immune function[270], which reflects the prominence of infectious disease as a driver of evolution[271]. For example, blood in man is neutrophil rich whereas that of mouse is lymphocyte rich[272]. Man has a ring of NALT aggregations at the pharyngeal inlet (most prominent at the nasopharyngeal tonsil or adenoid)[273], whereas in mice NALT aggregations are restricted to the opening of the Eustachian tube[274 275]. Some mucins expressed in human middle ear mucosa are not expressed in mouse[276]. Species and their microbiota have co-evolved and so pathogens are ecotropic: the human otopathogens *S. pneumoniae* and *NTHi* are not known to cause murine OM other than under experimentally induced conditions, whereas *M cattarhalis* is cleared rapidly from the mouse middle ear[277]. Differences outside of the immune system may also be important. Hominids, including man, have a complex posterior extension of the middle ear cleft in the form of a mastoid air cell

Table 2: Potential factors that differ between the laboratory mouse and man that could impact upon susceptibility to otitis media

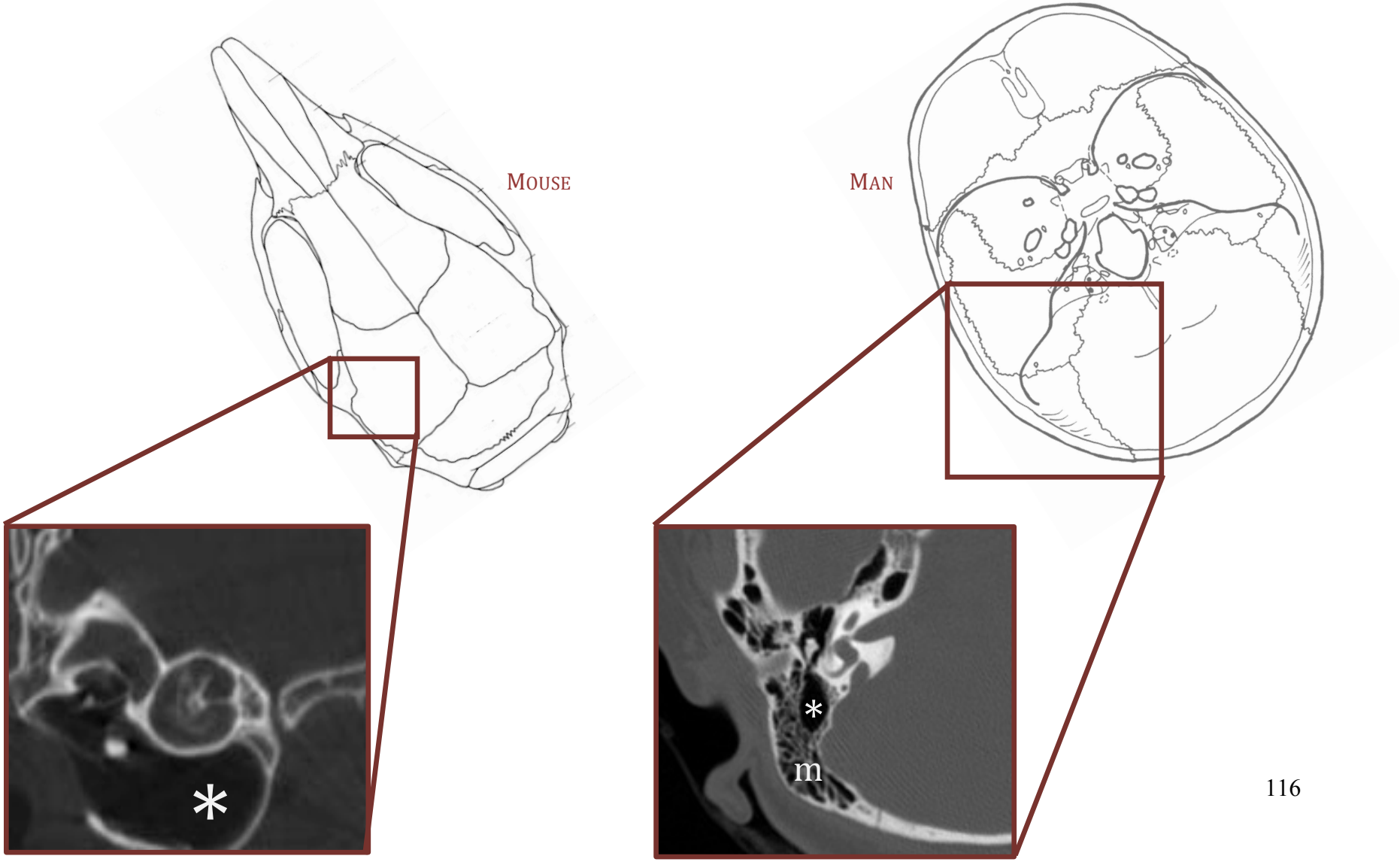
Type	Human	Laboratory Mouse	Notes
Common natural middle ear pathogens	*Steptococcus pneumoniae* Non-typeable *Haemophilus influenzae* (NT*Hi*) *Moraxella catarrhalis*[278]	Sendai virus *Mycoplasma pulmonis*[279]	*S. pneumo* and NT*Hi* can induce short-lived OM in mouse (but not *M. Cattarhalis*).
Natural incidence of otitis media	High (childhood)	Minimal[227]	A majority of B6;129 mice develop chronic OM in later life, with variable inflammatory infiltrate[280].
Inflammatory response	Variation in inflammatory response		e.g. Pn6A strain of *S. pneumoniae* is lethal in mice[241], but not humans
	Recognised mucoid effusion phenotype (OME)	No recognized mucoid effusion phenotype	*Muc7*, *Muc8*, *Muc1/12* and *Muc17* are not expressed in mouse[276]
Anatomy	Mastoid air cell system	Bulla only	A smaller mastoid air cell volume has been correlated to OM in humans[281 282]. Bulla size in wild mice shows regional variation[283], but this is of uncertain significance
	NALT in broad ring[273]	Nasopharyngeal lymphoid aggregates at Eustachian tube only[274 275]	-
Environment	Exposure to many pathogens	Often kept in high health status or pathogen free conditions	There is a variable health status of laboratory mice in different units

system[284], rather than the simple bulla found in most mammals (figure 3). A small mastoid volume is associated with otitis media in man[281 282] (probably because chronic or recurrent inflammation disrupts growth[285]), but bulla shape has not been correlated to OM susceptibility in mouse[245]. The overall impact on OM susceptibility arising as a result of species differences in immune or non-immune structure and function has not been characterized. Notably such inter-species immune differences have been considered important in other inflammatory disorders, fuelling the development of humanized mice[286].

Second, functional equivalence of genes cannot be assumed from homology in mouse and man, which may in part be due to species specific alternate splicing[287]. For example, mutation at *STAT3* or *ELA2* increases susceptibility to AOM in man, yet corresponding mouse models[25 27] demonstrate no immune dysregulation. Similarly monosomy X (Turner syndrome) is associated with AOM, but in the mouse phenotypic effects are mild with no OM reported[288 289]. Mutation at loci causing primary ciliary dyskinesia often leads to chronic middle ear effusion in man, but knockout mouse models show embryonic or early lethality[290].

Third, although mice have been used to identify highly penetrant loci underlying many Mendelian disorders[291], they have been less successful for complex trait analysis[268]. Contemporary mapping necessitates mice are inbred and housed in a controlled and pathogen free environment, and this is a deficient representation of the multitude of genetic and environmental factors impacting upon complex disease. Indeed, almost by definition, the effect of highly penetrant mutations in the mouse will be more severe than the small effect sizes predicted for allelic variants underlying complex disease in man. In addition, the relevance of genetic and environmental homogeneity of mouse models is further questioned by the sometimes profound effects upon penetrance that result from modification of these variables. For example both the *Jeff*[243] and *Junbo*[245] mouse develop chronic OM at an earlier stage and with

Figure 3. A comparison of the mouse and human middle ear cleft, as demonstrated by axial CT imaging. In the mouse, as in most mammals, the posterior extension of the middle ear space is limited to the bulla (*). In man the extension includes the mastoid antrum (*) and the mastoid air cell system (m). CT of mouse ear reproduced courtesy of Dr. Erik Ritman's lab, Mayo Clinic College of Medicine, Rochester.



greater severity if caged in conventional rather than specific pathogen free or gnotobiotic facilities[292]. Genetic background can also modify phenotype[293 294]: the *Hush Puppy*[199] and *Jeff* mutations are highly penetrant for OM on a C3He background but have lower penetrance on a C567BL/6 background, whereas the *Junbo* mutation is more severe on a C57BL/6 background than C3He or BALB/C (strain differences in phenotype could be due to differences in leucocyte response[294-297]). Methods are being developed to counteract the limitations of using inbred animals or using standardised mouse husbandry[225 298].

Finally, although the toolkit to manipulate the mouse genome has proven valuable, it is still a poor mimic of the allelic variations underlying common heritable disease. In particular the knockout approach produces amorphic rather than hypo or hypermorphic alleles. Most knockout models of OM display a plethora of anatomical and functional deficits as well as middle ear inflammation (table 2). In some cases pleotropic effects result from disruption of haematopoisis (*E2f4, Rpl38*), epithelial morphogenesis (*IκBαΔN*, *Sall4*, *p73*), ciliary function (*Dnahc5, Dnahc11*, *Cby1*), glycosaminoglycan storage (*Gus*, *Ids*, *Idua*, *Naglu*), or fibrin breakdown (*Plg*). In other cases the origin of effects is unknown. Knockout models can mimic some forms of syndromic OM (and indeed some knockout models have been created specifically to mimic syndromes), but where the mouse phenotype is diverse, the argument that this represents a good candidate locus for non-syndromic human OM susceptibility is less persuasive. ENU mutagenesis circumnavigates some of the problems of the knock-out approach, but 30% of ENU mutants are embryonic lethal, suggesting that ENU mutagenesis also produces unnaturally severe genotypic effects.

Give the limitations outlined above, clearly the mouse-to-man approach requires caution, but can nevertheless be successful. For example, loci found in mice have been successfully translated in identifying and characterizing alleles associated with risk of type 2 diabetes[299].

One strategy to tease out good candidate loci is by looking for instances where a particular biological pathway has been implicated by a number of mouse models, especially where a pathway is implicated by a mouse model with a phenotype more or less restricted to only middle ear inflammation. With this in mind, the models in table 2 have been grouped according to the pathways involved. This demonstrates that dysfunction of complement, toll-like receptor or TNF pathways can lead to prolonged inflammation following induced AOM (table 2), but as the inflammation nevertheless resolves in these models, such pathways may not play a critical role in signaling chronic inflammation (the focus of this thesis). Models of chronic spontaneous otitis media include models of ciliary defects or of lysosomal disorders, but these are models of syndromic otitis media. Those mouse models that do not correlate to a known human syndrome implicate a potential role for TGF-ß, NF-κB, or EYA pathways in driving chronic inflammation (table 2), and these pathways are considered further below.

EYA pathways

Mutation at *EYA1* in humans[300] causes Branchio-oto-renal (BOR) syndrome, characterised by hearing loss, auricular malformations, pharyngeal arch remnants, and renal anomalies, and with an incidence of 1 in 40,000[301]. The *Eya1* knockout mouse[247] displays a similar phenotype but also develops spontaneous chronic OM. Although otitis media has certainly been reported in human BOR syndrome[301 302], data from imaging studies[303-305] and exploratory tympanotomy[306] suggest it is infrequent.

EYA4 mutations cause the disease DFNA10, with late-onset sensorineural hearing loss[307-309] but with no reported association with middle ear disease. In contrast *Eya4* knockout mutants develop chronic OM in addition to sensorineural hearing loss[248].

EYA genes are transcriptional activators and so have diverse effects. In mice mutations in *Eya4* alter signalling in the innate immune response[310], and presumably *Eya1* may have similar functions, although this has not been demonstrated[311]. However, given species differences in phenotype due to *EYA* mutation, these genes likely have divergent functions in man and mouse, and so are not compelling candidate loci for human OM susceptibility.

NF-κB pathways

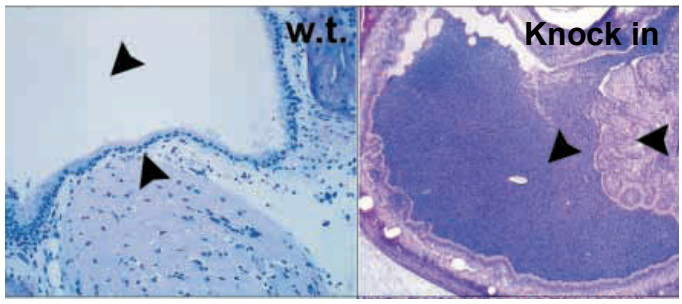
The *Junbo* mouse[245] (figure 4) was recovered from an ENU mutagenesis hearing screen[312] in our lab. It carries a A2288T transversion at *Evi1* (which is part of the *Mds1-Evi1* cluster, also known as *Mecom*), resulting in a non-conservative Asn763Ile change in the second of two zinc-finger domains. The heterozygote mutant develops spontaneous chronic OM usually by 20 days after birth, and with ~90% penetrance (although in 10% of these cases the OM is unilateral)[313]. The effusion is hypercellular ($7.1\pm1.7\times10^6$ cells per μl)[313], and contains a mix of neutrophils and macrophages. The *Junbo* mouse also develops rhinitis and occasional pneumonia when housed in conventional facilities, but extra-aural inflammation does not occur when it is housed in specific pathogen free or gnotobiotic conditions. There is no evidence of systemic inflammation or immune dysfunction[245]. The only other anomaly noted is an extra digit.

EVI1 is a nuclear transcription factor that plays a diverse role in embryogenesis, but is particularly known for its role in haematopoetic stem cell differentiation. *EVI1* over-expression, often due to chromosomal translocation or inversion, is associated with myeloid dysplasia and neoplasia[314]. The role of *EVI1* post-embryogenesis is less well understood, but polymorphism at this locus does predispose to nasopharyngeal carcinoma[315], which is thought to result from a chronic inflammatory response to latent Epstein Barr Virus infection[316]. EVI1 has interaction with several signaling pathways, notably with TGF-ß[317 318] and JNK[319] pathways through its first zinc-finger domain. However more recent work suggests that the second zinc-finger domain of

Figure 4: The *IκBαΔN* knock-in mouse, the *Jeff* and *Junbo* ENU mutants, and the *Tgif1* knockout implicate Nf-κB and TGF-ß pathways in chronic middle ear inflammation. Images show gross morphology of the mutant and axial histological sections (H&E stain) through the middle ear, compared to wild type littermate controls. *IκBαΔN* images from Schmidt-Ullrich et al, 2001[246]; *Junbo* images supplied by M Cheeseman (MRC Harwell); *Jeff* images from Hardisty-Hughes et al, 2006[244]; *Tgif1* images supplied by H Tateossian (MRC Harwell).

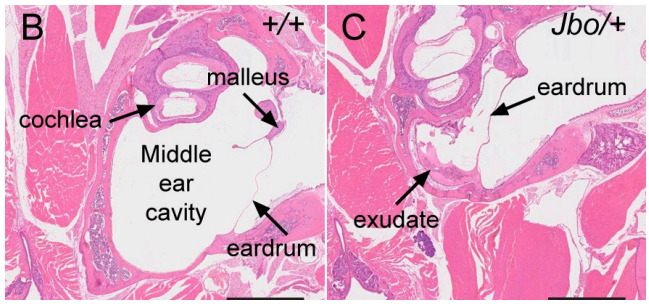
***IκBαΔN* knock-in**





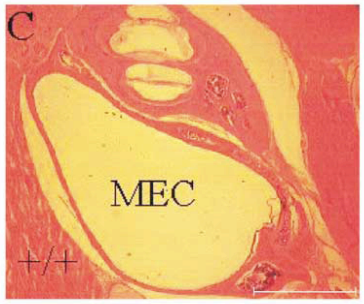
***Junbo* heterozygote**

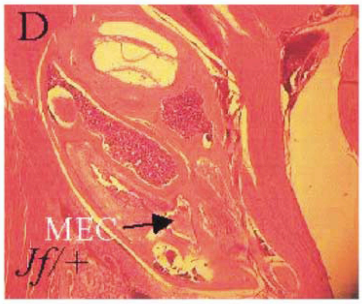




***Jeff* heterozygote**

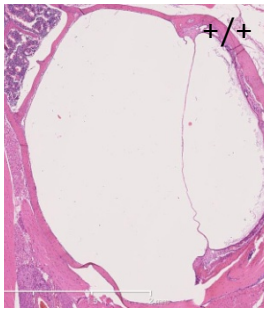


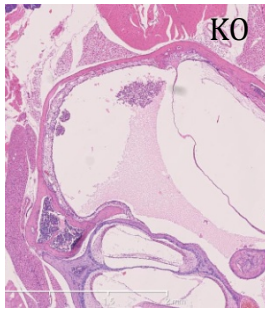




***Tgif1* knockout**

Macroscopic picture not available





EVI1 acts as a suppressor of NF-κB signaling by inhibiting its binding to DNA[320]. The mutation in *Junbo* is predicted to result in over-expression of NF-κB, which presumably leads to non-resolving inflammation in the middle ear. Of note, *Evi1* has several splice variants[321], and in particular exon 9 and 15 of this gene are alternately spliced in mouse and man[322]. This leads to a murine Δ105 transcript (with an additional 9 amino acids in the repressor domain and a 105 amino acid truncation from the C-terminus), which is abundant in many tissues in mouse, but absent in man. The Δ105 transcript is known to bind DNA, but its functional role is not known.

Otitis media is also reported in a knock-in mouse for *IκBαΔN*[246] (figure4), a repressor of NF-κB. Suppression of NF-κB in this mouse leads to diffuse embryonic disruption, with malformation of epithelial appendages and defective lymphatic development, including only rudimentary development of secondary lymphoid organs. Chronic otitis media develops 28-42 days after birth, with recovery of *Staphylococcus aureus* from middle ear effusions. It is likely that OM in this model is the regional manifestation of a more generalized defect in lymphocytes, but still suggests that pertubation of NF-κB signaling may play a role in chronic middle ear inflammation.

Finally, knockout of *Cyld*, a negative regulator of NF-κB, leads to prolonged middle ear inflammation induced by injection of NT*Hi*[240], but inflammation that nevertheless eventually resolves.

The NF-κB family of proteins are rapid acting transcription factors that play an important role in acute inflammation, and coordinate innate and adaptive immune response[323]. Taken together, the *Junbo* mutant, *IκBαΔN* knock-in, and *Cyld* knockout mouse models suggest a possible but somewhat conflicting role for NF-κB in chronic middle ear inflammation. Failure to suppress NF-κB signaling in the *Junbo* mutant or *Cyld* knockout leads to chronic or prolonged OM, whereas suppression of NF-κB in the *IκBαΔN* knock-in also leads to chronic OM. This apparent

conflict may reflect different roles and effects of NF-κB alteration in embryonic and post-embryonic tissues (lymphogenesis versus inflammation). In vitro experiments show that NF-κB protein levels are increased in middle ear epithelium in the acute inflammatory response to LPS[324], NT*Hi*[325], or cigarette smoke[326 327] exposure, although not all of these studies have ascertained the activation status of NF-κB as well as its abundance. There are no studies of levels of NF-κB in the chronically inflamed middle ear.

TGF-ß pathways

Like the *Junbo* mouse, the *Jeff* mouse[243] (figure 4) was recovered from an ENU mutagenesis hearing screen in our lab, and carries a A1472T transversion in *F*bx*o11*[244] leading to a non-conservative Glu491Leu change in the second of three CASH domains. The heterozygote develops spontaneous chronic middle ear inflammation with a hypocellular effusion ($55\pm25\times10^3$ cells per μl) and ~75% penetrance (~30% of these are unilateral OM)[313]. The homozygote shows perinatal lethality and facial clefting. The *Jeff* heterozygote is 20% smaller that WT and also develops an additional age-related sensorineural hearing loss, possibly as a consequence of chronic OM. There is also a mild craniofacial abnormality on a C3H/HeH background, but this is lost on a mixed C3H/HeH; C57BL/6J background, whereas chronic OM is still present.

Fbxo11 is expressed in a number of tissues in late embryology and early post-natal life, including middle ear mucosa[244]. It is part of the F-box family of molecules, which are specificity factors for the SCF E3 ubiquitin ligase complex, leading to targeted protein degradation[328]. Ubiquitination targets of Fbxo11 have not been comprehensively characterized, but Fbxo11 has also been suggested to neddylate and so inhibit activity of p53 (but without degradation)[329]. p53 is a transcription factor that responds to cellular stress to limit proliferation and promote apoptosis[330]. Computational predictions of protein structure suggest that the *Jeff* mutation

disrupts Fbxo11 binding of p53 (Hilda Tatteosian, personal communication), which would lead to abundant activity of p53. Amongst many functions, p53 is known to be a partner of Smad2[331] in the activation of TGF-β target genes. By way of confirmation, we find nuclear accumulation of pSmad2 in the *Jeff* homozygote mouse[332]. However, there are also some contradictory results: work from our lab has failed to demonstrate a direct interaction between Fbxo11 and p53, and we find reduced rather than elevated levels of p53 in the *Jeff* homo and heterozygote mouse[332]. These findings seem difficult to reconcile, and it is clear that the role of *FBXO11* is not well understood.

The *Junbo* mouse[245] (see previous section) may also signal chronic middle ear inflammation through TGF-β pathways. The first zinc finger domain of EVI1 is known to interact with and inhibit activity of an array of SMAD proteins[317] (the orchestrators of TGF-β response). Although the first zinc finger domain is not mutated in the *Junbo* mouse, the mutated second zinc finger domain could induce conformational changes in protein structure that do affect SMAD binding.

Based upon the discovery of the *Jeff* and *Junbo* models in our unit we have hypothesized that TGF-ß is a key regulator of chronic OM, and so we have recently become interested in the *Tgif1* knockout mouse. (figure 4) TGIF1 functions in a feedback loop, whereby TGF-ß promotes transcription of *Tgif1*, but TGIF1 then represses transcription of Smad2[333]. *Tgif1* knockout would therefore be predicted to lead to persistent TGF-β signaling. Our analyses show spontaneous chronic OM in the *Tgif1* knockout mouse, with onset within 21 days after birth, and ~70% penetrance (Hilda Tatteosian, personal communication). However, it is worth noting that TGIF1 does not have full functional equivalence in mouse and man; haploinsufficiency of *TGIF1* leads to holoprosencephaly in man, but not in mouse[334].

TGF-β is a key regulator of inflammatory response in a number of organs, through regulation of chemotaxis, activation, and survival of lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells, and granulocytes[335] [336]. The *Jeff* mutant and *Tgif1* knockout mouse provide strong evidence that TGF-β signaling is also an important regulator of chronic inflammation in the middle ear (whether this pathway is also relevant to the *Junbo* mouse is uncertain). Human studies in children[337] and in adults[338] show that TGF-β1 is consistently found in middle ear effusions, and that levels of this protein correlate to the duration of effusion. SMAD proteins bind to the promoter site of *Muc5ac* (which codes the major mucin found in human middle ear effusion[339]) and disruption of SMAD4 binding significantly impairs induced gene expression[340]. TGF-β is also upregulated in experimental acute OM[341], although in acute inflammation TGF-β may downregulate *Muc5ac* transcription[342].

Conclusion

At present the use of mouse models seems the most promising method to identify candidate loci underlying susceptibility to chronic otitis media. Mouse models have suggested that persistent NF-κB or TGF-β signaling could be two mechanisms leading to chronic OM. The evidence that NF-κB leads to chronic otitis media is somewhat uncertain, but the *Junbo* mouse develops isolated chronic otitis media and the *Evi1* locus seems a reasonable candidate gene. For TGF-β, the *Jeff* mouse implicates *Fbxo11* as a potential candidate, and the *Tgif1* knockout suggests *TGIF1* as a locus. Given the interaction of FBXO11 and TGIF1 with SMAD proteins, polymorphism at some *Smads* could also conceivably be relevant (chapter 4.1).

Clearly many more models of OM remain undiscovered, and ongoing mutagenesis screens will no doubt highlight new models and new pathways. Two methods that could aid identification of more candidate loci are discussed in section 2: otoendoscopy as a new screening tool for chronic OM, and mining of the Down syndrome library to identify a candidate region for chronic OM.

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Chapter 1.4
Existing association studies

In the last decade a number of human genetic association studies have been reported for otitis media. These include linkage analyses, candidate gene studies, and (unpublished) genome-wide association studies[1]. The populations studied have encompassed a variety of otitis media phenotypes.

Linkage analyses

Linkage mapping has been reported in three studies of OM[2-4] (table 1). However, the LOD scores for most regions are low, and results have not been replicated in separate cohorts[3 5]. In general, linkage analysis has proven to be a less successful method for non-Mendelian characteristics[6 7].

Table 1: Linkage Analyses for regions conferring otitis media susceptibility.

Phenotype	Linkage Region	Maximum LOD score	No of families (individuals) tested	Reference	Notes
Signs of or treatment for OM[8]	10q26.3	3.78 (p=3.0 x 10^{-5})	133 (591)	Daly et al, 2004[2]	Not replicated by Casselbrant et al, 2009[3], Replicated by Rye, 2009[5]
	19q13.43	3.75 (p=1.6 × 10^{-5})	139 (607)	Chen et al, 2011[4]	Not replicated by Casselbrant et al, 2009[3], or Rye, 2009[5]. No individual SNPs in region show association[4].
Ventilation tube surgery	17q12	2.83 (p=0.0007)	429 (1910)	Casselbrant et al, 2009[3]	Results not significant after Bonferroni adjustment
	10q22.3	*p=0.00181			

*LOD score not stated

Candidate gene studies

Several candidate gene association studies for OM have been reported, with positive findings summarized in table 2. For the most part, candidate genes have been selected by extrapolation of pathways demonstrated to be active in human or animal models of OM, or by extrapolation of findings from other respiratory infectious diseases.

There are several problems with the reported findings. Studies have been small (sometimes very small), making findings liable to false-discovery due to multiple hypothesis testing[9], but also meaning they are under-powered to detect polymorphisms conferring low or moderate risk of disease. Most studies have been a case-control design, and again may be false positives due to confounding by population admixture[10]. Reported significance levels have in general been low, and have not always provided sufficient detail of the rationale for testing, making it difficult to ascertain if reported significance levels should have been subject to Bonferroni adjustment. In common with genetic association studies in other diseases[11], replication of findings has either not occurred, or if it has occurred, has failed to confirm or has even contradicted previous findings.

The exception is the recent Australian study[12] showing association of disease susceptibility with the major A allele at SNP rs12712997 of *FBXO11*, with replication of this finding in an independent cohort. This locus was targeted because of the *Jeff* mouse model[13] (see chapter 4), and SNPs were tagged with a minimal allele frequency (MAF) of ≥0.2 (meaning only 8 SNPs were tested). The population studied was a predominantly rAOM phenotype (consistent with clinical definition of disease), but also included COME. Comparison between pure phenotypes was not possible (due to small numbers), but there was suggestion of greater association with the rAOM phenotype than COME[5]. This

Table 2: Reported significant associations in human candidate gene studies for otitis media (Ca = Cases; Co = Controls; OR = Odds Ratio; RSV = Respiratory Syncytial Virus, RV = Rhinovirus). Adapted from Rye, Bhutta, Cheeseman et al, 2011[14].

Locus	Polymorphism	Population	Phenotype	Sample Size	Reported Results	Reference
FBXO11	rs2134056	N. American	Signs of or treatment for OM[8]	142 families	p=0.02, not replicated in separate cohort[12]	Segade et al. 2006[15]
	rs12712997	Australian	Signs of or treatment for OM	Ca=253; Co=866	$p=6.9\times10^{-6}$	Rye et al. 2010[12]
		Australian	≥3 AOM by age 3 and ventilation tube insertion recommended	434 families	$p=9.0\times10^{-3}$	
	rs330787				p=0.053; not replicated in two other cohorts[12 15]	
SMAD2	rs1792658				p=0.038; not replicated in separate cohort	
SMAD4	rs10502913				p=0.048; not replicated in separate cohort	
TNFα	238 G/G	Dutch	2-3 AOM/yr vs ≥4 AOM/yr	Ca=120; Co=222	OR=2.13, p=0.03	Emonts et al. 2007[16]
	376 G/G				OR=3.1; p=0.05	
	308 G/G	N. American	AOM before age 6 months, ≥3 AOM/6 mo or ≥4 AOM/yr	Ca=192; Co=192	OR=1.6; p=0.05; not replicated in two other cohorts[17 18]	Patel et al. 2006[19]
IL6	174 C/C				OR=1.57; p=0.03, *increased* susceptibility	
		N. American	≥3 AOM/6 mo, ≥4 AOM/yr or ≥6 AOM by age 6	Ca=72; Co=169	p<0.01; *decreased* susceptibility	Revai et al. 2009[17]
		Dutch	≥2 AOM/yr	Ca=347; Co=460	OR>1.45; p=0.02; *decreased* susceptibility	Emonts et al. 2007[16]
IL10	High producer genotype	N. American	AOM episode following rhinovirus	Ca=42; Co=125	OR=0.47; p<0.01, *increased* susceptibility	Alper et al. 2009[20]
			AOM episode following rhinovirus	Ca = 34; Co = 123	OR=1.6; p=0.05. No association at locus in separate cohort[21]	
			AOM episode following RSV	Ca=8; Co=35	OR= 2.9; p=0.05; not replicated in separate cohort[18]	

TLR4	299 G	Dutch	2-3 AOM/yr vs ≥4 AOM/yr	Ca=120; Co=222	OR=0.5; *p*=0.04	Emonts et al. 2007[16]
		Turkish	Tympanosclerosis in those with chronic OM	Ca=50; Co=50	p<0.05	Alpay et al 2010[22]
	rs2770146	N. American	Signs of or treatment for OM[8]	142 families	OR=0.74; *p*=0.04; not replicated in separate cohort	Sale et al. 2011[21]
SCN1B	rs8100085				OR=0.64, *p*=0.01; not replicated in separate cohort	
SFTPD	rs1051246				OR=0.50, *p*=0.04; not replicated in separate cohort	
MUC2	rs7396030				OR=1.57, *p*=0.05; not replicated in separate cohort	
MUC5AC	rs2735733				OR=0.65; *p*=0.002; not replicated in separate cohort	
	rs2075859				OR=0.74; *p*=0.04; not replicated in separate cohort	
	long form	N. American	≥3 AOM/6 mo or OME ≥3 mo duration	≥3 AOM=20; OME=20; Co=40	OME vs ≥3 AOM; *p*=0.025	Ubell et al. 2010[23]
IFNγ	874 T/T or T/A	N. American	OM following RSV infection	Ca=20; Co=57	*p*=0.04	Gentile et al. 2003[18]
IL1α	889 C/T	Finnish	≥6 AOM/yr or ≥10 AOM/lifetime, and without history of allergy	Ca=17; Co=400	*p*=0.03	Joki-Erkkila et al. 2002[24]
CD14	159 C/T	Dutch	2-3 AOM/yr vs ≥4 AOM/yr and aged 1-2 yr	Ca=22; Co=12	*p*=0.004, No association at locus in separate cohort[21]	Wiertsema et al. 2006[25]
MBL2	G5-42	Belgian	≥3 OM/year	Ca=17; Co=172	OR=2.9 not replicated in separate cohort[18 26]	Nuytinck et al. 2006[27]

	non YA/YA haplotype	Dutch	≥2 AOM/yr and aged 1-2	Ca=81; Co=32	p=0.027. No association at locus in separate cohort[21]	Wiertsema et al. 2006[26]
SP-A1	$6A^4/1A^5$ haplotype	N. American	OM in first year of life & asthma	Ca=258; Co=97	OR=0.34, *decreased* susceptibility. No association at locus in separate cohort[21]	Pettigrew et al. 2006[28]
		Finnish	AOM requiring adenoidectomy and/or ventilation tubes	Ca=147; Co=278	p=0.03, *increased* susceptibility. No association at locus in separate cohort[21]	Rämet et al. 2001[29]
TGF-β1	not stated	N. American	OM episode following RSV	Ca=73; Co=31	p<0.05, possibly not replicated in separate cohort[18]	Patel et al. 2006[30]
HLA-A	HLA-A2	Swedish	≥6 AOM/yr	Ca=45; Co=22	p<0.01, not replicated in COME[31]	Kalm et al. 1991[32]
	HLA-A3				p<0.05, not replicated in COME[31]	
PAI-1	rs1799889	Dutch	controls vs ≥4 AOM	Ca=226; Co=463	OR=1.87; p=0.02	Emonts et al. 2007[33]
IGH@	G2m(23) allotype	Swedish	≥6 AOM/yr	Ca=20; Co=47	p=0.05	Prellner et al. 1985[34]

seems counter-intuitive given that *Fbxo11* confers susceptibility to chronic OM in the *Jeff* mouse. However, functional analyses have not been undertaken, and the reported variant could be tagging a hypomorph. The replication for this study was using a longitudinal population cohort, where the otitis media phenotype was less clearly defined.

In the context of the positive association with *FBXO11* from the Western Australia study, the finding by Segade et al[15] of nominal association at this locus is also worth noting. This was at the SNP rs2134056, with a p value of 0.02, but without replication. The findings at *FBXO11* are discussed further in chapter 4.2, together with my data on association testing at this locus.

Genome-wide Association Studies (GWAS)

To date two (unpublished) GWAS studies for OM susceptibility have been performed, but both have been significantly underpowered to detect alleles with moderate risk of disease susceptibility (table 3). The hits from these studies have either fallen below conventional thresholds for significance or have not been replicated, which suggests these results may be false positives.

Subject to funding, my future plans are to undertake a multi-centre international GWAS study on chronic OM, as an expansion of my current candidate gene study in the UK (chapter 4.1).

Table 3: Genome-wide Association Studies for otitis media (both studies unpublished)

Phenotype	Loci most strongly associated	No of individuals tested	Reference	Notes
Signs of or treatment for OM[8]	SNP rs10497394 (intergenic)	381 cases	Sale et al, 2011[35]	Not significant after Bonferroni adjustment
Signs of or treatment for OM (Raine study[12])	*GLANT14*, *CAPN14*, *GLANT13*, *BMP5*, *NELL1*, *TGFβ3*, *PLUNC*	416 cases, 1075 controls	Sarra Jamieson, personal communication	Not replicated in a separate cohort

Limitations of current studies

An issue pertinent to all reported studies is one of phenotype definition. Some studies have extracted genotype information from probands recruited to other epidemiological studies, for example looking at AOM following URTI[17-20], AOM in those part of a vaccine trial[16 25 26 33 36], or rAOM only in children with asthma[28]. There are no heritability estimates for these particular phenotypes, and (aside from the other methodological issues outlined earlier) the results of these studies may not be generalisable. Where recruitment to a study has been specifically for a candidate gene study for OM[16 33], disease definition has not always followed clinical definitions. Some have used disease definitions that are not sufficiently discriminatory, including lifetime history of AOM[24], history of surgery for OM (any phenotype)[29], chronic OM complicated by tympanosclerosis[22], or scoring of a combination of signs of current or prior OM[8 27]. Affectation status has been defined consistent with clinical definitions (chapter 1.1) in only two cohorts, the Australian cohort[12] and a small Swedish cohort[32 34], but these both used retrospective case identification, which introduces a source of error.

Phenotype definition and misclassification is not a trivial matter. There is a balance to be struck between choosing a narrow or broadly defined phenotype[37] [38]. A narrow definition of phenotype (high specificity) reduces clinical variability and ensures heritability of disease is maintained, but sample sizes may be small. A broad definition of phenotype (high sensitivity) suggests wider applicability of results, and engenders larger sample sizes, but at the risk of false positive or false negative findings. The conflict of specificity versus sensitivity in phenotype definition has been statistically modeled. These models demonstrate that, in almost all circumstances, a narrow definition of disease is to be preferred. In a family-based association study, a 5% misclassification of phenotype in a disease with 1% prevalence requires a 39-fold increase in sample size to maintain power[39]. The reduction in power is even more profound in case-control study designs[40].

The Otigen consortium[41] (chapter 4.1) has recently agreed definitions of rAOM and COME for future genetic association studies[42] (definitions correspond to clinically agreed definitions, see table 1 in chapter 1.1). Agreed disease definitions should assist future replication of findings, or meta-analyses, but the consortium recognizes that at present there is significant phenotype heterogeneity between studies. This may limit grouped analysis, although statistical methods have been developed to ascertain whether such grouping is valid[43].

Conclusion

Most previous human studies in otitis media have serious methodological flaws, and so their results may be unreliable. The exception is findings from the Australian study, which suggest disease association with polymorphism at the *FBXO11* locus.

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Chapter 2.1
Otoendoscopy: A new platform to phenotype otitis media in the mouse

The advantages (and limitations) of exploiting mouse models to dissect genetic susceptibility to OM were discussed in chapter 1.3. Large-scale mouse mutagenesis programmes, encompassing both the ENU mutagenesis and gene knockout approach, are currently underway. There is tremendous potential for these to generate further models of OM, but this is limited by the lack of a robust and scalable phenotyping procedure for identifying chronic otitis media in the mouse.

Here I explore the potential of otoendoscopy for phenotyping otitis media in the mouse. I used a population of double mutant mice carrying the *Jeff* mutation along with mutations in either the *Smad2* or *Smad3* locus. The development of a partially penetrant chronic OM in these mice provides the opportunity to assess the reliability and accuracy of oto-endoscopy in a blinded trial. I assess the presence or absence of OM using oto-endoscopy and compare this to a gold standard of histopathology.

Materials and Methods

Animals and endoscopic assessment

As part of the mutagenesis and breeding programme within our group, I obtained a number of mice with heterozygous mutations in *Smad2*, *Smad3* or *p53*, crossed with mice heterozygous for the *Jeff* mutation. Heterozygote or compound heterozygote mice formed the study sample. Mice were culled using an intra-peritoneal injection of pentobarbital and immediately assessed for the presence of otitis media by endoscopy.

I utilised a 0.9mm diameter rigid endoscope, 50mm length with a 5mm to infinity focal length, and 0-55^0 viewing field, connected to a metal halide light source, a miniature

analogue colour video camera, and a display screen (all equipment supplied by Optimax, Northampton, UK, figure 1). The endoscope was inserted through the external auditory canal and navigated until the tympanic membrane was visualised. Still and dynamic images were recorded of both ears.

Rating of endoscopic appearance

Three members of our team independently evaluated anonymised recorded images. Each had previously been shown endoscopic still images of otitis media phenotypes in humans. They were asked to dichotomise the presence or absence of the appearance of OM (yes/no), taking account of tympanic opacity, hyperaemia or tympanic membrane deformation (bulging or retracted). Where there was disagreement, majority opinion was used for final analysis, i.e. if two assessors suggested that OM was present but one did not, the otoscopic rating was coded as "disease present".

Histological assessment

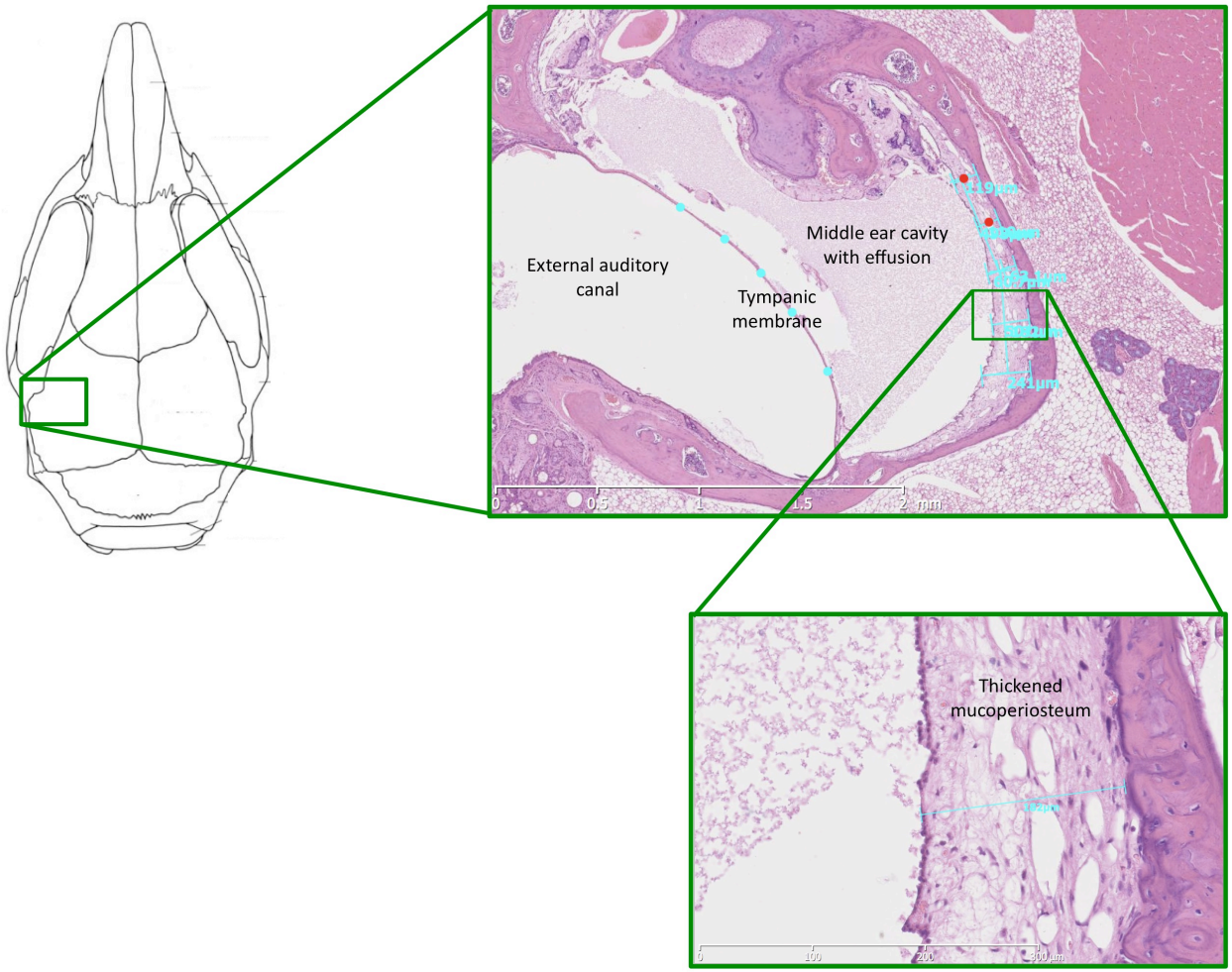
Mouse heads were fixed for 48 hours in 10% buffered formaldehyde, decalcified and embedded in paraffin. Sections of 3μm were obtained, de-paraffinized in xylene substitute and rehydrated via a graded ethanol, before staining with haematoxylin and eosin.

I assessed sections for histological evidence of otitis media, using thickness of the middle ear mucoperiosteum, thickness of the tympanic membrane, and the presence of middle ear effusion as markers of inflammation. On a representative section I measured mucoperiosteal thickness on the medial wall of the middle ear directly opposite the centre of the tympanic membrane (figure 2). I took two further measurements of mucoperiosteal thickness 250 μm and 500 μm anterior to this point, and a further two measurements 250 μm and 500 μm posterior to this point. I took a mean of these

Figure 1: Oto-endsocope, camera and viewing platform



Figure 2: Histological section through an inflamed left middle ear demonstrating the technique used for assessment of mucoperiosteal and tympanic membrane thickness. Measurements of the mucoperiosteum are taken at five points 250μm apart, centered on a point opposite the middle of the tympanic membrane. Measurements of the tympanic membrane are taken at five points equidistant along the length of the membrane. Note presence of middle ear effusion.



measures to give an aggregate measure of mucoperiosteal thickness. For tympanic membrane thickness I used the mean of five measures at equidistant points along the length of the membrane. All sections were also rated for the presence or absence of middle ear effusion. Histological assessments were undertaken blinded to the results of otoscopic assessment.

Ethical approval

Procedures were performed by specific amendments to UK Home Office project licence PPL30/2530 and to my personal licence PIL30/8687.

Results

Animals assessed

The genetic background of the 36 mice (72 ears) used in this study is shown in table 1. These mice were generated as part of our work to investigate the effects of the *Jeff* mutation on TGF-ß signalling[1]. A significant number of these mice develop chronic otitis media in adulthood, but some lines develop only unilateral disease, or no disease. Consequently, these populations are an excellent sample on which to test a blinded phenotyping platform for murine otitis media.

Table 1: Genetic background of mice used in this study

Genetic background	Number
Smad3/+ Jeff/+ compound heterozygote	23
Smad2/+ Jeff/+ compound heterozygote	11
p53+/-	2

Endoscopic assessment

Most ears could be assessed in around 30 seconds. Examples of still images are shown in figure 3, but we found that dynamic video images were easier to evaluate than static images (videos are available with the online edition of the paper generated from this work[2]). In one case, and early in our endeavours, a tympanic membrane was accidently perforated by the end of the endoscope. Data for this ear were disregarded, and so 71 ears contributed to the final analysis. There were no other complications. In conclusion, the procedure of oto-endoscopy in culled mice was straightforward, quick and with a low incidence of complications.

As proof of principle, I have subsequently trialled this technique on twelve wild-type mice anaesthetised by intraperitoneal injection of ketamine/xylazine, Again the procedure was without intra or post-operative complications.

Rating for appearance of otitis media

On oto-endoscopic appearance three assessors coded 31 ears of culled mice as "otitis media present" (OM+), and 40 as "otitis media absent" (OM-). There was 95.3% agreement between assessor ratings, with a free-marginal kappa coefficient of 0.91.

Histological findings

I found a significant difference in the mean mucoperiosteal thickness of mice rated as OM+ versus OM- (figure 4, $p<0.001$, Kruskall-Wallis test), and also in the mean tympanic membrane thickness (figure 5, $p<0.001$, Kruskall-Wallis test). I undertook linear regression analysis of mean tympanic membrane thickening and mean mucoperiosteal thickening and found that, as expected, the two variables were related (figure 6, $r^2=0.486$, $p<0.001$).

Figure 3: Oto-endoscopic appearances of the mouse tympanic membrane, with and without middle ear effusions due to otitis media. A. Normal. B. Cloudy appearance. On histology a granulocyte deplete effusion was found. C. Opaque appearance. On histology a granulocyte rich effusion was found. m = manubrium of malleus. a = tympanic annulus. Video images can be found in the online version of the paper generated from this work[2].

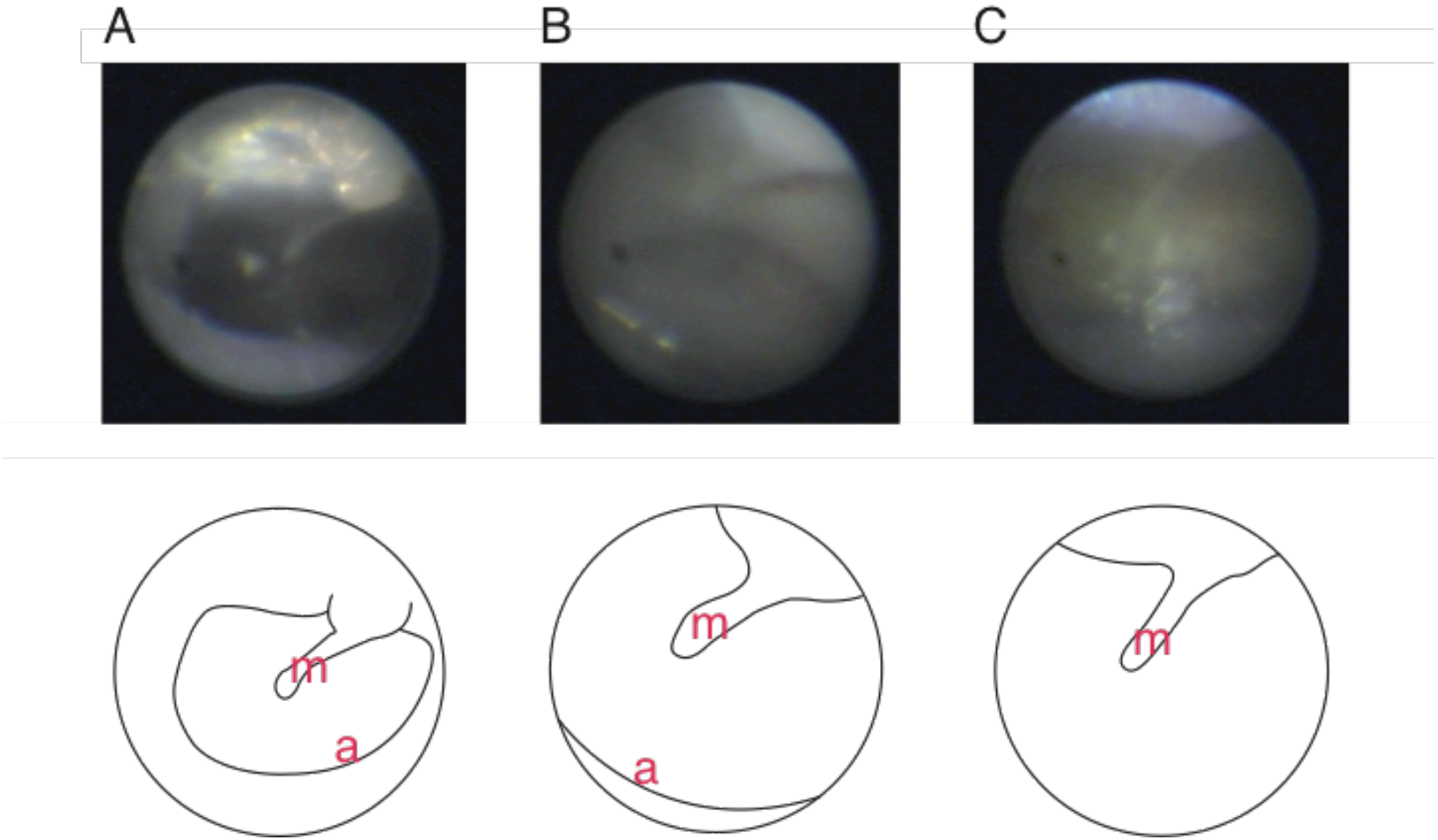


Figure 4: Box and whisker plot comparing mean mucoperiosteal thickness in ears rated as OM+ on otoscopy versus those rated as OM-. p value is for Kruskall-Wallis test.

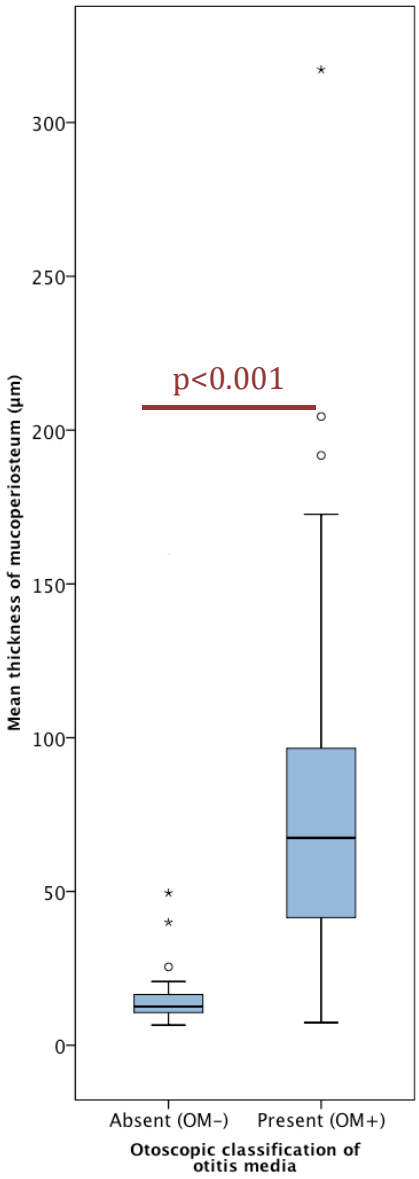


Figure 5: Box and whisker plot comparing mean tympanic membrane thickness in ears rated as OM+ on otoscopy versus those rated as OM-. p value is for Kruskall-Wallis test.

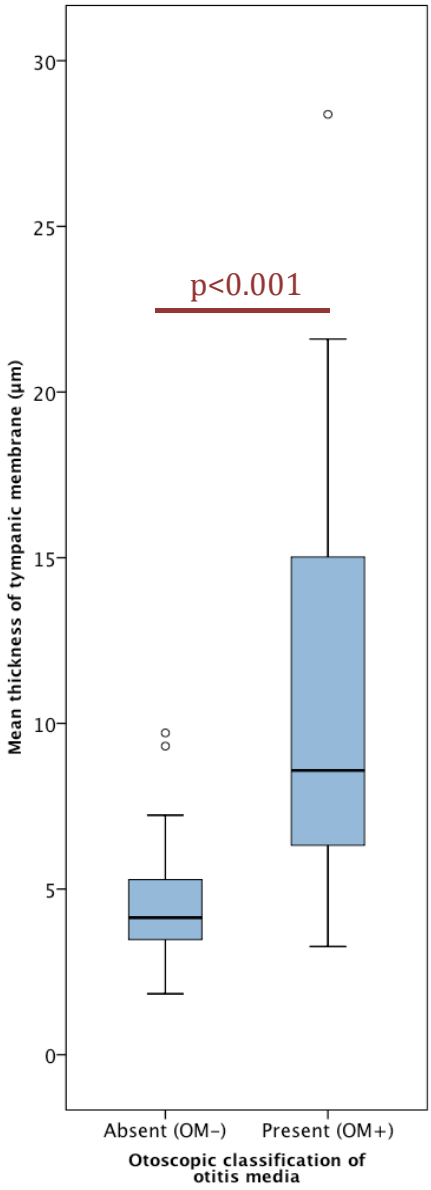
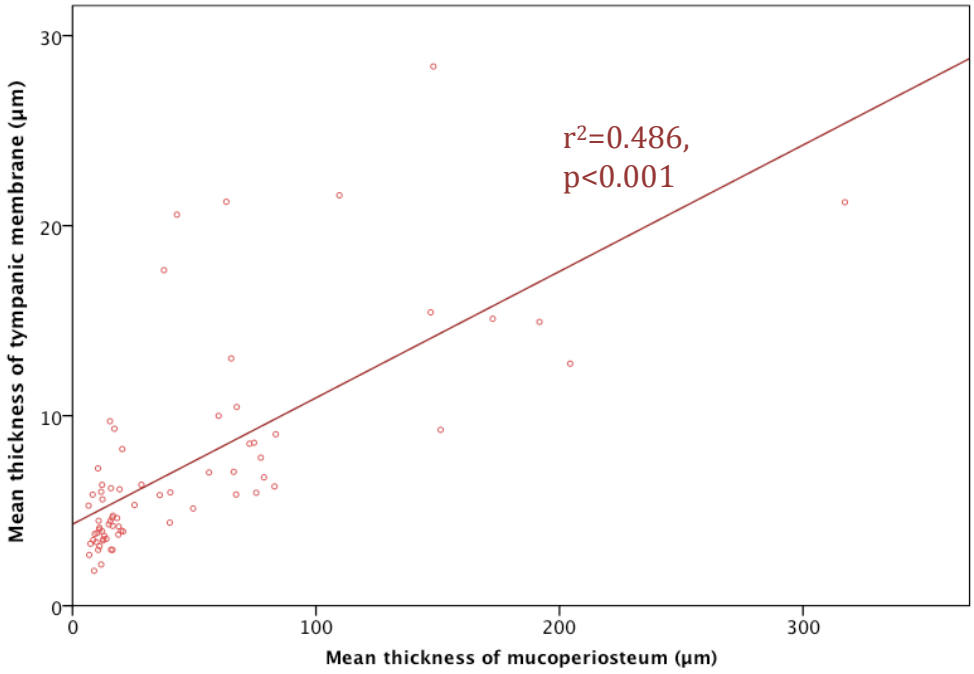


Figure 6: Scatter plot and linear regression analysis of mean tympanic thickness to mean mucoperisoteal thickness.



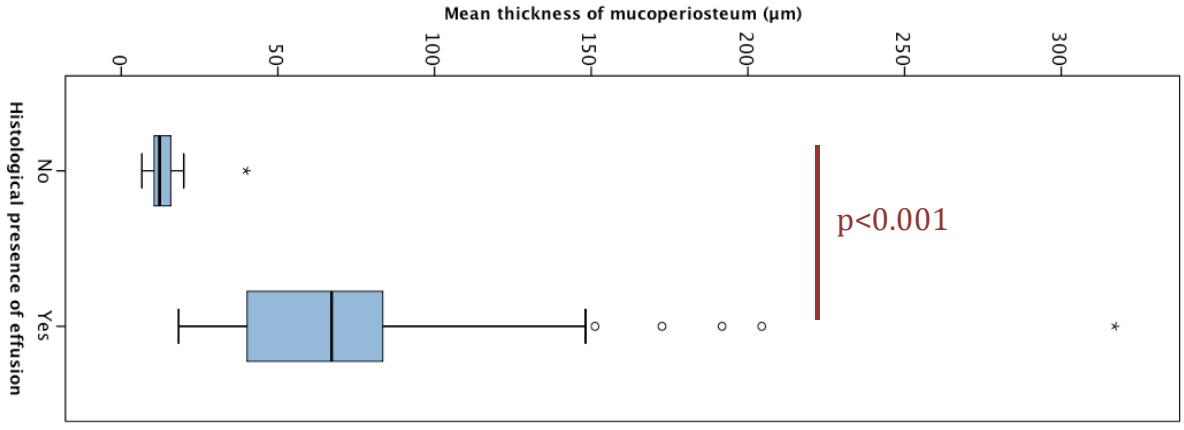
I also compared OM+ versus OM- ratings from oto-endoscopy with histological evidence of the presence of middle ear effusion. Overall 33/71 (46.5%) of ears had histological evidence of middle ear effusion, although I did not feel it was possible to accurately quantify volume due to processing artefact, and it is possible that some small volume effusions have been missed. When tabulating the presence of effusion with otoscopic rating of otitis media (table 2), I found that the two variables were significantly associated (chi-squared $p<0.001$). When calculating the validity of otoscopic rating as a marker of middle ear effusion, I calculated 88% sensitivity and 97% specificity, with a positive predictive value of 97% and a negative predictive value of 90%. This gives an accuracy of 93%. Only one ear that was rated as OM+ did not have a middle ear effusion, and I surmised that this case may have been misclassified because of significant TM thickening which could mimic opacity due to middle ear effusion. However the data for this case showed the TM was not thickened.

Table 2
Cross-tabulation of oto-endoscopic rating of otitis media and histological presence or absence of effusion. Sensitivity is 29/(29+4) = 88%. Specificity is 37/(37+1) = 97%. Positive predictive value is 29/(29+1) = 97%. Negative predictive value is 37/(37+1) = 90%.

		Effusion		
		Present	Absent	
Oto-endoscopic rating	OM+	29	1	30
	OM-	4	37	41
		33	38	71

Finally, I tested the relationship between mucoperiosteal thickness and the presence or absence of middle ear effusion. I found that, unsurprisingly, the mucoperiosteum was on average much thicker in ears with an effusion than those without (figure 7, $p<0.001$, Kruskall-Wallis test). A mucoperiosteal thickening greater than 20μm was associated with a middle ear effusion in 97% of cases (31/32), whereas a mucoperiosteal thickening less than 20μm was associated with effusion in only 5% (2/40).

Figure 7: Box and whisker plot comparing mean mucoperiosteal thickness in ears with histological presence of effusion to those without. p value is for Kruskall-Wallis test.



Discussion

Current screening methods for otitis media

Present screening tests for otitis media in the mouse are reliant upon audiological assessment, on the premise that inflammatory middle ear effusion will cause a conductive hearing loss. Mice are screened for elevated hearing thresholds based upon click-box assessment of the Preyer reflex, a startle response to a loud high frequency noise (90dB, 20KHz, 30cm from the pinna)[3]. However, although the absence of Preyer reflex may be a good marker of the often severe hearing loss associated with cochlear deformity or malfunction, because this is a supra-threshold response the test often fails to detect mild or moderate hearing impairment[4]. Otitis media rarely causes thresholds raised greater than 40dB in the human, or in the mouse. Consequently, the Preyer reflex may still manifest in the presence of a middle ear effusion, and so may be insensitive to the presence of OM. An alternative screen is to use Auditory Brainstem Response (ABR) to characterise mild or moderate degrees of hearing loss, and although this has been utilised in auditory phenotyping pipelines, the technique is time-consuming, requires general anaesthesia, and may still miss less severe cases of OM.

Other methods exist for diagnosing OM in children, and could potentially be extended to the mouse. Abnormal tympanometry[5] has a sensitivity of 94% and specificity of 80% for detection of a middle ear effusion, although performance varies depending upon the parameters classed as abnormal[6]. Tympanometry measurements in the mouse have been attempted, under general anaesthesia. Zheng et al reported variation in tympanometry between 61 different strains of mice[7], and correlated the tympanogram to histological evidence of middle ear effusion in one strain: the LP/J mouse (which has propensity to OM). They reported greater compliance of the tympanic membrane in ears with OM than those without[7], which is the opposite of what would be expected from

human data[6]. An abnormal type B or type C trace was found in six other mouse strains, but no attempt was made to correlate this to the presence or absence of otitis media. In 9 (15%) strains the tympanogram was inconsistent and could not be classified. In the mouse, there is as yet insufficient evidence that tympanometry can be a reliable and valid screen for otitis media. The technique can also be difficult to perform, owing to small volumes both of the external and middle ear.

Visualisation of the tympanic membrane is another method of diagnosing OM. In children with non-suppurative effusions the handheld pneumatic otoscope has a sensitivity of 93.8% and a specificity of 80.5% for detecting that effusion[6]. Visualisation with a microscope gives sensitivity of 92.9-100% and specificity of 61.5%-93%[8-10], and video oto-endoscopy gives sensitivity of 97.8% and specificity of 100%[11]. Suppurative OM is more difficult to diagnose[12], and visual diagnosis of AOM is more variable, but an abnormal colour of the tympanic membrane has a 94-98.9% sensitivity and 73.5-79.8% specificity for the presence of a middle ear effusion[13].

Endoscopic assessment reliability and validity

I have undertaken studies to explore visualisation of the tympanic membrane as a valid method of diagnosing OM in the mouse. Recent advances in engineering have enabled endoscopes with a diameter smaller than the mouse external auditory canal. Mouse oto-endoscopy has been described before[7], but not validated. I have shown that this is reliable and valid, with a sensitivity and specificity comparable to that in diagnosis of human OM. Specifically, independent rating of the middle ear for evidence of middle ear inflammation showed excellent inter-observer reliability, and was strongly correlated to the presence of a thickened middle ear mucoperiosteum with 93% accuracy in predicting middle ear effusion. We must be cautious to apply Bayes theorem: in my study the prior probability of effusion in the population tested was high. If oto-

endoscopy is used to screen a population of mice with a low prior probability of OM, the positive predictive value of this technique will be lower. However, uncertainties can of course be verified by histology.

Sometimes middle ear mucosal inflammation can occur in the absence of significant effusion (chronic silent otitis media[14], chapter 1.1), and arguably is a more valid measure of OM. Here I used mucoperiosteal thickness as a proxy of inflammatory mucosal proliferation and oedema, and showed that oto-endoscopy is still a valid way of diagnosing such thickening. In any case, mucosal thickening less than 20μm was rarely (5%) associated with effusion, but thickening greater than 20μm was almost always (97%) associated with effusion.

Potential Applications

Oto-endoscopy has significant potential for screening for OM in the mouse. Given its rapidity and reliability it could be integrated into current pipelines utilised for cohorts of mouse kockouts[15] or ENU mutants generated as part of international programmes. Oto-endoscopy could be applied at various junctures in the pipeline when the mouse is under anaesthetic, or the technique could be applied as a terminal procedure. If a relevant genetic model is identified, the technique could be used to gauge features such as age of onset, or to assess the effect of therapeutic interventions.

Conclusion

In conclusion, I have shown the oto-endoscope to be a valid method for identifying mouse models of OM. With the increasing use of the mouse in the study of middle ear inflanmation[16 17], oto-endoscopy should prove a valuable addition to the mouse toolkit.

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Chapter 2.2
Mining the Down Syndrome mouse library for chronic otitis media

Down syndrome (DS) is the most common human chromosomal abnormality, occurring in 1/750 live births, and due to partial or total trisomy HSA21. There are variable clinical features including skull and midface malformation, cardiac anomalies, hypotonia, delayed growth, developmental disorders, thyroid disease, obesity, immunodeficiency and also chronic otitis media.

Most children with Down syndrome develop OME, which is often persistent (COME), and leads to a hearing loss that can compound learning difficulties[1 2]. A longitudinal study of 79 children with DS reported that OME was diagnosed in 93% at age one, falling to 68% by age five[3]. Similar findings have been reported in other series [4-11]. COME may persist, and at least 10-20% of adults with DS have a conductive hearing loss (often undiagnosed)[12 13]. There may also be subclinical middle ear inflammation[14]. Several authorities advise routine and regular audiological screening of children with DS[15 16].

Standard treatments for COME are not as effective in children with DS. Grommets (ventilation tubes) in this population may not significantly improve hearing, have little if any effect on disease recurrence, and are not infrequently complicated by infection[17-19]. Guidance in the UK suggests hearing aids as the preferred treatment for COME in DS[20], and some advocate bone-anchored hearing aids[21 22].

An understanding of the pathogenic mechanisms leading to chronic middle ear inflammation in Down syndrome offers potential for targeted and more effective therapy of COME in this population. DS is presumed to arise as a consequence of either an extra copy of protein-coding sequences that are dosage sensitive, or an extra copy of nonprotein-coding sequences that are regulatory or otherwise functional[23-27]. Importantly, discovery of a locus or region that underlies chronic OM in DS may, in addition, give insights into mechanisms of chronic OM in

non-syndromic disease. Specifically trisomy in DS could mimic hypermorphic polymorphisms in non-syndromic children with COME. Given the difficulties inherent in interrogating the genetic basis of non-syndromic chronic OM (chapters 1.3 and 1.4), understanding DS pathobiology could be very fruitful.

HSA21 is the smallest human autosome, with only around 300 genes, and so only a handful of genes are likely to play a role in the phenotypic manifestations of DS. This makes exploration of genotype-phenotype correlation feasible, and several methods have been used for such exploration[27 28].

Individuals with partial trisomy of HSA21 offer serendipitous clues to identify critical regions for DS phenotypes. Initial analyses of such individuals suggested that all or most phenotypic manifestations of DS were due to a short critical region[29 30], but more recent analysis refutes this, and suggests that loci determining the DS phenotype are scattered across HSA21[31]. This analysis has not specifically been undertaken for the OM phenotype, but given the rarity of partial trisomy HSA21, and the lack of a large database of genotype-phenotype correlation for aneuploidies, such analysis is at present difficult.

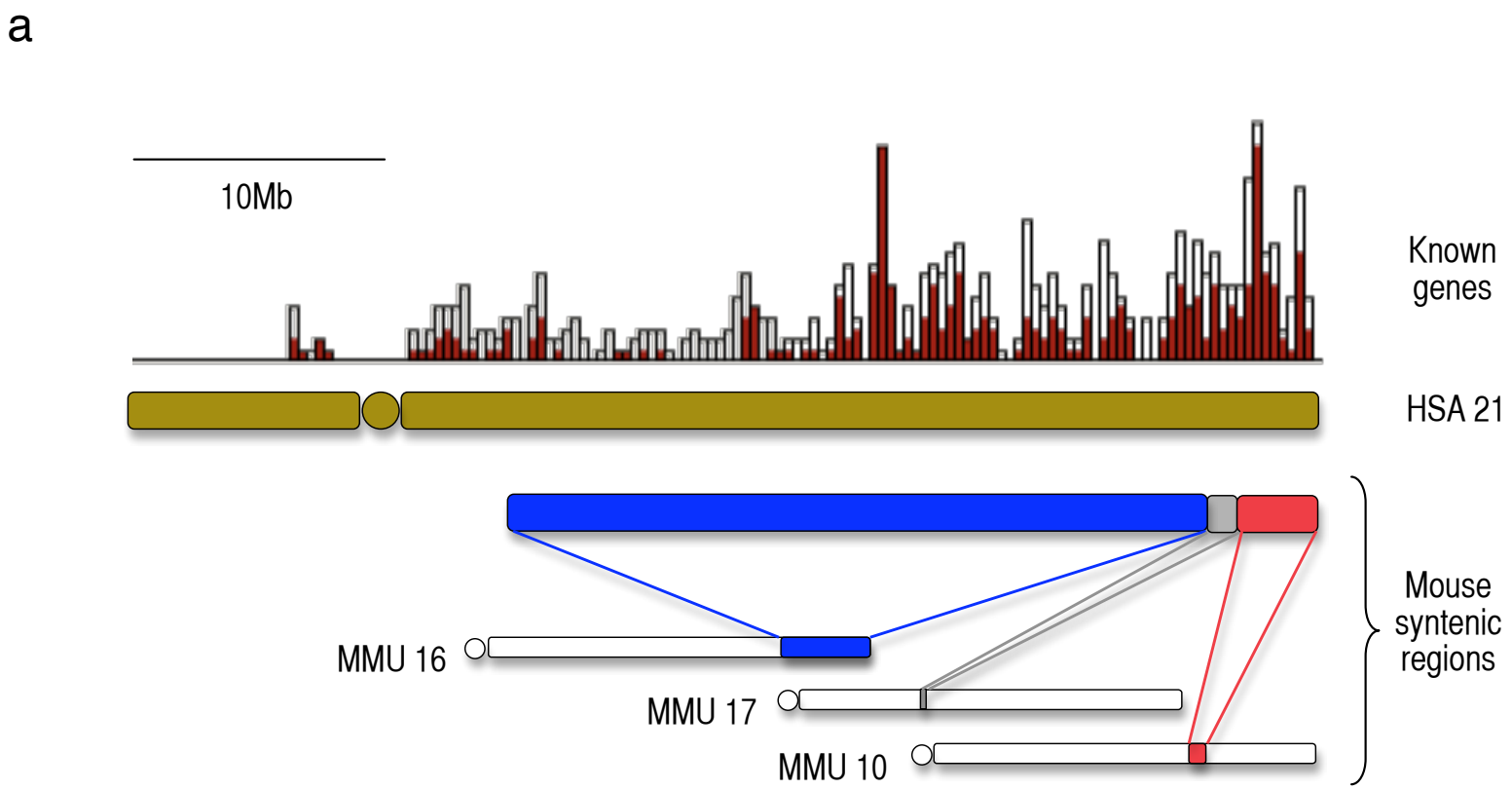
Readouts of the transcriptome of HSA21 have also been used to define critical genes. Data from human[32] and mouse[31] show that only around a third of the genes on HSA21 are transcribed at more that the theoretical 1.5 fold increase, suggesting that the majority of trisomic loci are compensated for by negative feedback that modulates transcriptional activity or mRNA stability. Although this does appear to narrow the possibilities, the method is not entirely reliable. Data of the transcriptome from mouse and human differ, as do data from different tissue types[31]. In addition transcription is only a component of gene expression[33], and so small changes in transcription could have large phenotypic effects for some loci, and large changes in

transcription have little or no phenotypic effect for other loci.

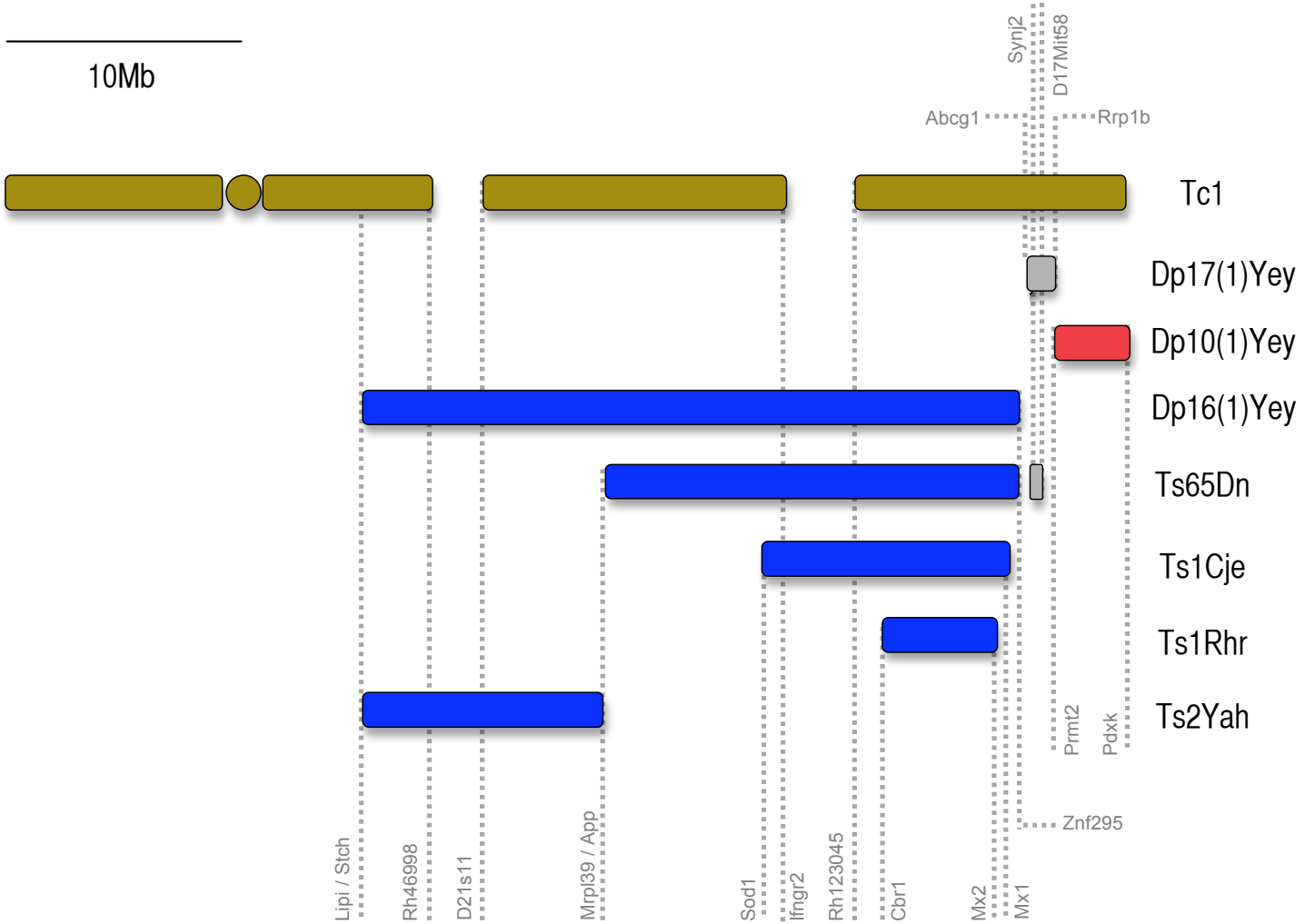
Given the advantages from the ability to genetically engineer mice, and easy access to tissues of interest (chapter 1.3), there has been increasing interest in using mouse models to unravel pathobiology in Down Syndrome. The syntenic regions to HSA21 are scattered across the mouse genome on MMU16, MMU17, and MMU10, which makes genetically modelling more difficult[34]. There is however a large and growing library of mouse models of Down Syndrome. The Ts65Dn mouse[35], Ts1Cje mouse[36], Ts2Yah mouse[37], Ts1Rhr[38] mouse, Dp16(1)Yey mouse[39], Dp10(1)Yey mouse[40], and Dp17(1)Yey mouse[40] each carry a partial trisomy for the homologous genes of HSA21. Together these models cover almost all regions triplicated in DS (figure 1). In addition, the Tc1 mouse is a transchromosomic model, carrying most of HSA21 in addition to the full complement of mouse chromosomes[41].

This mouse library has been mined to identify loci responsible for some of the phenotypic manifestations of DS, but has never been systematically interrogated for the presence of otitis media. The Ts65Dn mouse[35] (which carries a 15.6 megabase segmental trisomy of MMU16) has been reported to develop spontaneous chronic otitis media[42], and recently the Tc1 mouse has been reported to *not* develop chronic OM[43]. There are no reports of the presence or absence of OM in the other murine models of DS. In this study I systematically screened the murine library of DS models to identify histological presence of chronic OM, with the aim of narrowing and defining a critical region or set of loci underlying this phenotype.

Figure 1
Map of (a) murine synteny to human chromosome 21 and of (b) trisomic regions in mouse models of Down Syndrome used in this study (showing genes at chromosome breakpoints). The sytenic regions to HSA21 are on MMU16 (76Mb-98Mb), MMU17 (31Mb-32Mb), and MMU10 (78Mb-76Mb). The Tc1 mouse carries an incomplete HSA21 in addition to the normal mouse genome, whereas all other models are trisomic for murine DNA. Known genes are derived from the Ensembl web browser[44].



b


10Mb
Synj2
D17Mit58
Abcg1
Rrp1b
Tc1
Dp17(1)Yey
Dp10(1)Yey
Dp16(1)Yey
Ts65Dn
Ts1Cje
Ts1Rhr
Ts2Yah
Prmt2
Pdxk
Znf295
Lipi / Stch
Rh46998
D21s11
Mrpl39 / App
Sod1
Ifngr2
Rh123045
Cbr1
Mx2
Mx1

Materials & Methods

I obtained cadaveric specimens of almost the entire reported library of Down syndrome mouse models from a number of sources. Specimens of the Ts65Dn mouse, the Ts1Cje mouse, the Ts2Yah mouse, and the Ts1Rhr mouse were obtained in collaboration with Professor Yann Herault at the Institut Clinique de la Souris, Strasbourg. Specimens of the Dp16(1)Yey mouse, the Dp10(1)Yey mouse, and the Dp17(1)Yey mouse were obtained in collaboration with Professor Yuejin Yu at Roswell Park, New York. Specimens of the Tc1 mouse, and additional specimens of the Ts65Dn mouse, were obtained in collaboration with Professor Elizabeth Fisher at University College London. The number and age of mice supplied depended upon local availability. All mice were supplied with WT littermate controls, and all were genotyped at source. All mice had been kept in high health status facilities, but microbes had been isolated from the colonies from which they were derived (table 1).

Table 1: Microbes isolated at source from colonies that included the mice used in this study.

Source	Mouse lines	Microbes isolated from colony
Institut Clinique de la Souris	Ts65Dn, Ts1Cje, Ts2Yah, Ts1Rhr	Norovirus, streptococci, helicobacter, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Entamoeba muris*
Roswell Park	Dp10(1)Yey, Dp16(1)Yey, Dp17(1)Yey	Norovirus, helicobacter
University College London	Tc1, Ts65Dn	*Helicobacter hepaticus*, *Tritrichomonas*

Mouse heads were fixed in 10% neutral buffered formalin, decalcified, and embedded in paraffin. Serial cross sections of 3μm depth were stained with haematoxylin and eosin, and the status of the middle ear assessed by an experienced pathologist (Michael Cheeseman).

Results

A total of 532 ears from eight mouse lines were analysed (table 2). Statistically, only the Dp16(1)Yey mouse line reliably developed chronic OM (19 ears in 10 of 13 mice). Histology revealed thickened polypoidal mucoperiosteum with neutrophil and macrophage infiltration, and an effusion of variable cellularity (figure 2). In this line disease was present from the age of 2 months. Some of the older (>7 months old) WT littermate controls for this line also developed chronic OM (7 ears in 4 mice).

OM in other models was not present, or was rarely present. A unilateral small volume exudate and inflammatory polyp formation was noted in one of the Ts65Dn mice from University College London (figure 3). There was also mild unilateral oedema in the middle ear mucosa of two Ts65Dn mice from Institut Clinique de la Souris, but without a leucocyte infiltrate (not shown). There was bilateral otitis media in one Ts1Cje mouse, with infiltration by macrophages and neutrophils (figure 4).

Discussion

Otitis media in Down syndrome

The propensity to chronic middle ear inflammation in Down syndrome is not understood. A popular explanation is that an anatomical anomaly of the Eustachian tube accompanies the midface and skull base defects in this syndrome[45], and that consequently there is an inability to ventilate the middle ear, which leads to effusion[46]. There is little evidence that ventilatory dysfunction of the Eustachian tube is the primary mechanism leading to COME in non-syndromic children[47 48] (see also chapter 1.3), but in Down syndrome the Eustachian tube is

Table 2: Nature of mice used in this study, their source, and presence or absence of otitis media (OM) on histology (denominator is for number of ears). The p-values relate to Fisher's exact test for the difference in OM prevalence in mutants versus wild type (WT) littermates. UCL = University College London, ICS = Institut Clinique de la Souris.

Mouse line	Background strain	Trisomic segment	Source	Age range (months)	OM in mutant	OM in WT controls	p-value
Dp17(1)Yey	B6/J; 129Sv	MMU17 *Abcg1-Rrp1b*	Roswell Park	2-3	0/28	1/32	1
Dp10(1)Yey	B6J; 129Sv	MMU10 *Prmt2-Pdxk*		2-4	0/28	0/28	1
Dp16(1)Yey	B6J; 129Sv	MMU16 *Lipi - Znf295*		2-15	19/26	7/24	0.004
Ts1Cje	B6/C3B	MMU16 *Sod1- Znf295*	ICS	2	2/28	0/28	0.491
Ts1RhR	B6/N10	MMU16 *Cbr1 – Mx2*		2	0/36	0/36	1
Ts2Yah	B6/N10	MMU16 *Stch - App*		2	0/34	0/34	1
Ts65Dn	B6/C3B	MMU16 *Mrpl39 - Znf295* & MMU17 *Synj2-D17Mit58*		2	2/40*	0/40	0.494
Tc1	129sv ; B6	HSA21 **except** *Rh46998-D21s11* & *Ifngr2-Rh120345*	UCL	11-16	1/12**	0/22	0.353
				2-3	0/30	0/26	1

*Two ears in this cohort of Ts65Dn mice showed mild oedema of the middle ear mucoperiosteum, but without leucocyte infiltration.
** One ear showed a small middle ear exudate with micropolyp formation

Figure 2
Histological section through the middle ear of a Dp16(1)Yey mouse affected by chronic otitis media (H&E stain). There is polypoidal hyperplasia of the mucoperiosteum (m) with an effusion (e) infiltrated by neutrophils and macrophages. This was noted in 19/26 (73%) of Dp16(1)Yey mice examined.

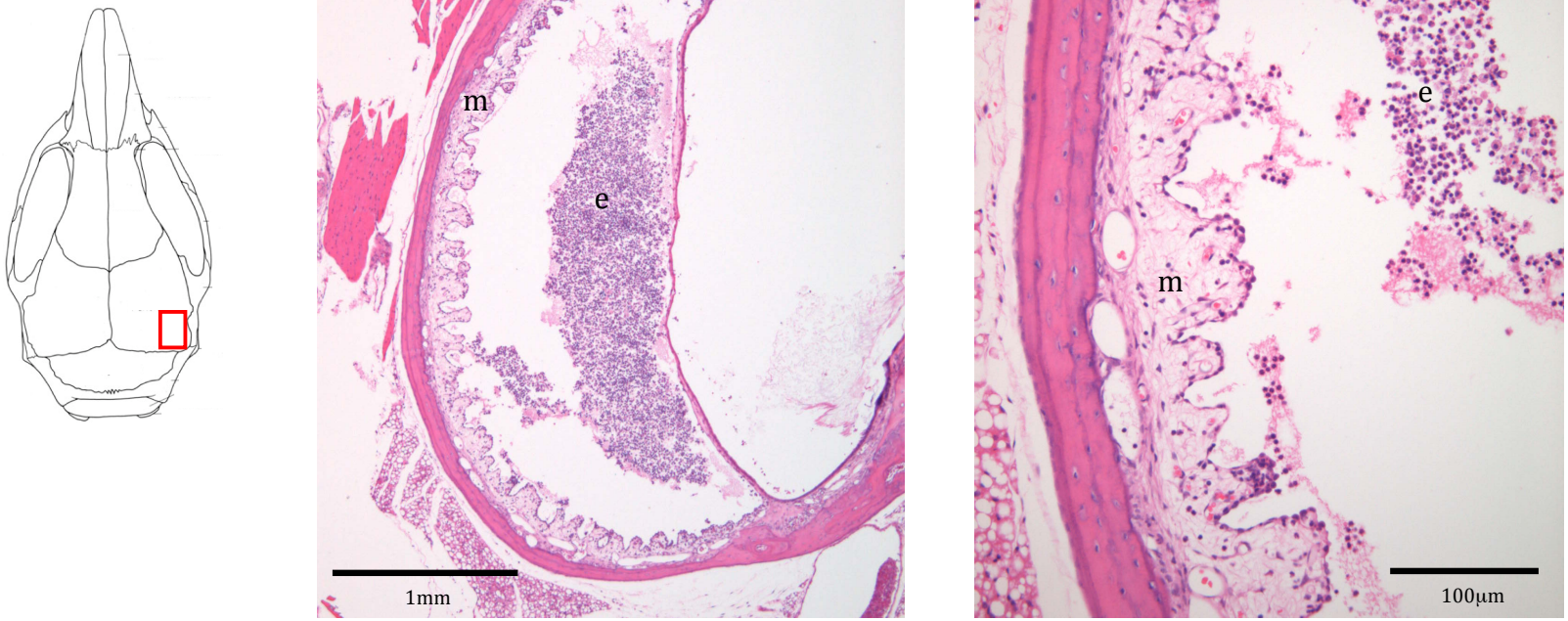
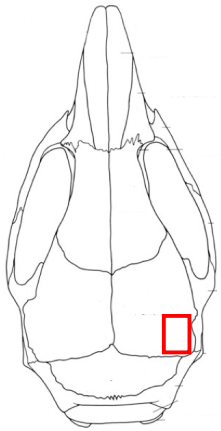
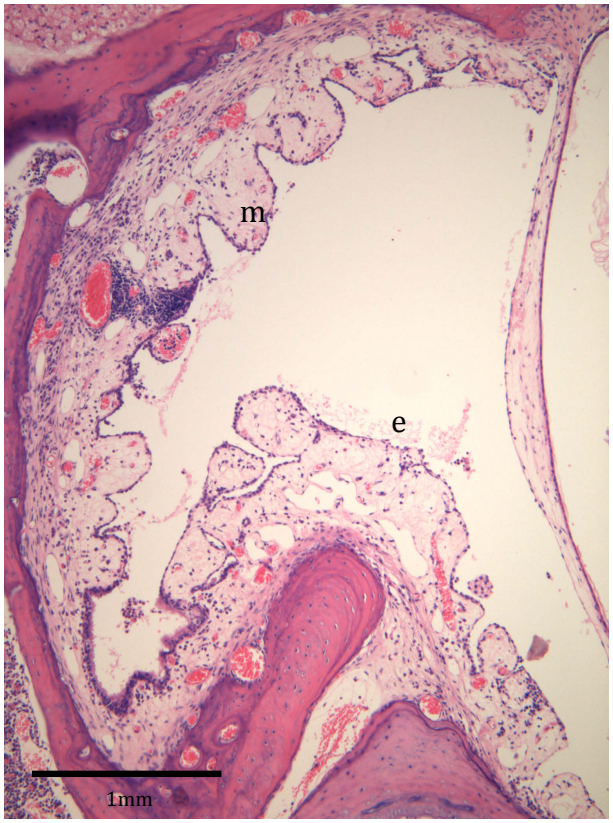
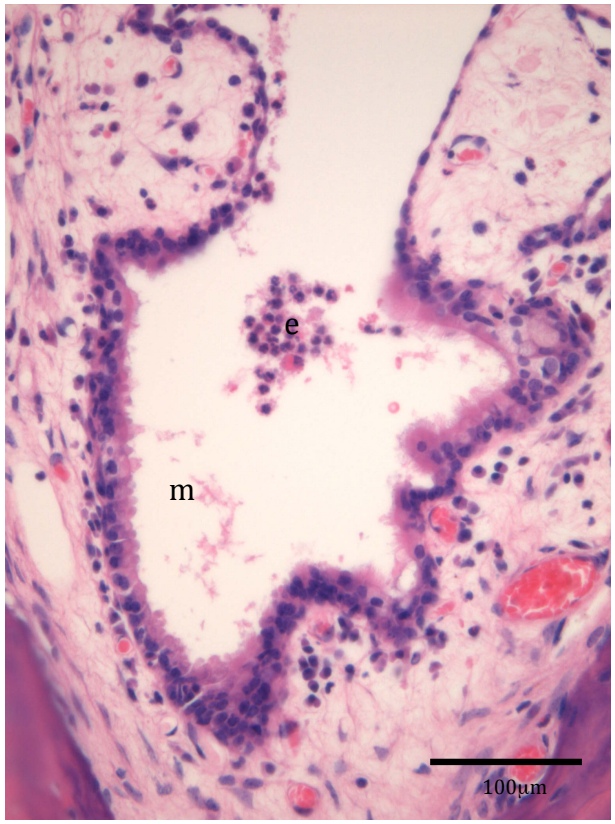


Figure 3
Histological section through the middle ear of a Ts65Dn mouse affected by chronic otitis media (H&E stain). There is polypoidal hyperplasia of the mucoperiosteum (m) with an effusion (e) infiltrated by neutrophils. Only one mouse was affected in this way, although two mice from a separate colony has mild oedema of the mucoperiosteum.



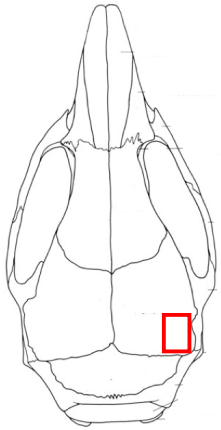


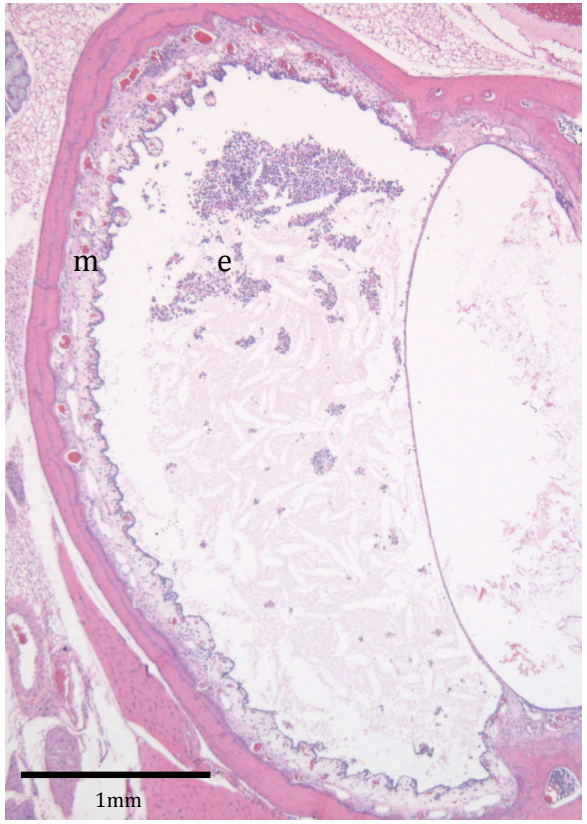
x 40



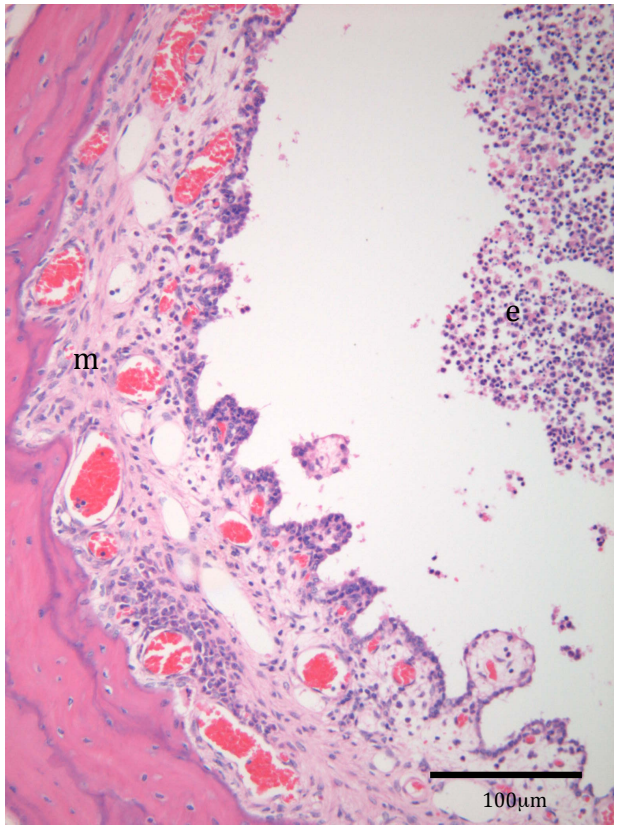
x 200

Figure 4
Histological section through the middle ear of a Ts1Cje mouse affected by chronic otitis media (H&E stain). There is polypoidal hyperplasia of the mucoperiosteum (m) with an effusion (e) infiltrated by neutrophils and macrophages. This was noted to be present (bilaterally) in only one of the 14 Ts1Cje mice examined.





x 40



x 200

angulated differently[49], and perhaps contains less cartilage[50] when compared to the normal population. However, the only published study of Eustachian tube patency in DS did not demonstrate this tube to be obstructed[51].

An alternative explanation may lie in defective mucosal immune response. Indeed, immune dysfunction is well recognised in Down Syndrome[52], manifesting as infection, haematological malignancy, or autoimmune disease. Children with Down syndrome have more frequent and more severe lower respiratory tract infections[53], with one in ten suffering more than three such infections in a year[54-57]. No defects in mucociliary function have been demonstrated[58 59], but immunological dysfunction in T-cells[52] (secondary to thymic dysfunction[60]) has been shown. The molecular basis of this anomaly is not understood, but presumably relates to disrupted thymic ontogenesis, which may be caused by over-expression of genes such as *RUNX1*, *AIRE*, *IFNGR2*, *ITGB2* or *UBASH3A*[61].

Chromosomal localisation of OM

In this study I screened the Down syndrome mouse library to identify a critical region for propensity to chronic OM. The Dp16(1)Yey mouse, which is trisomic for the entire region of MMU16 syntenic to HSA21, was found to reliably develop chronic OM. Here, OM was of early onset (by 2 months) and with penetrance of 0.73 (19/26 ears). Some of the WT littermate controls of the Dp16(1)Yey mice also developed OM, but only when older than 7 months. Chronic OM is common in older laboratory mice[62] (although the reasons are unclear), and so the pathology in older WT mice is not unexpected.

Of note, there was no evidence of OM in the Dp17(1)Yey or Dp10(1)Yey models, suggesting that only genes found on MMU16 are responsible for the OM phenotype. There was also no OM in the Tc1 mouse, even though this model carries many of the genes found in trisomy in DS (in the

form of mouse and human chromosomes). The Tc1 mouse has been reported to lack many other elements of the DS phenotype, and this has been hypothesised to perhaps be due to species differences in genetic regulatory elements, or because of genetic mosaicism in this model[28].

My findings strongly implicate a region of MMU16 to underlie the chronic OM phenotype. Empirically this region could be narrowed further by analysis of the Ts65Dn, Ts1Cje, Ts1Rhr, and Ts2Yah mice which each carry shorter segment trisomy of MMU16 (figure 1).

Localisation of OM within MMU16

Han et al reported a highly penetrant chronic OM in the Ts65Dn model (in 11/15 mice), with a variable cellular infiltrate and effusion, and with audiological evidence of disease onset by the age of two months[42]. Here I found definite OM in only one ear of the 52 Ts65Dn ears screened, although there was mild mucosal oedema in two other Ts65Dn mouse ears (which may be indicative of early or mild inflammation). The variable penetrance of OM in the Ts65Dn mouse suggests that development of chronic OM in DS is not solely determined by a critical genetic region in trisomy, but also factors important modifiers of phenotype, whether genetic or environmental.

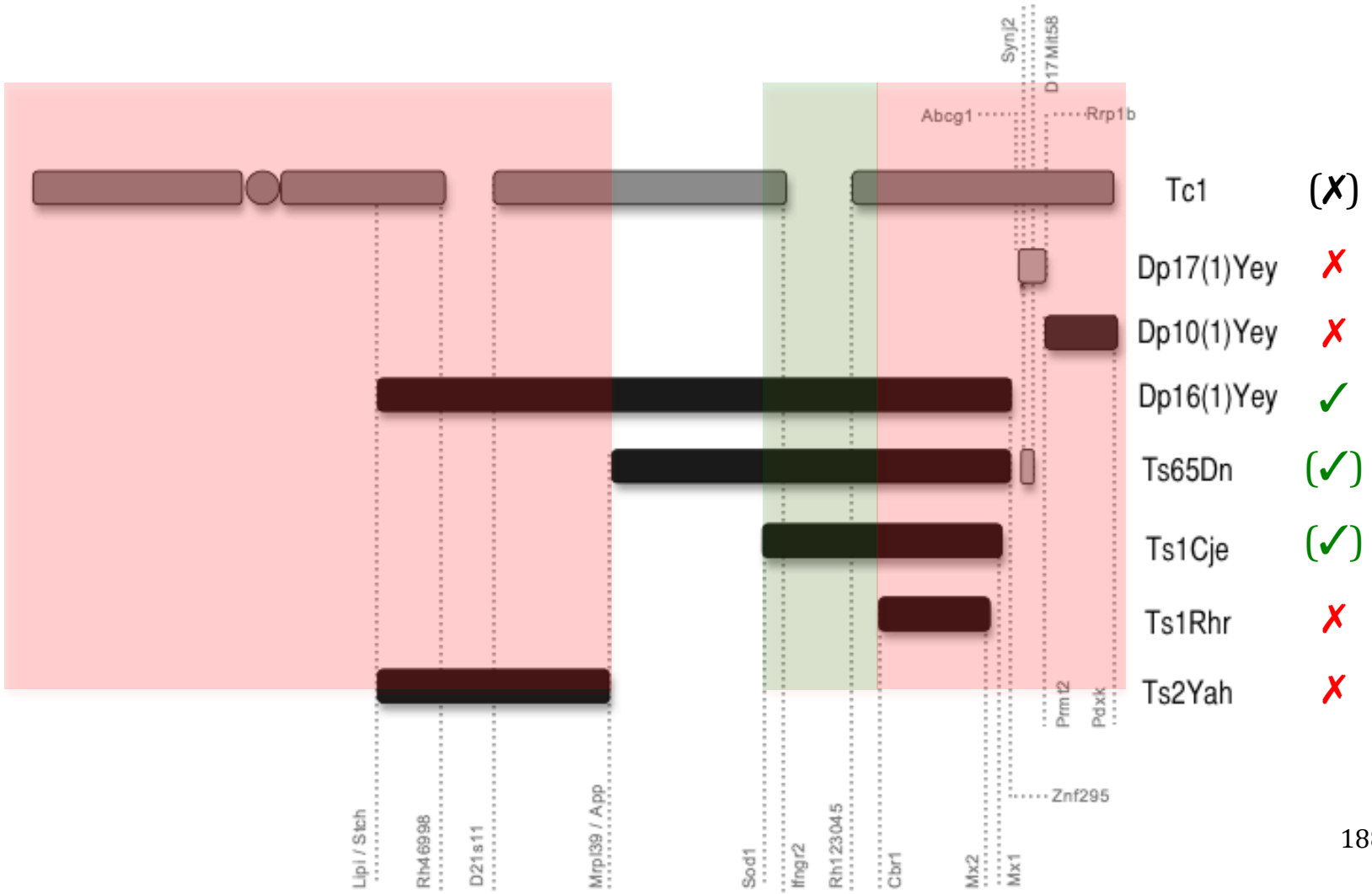
Allelic or structural genomic variants could determine a critical threshold for phenotype manifestation, whether within the hypothesised gene or locus critical for the OM phenotype, or in a disomic or trisomic gene in epistasis with this region. The Ts65Dn mice in my study were maintained on a B6/C3B background through breeding with local stock. The Ts65Dn mice reported by Han et al[42] were similarly maintained by local breeding on a B6/C3B background. However, allelic or structural genomic variations in C57BL/6 can occur between laboratories[63], and so presumed isogenicity of these mice is not assured. Even small genomic variations can play a pivotal role in phenotype penetrance in DS[24]. For example allelic variation in *CRELD1* on

HSA3 modifies the risk of cardiac malformation[64] in children with DS. In the mouse, breeding the Ts1RhR mouse model onto a mixed B6;C3H;129 background as opposed to pure B6, leads to loss of the Down Syndrome craniofacial phenotype[65]. It is possible that the Ts65Dn mice used in this study carry an unidentified mutation that rescues the chronic OM phenotype. However, it seems unlikely that the Ts65Dn mice both from Institut Clinique de la Souris and from University College London would carry such a mutation.

A more likely explanation is that an environmental antigen, such as a bacterial pathogen, triggers the onset of OM. Variation in housing conditions could lead to differences in environmental exposure and so explain variable phenotype penetrance. Han et al[42] reported coagulase-negative *Staphylococci*, *Burkholderia cepacia* and *Bordetella avium* in middle ear effusions of their Ts65Dn mice, but also *Klebsiella oxytoca*, *Streptococcus viridans* and *Bordetella avium* in wild type controls. I did not culture bacteria from effusions in this study (only fixed tissues were available), but because a number of microbes have been isolated at the source of these animals (table 2), it is possible that one or more of these microbes, or other unidentified microbes, plays a pivotal role in disease onset.

It is difficult to draw firm conclusions from the data on other partial trisomy mouse models. I found no OM in the Ts1Rhr or Ts2Yah mice, but one Ts1Cje mouse developed severe bilateral OM. Consequently, it is tempting to suggest that the critical region for developing OM is in the region that is trisomic in the Ts1Cje mouse but not Ts1Rhr or Ts2Yah (*Sod1-Cbr1*, figure 5), but this conclusion could be unsound given that it is based upon disease presence in only one animal.

Figure 5
The lack of otitis media in cohorts of Dp17(1)Yey, Dp 10(1)Yey, Ts1Rhr, and Ts2Yah mice suggests that the regions that are trisomic in these mice (shaded red) do not underlie the chronic OM phenotype. This narrows the candidate region for OM to *App-Cbr1* on MMU16. The presence of OM in the Dp16(1)Yey mouse (high penetrance), and in the Ts65Dn and Ts1Cje mice (low penetrance), suggests that the critical region may actually be *Sod1-Cbr1* (shaded green). The findings in the Tc1 mouse are less assured (see text).



Further defining the critical region using gene expression and function data

My data, and those of Han et al, propose that the critical region for developing chronic OM is in genes that are found in trisomy in both the Dp16(1)Yey[39] mouse and in the Ts65Dn mouse[66 67]. One way to narrow down this list to a critical trisomic locus/region is by using data on gene expression and gene function. However, this approach is speculative and must be undertaken with caution; because it is possible that regions other than those identified are involved, because it is possible that genes in the identified region have as yet unrecognized roles in inflammation, and because gene expression comprises more than transcriptional up or down-regulation.

Interrogation of the Ensembl database[44] for loci trisomic in Dp16(1)Yey and Ts65Dn recovers 92 known genes and open reading frames (table 3). Published expression analyses from the Ts65Dn mouse[31] show that a significant minority of trisomic genes are not transcriptionally upregulated, presumably due to regulatory or feedback pathways. Genes that are not significantly transcriptionally upregulated (which here I define as transcript fold change <1.2) will be unlikely to contribute to phenotypic manifestations. Excluding such loci from our list reduces the number of candidate genes to 82 (table 3).

We can also look at the known or hypothesized function of genes in this list. The majority of genes seem to encode structural or metabolic proteins, and although some genes may have unknown pleoitropic functions, on principles of ontology and physiology it seems unlikely they have adopted a central role in inflammation. For other genes, their function is unknown. Conversely, genes known to be involved in pharyngeal or leucocyte embryology, or those involved in inflammatory signaling are good candidates for causing chronic middle ear inflammation. On this premise, eight genes are highlighted (table 3): *JAM2*, *RUNX1, MX1, RIPK4*, *IFNAR2, IL10RB, IFNAR1,* and *IFNGR2*.

Table 3: Potential candidate genes underlying the chronic OM phenotype in Down Syndrome. The list is derived from genes that are trisomic in the Ts65Dn mouse and in the Dp16(1) Yey mouse, and that have human homologues (data from Ensembl[44]). Relative transcript levels refer to those in adult lung of Ts65Dn mice[31] (which is close in structure and function to middle ear mucosa), where transcript levels are <1.2 fold (shaded grey), the gene is not considered to be a candidate. Where gene function (data from GeneCards v3[68]) is known and does not involve leucocyte or pharyngeal arch biology (shaded grey), genes are also not considered candidates. Consequently candidate genes are those that satisfy the criteria of being present in Ts65Dn and Dp16(1)Yey mice, present in man, transcriptionally upregulated >1.2 fold in Ts65Dn mice, and having a function that may link to inflammation pathways (or function that is unknown). Genes between *SOD1* and *MX1* are also found in the Ts1Cje mouse.

Gene	Relative Transcript Level	Function	Candidate
MRPL39	-	Mitochondrial ribosomal protein	
JAM2	-	Immune cell trafficking	✓
ATP5J	-	Catalyst for ATP synthesis	
GABPA	1.24	Activation of cytochrome oxidase in mitochondria	
APP	1.33	Receptor for neurite growth and neuronal adhesion	
CYYR1	-	Unknown	✓
ADAMTS1	1.09	Metalloprotease. Possibly associated with inflammation	
ADAMTS5	1.23	Cleaves aggrecan. Involved in cartilage turnover	
N6AMT1	1.25	Methyltransferase	
LTN1	1.40	E3 ubiquitin ligase	
RWDD2B	1.39	Unknown	✓
USP16	1.50	Deubiquitinating enzyme	
CCT8	1.2	Molecular chaperone. Assists protein folding	
C21orf7 / ORF63	0.98	Possible role in TGF-β signaling transduction	
BACH1	1.41	Transcription factor	
GRIK1	-	Unknown	✓
CLDN17	-	Component of tight junctions	
CLDN8	1.33	Component of tight junctions	
KRTAP cluster	-	Components of hair shaft	
TIAM1	1.10	Connects extracellular signals to cytoskeletal activities	
SOD1	1.37	Binds ions and destroys free radicals	
SCAF4	1.19	Splicing factor	
HUNK	1.57	Tumour associated kinase	
MIS18A	1.53	Unknown	✓
MRAP	-	Modulates signaling of melanocortin receptors	
URB1	-	Unknown	✓
C21orf63	1.37	Unknown	✓
C21orf59	1.28	Unknown	✓
SYNJ1	1.22	Regulates synaptic transmission and membrane trafficking	
GCFC1	1.54	Probable regulation of transcription	
C21orf62	-	Unknown	✓
OLIG2	-	Regulator of ventral neuroectodermal embryogenesis	
OLIG1	-	Promotes maturation of oligodendrocytes	
IFNAR2	1.26	With IFNAR1 forms receptor for interferons	✓
IL10RB	1.54	Receptor subunit for activation of class 2 cytokines	✓
IFNAR1	1.32	With IFNAR2 forms receptor for interferons	✓
IFNGR2	1.37	Receptor subunit for interferon gamma	✓
TMEM50B	1.43	Brain development	

DNAJC28	-	May act in protein folding or as a chaperone	
GART	1.46	Purine biosynthesis	
SON	1.77	mRNA splicing cofactor	
DONSON	1.35	Unknown	✓
CRYZL1	1.40	Unknown	✓
ITSN1	1.42	Coordinates endocytic membrane traffic	
ATP5O	1.40	ATP synthesis	
MRPS6	1.28	Mitochondrial protein synthesis	
SLC5A3	0.91	Prevents intracellular accumulation of myo-inositol	
KCNE2	1.32	Component of voltage-gated potassium channels	
FAM165B	1.39	Unknown	✓
KCNE1	-	Component of voltage-gated potassium channels	
RCAN1	0.79	Inhibits calcineurin-dependent signaling pathways	
CLIC6	-	Probable chloride ion channel	
RUNX1	1.31	Functions in haematopoeisis, interacts with TGF-β	✓
SETD4	1.32	Unknown	✓
CBR1	1.44	Ubiquitous reductase enzyme	
RIMKLBP1	-	Unknown	✓
MEMO1P1	-	Unknown	✓
CBR3	1.59	Reductase enzyme	
DOPEY2	1.41	Possibly involved in protein trafficking	
MORC3	1.36	Nuclear protein of unknown function	
CHAF1B	1.54	Chromatin assembly	
CLDN14	-	Component of tight junctions	
SIM2	-	Transcription factor in embryogenesis	
HLCS	1.48	Metabolic enzyme catalysing binding of biotin	
DSCR6	-	Unknown	✓
PIGP	1.46	Glycosylphosphatidylinositol biosynthesis	
TTC3	1.34	E3 ubiquitin-protein ligase for Akt	
DSCR3	1.43	Unknown	✓
DYRK1A	1.15	Probable regulation of cell proliferation	
KCNJ6	-	Potassium channel	
KCNJ15	1.19	Potassium channel	
ERG	0.91	Transcriptional regulator	
ETS2	1.05	Transcriptional regulator	
PSMG1	1.36	Chaperone protein for assembly of the 20S proteasome	
BRWD1	-	May facilitate formation of multiprotein complexes	
HMGN1	1.30	Maintains chromatin conformation	
WRB	1.39	Unknown	✓
LCA5L	1.43	Unknown	✓
SH3BGR	-	Unknown	✓
B3GALT5	-	Membrane glycoprotein with diverse enzymatic functions	
IGSF5	1.63	Cell adhesion molecule	
PCP4	1.53	Unknown	✓
DSCAM	-	Promotes repulsion of neuronal processes	
BACE2	1.72	Proteolytic processing of the amyloid precursor protein	
FAM3B	1.70	Induces apoptosis of pancreatic alpha and beta cells	
MX2	1.57	Interferon α inducible GTPase, function in man unknown	✓
MX1	2.03	Interferon inducible protein, protection against viruses	✓
TMPRSS2	1.70	Serine protease with diverse functions	
RIPK4	1.40	Serine/threonine protein kinase. May activate NF-κB.	✓
PRDM15	0.97	May be involved in transcriptional regulation	
C2CD2	1.23	Unknown	✓
ZNF295	0.90	Transcription repressor	

JAM2 is a member of the junctional adhesion molecules[69], which in turn are part of the immunoglobulin superfamily. *JAM2* is upregulated in arterioles within or around foci of lymphocytic inflammation in diverse tissue types[70], and plays a critical role in T-lymphocyte migration across endothelium[71]. Experiments in sertoli cells suggest that TGIF is a transcriptional promoter of *JAM2,* but this activity can be repressed by SMAD proteins[72]. These interactions could be important, given the role of TGF-β signaling in some mouse models of chronic otitis media (chapter 1.3). The role of JAM2 in otitis media has not yet been investigated.

RUNX1 plays a role in embryonic and adult haematopoesis, including lymphoid differentiation[73]. SNPs in *RUNX1* or binding sites of RUNX1 possibly increase susceptibility to the chronic autoimmune inflammatory diseases rheumatoid arthritis[74 75], psoriasis[76], and systemic lupus erythematosis[77]. RUNX1 translocations can be found in malignant haemopathies[78]. There is no known role in otitis media.

MX1 protects against infection from a wide range of viruses, although mechanisms are unclear[79]. Empirically over-expression of *MX1* should not interfere with this function, and so seems unlikely to factor prominently in chronic OM.

RIPK4 displays dose-dependent activation of NF-κB signaling pathways[80 81], but little is known of underlying mechanisms[82]. Given that over-expression of NF-κB is probably responsible for chronic OM in some mouse models (chapter 1.3), this could be the mechanism of chronic OM in DS.

The *IFNAR2*, *IL10RB*, *IFNAR1,* and *IFNGR2* cluster constitute the class II cytokine receptor family, which plays a central role in regulating inflammatory response in a diverse range of tissues.

These highly conserved genes share sequence similarity[83] and tertiary protein structure[84]. IFNAR2, IFNAR1, and IFNGR2 bind the interferons IFN-λ and IFN-γ. *IFN-γ* transcripts are not significantly upregulated in the *Jeff* and *Junbo* mouse models of chronic OM[85], and although IFN-γ may be found in human middle ear effusion[86-88], it does not seem to play a prominent role. In contrast *Il10rb* transcripts are 9-36 fold upregulated in the *Jeff* and *Junbo* mouse models[85], and transcripts of the main ligand for this receptor, IL10, are 21-55 fold upregulated, although IL10RB can also bind IL22, IL19, IL20, IL24, IL26, IL28 and IL29[84]. Together with TGF-β, IL10 is a key regulator of T-cell response[89], and initially downregulates inflammation but becomes pro-inflammatory if persistently expressed[90]. Levels of IL10 in human middle ear effusions correlate to duration of disease[91]. Transgenic over-expression of IL10 in mouse lung invokes inflammation and mucus metaplasia[92] (although this response has not been assessed in the middle ear). In mouse models of acute OM, the role of IL10 is less clear: knockout mice show reduced inflammatory response to bacterial inoculation of the middle ear[93], but inoculation of IL10 into the middle ear reduces inflammatory response to LPS[94].

It is also interesting to note that the class II cytokine receptor family, including the *Il10rb* gene, is trisomic in the Ts1Cje mouse, but not in the Ts2Yah or Ts1Rhr mouse. This could explain my findings of OM in the Ts1Cje mouse, but not in Ts2Yah or Ts1Rhr.

A model for pathogenesis of chronic OM in Down Syndrome

The data presented here suggest that aetiology of chronic OM in Down Syndrome is complex. Given the variable penetrance of disease in inbred mice, it seems likely that there is an environmental trigger causing onset of middle ear inflammation. In mice I have been unable to ascertain if a particular microbe may be responsible. In human disease, children with DS are known to have immune dysfunction, which presumably leads to increased susceptibility to

AOM, but there are no data on whether the pathogens responsible for AOM in Down Syndrome are the same as those in the non-Down syndrome population.

Once inflammation has been triggered, certain loci subsequently drive persistence of middle ear inflammation. It is unclear what these loci are, but I believe the most likely are *JAM2*, *RIPK4*, or *IL10RB* - with the latter the most interesting candidate.

Further work in this area could aim to purposefully expose models of DS to potential pathogens, with the hope that this would trigger chronic disease, and so give a highly penetrant phenotype. If this strategy was successful, a conclusive test that a single gene or region underlies propensity to chronic OM would be to demonstrate such pathology in a mouse engineered to be trisomic only for this region, or to show loss of phenotype in a trisomic mouse engineered to restore diploidy only of this hypothesised critical region[28]. Any critical region so identified could then be tested for polymorphism in DNA databases of non-syndromic human chronic OM (see section 4), or gene expression analyses could be undertaken from effusions in children with Down Syndrome.

Conclusion

Extrapolation of results from screening the library of mouse models of DS supports a model of chronic OM pathogenesis requiring a trigger for disease onset, with subsequent over-expression of a single or handful of trisomic genes causing persistence of inflammation. It is most likely that the locus (or loci) of interest are in the region of *Sod1-Cbr1* on MMU16.

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Chapter 3.1
Myringotomy alleviates inflammation and cellular hypoxia in the *Junbo* mouse

The *Jeff*[1] [2] and *Junbo*[3] mouse models can be exploited to explore the pathobiology of chronic otitis media. In particular we have become interested in the role of cellular hypoxia in the inflamed middle ear.

Ontogeny and physiology of hypoxia signalling

Oxygen has been a major driver of evolution[4]. A proteo-bacterial species survived engulfment by a unicellular organism 1.7 billion years ago and so created the mitochondrion, offering energy-rich aerobic respiration. Subsequent oxygenation of the Earth's environment favoured descendants of this organism, and led to the evolution of aerobic eukaryotic multicellular organisms. With increasing complexity and consequent energy demands, most extant multicellular organisms have become wholly reliant on aerobic respiration, including the development of mechanisms to deliver oxygen to tissues through a circulatory system. In contrast prokaryotes have retained metabolic adaptability, and many can survive or even thrive in an anaerobic environment[5] (amongst the otopathogens, *S. pneumoniae* and NT*Hi*, but not *M. catarrhalis*, are facultative anaerobes).

Inflammation increases cellular energy demands, but at the same time distances inflammatory cells from blood vessels due to cellular oedema and hyperplasia[6], and so regional hypoxia is a common feature of inflamed microenvironments[7] [8]. Transcriptional regulation in eukaryotes can compensate for the hypoxic environment, and allow continued immune function[9] and some restoration of tissue homeostasis[10]. In metazoans[11] the central transcriptional regulator of hypoxia response is hypoxia-inducible factor (HIF)[12], which regulates a plethora of genes[13] involved in transcription, biosynthesis, extracellular matrix formation, glycolysis, energy production, cell survival, and cell stress. However, persistent hypoxia signaling is maladaptive and contributes to continuing inflammation and tissue damage[14], and so offers a potential therapeutic

target in chronic inflammatory disease[15].

Hypoxia in the chronically inflamed middle ear

In a recent paper I co-authored[16] (I contributed to the intellectual rather than the experimental content of this paper), we have demonstrated cellular hypoxia in the chronically inflamed middle ear environment in our *Junbo and Jeff mouse* models (Appendix). Pimonidazole (PIMO) is a marker of tissues with an oxygen tension below 10 torr (≈1.5% O_2)[17]. Systemic administration of PIMO to the *Jeff* and the *Junbo* models labels both the inflamed middle ear mucosa and leucocytes within the inflammatory exudate. Fluorescence-activated cell sorting (FACS) confirms hypoxia in viable and apoptotic polymorphonuclear cells in the effusion, and transcriptome analysis of these cells reveals upregulation of a number of HIF responsive genes, notably in the signaling protein Vascular Endothelial Growth Factor (VEGF, 128-145 fold upregulation), a key executor of HIF response. Treatment with the experimental VEGF receptor inhibitors BAY 43-9006, SU-11248 and PTK787/ZK 222584 improves inflammation and moderates hearing loss in both Junbo and Jeff mice. Similar results are found on treatment with 17-DMAG, an inhibitor of HSP90, a chaperone for HIF-1α.

As discussed in chapter 1.3, pertubations in either (or both) TGF-β and NFκB pathways are implicated in chronic OM in the *Jeff* and *Junbo* models. Both TGF-β[18] [19] and (especially) NFκB[20] pathways cross-talk with hypoxia pathways, and so may contribute to upregulation of VEGF or other hypoxia responsive genes. Regardless, our findings evidence cellular hypoxia to be a common finding in the chronically inflamed middle ear, and suggest antagonising hypoxia signaling as a novel mechanism to moderate inflammation in this environment.

A number of other lines of evidence confirm hypoxia pathways are activated in otitis media. In animal models of AOM, injection of endotoxin or NT*Hi* into the middle ear upregulates *Vegf* and its receptors[21-23], and injection of recombinant VEGF leads to inflammation, neovascularisaton, and middle ear effusion[23 24]. Although there is no evidence of generalized hypoxia within middle ear effusions in children with COME[25 26], the finding of elevated VEGF levels in such effusions does suggest cellular hypoxia[22 27]. The promoter region of the *MUC5AC* gene, which encodes one of the major mucins found in COME[28], contains a highly conserved HIF binding site (together with a SMAD4 binding site), and experimental disruption of these binding sites abolishes stimulated mucin secretion[29].

In CSOM less is known of pathobiology, but microbial culture of the effusion[30] commonly isolates obligate anaerobes such as *Bacteroides* and *Fusobacterium*. The additional isolation of the species *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which have traditionally been classified as aerobes, may suggest that hypoxia cannot be widespread in CSOM, but recent work[31 32] shows that both these species are metabolically able to adapt to hypoxic environments (in fact *P. aeruginosa* preferentially migrates to deep within the bronchial mucus of patients with cystic fibrosis, which is markedly hypoxic[33]).

Middle ear ventilation and hypoxia signaling

Our demonstration that cellular hypoxia is a driver of chronic otitis media in the *Jeff* and *Junbo* mouse models may have implications for surgical therapy of chronic otitis media, and in particular the insertion of ventilation tubes (grommets). Grommets are the only treatment known to reliably lead to resolution of effusion in COME[34] (whilst they remain patent). Grommets may also reduce the risk of developing subsequent cholesteatoma[35].

The therapeutic effect of grommet insertion is usually assumed to be rheological, whereby creation of a vent reduces fluid inertia, allowing effusion to be cleared down the Eustachian tube by the ciliary apparatus of the protympanum. Aspiration of effusion at the time of operation is not necessary[36], but myringotomy alone does not lead to long-term resolution of effusion[37]. This suggests that grommet may moderate the inflammatory process in addition to, or in place of, any rheological effect that may occur (although there is limited evidence that grommets effect the long-term outcome of COME). The mechanism of such moderation is not known. There is no convincing evidence of modulation of bioelectric properties of the middle ear mucosa as a result of altered oxygenation[38 39]. There is no evidence that grommets alter ventilatory function of the Eustachian tube in the short[40], medium[41-43], or long-term[44].

Here, and in a preliminary study, I use the highly penetrant *Junbo* mouse model to investigate a novel hypothesis that the long-term beneficial effect of middle ear ventilation may be through alleviation of cellular hypoxia in the chronically inflamed middle ear. Specifically, I demonstrate that operative myringotomy (with aspiration of effusion) leads to a variable improvement in mucosal inflammation, and loss of PIMO labelling.

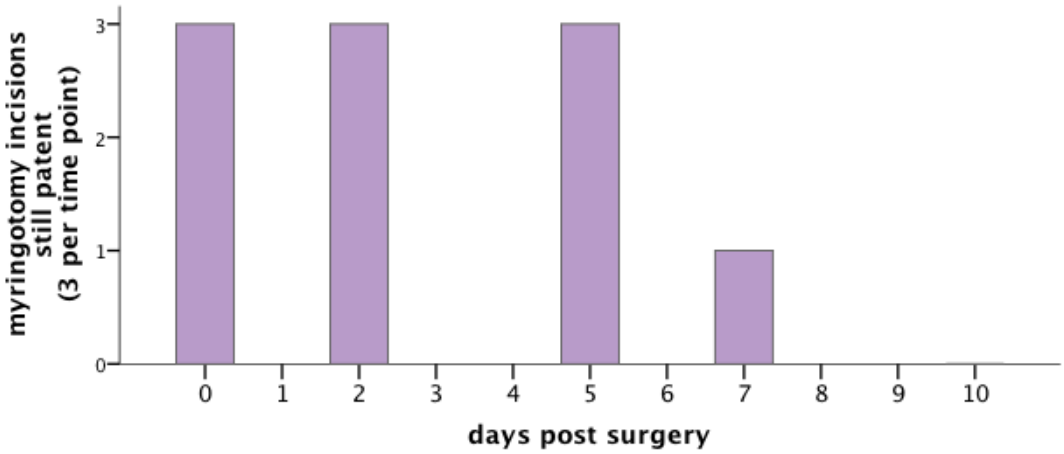
Materials and Methods

Establishing duration of myringotomy patency in the mouse

Surgical myringotomy has not previously been reported in the mouse. I performed myringotomy upon wild-type (WT) C3H/HeH mice (aged 6-9 weeks, mean 49 days) to establish normal duration of patency. Mice were anaesthetized by intraperitoneal

injection of 10mg/kg xylazine and 100mg/kg ketamine. The posterior pars tensa of the left ear was incised using a disposable myringotome (Exmoor plastics, UK) under direct vision and using an operating microscope. Anaesthetic was reversed with 5mg/kg atipamezole hydrochloride. Subsequently mice were culled with an overdose of intraperitoneal anaesthesia at time points of 3, 5, 7, and 10 days post-operatively (three mice for each time point). Ears were microscopically examined, and I established that myringotomy was reliably patent at up to five days post-op (figure 1)

Figure 1: Macroscopic patency of myringotomy at up to 10 days after incision. Data are for the wild-type C3H/HeM mouse strain, with three mice used for each time-point. These data suggest patency is reliably maintained for 5-6 days after surgery.



Myringotomy and assessment of Junbo mice

I subsequently performed myringotomy and aspiration of effusion on the left ear of 17 *Junbo* mice and 11 WT littermate controls (aged 6-10 weeks, mean 54 days), using the same protocol. The right ear was not operated to act as a control. All mice were assessed for visible evidence of middle ear effusion prior to operation. Five days later, under terminal intraperitoneal anaesthesia, mice were labeled by intraperitoneal

injection of 60 mg/kg Pimonidazole (PIMO) (Hypoxyprobe, HPI Inc) dissolved in 100ml of PBS. Mouse heads were fixed for 48 hours in 10% neutral buffered formalin, decalcified with Immunocal (Decal Corp) for 72 hours, and embedded in paraffin. 3μm serial cross sections were stained with haematoxylin and eosin or immunostained for PIMO. Mucoperiosteal thickness was measured as the mean of five equidistant points on a representative 1cm section of the medial wall of the middle ear (opposite the centre of the tympanic membrane, see chapter 2.1). Histology was compared between the operated left ear and the unoperated right ear.

Ethical approval

Procedures were performed under specific amendments to UK Home Office project licence PPL30/2530 and to my personal licence PIL30/8687.

Results

There were no significant complications or identifiable adverse effects from myringotomy. In WT mice myringotomy did not induce mucosal inflammation (mean mucoperiosteal thickness in operated ear 12.9μm, unoperated ear 14.0μm, p=0.51 paired samples t-test). In *Junbo* mice, one mouse had pre-operative evidence of OM in only one ear, and so was excluded, leaving 16 *Junbo* mice for subsequent analyses.

On qualitative histological assessment there was a variable response to surgery. In most (12/16) cases effusion resolved and there was reduction in inflammation, sometimes markedly so (figure 2). In a significant minority (4/16) of cases there was little obvious effect upon effusion or inflammation. Due to variable success of immunohistochemistry

we were unable to visualize anti-PIMO labeling in many of our sections, but in the few mice where this was successful we demonstrated loss of staining (suggesting resolution of hypoxia) in those cases where inflammation had improved in response to myringotomy (figure 3).

I found that data for mucoperiosteal thickness were normally distributed (Kolmogorov-Smirnov test). Mean mucoperiosteal thickness in the (right) unoperated ear was 104.6μm and in the (left) operated ear was 84.7μm (figure 4). The difference in mucoperiosteal thickness between the operated and unoperated ear was significant at $p<0.05$ on paired-samples T-test. The difference in mucoperiosteal thickness between operated (32.2μm) and unoperated (34.3μm) ears in WT mice was not significant.

Discussion

This study demonstrates that myringotomy and aspiration of effusion reduces inflammation in the Junbo mouse model, with qualitative evidence (PIMO labeling) that this is with alleviation of cellular hypoxia. Grommets expose the middle ear space to relative hyperoxia[45] (table 1), and so the data presented here are consistent with the idea that an important biological mechanism for their action may be through alleviation of cellular hypoxia in inflamed middle ear tissues.

These results are preliminary. In particular the duration of therapy (five days) is relatively short. It is possible that the therapeutic benefit observed arises only from the aspiration of effusion rather than myringotomy, and if so, long-term alleviation of OM may not be seen. It is also possible that long-term ventilation could have a greater

Figure 2: Sections of the middle ear of *Junbo* mice five days post myringotomy and aspiration of effusion (x40, H&E stain). (A) In the majority of cases there is minimal reaccumulation of effusion (e) and there is reduction in mucosal (m) inflammation. (B) In a minority of cases there is no apparent effect of surgery. Note squamous hyperplasia at the site of surgical myringotomy (s).

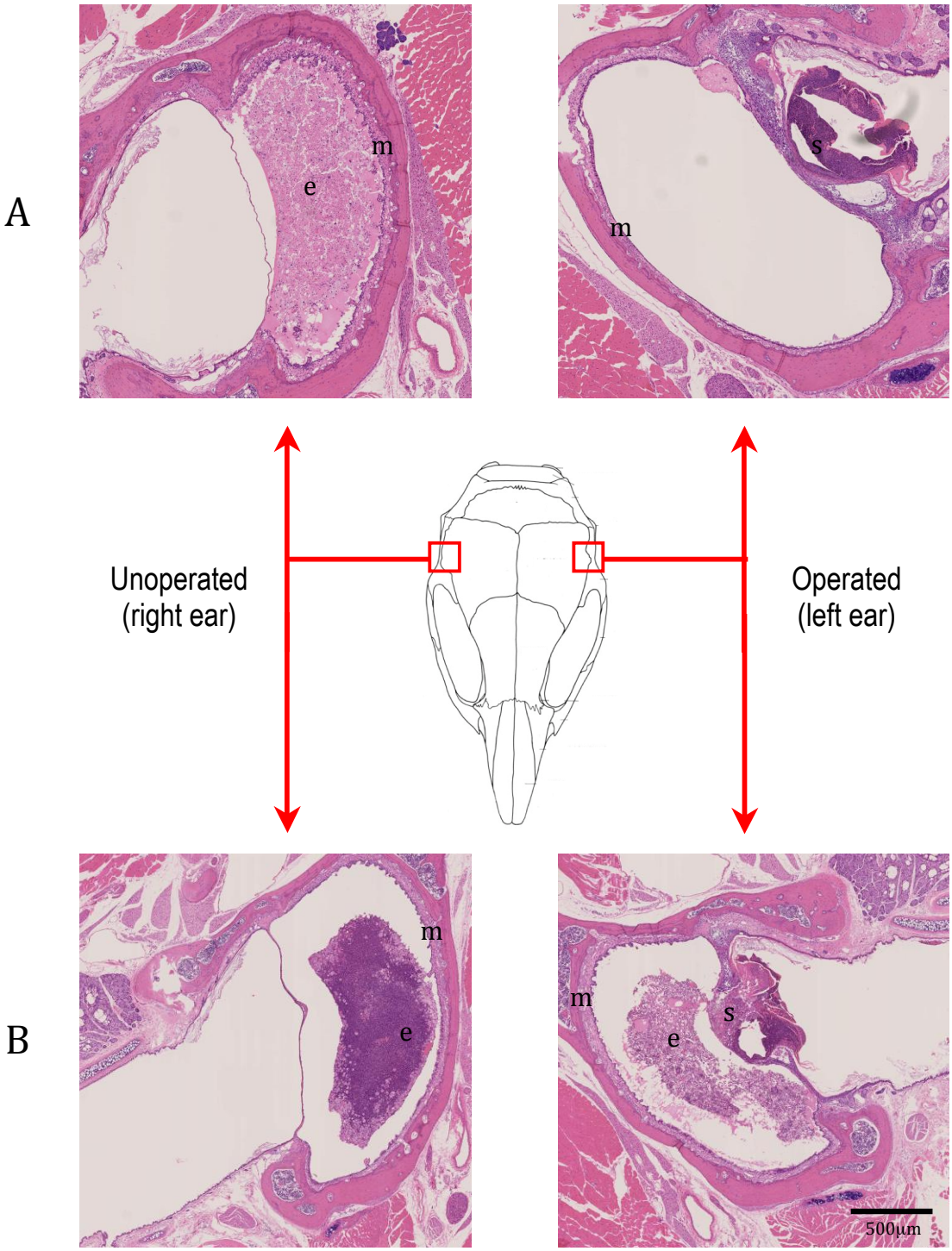
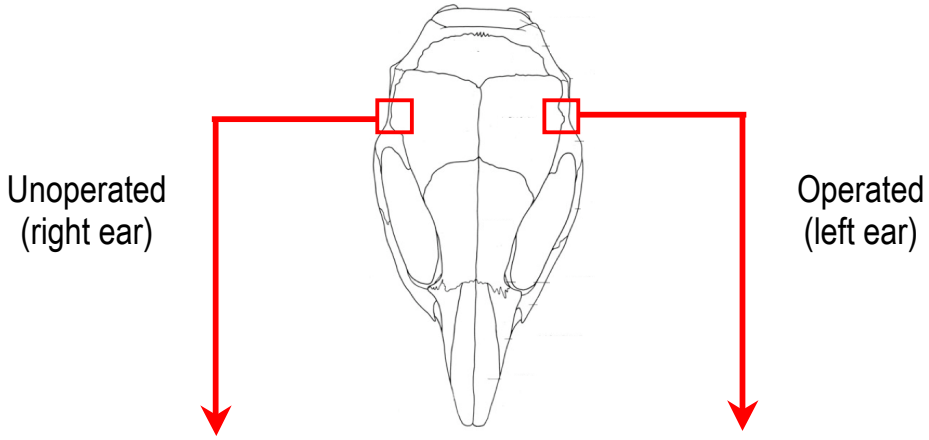
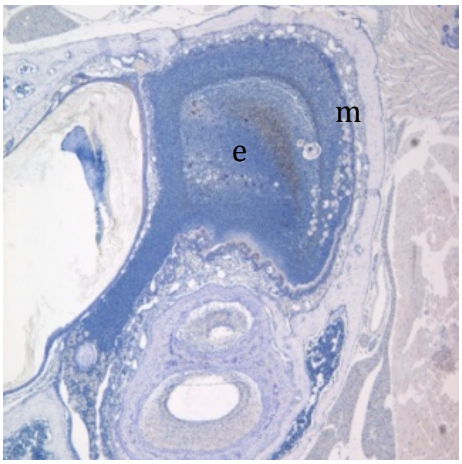
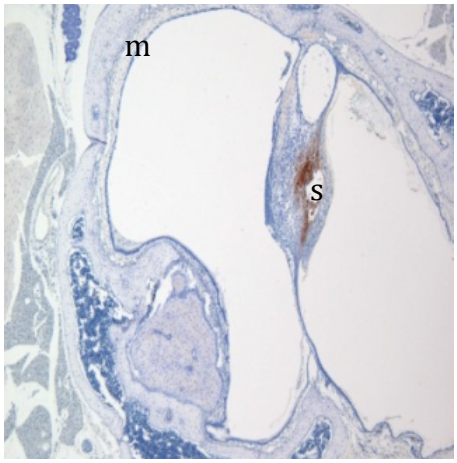


Figure 3: Anti-PIMO labeling of the left middle ear of a *Junbo* mouse five days post myringotomy and aspiration of effusion. In the unoperated ear PIMO staining is demonstrated in the middle ear mucosa (m) and in leucocytes within the effusion (e). In the operated ear there is little effusion or inflammation, and the only labeling is of the healing surgical myringotomy (s).

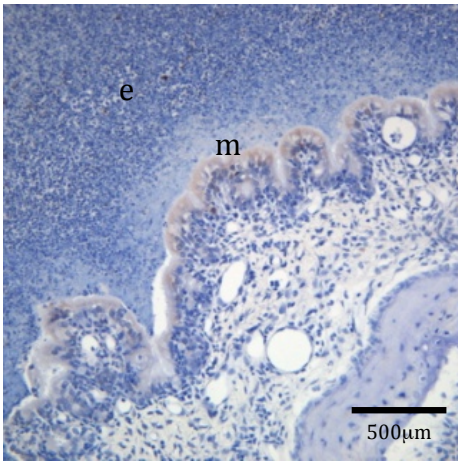


x 40





x 200



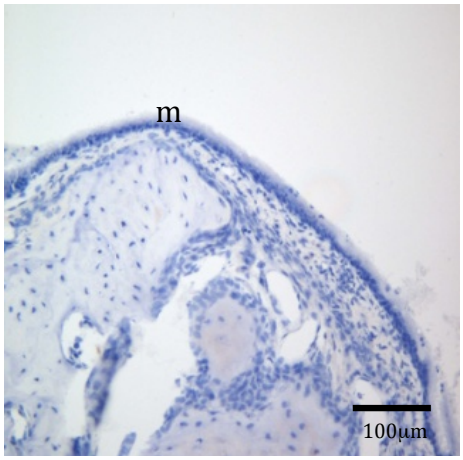


Figure 4: Mucoperiosteal thickness in the operated and unoperated ears of *Junbo* mice five days post myringotomy.

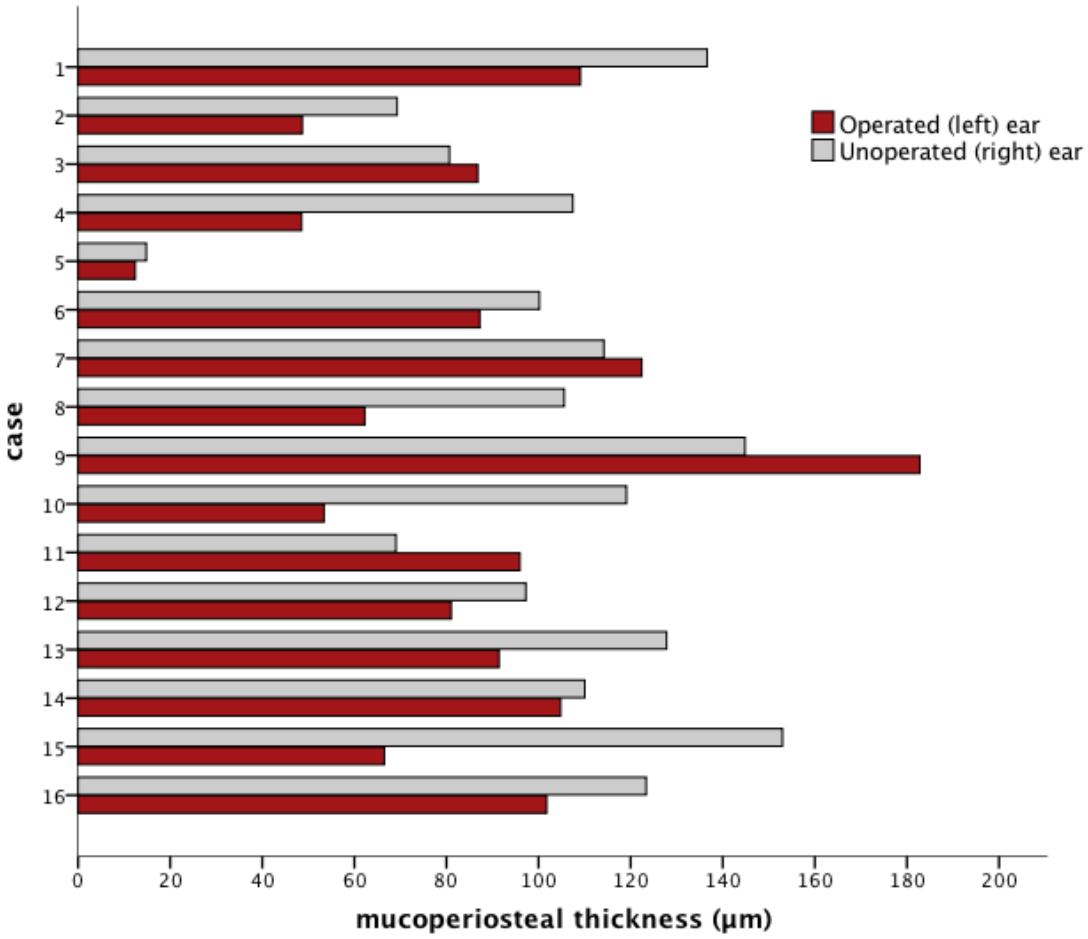


Table 1: Partial pressures (mm Hg) of gases in the healthy and surgically ventilated middle ear space. In the healthy ear gaseous pressures largely equilibrate with those in venous blood, as a result of trans-mucosal gas exchange[46]; in the ventilated ear they largely equilibrate with the atmospheric air. Oxygen and carbon dioxide within cells (in middle ear mucosa, or leucocytes in effusion) is difficult to measure, but presumably will somewhat mirror loco-regional gaseous constitution. Figures derived from from Felding et al, 1987[45].

	Venous blood	Healthy middle ear	Air (37°C)	Middle ear after myringotomy
pO_2	38	39	150	138
pCO_2	44	48	0	15
H_2O	47	47	47	47
Inert gases	704	760	563	570
Total	704	760	760	760

therapeutic effect than the inconsistent (and relatively mild) effects on inflammation seen here.

Extrapolation from human data would support the latter. In a chinchilla model of induced COME, grommets eliminated effusion but did not improve inflammation in the short-term[47]. In human studies of COME, resolution of inflammation on endoscopic[41] or histological[48 49] assessment is proportional to the duration of ventilation by grommets. Prolonged ventilation is by no means a panacea, and 20-30% of children have recurrent effusion once grommets extrude[34 50-52], but retrospective case series suggest that risk of recurrence is inversely related to duration of ventilation[53 54]. Ventilation appears to be a less successful treatment in more established forms of chronic otitis media (for example there is no evidence of long-term benefit in advanced tympanic membrane retraction[55]), but in some of these cases middle ear tissues may be irreversibly damaged. Subepithelial fibrosis is a feature of persistent inflammation[56], and if alleviation of hypoxia is important for disease resolution, it is possible that such fibrosis permanently interferes with gas exchange[57] and consequently the main route of oxygenation of the middle ear mucosa and air space.

There are a number of ways that these investigations could be extended, both in terms of improving the therapeutic effect and in terms of improving readouts.

A longer duration of ventilation would be useful. This could be achieved by laser myringotomy[58-65] or by application of mitomycin-C[66-70] to the incision, but these methods may only slightly prolong ventilation and may in themselves contribute to inflammation. An alternative is to place an indwelling device. In collaboration with the Micro and Nano Technology Centre at the Rutherford Appleton Laboratory (Professor Bob Stevens) and the Advanced Imaging and Measurement Laboratory at the University

of Warwick (Dr Simon Leigh), I have designed a prototype micro grommet for the mouse tympanic membrane (figure 5). Due to its small size (500μm diameter) this has proved technically challenging to surgically implant. We are also pursuing a prototype micropatch for trans-tympanic ventilation (figure 5). These techniques have not yet been trialed *in-vivo*.

It would also be desirable to quantitatively verify that hypoxia signaling is down-regulated as a result of middle ear ventilation. Standing alone, the data presented on PIMO labeling do not show this with confidence. In our group we have crossed the *Junbo* mutant with a reporter mouse to enable *in-vivo* bioluminescent imaging[71] of *Vegf* transcription, but results have so far been inconclusive. It may also be possible to perform mucosal transcript assays for hypoxia response genes, but harvesting murine middle ear mucosa is technically challenging. We have attempted to immunostain our sections for VEGF protein, but this has so far not proven successful.

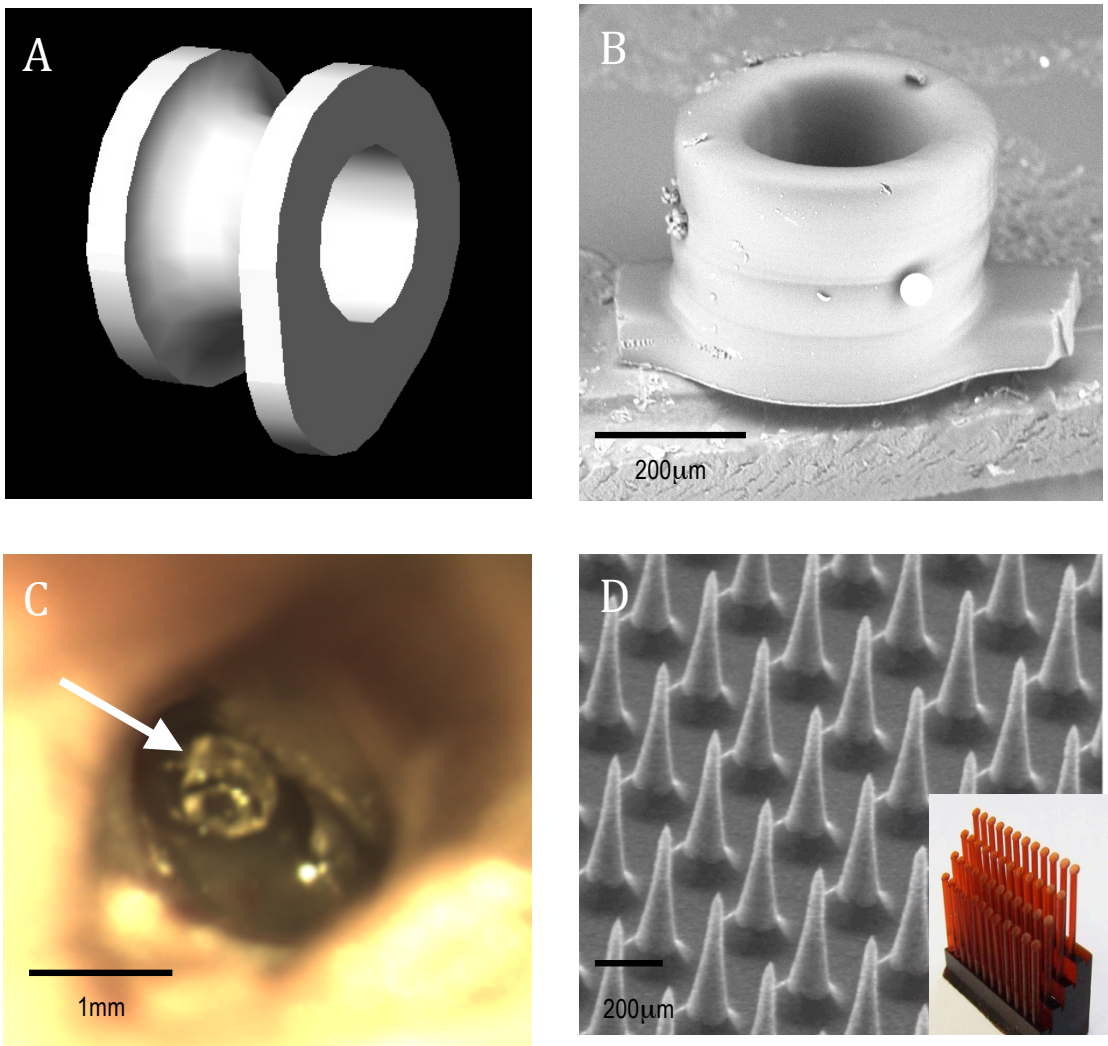
The results presented here are also based upon small numbers, and need verification. At the time of writing we are repeating experimental myringotomy in a further cohort of 8 week old *Junbo* heterozygote mice, but also without aspiration of effusion.

Conclusion

Our mutant mouse models of spontaneous chronic otitis media provide a resource to explore the pathobiology of chronic middle ear inflammation, and continue to offer tremendous potential to investigate current and future therapies, including those focused on cellular hypoxia. The results here show that ventilation downregulates

inflammation in the *Junbo* mouse model. This may be due to alleviation of tissue hypoxia, but further studies are required to verify this hypothesis.

Figure 5: Potential micro-technology solutions for prolonged ventilation of the mouse middle ear.
A-C: Micro-grommet. A: 3d CAD model, from my specifications. The design is based upon the mini-Shah grommet (Exmoor plastics) scaled down to a 500μm diameter. B: Scanning electron microscope image of the micro-grommet, sculpted in polyethylene glycol using micro-stereolithography (Simon Leigh, University of Warwick). C: Micro-grommet (arrow) placed into the posterior right tympanic membrane of a culled mouse. Access necessitated a linear incision along the ventral aspect of the external auditory canal. D An alternative is to engineer a ventilating patch based upon microneedle arrays that puncture the tympanic membrane (image from McAllister et al, 2003[72]). Inset: My design for a prototype mouse patch applicator (Simon Leigh, University of Warwick).



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Chapter 3.2
Hypoxia responsive transcripts are upregulated in chronic otitis media in man

At MRC Harwell we have demonstrated hypoxia to feature in the chronically inflamed middle ear of the *Junbo* and *Jeff* mouse models[1] (as detailed in the previous chapter). Furthermore, using experimental compounds to inhibit the VEGF receptor downregulates inflammation in these mouse models. This suggests the possibility that hypoxia may also be a finding in human chronic otitis media, and that targeting the VEGF pathway may be a new therapeutic opportunity.

To date there has been limited investigation of hypoxia pathway activation in human chronic otitis media. Jung et al reported over-expression of *VEGF* transcripts in middle ear fluids from eight patients with OME (four adults and four children), and in middle ear mucosal cells and capillaries in biopsies from four adult patients with chronic OM[2]. Sekiyama et al reported that VEGF protein was present in all (46/46) OME fluids collected from 33 children (aged 1-12 years)[3]. They also found that VEGF levels were higher in mucoid (2321 ± 2058 pg/ml) versus serous effusions (678 ± 250 pg/ml).

However these two human studies are limited. VEGF exists in several variants, classed into the VEGF-A family and the VEGF-B family. Although VEGF-A and VEGF-B have 94–98% sequence homology, they have opposing functions: VEGF-A is pro-angiogenic, but VEGF-B is anti-angiogenic[4]. The two previous studies on otitis media have failed to differentiate whether VEGF-A and/or VEGF-B transcripts or proteins are being quantified.

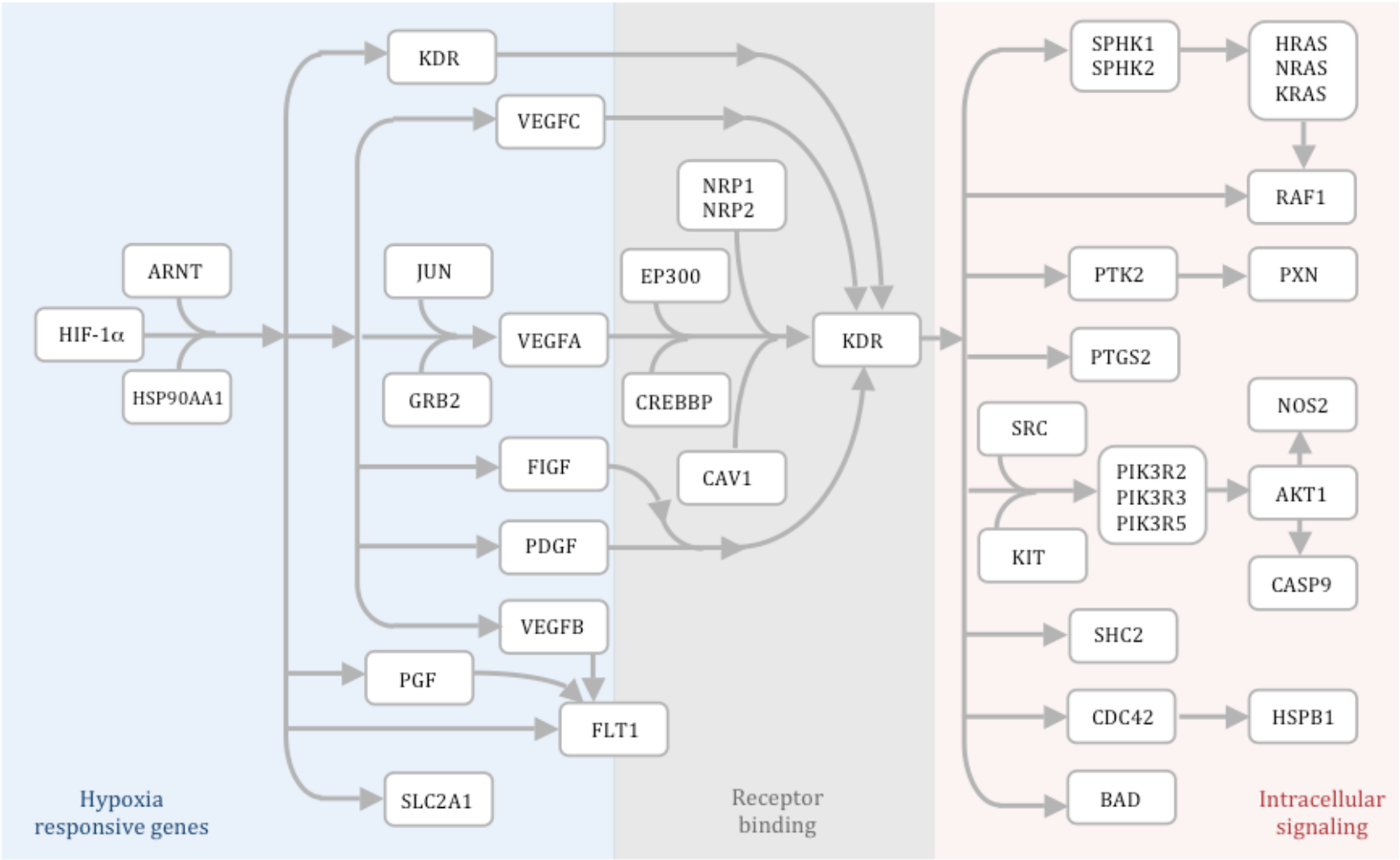
These two previous studies have also not examined what is driving *VEGF* expression. Although hypoxia, through HIF-1α, is the canonical path for VEGF transcriptional upregulation, alternate mechanisms exist. The Wnt/β-catenin[5], TGF-β/SMAD[5], or the PGC-1α/ERR-α[6] pathways can all upregulate VEGF-A transcription independent of HIF-1α, as can the unfolded protein response[7], and some microRNAs[8].

Here, our group has set out to undertake a more complete analysis of hypoxia and *VEGF* gene expression in childhood COME. Specifically we looked at key genes in the canonical pathways for HIF-1α and its partners, for agonists and antagonists of the VEGF receptors FLT1 (VEGFR1) and KDR (VEGFR2), and for genes involved in the downstream response of KDR (VEGFR2) stimulation (figure 1). We also looked at levels of *FBXO11* and *EVI1* (*MECOM*), the genes mutated in our two reported models of chronic OM, *Jeff*[9 10] and *Junbo*[11].

Our aim is to collect aspirates of middle ear effusions in approximately 50 children with COME, looking at transcript levels (utilizing Real-time PCR) and at protein levels. We assume these values by and large reflect gene expression in leucocytes in the effusion. We compare our results to those from venous blood, on the assumption that this reflects leucocyte gene expression in a non-inflammatory state. At the time of writing we have recruited and analysed transcript levels in only a handful of probands, but recruitment is ongoing, and we plan to start protein quantification imminently. Consequently the results presented here are preliminary.

I conceptualized this project, and helped to raise funding and to gain ethical approval (with Michael Cheeseman and James Ramsden). I also collected a proportion of the operative samples. However, laboratory preparation and analysis of all samples was undertaken by colleagues (Jane Lambie, Lindsey Hobson, Hayley Tyrer, Debbie Williams, and Michael Cheeseman).

Figure 1: Genes analysed in this study and their function in hypoxia pathways.



Materials and Methods

Study Literature and Ethics Approval

I designed Participant Information Sheets (PIS), and consent forms in accordance with National Research Ethics Service (NRES) guidelines[12]. I calculated Flesch-Kincaid Reading Ease for each PIS, using a web-based tool[13]. We sought research ethics approval from Oxfordshire Research Ethics Committee C[14], with James Ramsden as the named principal investigator.

Sample collection

We aimed to recruit 50 children (age<10, non-syndromic) undergoing grommet insertion for bilateral COME at the John Radcliffe Hospital in Oxford. Participants underwent myringotomy and immediate aspiration of middle ear effusion using a Juhn Tymp-Tap device (Medtronic Inc, Minneapolis). Consistency of effusions was visually assessed as mucoid or serous by the operating surgeon. Samples of effusion were collected from both ears and one side chosen at random for RNA extraction. The effusion sample from the other side was stored for planned future protein quantification. A contemporaneous sample of venous blood was taken to serve as a control for gene and protein expression.

Sample processing

A sample of the middle ear aspirate was used to make a cellular smear. The smear was stained using Diff-Quick (Merck KGaA, Darmstadt) and examined by light microscopy for cellular content. The remainder of the middle ear aspirate was frozen in dry ice shortly after aspiration, and stored at -80^{o}C. The aspirate was subsequently thawed and RNA extracted using a Nucleospin RNA/protein isolation kit (Macherey-Nagel), following the manufacturers

instructions but with the addition of Glycoblue (Applied Biosystems) and 1ml of lysis buffer to enhance extraction.

For blood, 2.5mls was added to a PAXgene tube (PreAnalytiX, Hombrechtikon) and kept at room temperature for up to 72 hrs, before being stored at -20^{0}C. The sample was subsequently thawed and RNA extracted using the PAXgene manufacturer's protocol.

RNA in blood and middle ear aspirate was quantified by 260nm:280nm ratio on spectrophotometry. cDNA was generated using 700ng of RNA and MultiScribe Reverse Transcriptase (Applied Biosystems), following the manufacturer's instructions.

Transcript Analysis

cDNA analysis was undertaken on a custom microarray (Applied Biosystems), following manufacturer's protocol. Plates were run in a 7500 Fast Real-Time PCR System (Applied Biosystems) and analysed using the 7500 Fast Software v2.0.1 (Applied Biosystems). β-actin (*ACTB*), *18S* RNA and *PPIA* were used as normalising reference genes. Transcript quantification was compared in middle ear aspirates and blood to determine relative quantity (RQ).

Results

NRES approval for the study was granted on 18th May 2011 (study reference 11/SC/0057). Approved PIS, consent forms and enrolment forms are in appendix 2. Flesch-Kincaid Reading Ease scores for the PIS were age appropriate (table 1).

Table 1: Flesch-Kincaid Reading Ease scores (0-100) for PIS used in this study.

PIS	Flesch-Kincaid Reading Ease
Adult	64.7
Child 6-10	84.8
Child <5	91.4

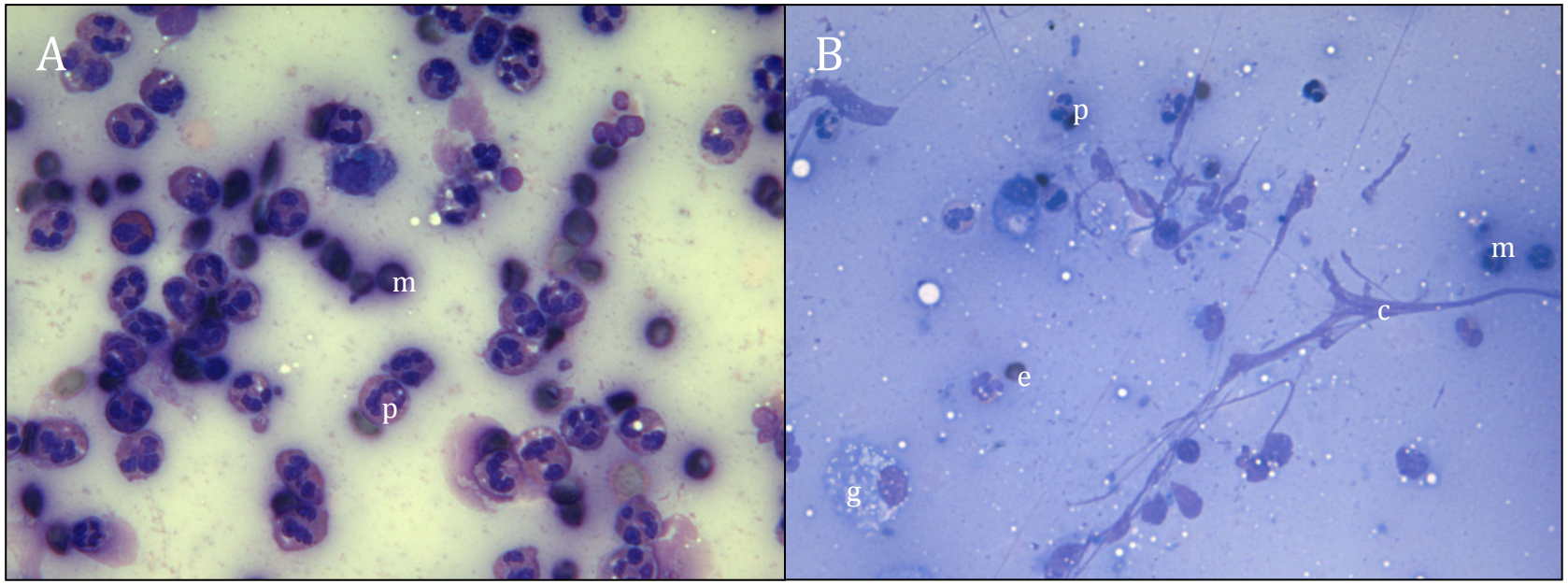
Table 2
Details of samples analysed in this study, showing participants' age and sex (M=male, F=female), and which ear (R=right, L=left) was selected for RNA extraction. Also shown are results of quantification of total RNA in participants' blood and in their effusion aspirate.

ID	Age	Sex	Ear	Blood			Effusion		
				260:280 ratio	[RNA] (ng/ul)	amount RNA (ng)	260:280 ratio	[RNA] (ng/ul)	amount RNA (ng)
1	5	M	R	2.18	46.2	3233	1.98	29.7	892
2	4	F	R	2.06	98.6	6901	1.85	49.2	1476
3	4	M	R	2.2	107.5	7525	2.13	89.7	2691
4	8	M	L	1.96	41.9	2936	2.04	66.8	2004
5	3	F	L	2.11	105.7	7399	2.13	98.3	2948

At the time of writing samples from five participants had been analysed (table 2). These samples were from children aged 3 to 8 (mean 4.8). All participants had bilateral mucoid middle ear effusions.

Cytology of middle ear aspirates showed a variable appearance (figure 2). In some cases there was an abundance of polymorphonuclear leucocytes, but in other there was a sparse or mixed cellular infiltrate.

Figure 2
Variable cytological appearance of smears of middle ear aspirates. A: sample from participant 4. There is an abundance of polymorphonuclear leucocytes (p), with occasional monocytes (m). B: sample from participant 2. There is a relative paucity of cells, with only a few polymorphonuclear leucocytes (p) and monocytes (m). There are also exfoliated goblet cells (g) from the middle ear lining, and occasional erythrocytes (e), which are probably contaminants from the operative procedure. In addition this sample shows chromatin streaming (c) due to cellular rupture. Both images stained with Diff-Quick, x600 magnification.



Results of custom microarray quantification of RNA transcripts are shown in table 3. The majority of hypoxia responsive genes show transcriptional upregulation (*FLT1, HIF1α, HSP90AA1, JUN, KDR, PGF, SLC2A1, VEGFA,* and *VEGFB*). Of note, *HIF1α* was upregulated in all samples, with transcript levels 2-5 fold higher than in blood, except for participant 4 where *HIF1α* transcripts were 27.5 fold those found in blood. This participant had an abundant polymorphonuclear leucocyte infiltrate on cytology of the effusion (figure 2A).

The VEGF receptors KDR and FLT1 have five agonists (VEGFA, VEGFB, VEGFC, PLGF, and PDGFC), and of these VEGFA was upregulated to the greatest extent (figure 3). Mean transcript levels of VEGFA were nearly 50 times those found in blood. Of the VEGF receptors themselves, *FLT1* is consistently upregulated, as are the co-receptors *NP1* and *NP2* (figure 4). Genes downstream of KDR activation show less consistent changes in transcripts. Only *HSPB1, KIT, SRC*, and *SPHK1* are consistently upregulated.

Of the genes mutated in the Jeff and *Junbo* mouse models, *FBXO11* was not reliably upregulated, whereas *EVI1* was upregulated, but to a variable extent.

Table 3 .Results of Real-time PCR on RNA extracts from blood and middle ear effusion in the five participants in this study. C_t = cycling threshold, RQ = Relative Quantity, U = undetermined, CI = confidence interval. Genes with mean RQ > 3 are highlighted in red.

Gene	Participant 1 C_t			Participant 2 C_t			Participant 3 C_t			Participant 4 C_t			Participant 5 C_t			Mean RQ (95% CI)
	Blood	Effusion	RQ	Blood	Effusion	RQ	Blood	Effusion	RQ	Blood	Effusion	RQ	Blood	Effusion	RQ	
Control genes																
18S	9.57	13.3	1.25	11.0	12.1	0.75	10.4	10.8	0.47	9.55	10.3	1.3	9.56	11.7	0.46	0.85 (0.49, 1.20)
ACTB	20.5	25.5	0.51	23.0	23.4	1.26	21.3	21.9	0.43	21.0	19.6	6.07	21.3	23.3	0.5	1.75 (-0.38, 3.89)
PPIA	21.9	26.0	U	23.8	24.5	U	22.9	22.3	U	22.4	23.6	U	23.0	23.9	U	U
Hypoxia responsive genes																
ARNT	27.1	30.7	1.28	28.4	28.4	1.71	27.8	27.3	0.94	27.9	27.9	2.15	28.4	28.6	1.76	1.57 (1.16, 1.98)
FIGF	37.2	U	U	U	32.9	U	U	32.9	U	U	35.6	U	36.6	33.4	17.4	U
FLT1	32.8	33.7	8.46	34.3	31.9	8.88	33.0	28.0	20.4	33.6	30.0	28.2	32.9	29.6	19.7	17.13 (9.76, 24.49)
GRB2	25.2	28.4	1.8	26.0	26.2	1.42	25.5	25.0	0.96	26.0	24.4	6.52	26.2	26.6	1.55	2.45 (0.44, 4.46)
HIF1α	27.9	29.6	5.03	28.7	28.3	2.14	28.7	26.6	2.83	28.6	25.0	27.5	29.1	28.6	2.84	8.07 (-1.51, 17.65)
HSP90AA1	26.1	28.1	4.07	28.0	24.9	14.0	27.4	25.0	3.46	27.7	25.4	11.0	27.3	25.7	5.87	7.67 (3.64, 11.70)
JUN	29.7	27.8	61.0	30.0	26.9	13.9	30.7	27.2	7.3	29.6	26.3	22.4	30.6	27.8	13.3	23.59 (4.66, 42.53)
KDR	33.8	34.3	11.1	34.8	36.0	0.76	32.6	33.7	0.32	U	U	U	35.9	U	U	4.07 (-2.85, 10.98)
PDGFC	30.6	34.0	1.53	31.7	32.0	1.32	30.6	28.4	2.79	31.4	30.9	3.34	31.4	33.4	0.51	1.90 (0.89, 2.90)
PGF	37.0	35.8	38.0	U	33.2	U	31.1	35.4	0.03	37.0	34.2	15.6	36.3	33.5	13.7	16.85 (1.44, 32.26)
SLC2A1	28.6	31.8	1.76	29.8	27.3	9.31	28.5	27.2	1.52	29.0	28.0	4.49	28.6	28.7	1.87	3.79 (0.89, 6.69)
VEGFA	29.5	28.1	41.4	31.2	26.1	58.4	30.9	27.1	9.3	30.0	24.8	85.4	32.0	27.2	54.1	49.71 (25.43, 73.99)
VEGFB	28.0	29.7	4.76	30.0	27.9	7.06	29.0	26.5	3.62	28.8	27.8	4.4	29.1	28.6	2.67	4.50 (3.06, 5.94)
VEGFC	31.9	35.8	1.09	34.1	U	U	33.2	37.1	0.04	33.2	34.1	1.17	32.8	U	U	0.77 (0.15, 1.38)

VEGF receptors and co-receptors																
CAV1	31.2	35.7	0.69	32.3	35.9	0.14	32.9	35.1	0.14	31.6	34.4	0.32	32.5	35.3	0.28	0.31 (0.12, 0.51)
CREBBP	25.8	29.7	1.08	26.5	27.2	1.07	25.6	26.3	0.39	26.7	25.0	7.51	26.9	27.9	0.97	2.20 (-0.41, 4.82)
EP300	27.1	30.3	1.72	28.0	28.1	1.54	27.0	26.8	0.72	28.0	26.1	8.32	28.2	28.8	1.33	2.73 (-0.03, 5.49)
FLT1	32.8	33.7	8.46	34.3	31.9	8.88	33.0	28.0	20.4	33.6	30.0	28.2	32.9	29.6	19.7	17.13 (9.76, 24.49)
KDR	33.8	34.3	11.1	34.8	36.0	0.76	32.6	33.7	0.32	U	U	U	35.9	U	U	4.07 (-3.80, 11.93)
NRP1	32.3	31.7	25.5	34.1	28.9	59.4	32.9	26.1	70.3	35.1	27.3	504	35.0	29.6	78.7	147.75 (-28.11, 323.61)
NRP2	31.3	29.8	45.4	33.0	26.6	145	32.1	24.8	101	32.6	26.7	138	32.9	26.2	211	128.33 (74.81, 181.84)

VEGF receptor downstream signalling																
AKT1	25.7	29.2	1.45	27.1	27.9	0.95	26.7	26.7	0.66	26.1	26.2	2.06	27.0	27.3	1.58	1.34 (0.86, 1.82)
BAD	31.4	33.6	3.63	32.5	33.5	0.82	32.7	31.9	1.13	31.3	30.9	2.86	31.8	32.9	0.86	1.86 (0.72, 3.00)
CASP9	33.0	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
CDC42	22.1	26.0	1.09	23.3	24.2	0.9	22.9	23.0	0.58	22.9	22.4	3.35	23.4	25.0	0.65	1.31 (0.30, 2.33)
HRAS	31.0	33.8	2.33	32.1	32.3	1.56	31.5	30.4	1.35	30.7	31.0	1.82	31.2	31.3	1.81	1.77 (1.45, 2.10)
HSPB1	27.7	27.9	14.3	29.0	25.6	16.9	28.5	25.8	4.33	28.5	26.5	9.25	28.0	26.1	7.2	10.39 (5.89, 14.90)
KIT	32.5	34.1	5.46	34.0	32.8	3.76	32.8	31.8	1.34	33.8	32.6	5.2	33.6	33.8	1.72	3.50 (1.82, 5.17)
KRAS	27.8	31.4	1.31	29.0	29.3	1.35	28.1	27.4	1.08	28.6	26.6	9.57	29.0	30.1	0.91	2.84 (-0.46, 6.14)
NOS2	37.1	33.7	171	33.2	36.5	0.17	35.4	35.8	0.52	U	36.3	U	34.9	34.4	2.71	43.49 (-39.53, 126.51)
NRAS	26.9	30.0	1.96	28.3	27.9	2.32	27.7	26.3	1.71	27.7	27.4	2.76	28.0	27.9	2.01	2.15 (1.80, 2.51)
PIK3R2	28.6	31.9	1.57	29.9	30.4	1.14	28.5	28.7	0.57	29.4	30.0	1.69	29.4	30.6	0.86	1.17 (0.75, 1.58)
PIK3R3	29.9	33.7	1.2	33.2	33.6	1.27	32.2	32.3	0.61	31.3	32.3	1.12	32.7	32.9	1.73	1.19 (0.84, 1.54)
PIK3R5	25.8	28.8	2.06	26.6	26.9	1.33	25.9	26.1	0.54	25.9	24.4	6.39	26.4	27.5	0.91	2.25 (0.16, 4.34)
PTGS2	29.2	31.8	2.61	29.5	29.6	1.62	29.0	30.0	0.31	30.5	26.3	40.4	30.1	31.9	0.57	9.10 (-6.25, 24.46)
PTK2	30.0	34.0	1	31.7	32.7	0.83	30.6	30.5	0.72	31.0	31.5	1.52	30.8	31.2	1.44	1.10 (0.79, 1.42)
PXN	24.9	29.8	0.55	25.5	27.4	0.45	25.0	26.0	0.32	25.7	23.7	8.86	25.9	29.4	0.18	2.07 (-1.26, 5.40)
RAF1	27.7	32.1	0.81	28.5	28.8	1.35	27.8	28.3	0.47	28.4	27.8	3.44	29.0	30.2	0.87	1.39 (0.35, 2.43)
SHC2	34.5	34.4	17.6	35.2	37.0	0.49	33.4	34.8	0.24	33.9	U	U	33.8	35.0	0.87	4.79 (-3.56, 13.14)

SPHK1	31.6	31.1	23.5	32.9	30.0	13.2	32.0	28.2	8.49	31.4	27.4	36.0	31.8	29.4	10.6	18.35 (8.33, 28.37)
SPHK2	30.2	33.9	1.22	31.6	32.4	1.02	30.8	31.0	0.59	30.2	30.3	2.08	30.8	32.3	0.68	1.12 (0.60, 1.64)
SRC	28.2	30.0	4.8	29.9	28.5	4.41	29.2	26.8	3.46	29.4	27.5	7.95	29.4	27.5	7.39	5.60 (3.88, 7.32)
Loci from mouse models																
FBXO11	26.6	30.4	1.21	27.2	27.9	1.04	26.6	26.2	0.8	27.1	26.3	4.14	27.4	28.2	1.07	1.65 (0.43, 2.88)
MECOM	35.9	33.2	107	35.9	34.8	3.72	37.0	34.9	2.84	35.0	35.7	1.44	36.8	35.2	6.13	24.18 (-16.32, 64.69)

Figure 3: Relative quantity of transcripts in middle ear effusions when compared to blood, for ligands of VEGF receptors. The dotted line shows the mean. Note that *VEGFA* shows consistent and marked upregulation.

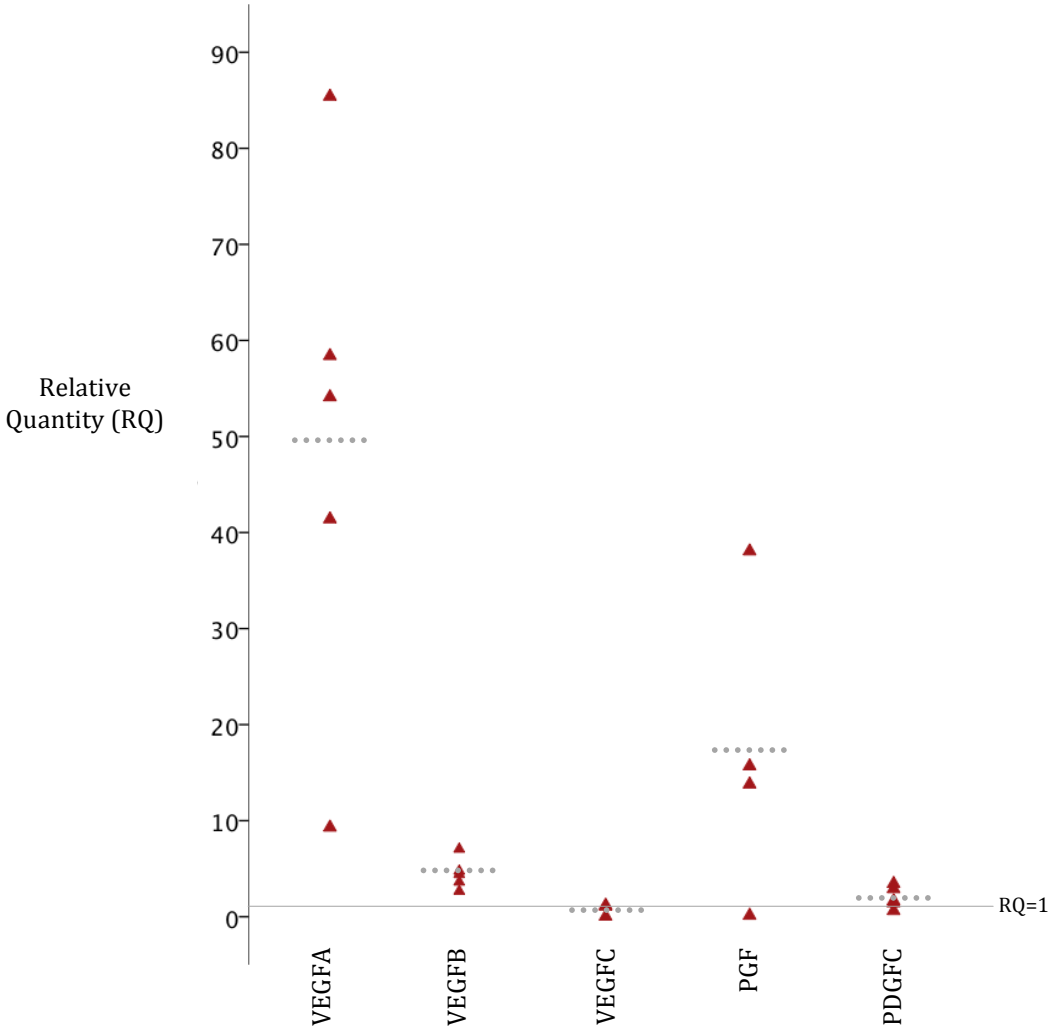
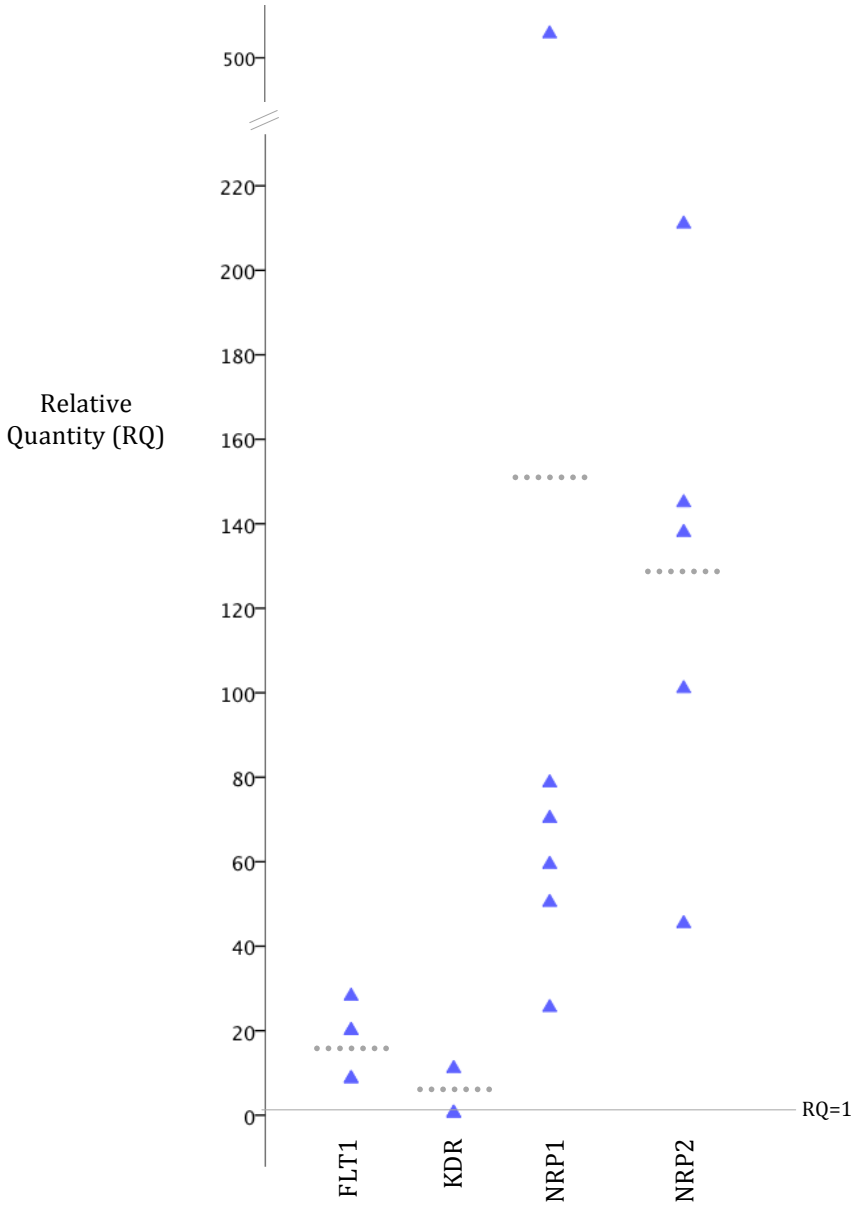


Figure 4: Relative quantity of transcripts in middle ear effusions when compared to blood, for VEGF receptors and co-receptors. The dotted line shows the mean. *FLT1, NRP1* and *NRP2* are consistently upregulated.



Discussion

Under conditions of normoxia HIF-1α protein is hydroxylated by prolyl hydroxylase domain-containing (PHD) enzymes, which marks it for proteasomal destruction[15]. Under hypoxia PHDs function less well (because they utilize oxygen as a co-substrate), resulting in stabilization and relative abundance of HIF-1α protein. Stabilised HIF-1α binds to ARNT (HIF-1β) to form HIF1. HIF1 acts as a transcriptional regulator, binding to a hypoxia responsive consensus sequence to promote transcription of up to 70-200 genes[16].

The data presented here are based upon a small sample, but nevertheless show that a number of HIF responsive genes (figure 1) are transcriptionally upregulated (table 3). This is good. but only preliminary, evidence that hypoxia pathways are active in human COME, and that leucocytes in the middle ear effusion exist in a hypoxic environment.

There may however be less parsimonious explanations for these findings. HIF1 can be present in a normoxic environment if HIF1α transcription and translation is upregulated to an extent that overcomes PHD hydroxylation. Transcription of *HIF1α* can for example be induced by cytokines[17], including NF-κB[18]. It can also be induced by growth factors, vascular hormones, and viral proteins[17]. Here we found that *HIF1α* transcripts are indeed higher than in venous blood in all the samples (table 3), but in most samples the transcript levels were only marginally higher. It seems unlikely that transcriptional upregulation of HIF (rather than HIF protein stabilisation) is responsible for hypoxia pathway activation. We do not (at present) have data to quantify HIF protein in effusions.

It is also possible that hypoxia responsive genes are upregulated by mechanisms other than by HIF1 transcriptional promotion. Indeed a number of these proteins can be upregulated by other cytokines. However, we did find that *VEGFA* transcripts are upregulated 9 to 85 fold (figure 3), and although a number of molecules can promote *VEGF* transcription, HIF is the most potent of these[19], and so the likely culprit.

Regardless, the finding in this small sample that *VEGF* transcripts are upregulated in chronic middle ear effusions confirms the findings of others[2 3]. Our data are however the first to show that it is *VEGFA* that is most abundant (figure 3). VEGFA binds to both the FLT1 (VEGFR1) and KDR (VEGFR2) receptor. Our data show that *FLT1* is upregulated (figure 4), and indeed *FLT1* is known to be HIF responsive[20]. The data on transcript levels of KDR are less conclusive. Of course receptors can be activated regardless of whether or not they are transcriptionally regulated. Of note, the neuropilins NRP1 and NRP2 are abundant in middle ear effusions. One of the function of neuropilins is as co-receptors to enhance VEGF binding to VEGF receptors[21].

The downstream effects of FLT1 or KDR stimulation are not readily discernible from our data. Downstream pathways of KDR activate pathways involved in cellular survival, proliferation, and migration, and vascular permeability and proliferation[20]. A number of molecules downstream of KDR are transcriptionally upregulated (table 3), but these may also be upregulated through other pathways. Downstream effectors of FLT1 stimulation are less well characterized, but outcomes include increased vascular permeability and monocyte/macrophage recruitment.

Levels of *FBXO11* and *MECOM* (*EVI1*) transcripts are variable in our data. This sheds little light on the role of these molecules in human chronic OM.

One question that remains about our data is the cell type being sampled. The assumption is that transcript abundance relates to leucocytes in the effusion, but cytology shows that some samples were contaminated by mucosal cells and erythrocytes (figure 2). It is possible that the variations described in transcript abundance between blood and effusions are due to baseline differences in gene expression in different cell types. For example, neuropilins are known to be expressed in normal respiratory mucosa (in the lung)[21], and if they are also highly expressed in middle ear mucosal cells (not known), this could be an explanation for their apparent abundance in middle ear effusion. However, the relative paucity of mucosal cells makes such an explanation less likely, and in addition patient 4 showed very high number of leucocytes in the effusion (figure 2) with comparable marked upregulation of *HIF-1α, VEGFA*, and the neuropilins, suggesting that leucocytes are indeed the source of these molecules.

The data presented here are preliminary, and based upon only five participants. We plan to extend our study to at least 50 participants, and to also undertake parallel protein quantification in blood and effusions, using Enzyme-linked Immunosorbent Assay (ELISA). We also plan in the future to undertake transcript and protein analyses on biopsies of middle ear mucosa in COME, and to extend our analyses to other pathways that are potential druggable targets.

In spite of these limitations, the initial data presented here do suggest that hypoxia pathways are active in COME, and that VEGFA may be a central orchestrator of this response. If these results are upheld with analysis of the full intended 50 recruits, we would be encouraged to look in more detail at sites of expression of hypoxia pathway genes and their protein levels, as a prelude to potential targeted therapy in man. This could mirror our success in mouse models of treating chronic middle ear inflammation with VEGF receptor inhibitors[1], but would likely need to be combined with a method of delivery of drug into the middle ear space in order to minimise

systemic side-effects. How this would occur is not yet clear, but encouraging recent work has shown that chemical permeation enhancers such as bupivacaine, limonene, and sodium dodecyl sulfate can effect sustained delivery of antibiotics (ciprofloxacin) across the tympanic membrane[22],

Conclusion

Our preliminary data suggest that hypoxia pathways are active in human chronic otitis media. If this is borne out by further analyses, hypoxia pathways may be a novel therapeutic target in man.

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Chapter 4.1
Design and recruitment to a human association study

A number of previous studies have assessed genetic susceptibility to otitis media, but most have been small and with unclear phenotype definition (chapter 1.4). In addition, all have focused on rAOM, and none specifically at chronic OM phenotypes.

This chapter outlines design and recruitment to a genetic database of families affected by otitis media. Subsequent chapters describe interrogation of this database in a candidate gene association study (from candidate loci suggested by mouse models). Throughout I report methods and results in accordance with the STREGA statement for reporting of genetic association studies[1].

Rationale

I focused on the COME phenotype because it is the prototypical and most prevalent form of chronic OM in the UK. It is also the probable precursor to most other forms of chronic OM (chapter 1.1). I prospectively recruited patients listed for grommet (ventilation tube) surgery, a treatment both for COME[2] and, less often, rAOM[3], and recruited the entire nuclear family to enable case-parent triad analysis (chapter 4.3).

Phenotype misclassification significantly reduces study power, and has been a major limitation of previous studies in this field (chapter 1.3). For pragmatic reasons a clinical history of rAOM was used to define this phenotype (table 1). For COME, phenotype was defined by a clinical history of at least three months of effusion (consistent with UK guidelines for operative intervention[4]), but with effusion confirmed at myringotomy (table 1). Some may argue that this is unduly stringent, but clinical assessment of effusion may be inaccurate[5], with 12% of children diagnosed with COME found to have a "dry tap" at operation[6]. It is possible that such lack of effusion is caused by displacement from gaseous anaesthetic agents[7-18], but more likely reflects misdiagnosis[19 20], resolved effusion, or possibly less severe disease[6 21 22]. A definition of COME that excludes participants with a "dry tap" mitigates risk of phenotype misclassification.

Table 1: Definitions and ascertainment of phenotypes for this study

Phenotype	Definition	Ascertainment
COME	Effusion present for ≥ 3 months	History of duration of disease. Effusion confirmed at myringotomy
rAOM	> 3 episodes AOM in 6 months or ≥ 4 episodes in 12 months	Clinical history. Undergoing grommet surgery

The otitis media phenotype is a continuous variable (chapter 1.1) and the dichotomisation of the COME phenotype I have opted for here (present or absent depending upon intraoperative presence of effusion) risks reducing power and precision of association testing[23]. However dichotomisation was necessary because of the difficulties in ascertaining accurate clinical history for a disease that often has insidious onset, and in attempting quantitative or qualitative assessment of viscosity or volume of effusion (although data from other sources suggests the effusion will be high volume[24]).

Materials and Methods

Power calculation

The sample size sufficient for 80% power was estimated using the Harvard Genetic Power Calculator for TDT for discrete traits[25 26]. Disease prevalence was set to 0.05, genotype relative risk to 1.3 (the median risk of disease associated alleles[27 28]), D-prime to 0.8, and type 1 error rate to 0.05.

Study Literature and Ethics Approval

Participant Information Sheets (PIS), and consent forms were designed in accordance with National Research Ethics Service (NRES) guidelines[29]. I calculated Flesch-Kincaid Reading Ease for each PIS, using a web-based tool[30]. An enrolment form and parallel electronic database was created (database script by Simon Greenaway). I also designed a website for participants[31], and analysed traffic using Google Analytics[32] (technical expertise by Andy Blake). I sought national research ethics approval for the study from Oxfordshire Research Ethics Committee B[33], and local approval from each participating hospital.

Recruitment Centres

The study was initially located in National Health Service (NHS) hospitals in the Oxford Deanery for ENT surgery[34], and funded through a Wellcome Trust Clinical Research Fellowship. The Thames Valley Comprehensive Research Network provided additional funds for a regional research nurse and local study administrator.

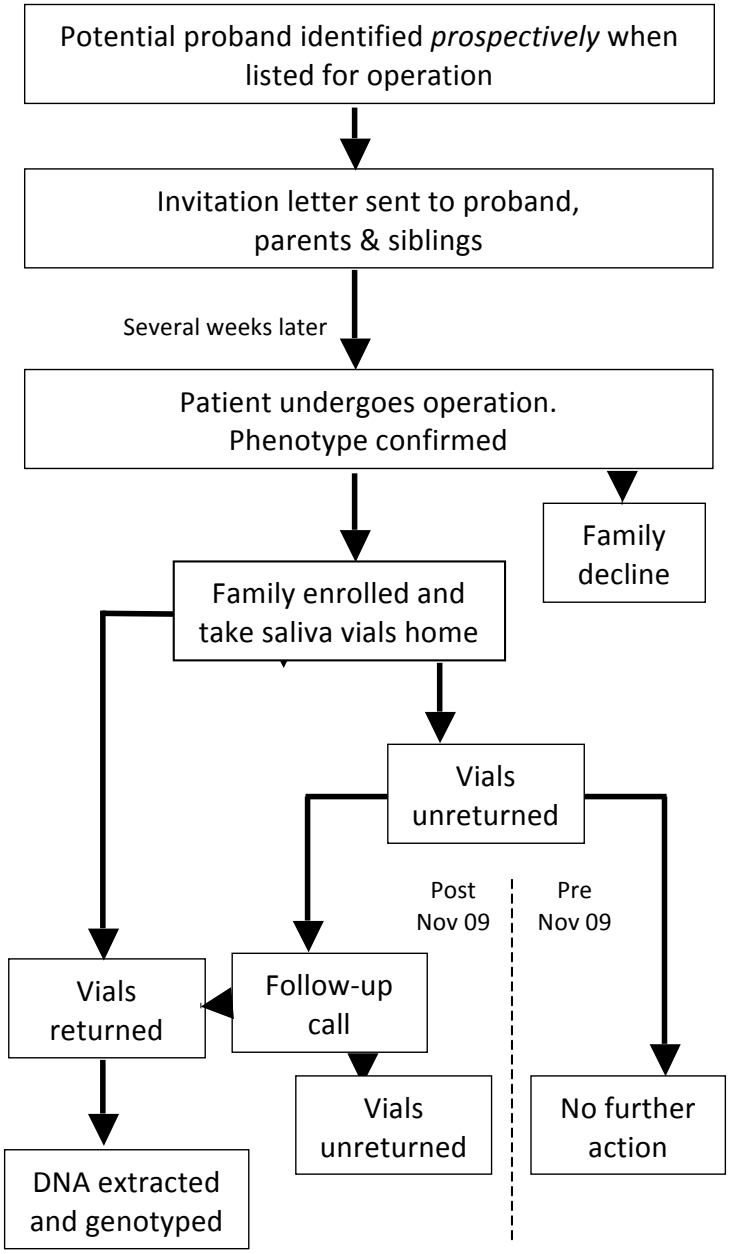
I applied for inclusion of the study in the NIHR portfolio[35], as a means to invite further NHS hospitals to join. For each additional centre a part-time research nurse was identified to participate in local recruitment. Further funds were sourced to meet the cost of additional centres, by application either to national funding bodies or to loco-regional funds.

Study protocol

The protocol for recruitment is shown in figure 1. All participants were recruited prospectively to ensure they met the phenotype definition (table 1). The entire biological nuclear family was recruited wherever possible. Data was also collected on potential environmental risk factors for disease, and on whether parents or siblings had

previously had grommets (see enrolment form, Appendix). Children over ten years old or those with syndromic OM were excluded, because this group may represent different disease aetiology to that of non-syndromic OM in early childhood.

Figure 1: Recruitment protocol



DNA was collected using saliva vials (Oragene® OG-250 pot, DNA Genotek Inc, Ontario, Canada), enabling self-collection and return by post (pre-paid Royal Mail). Due to low return rates for saliva vials, 17 months into the study an approved amendment was instigated for a reminder phone-call to participants who failed to return their DNA.

Identification of replication cohorts

Given the necessity to replicate findings in association studies, I co-founded a consortium called "Otigen"[36]. This consortium comprises research groups with an interest in human genetics of OM. I created content for an Otigen website, to encourage new members (technical expertise by Andy Blake). We sought to identify potential replication cohorts within the Otigen consortium, and also sought details of any OM phenotypes within birth cohorts. The Western Australia group have published details of a replication cohort from the Australian Raine study[37 38]. I contacted the UK ALSPAC study[39] to explore OM phenotyping in this group.

Results

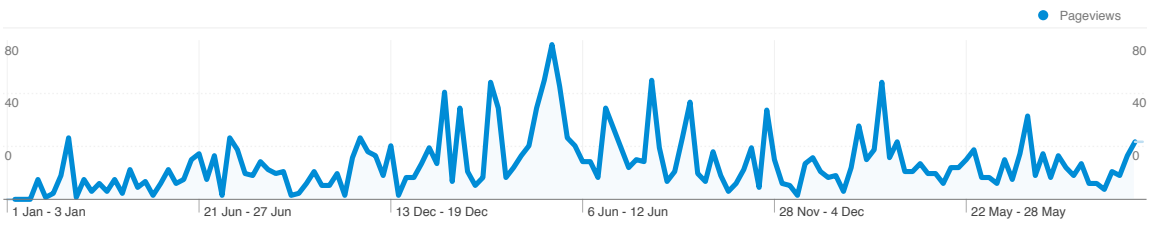
I ascertained a sample size of 897 family trios as giving 80% power. Because a significant minority of those recruited were predicted to have rAOM rather than COME, and because these phenotypes may differ in their genetic predisposition (chapter 1.2), I had a target sample size of 1,000 families.

NRES national approval for the study was granted on 3rd October 2008 (study reference 08/H0605/109). Approved PIS, consent forms and enrolment forms are in the Appendix. Flesch-Kincaid Reading Ease scores for the PIS were age appropriate (table

Table 2: Flesch-Kincaid Reading Ease scores (0-100) for PIS used in this study.

PIS	Flesch-Kincaid Reading Ease
Adult	64.1
Child 11-15	75.6
Child 6-10	85.3
Child <5	99.9

Figure 2: Study website traffic from inauguration in Jan 2009 until 1st November 2011. There were a total of 2,699 page views. Data from Google Analytics[40] (courtesy of A Blake).



2). The database front page is shown in figure 3, website homepage in figure 4, and a summary of website traffic in figure 2.

The study was adopted onto the NIHR portfolio (reference number 5376), and a significant number of additional centres contacted and joined the study as a consequence. Table 3 summarises data on the 22 NHS Trusts participating or waiting to participate as of 1st November 2011. Figure 5 shows the location of recruiting centres.

Figure 3: Database front page

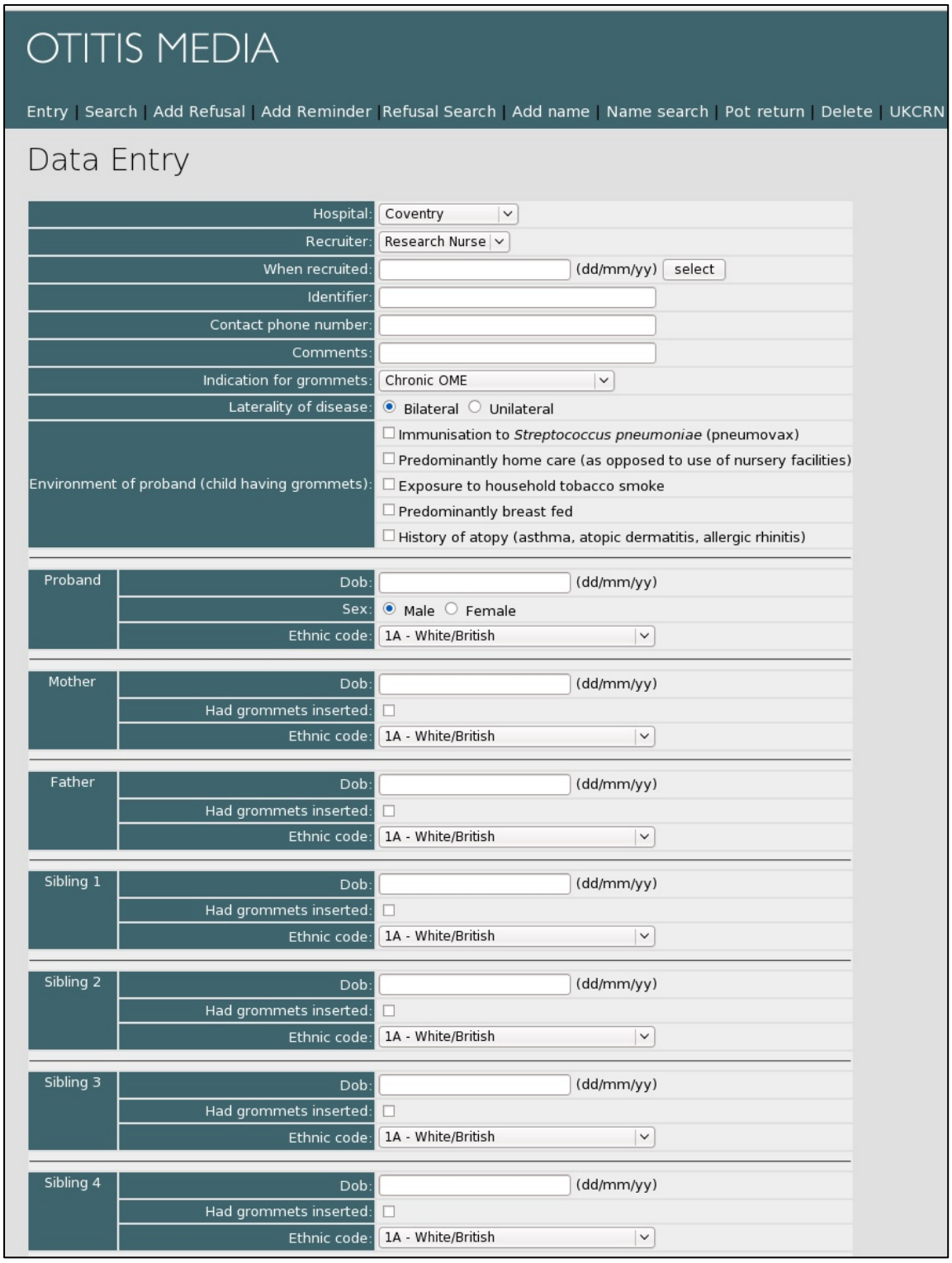


Figure 4: Front page of the study website (www.har.mrc.ac.uk/geneticsomstudy)



Table 3: Data for sites recruiting to this study, correct at November 1st 2011. 1329 families had been recruited from 20 NHS Trusts.

Hospital	Local Principal Investigator	Research Nurse(s)	Source of Funding	Approval granted	First enrolment	Families Recruited
Heatherwood & Wexham Park Hospital (Slough)	Chris Aldren	Jane Lambie	Wellcome Trust	11/11/2008	08/04/2009	49
Stoke Mandeville Hospital	Ian Bottrill			12/02/2009	23/04/2009	51
John Radcliffe Hospital (Oxford)	Martin Burton			03/10/2008	30/04/2009	133
Northampton General Hospital	Dermot Commins	Joy Margetts, Kate Smith, Rachel Hitchcock, Lucy Dudgeon		13/02/2009	12/05/2009	157
Great Western Hospital (Swindon)	Angus Waddell	Jane Lambie		23/10/2008	26/05/2009	31
Kettering General Hospital	Mohammed Latif	Mary Chapman		18/01/2009	01/06/2009	144
High Wycombe Hospital	Hamish Thomson	Jane Lambie		12/02/2009	04/06/2009	31
Milton Keynes General Hospital	Stephen O'Malley			29/07/2009	25/11/2009	21
Coventry & Warwick Hospital	Hisham Mehanna	Ruth Goalby, June Jones	Royal National Institute for Deaf People	09/10/2009	01/02/2010	118
Warwick Hospital	David Phillips	Nikki Clewes		18/12/2009	19/03/2010	21
Royal Surrey County Hospital (Guildford)	Deepak Prasher	Elizabeth Cattermole	CLRN	10/03/2010	04/05/2010	103
Newcastle upon Tyne Hospital	Janet Wilson	Sharon Bradley	Local Funds	21/05/2010	14/06/2010	144
Cumberland Infirmary (Cumbria)	Nick Murrant	Claire Hagon, Nicci Kelsall	CLRN	08/06/2010	17/09/2010	33
Epsom Hospital	Peter Robb	Elizabeth Cattermole, Tatiana Gutierrez	CLRN	17/06/2010	24/01/2011	19
Royal Hospital for Sick Children (Glasgow)	Haytham Kubba	Jadranka Zelenovic	Deafness Research UK	31/08/2010	27/01/2011	128
Blackpool Victoria Hospital	Megan Thomas	Jackie Bradley, Jacqui Woods	CLRN	22/11/2010	23/02/2011	46

Crosshouse Hospital (Kilmarnock)	Miss Mary Shanks	Claire Bell	Deafness Research UK	14/12/2010	24/02/2011	30
Aberdeen Royal Infirmary	Derek Mr Veitch	Jill Paxton, Lindsay Cameron		14/01/2011	30/03/2011	13
Worcestershire Acute Hospitals	Chris Ayshford	Julie Wollaston	CLRN	10/11/2010	20/04/2011	42
Royal Preston Hospital	John de Carpentier	Helena Prady	CLRN	22/09/2010	27/05/2011	15
Bradford Royal Infirmary	Chris Raine	awaited	Local Funds	28/09/2011	awaited	awaited

Royal Hospital for Sick Children (Edinburgh)	Mary-Louise Montague	Kay Riding	Deafness Research UK	awaited	awaited	awaited
Royal Sussex County Hospital (Brighton)	Prodip Das	Rebecca Allen, Lianne Windless	Wellcome Trust	awaited	awaited	awaited

Figure 5: Map of recruitment sites as of 1st November 2011. Centres marked with blue dots have joined the study, centres with red dots are waiting to join.



As of 1st November 2011, 2052 families had been invited to participate. Of these 723 (35.2%) were not recruited: 476 (23.2%) because they declined to participate, 199 (9.7%) because of a "dry tap", and the remaining 48 for a variety of predominantly logistical reasons. If we assume the same dry tap rate in families who refused to participate as those who agreed (9.7%), the predicted overall dry tap rate for this study is:

((476 x 0.097) + 199) / 2052 = 11.9%.

1329 families (5090 individuals) were recruited to the study (table 3, figure 6). However only 600 of these families (2319 individuals) returned saliva samples, giving a return rate of 45.1%. The reminder telephone call instituted in November 2009 significantly improved samples return from 26% (50/195) of families before the telephone reminder, to 48% (550/1134) afterwards (Fisher's exact test on the difference, $p<0.0001$).

Of the returned samples, 57% (342) of probands were male, and 94.2% (565) described their ethnicity as white (table 4). At recruitment, 41% (246) of probands were aged 4 or 5 (figure 7). Four probands were aged 10, meaning they did not meet the inclusion criteria, but were kept in the analysis regardless. The indication for surgery was COME in 80.5% (483) of probands, and rAOM in 19.5% (117). Of those having surgery for rAOM, 73.5% (86) had an effusion at operation. These data are comparable to previous data from the UK (where 15% of grommet procedures were for rAOM, and of these 74% had effusion at operation[6]).

The Otigen website is shown in figure 8. Several groups were identified with potential cohorts for replications (table5). The ideal replication cohort would be to find a group

Figure 6: Enrolment and DNA returns to the study from April 2009 to October 2011. There were a total of 1329 family enrolments, and 600 family DNA returns. Crosses on the x-axis signify the addition of an additional recruiting centre.

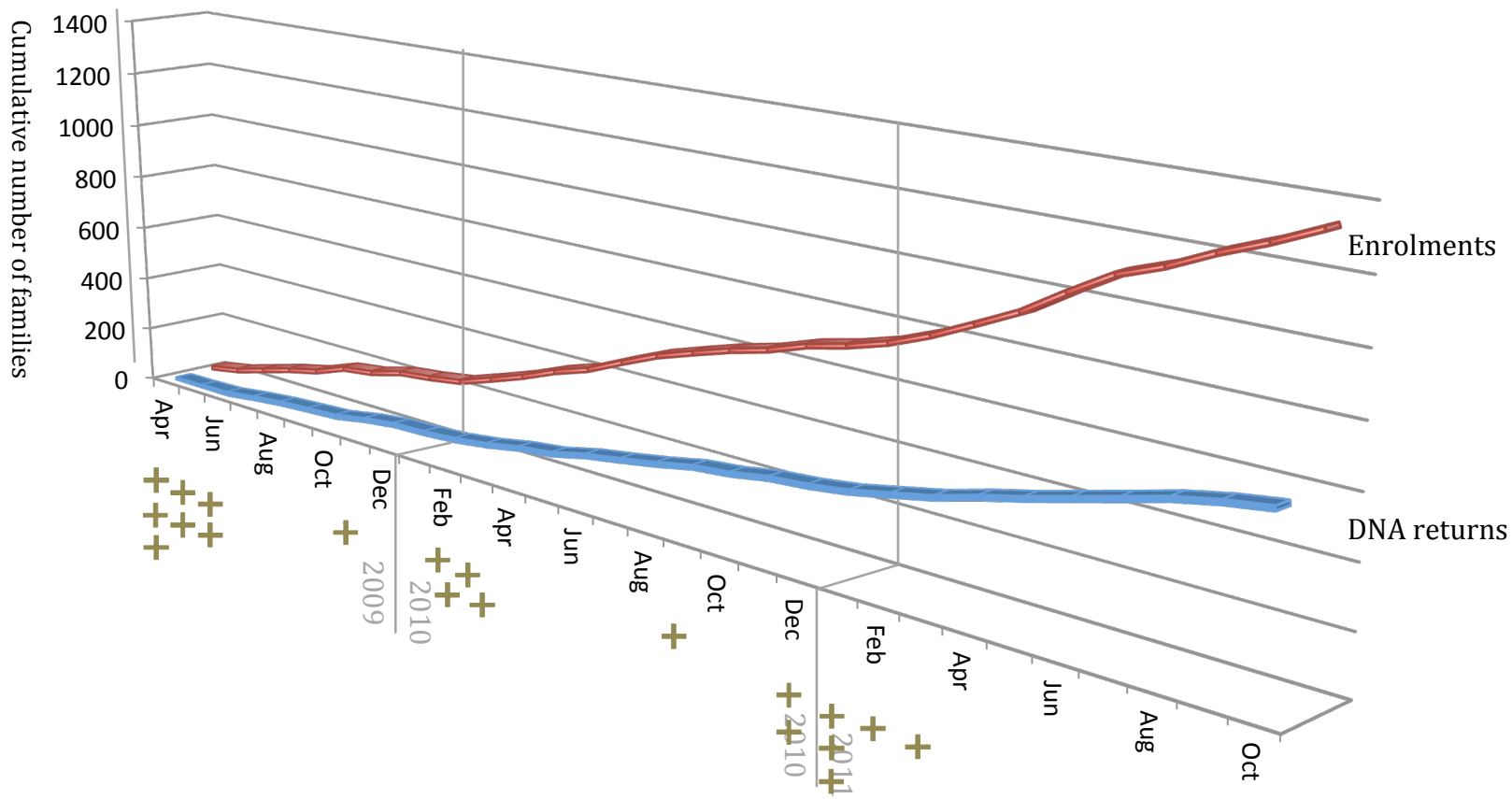


Table 4: Self-described ethnicity of probands in samples returned

Ethnicity	Number
White	94.2% (565)
Asian	2.2% (13)
Black	0.5% (3)
Mixed	3% (18)
Other	0.2% (1)

Figure 7: Age of proband at time of recruitment.

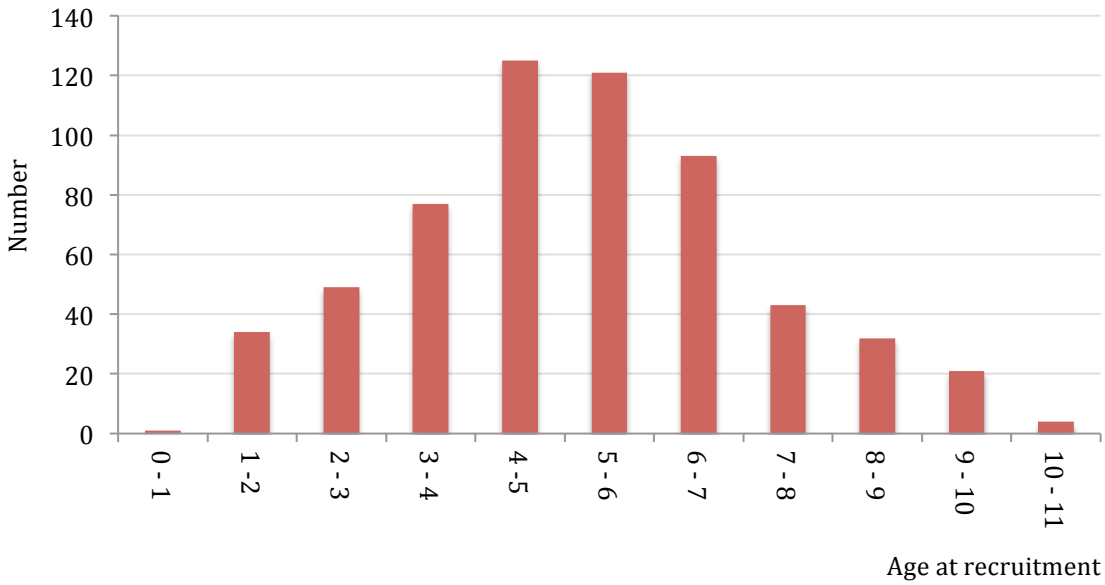
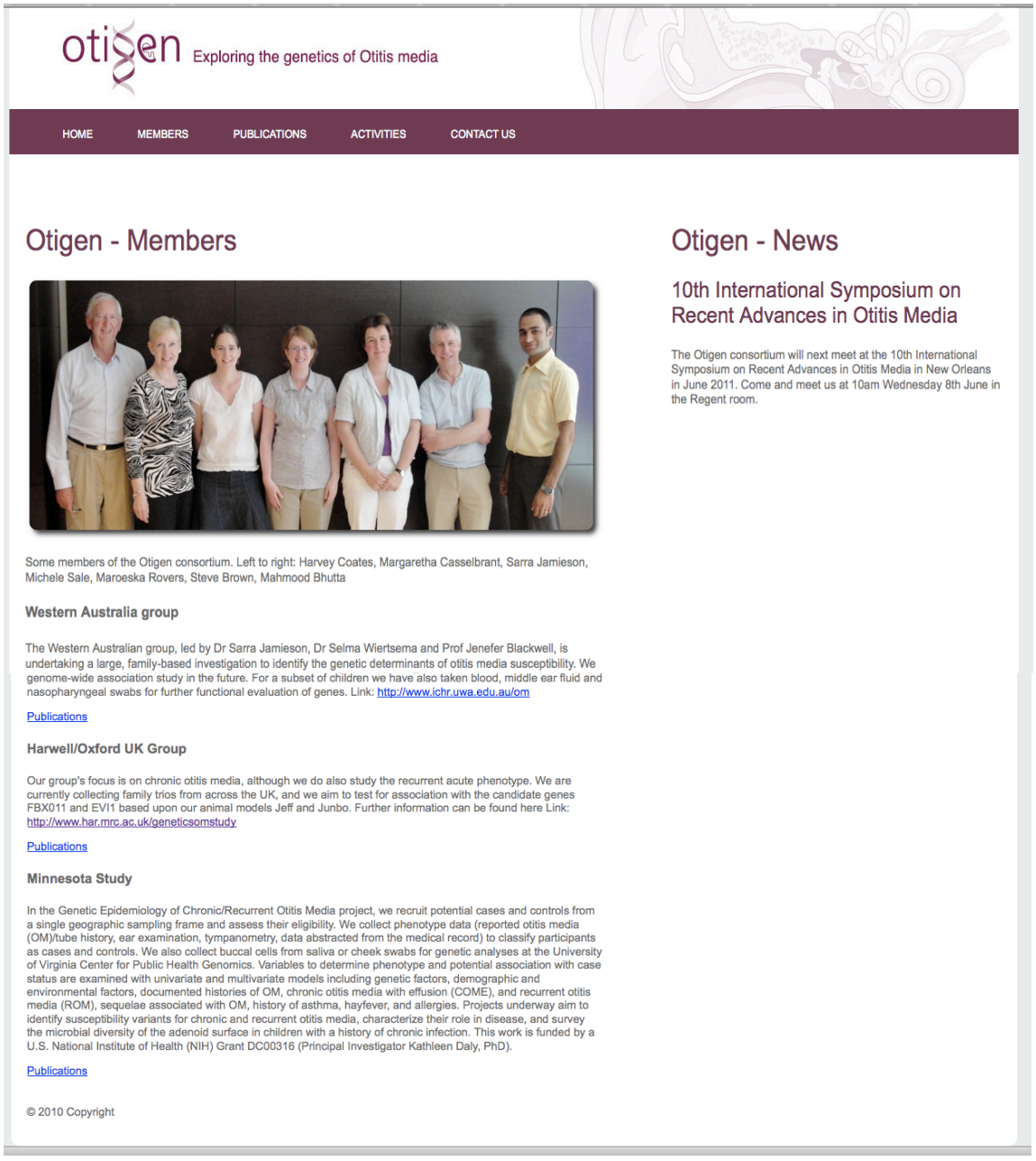


Table 5
Potential cohorts for replication from the Otigen consortium[36], the ALSPAC[39] group, and the Raine study[37].

	Location	Lead(s)	Phenotype	Phenotype definition	Number	Notes
OM specific cohorts	Western Australia*	Sarra Jamieson, Jennifer Blackwell, Harvey Coates	rAOM	≥3 AOM by age 3 and ventilation tube insertion recommended[38]	434 families (561 affected)	Recruitment ongoing
	Minnesota*	Michele Sale, Kathleen Daly	OM	Signs of or treatment for OM[41]	143 families (463 affected)	Number meeting clinical definitions of rAOM or COME not determined
	Pittsburgh*	Maragretha Casselbrant	rAOM & COME	Ventilation tube surgery[42]	403 families (377 affected sib pairs), 1431 individuals	Clinical indication for grommets not recorded. Grommet insertion more common in the US than the UK.
	Utrecht*	Maroeska Rovers, Anne Schilder	rAOM	Heterogenous cohort	-	Negligible DNA remaining
Birth cohorts	Bristol	ALSPAC (Amanda Hall)	COME	Subset with persistent type B or C2 tympanogram	142 cases, 945 controls	Operative intervention not known
	Perth	Raine	OM	Subset with signs of or treatment for OM[38]	253 cases, 866 controls	Number meeting clinical definitions of rAOM or COME not determined

* members of Otigen

Figure 8
Members page from the Otigen Website[36]



that is perfectly phenotype matched, i.e. white children undergoing grommet insertion with glue confirmed at operation. This was not available.

Discussion and Conclusion

The expansion of this study was extremely successful due to support from the NIHR portfolio. At the time of writing it is the largest recruiting study in the ENT specialty, and for this reason has been highlighted in the NIHR newsletter of February 2011 (see Appendix).

The increase in the number of recruiting centres has allowed me to surpass the target recruitment of 1000 families within the intended study period. Unfortunately this has been offset by a low return rate of 45.1%, meaning DNA samples are available for only 600 families. Previous studies using saliva self-collection have reported return rates of 59-95%[43-46]. However, these studies have required a sample to be returned only from an adult proband, whereas my study requires DNA from multiple family members, and this may explain the lower return rate I have seen here.

An extension to the study has been approved, enabling continued recruitment, and I aim to reach the target of 1,000 families with DNA returned by late 2012 or early 2013.

Through the Otigen consortium I have identified a number of cohorts for replicating any findings from my study. Because I have concentrated on the COME phenotype, the most appropriate replication is from the Pittsburgh cohort, or the (smaller) ALSPAC cohort, although even these do not have robust phenotyping for chronic OM.

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Chapter 4.2
DNA extraction, tag SNP derivation, & genotyping

I sought to undertake a candidate gene study for COME using my DNA database (chapter 4.1). Loci for testing were selected based upon selected candidates from mouse models (chapter 1.3), and associated pathways.

My initial strategy was to test for disease association only with the loci *FBXO11* and *EVI1* on the basis of the *Jeff*[1 2] and *Junbo*[3] models, and the Minnesota group report of nominal levels of association of OM susceptibility with SNPs at *FBXO11*[4]. This I have termed part 1 of the analysis. For this part I created tagging algorithms with the phase 2 release of HapMap[5 6], and I personally undertook genotyping on our in-house Pyrosequencing platform[7].

During the course of the study new data became available. Rye et al[8] reported association of susceptibility to rAOM with novel SNPs in *FBXO11*. In this study they also analysed loci in related pathways, specifically a number of *SMAD* genes in the TGF-β pathway. They reported associations approaching significance with SNPs in *SMAD2* and *SMAD4.* In our lab we discovered that the *Tgif1* knockout mouse spontaneously develops chronic otitis media (unpublished findings). Consequently *SMAD2*, *SMAD4* and *TGIF1* were included as additional loci for association testing. In addition a Phase 3 release of HapMap[9] provided higher resolution mapping, and so LD maps were rederived using Phase 3 data[8]. This I have termed part 2 of the analysis. Because all samples had to be genotyped afresh, and due to time pressures, genotyping for part 2 was outsourced.

Materials and Methods

DNA extraction

DNA was extracted from Oragene® OG-250 vials using an automated system (KBiosciences[10], Hoddesdon, UK). Cells were lysed and DNA was bound to silica particles mediated by guanidinium isothiocyanate. The solution was washed to remove contaminants and then DNA was eluted into a low salt buffer. DNA quantification was by spectrophotometry.

Tagging Algorithm

For each locus I interrogated the Ensembl database to assess overall polymorphism. Hypothesized regulatory elements (based upon the Ensembl algorithm) were also catalogued. For each locus no hypothetical regulatory elements were found more than 3kb upstream or downstream of protein-coding regions.

For part 1, *FBXO11* and *EVI1* linkage disequilibrium (LD) maps were interrogated from the HapMap Genome Browser[5 6] (phase 2, release 26) to identify tag SNPs. Tracks were configured to include the Untranslated Region (UTR) 3kb 5' and 3' of the protein-coding region. r^2 was set to 0.8, with a minor allele frequency (MAF) >0.05 in the Caucasian (CEU) population.

For part 2, tagging SNPs were rederived using Haploview 4.2 software[11 12] from LD maps in Phase 3 data of HapMap (release 28) for the loci *FBXO11*, *EVI1*, *SMAD2*, *SMAD4* and *TGIF1*. Tracks were again configured to include the UTR 3kb 5' and 3' of the protein-coding region, with r^2 set to 0.8 and MAF>0.05 in the CEU population. SNPs associated or approaching significance in published studies were include in the tagging algorithm (table 1).

Table 1: Tagging algorithm for loci in Part 2 of analysis. SNPs that were force included for *FBXO11* were rs2134056, reported by Segade et al[4] and rs330787, reported by Rye et al[8]. For *SMAD2* rs1792658 was included, which was approaching significance in the study by Rye et al[8]. For *SMAD4* rs10502913 was included, also approaching significance in the study by Rye et al[8].

Locus	Protein coding transcripts	Tagging algorithm configuration					Tagging results		
		Region (NCBI 36 coordinates)	MAF	Population	r^2	SNPs force included	Number of tag SNPs	Alleles captured	Mean max r^2
FBXO11	11	2:47882565..47991318	>0.05	CEU	>0.8	rs2134056, rs330787	17	44/44	0.96
*EVI1**	5	3:170282244..170349787	>0.05	CEU	>0.8	-	10	71/71	0.98
TGIF1	9	18:3399072..3451404	>0.05	CEU	>0.8	-	24	41/41	0.979
SMAD2	3	18:43610465..43713924	>0.05	CEU	>0.8	rs1792658	8	71/71	0.939
SMAD4	3	18:46807611..46863144	>0.05	CEU	>0.8	rs10502913	3	20/20	0.988

*EVI1 is part of the MDS1/EVI1 cluster, recently renamed by HUGO as MECOM.

Genotyping

For part 1 Pyrosequencing (Qiagen, Hilden) was utilised for genotyping[7], following the manufacturers instructions and manufacturers reagants. Sequences for selected tag SNPs were extracted from the dbSNP database[13] and amplification and sequencing primers designed using PSQ software (Version 1: Qiagen, Hilden). Validity of primers was tested on a panel of anonymous human DNA, and results subsequently optimised on a PCR temperature gradient. Genotype calling was based upon manufacturer's automated software reading of fluorescence peaks.

For part 2, I again extracted SNP sequences from the dbSNP database[13]. Primer design and genotyping was performed using KASPar[14] primer extension sequencing by KBiosciences[10]. Genotype calling was again based upon automated software reading of fluorescence (Kluster Caller[15]).

Results

As of 1st November 2011, DNA had been extracted from 1911 samples, with a median DNA yield of 15.3ng (figure 1). There was a lower median DNA yield in children (9.3ng) compared to adults (26.4ng/ul). This difference was statistically significant ($p<0.001$, Mann-Whitney U test).

Tag SNPs for part 1 at the *FBXO11* and *EVI1* loci are shown in table 2 (LD triangle plots not shown). For part 2 LD triangle plots for the loci *FBXO11*, *EVI1*, *SMAD2*, *SMAD4* and *TGIF1* are shown in figures 2-6, and tag SNPs in table 3.

Figure 1: DNA yield for children and adults from the 1911 samples extracted as of 1st November 2011. Note logarithmic scale. The median yield (vertical line) was significantly higher in adults than in children.

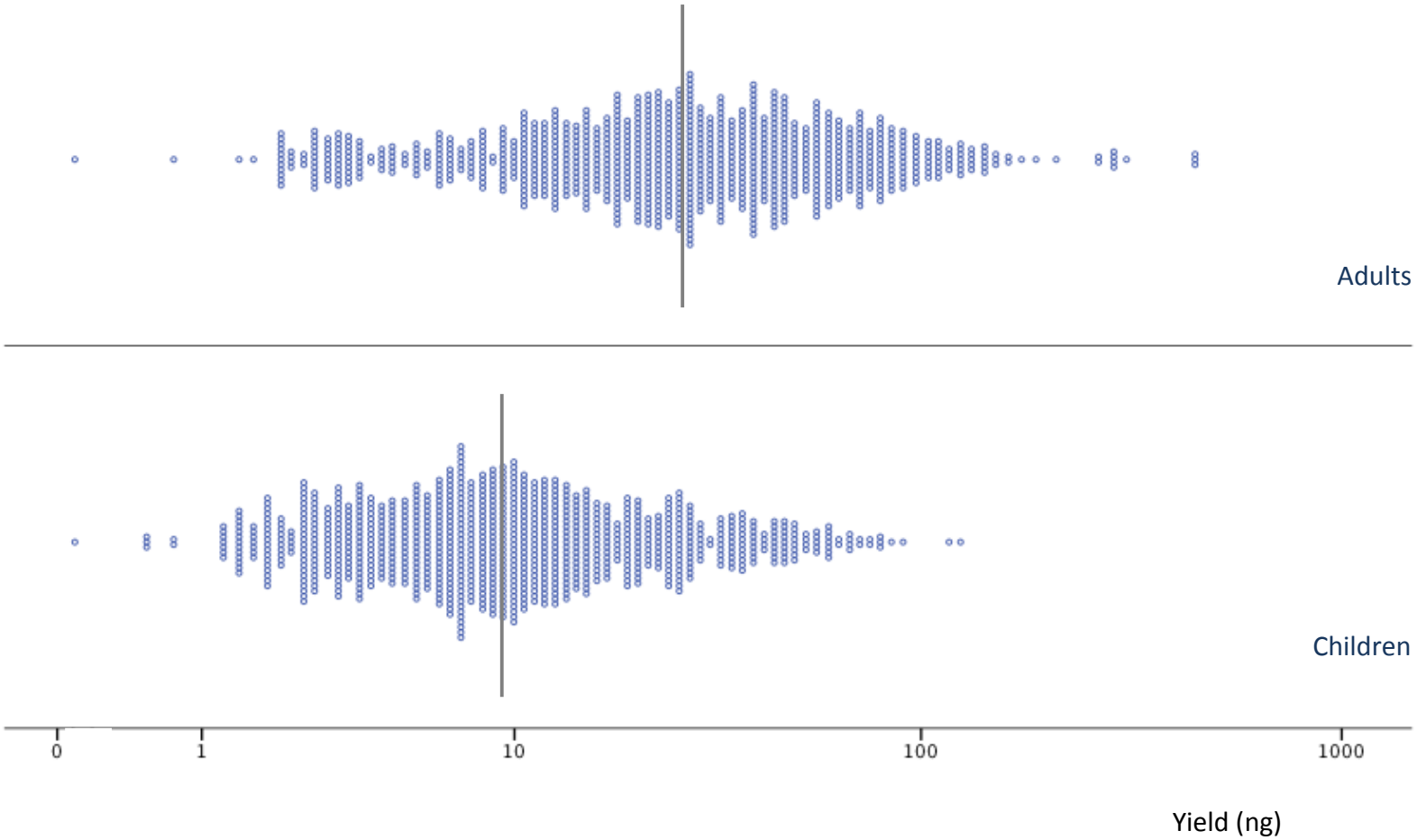


Table 2: Pyrosequencing PCR primers used in part 1 of the analysis. *= biotinylated

Locus	SNP	Location	Forward primer	Reverse primer	Sequencing primer	PCR Temp (°C)	Allele A	Allele B
FBXO11	rs7562048	2: 47885816	GCCTCAGACAGAATAATGTCA	GGGAAACTCCATCTCAAAA*	CATCACTTTTTAAGAACTGC	56	A	G
	rs3136367	2: 47887055	TTCGGTTTTTTGAGAGGG	ACCATGCATGCCTTAAGA*	TTTTGAGAGGGCACTT	53	C	G
	rs2537742	2: 47892548	GGGTGGGACATGTTAATGAT	TGATACTCCTGCCTTAGCC*	CTTAAAAGGACAGAACCA	60	A	G
	rs17323768	2: 47892727	GCCTGAGTTATTCCAAGTCC	TCATGAGGTTTTCCATATGC*	CAAGTGCTATTGCTAAGATG	60	G	T
	rs3732191	2:47893789	CCTAAACCAGTTCAACTTGATAA*	TTTCCATTATTCTTCCATATCTTT	ATCAATGACATTGCTAACTA	53	A	C
	rs330787	2:47894881	TGAGCATTTTTTTAGCGATAA*	CGTTCCAGACCACCATAATA	TTGCAATAAAGAGAGTCG	54	C	T
	rs17037002	2: 47931209	AGCCATATGATGTAAAGTGGACTA	GCCCACAGACATTTTTTACTTAT*	ATGATGTAAAGTGGACTATT	60	A	T
	rs960106	2: 47932342	AAGCAATATAGTAAGTGCCATACC	ATTAGGGACAGGCTAAATTGTT*	GGACACACAAATCGTCA	56	C	T
	rs12712997	2: 47938565	AGCTGGAAATACAAGAATCATAAG	GTTCTTTAATGCGATATTTCATCT*	AAATCGCCATTATTTTG	60	A	C
	rs10182633	2: 479 449 12	CGGTATTTCATGAGAAAACATATA	AGCCAATTCCTGTCTTTAA*	CATATAATGAAAGAGACAGA	60	A	T
	rs874869	2: 47947841	AGGTTCCAGATTTTGTCTAACA	AGTGGAGGATGATTCTATGGA*	TGCAGTAACAAATCCC	60	C	G
	rs6728843	2: 47957015	CTTTGTTTGCCACTGTATTATTT	AATAGTAAACCAGGGAGAAGGA*	TTTGCCACTGTATTATTTT	64	C	T
	rs17395881	2: 47957942	TTGCCCACACTGGTAATTT*	GTGGGAATAATTGATATTCAAGTG	GAACAATTTTAAAGTGTATG	60	A	C
	rs13035558	2: 47985440	CGATAGAGCGGAAGGGTA	CTGGGAAGGACCCATTAA*	AAATGGCCGATGGAT	56	C	T
EVI1	rs4955634	3: 170279757	TACTGTGAATAATGCTGCTATGA*	TCGTGATTTCACTTATGGGTATA	CTCAAAAGAATTAGCAGG	60	A	C
	rs12636548	3: 170292726	AGGGCAGTAAAAACAAGGAT	ATGGAGTTTCCCCCAAAT*	GCAGTAAAAACAAGGATG	54	G	T
	rs13094430	3: 170297603	GAAAACCCTGTTACAGTAAACG*	AGCATGGCAGAAACATAATC	CTAAAGTGTTTTGTGGGA	60	C	T
	rs16853191	3: 170321993	AGGCCTTTTTGATACCATTTA	TCCTCCAAATTTTTGTACACA*	ATTTAGAGTGACTTGAGTT	53	A	G
	rs3851383	3: 170325287	ATCCGCTCTCTAGTTTTGTGTA	TGGTTGGTACTTTTTCTTTTCTAA*	GCGCTCCACAATAAA	64	C	G
	rs6773653	3: 170334628	ACGTCAGGTCATTTTAAGAGAG*	GTTCACATTGGATTGAGTAAATCT	AGAGACATGATTAATAGTGG	60	A	G
	rs12631447	3: 170340920	GACTTGGGTATCTTTAATGGATCT	TATTCACCCCTGAAAAAATG*	TCTTTGGTATAGCTTCTTTC	53	C	T
	rs17468398	3: 170342189	TTCCCTTTTGTCTTGTCAATT	TAAACAACAAGGGAGGAAAGT*	TTTTGTCTTGTCAATTGG	53	C	T
	rs7633965	3: 170347337	TCAGCAATAATAGGCATTCAC	AGACCCATTTGGAAATCG*	GATCCCGAGCAACAT	64	A	C
	rs10936575	3: 170347845	CTTTCGCAACTCCAAGCT*	TGGTGCATCCATAATCAGTC	CAGTTCATGAAATAGAAACC	64	C	T

Figure 2: Linkage disequilibrium (LD) triangle plot for the locus *FBXO11.* Extracted from HapMap phase 3 (release 28) using Haploview 4.2 software. Values are for D' between SNPs. Colour coding is the standard scheme for Haploview, where white represents D'<1, LOD<2; blue represents D'=1, LOD<2; pink/red repres ents D'<1, LOD≥2, bright red represents D'=1, LOD≥2

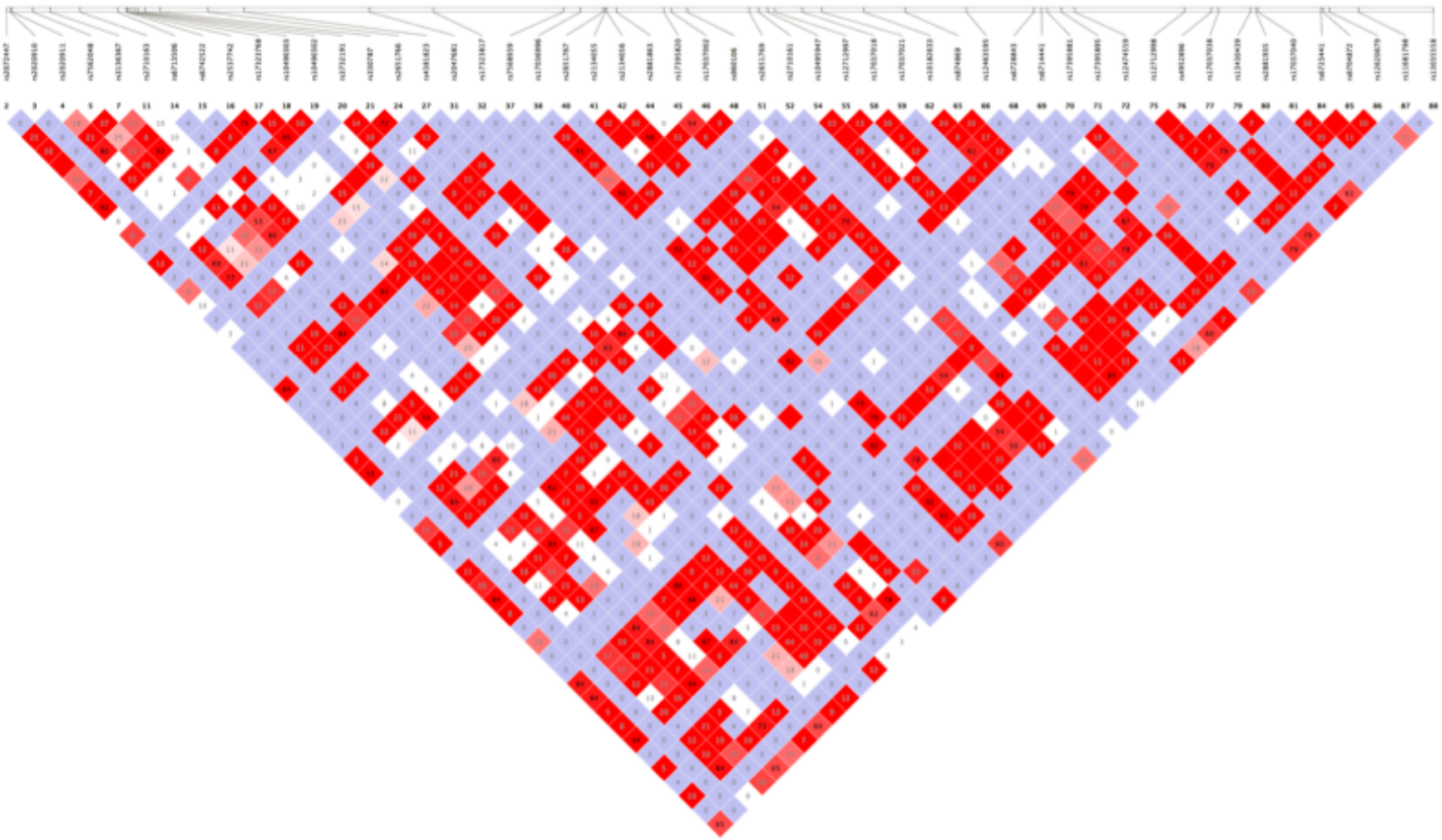


Figure 3: Linkage disequilibrium triangle plot for the locus *EVI1* (part of the *MECOM* locus). Extracted from HapMap phase 3 (release 28) using Haploview 4.2 software. Values are for D' between SNPs. Colour coding is the standard scheme for Haploview, where white represents D'<1, LOD <2; blue represents D'=1, LOD<2; pink/red represents D'<1, LOD≥2, bright red represents D'=1, LOD≥2

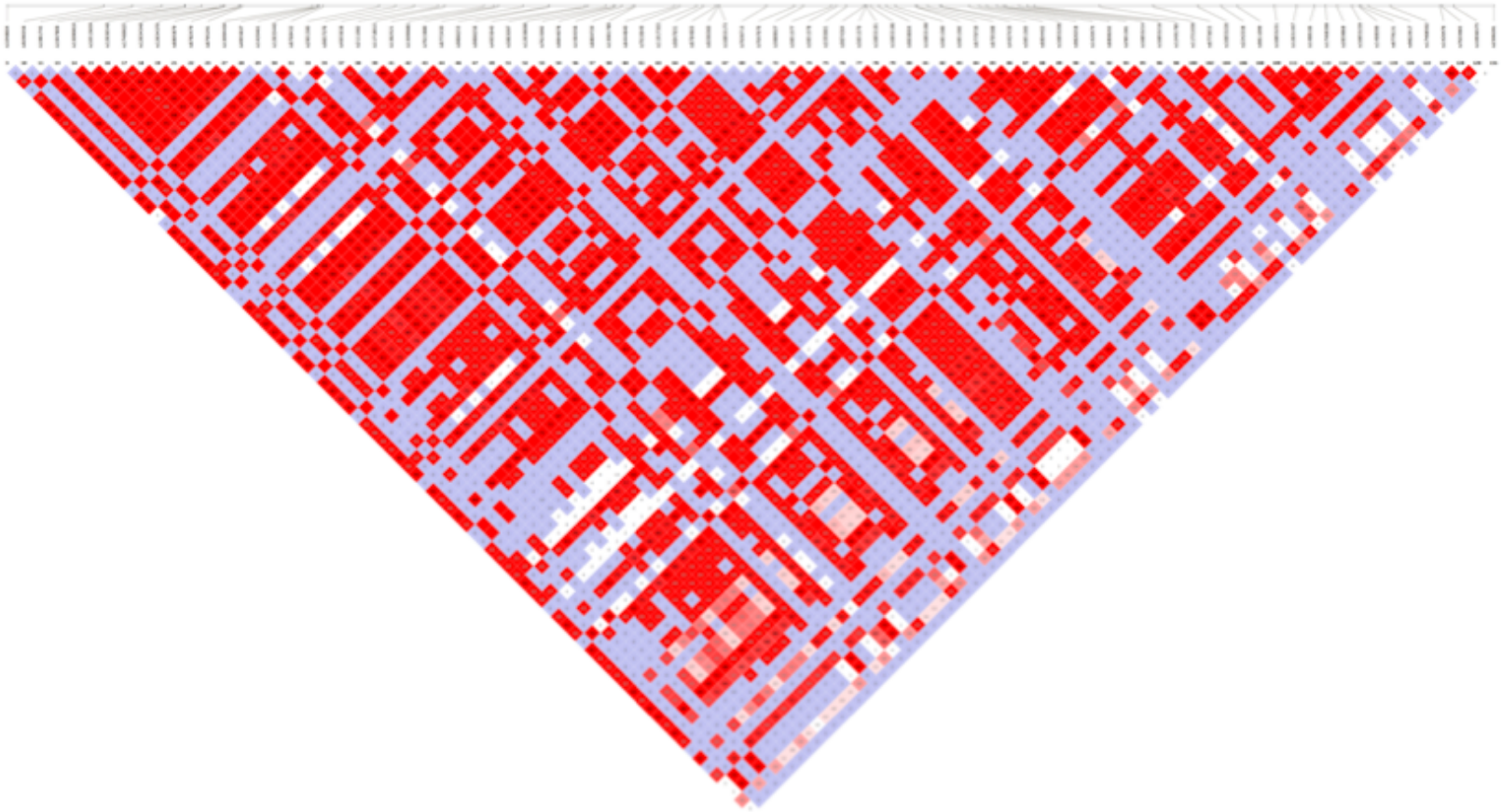


Figure 4: Linkage disequilibrium triangle plot for the locus *SMAD2.* Extracted from HapMap phase 3 (release 28) using Haploview 4.2 software. Values are for D' between SNPs. Colour coding is the standard scheme for Haploview, where white represents D'<1, LOD<2; blue represents D'=1, LOD<2; pink/red represents D'<1, LOD≥2, bright red represents D'=1, LOD≥2

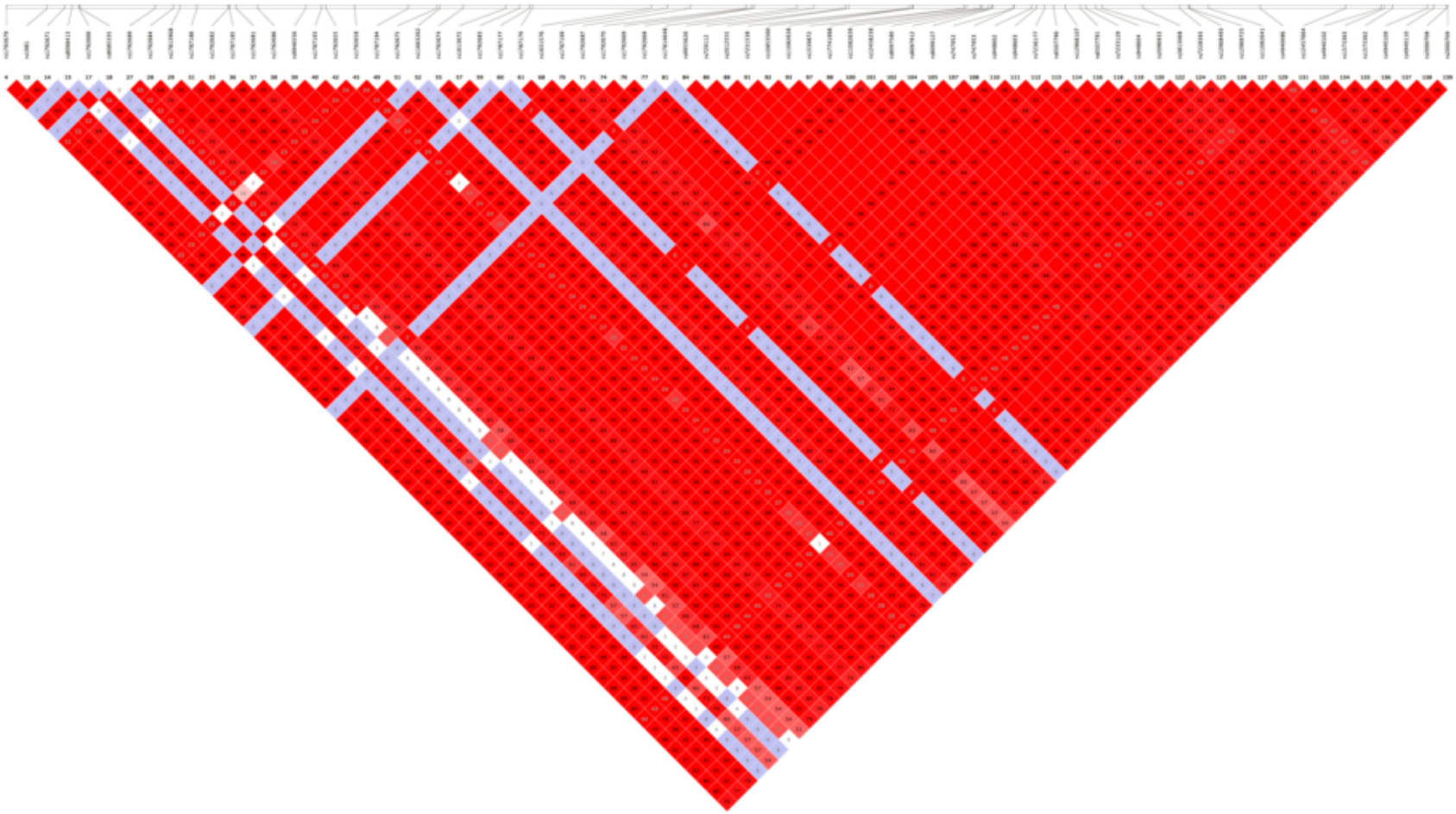


Figure 5*:* Linkage disequilibrium triangle plot for the locus *SMAD4.* Extracted from HapMap phase 3 (release 28) using Haploview 4.2 software. Values are for D' between SNPs. Colour coding is the standard scheme for Haploview, where white represents D'<1, LOD<2; blue represents D'=1, LOD<2; pink/red re presents D'<1, LOD≥2, bright red represents D'=1, LOD≥2

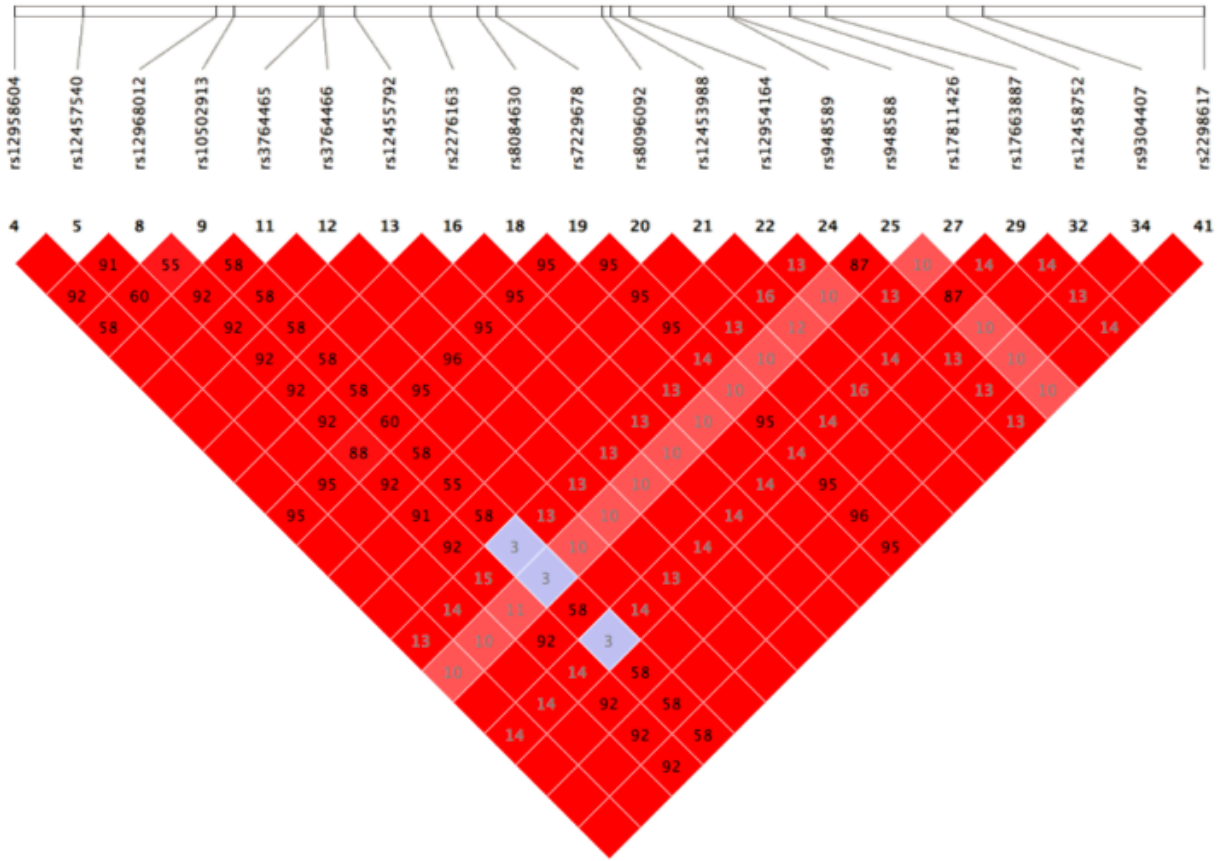


Figure 6: Linkage disequilibrium triangle plot for the locus *TGIF1.* Extracted from HapMap phase 3 (release 28) using Haploview 4.2 software. Values are for D' between SNPs. Colour coding is the standard scheme for Haploview, where white represents D'<1, LOD<2; blue represents D'=1, LOD<2; pink/red represents D'<1, LOD≥2, bright red represents D'=1, LOD≥2

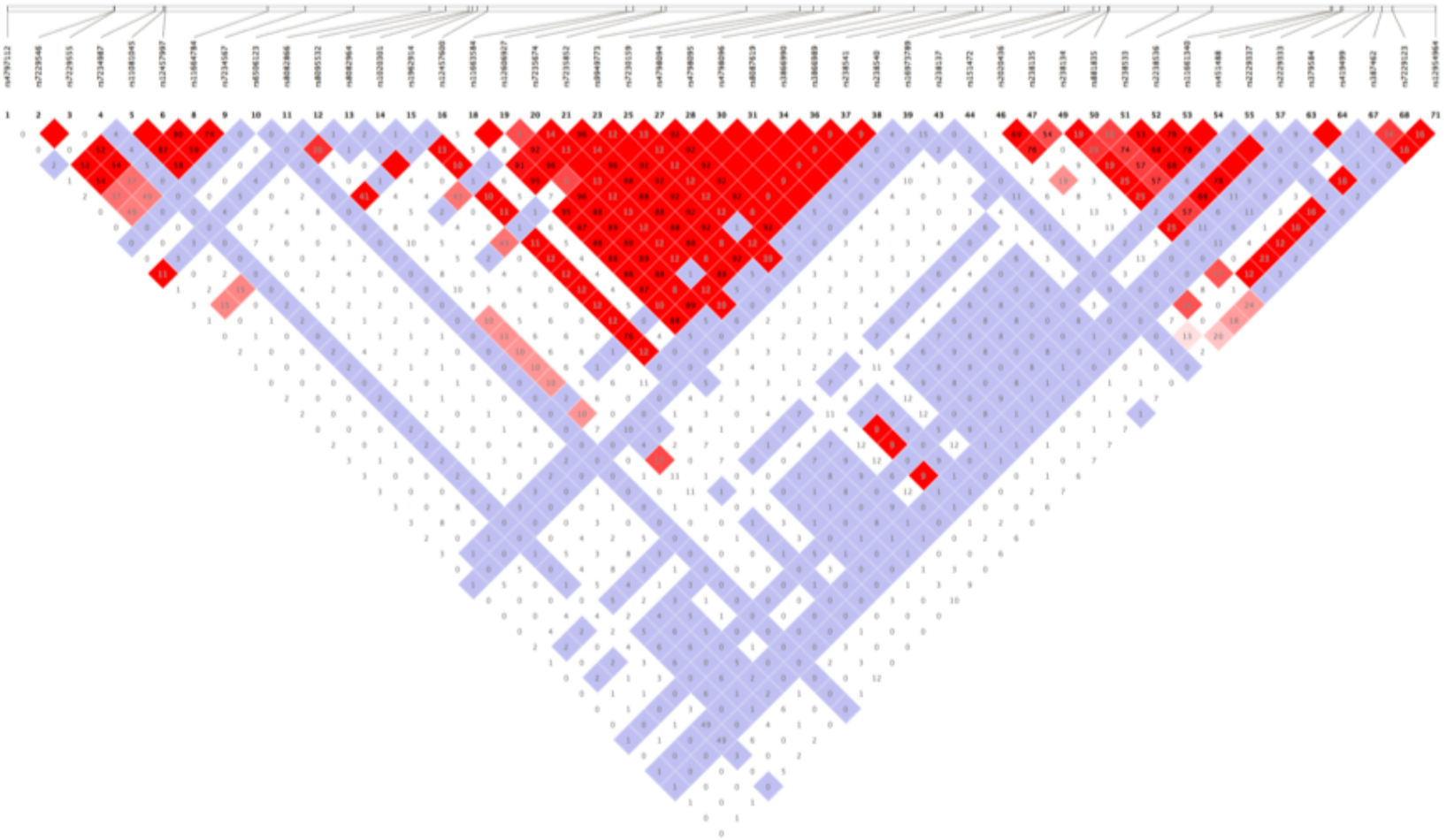


Table 3 : KASPar PCR primers used in part 2 of the analysis.

Locus	SNP	Location	Primer Allele A	Primer Allele B	Primer Common	Allele A	Allele B
FBXO11	rs330787	2:48041377	GAGTATTGCAATAAAGAGAGTCGCG	CGAGTATTGCAATAAAGAGAGTCGCA	GCTATTGCATACTTAATAGACTACAGTATA	C	T
	rs2134056	2:48073117	ACTTCTCCATAATTTCAGGAGGGG	AATACTTCTCCATAATTTCAGGAGGGA	CCACAAATGCATTTACATGTAGATCCATTA	C	T
	rs2072447	2:48030458	GTCTTCTTTATATAACACATTTCATTATAAGAC	GTCTTCTTTATATAACACATTTCATTATAAGAG	GGAGATCGTTGGACTGTAATTGAAAGTTA	C	G
	rs12712997	2:48085061	GCAGACTTTTAAAAATCGCCATTATTTTGTA	CAGACTTTTAAAAATCGCCATTATTTTGTC	GCCATCTGCTGTTCTTTAATGCGATATTT	A	C
	rs13430439	2:48118896	CAAGTGTTGGTGAAGATACGTAGAAG	ACAAGTGTTGGTGAAGATACGTAGAAA	TTTCTACCAACAGTGTATAAGGGGTTCAA	C	T
	rs960106	2:48078838	AAATTGTTTTCTTTTAAACTGTGTGCG	GCTAAATTGTTTTCTTTTAAACTGTGTGCA	AGTATAAAACAGGACACACAAATCGTCATA	C	T
	rs17395881	2:48104438	CCTTTACCTCCCTGAGGATCTTAA	CTTTACCTCCCTGAGGATCTTAC	CAAGTGAATCCTTAGTCTATAGGTGTAAAT	A	C
	rs13035558	2:48131936	GGAAATGGCCGATGGATTCC	GCTGGAAATGGCCGATGGATTCT	GGAAGGACCCATTAAATCAAGACCTTTAA	C	T
	rs874869	2:48094337	GTTTAAGCTGCAGTAACAAATCCCC	GTTTAAGCTGCAGTAACAAATCCCG	CTCAGGATAAACATGTAAAGTGAACAACAA	C	G
	rs7562048	2:48032312	TTAAAAACATCACTTTTTAAGAACTGCATAA	TAAAAACATCACTTTTTAAGAACTGCATAG	GCAACAAGAGGGAAACTCCATCTCA	A	G
	rs3732191	2:48040285	TTAATGATCAATGACATTGCTAACTAAGT	TAATGATCAATGACATTGCTAACTAAGG	GATAAGGAAAAAGACTTGAGATTCACTCAT	A	C
	rs2047681	2:48056231	CCACCACGAGGCAGTGTTTCA	CCACCACGAGGCAGTGTTTCG	CAGATCCCTTGAGCCCAGGAGTT	A	G
	rs17395820	2:48077179	GCACTGGTGGCAGGAGACC	GCACTGGTGGCAGGAGACG	GAGTTGTATTGTGCTGTAGCTCCCTT	C	G
	rs10182633	2:48091408	GTCTTTAATTATGAGTTGTTCAAAGGTAGT	GTCTTTAATTATGAGTTGTTCAAAGGTAGA	GTACTCACGGTATTTCATGAGAAAACATAT	A	T
	rs2537742	2:48039044	GTGTGAGCCACTGTGCCCAGT	GTGAGCCACTGTGCCCAGC	GGGGTGGGACATGTTAATGATACTTAAAA	A	G
	rs2881863	2:48073815	AATTTCACTGTTTTATTTTTTAATGCAGCTAAG	AATTTCACTGTTTTATTTTTTAATGCAGCTAAA	CCAGAAATAGAACGCAATCCACATATGTA	C	T
	rs10490302	2:48039744	TACATAGAATAGCTAGTTATTACTCTAACATA	CATAGAATAGCTAGTTATTACTCTAACATG	CATCACTGCTTGGCTGTGGTATTAAATTT	A	G
EVI1	rs6804720	3:168828996	CAGGTAGACTAAGATGTTCAAAGACAA	AGGTAGACTAAGATGTTCAAAGACAC	GGTAGAATGGAGAAAAACCAAAAAGGGAT	A	C
	rs7615880	3:168823078	GGGTAACTTGGCAGTTCTGTGAC	GGGTAACTTGGCAGTTCTGTGAT	CTAAGGTGTTGACTAGGGCTGATGTT	C	T
	rs13090810	3:168809291	ATAATGATATTTACTTGATGCAAATAAGAACTG	ATAATGATATTTACTTGATGCAAATAAGAACTC	TCAGAACCCAAATGGTGTGTTTTGTTCAA	C	G
	rs12631447	3:168858226	AGTGTCTTTGGTATAGCTTCTTTCAC	CTAGTGTCTTTGGTATAGCTTCTTTCAT	CTGACTGATATCTTTATTCACCCCTGAAA	C	T
	rs7633965	3:168864643	TTCAAACGATCCCGAGCAACATTTA	CAAACGATCCCGAGCAACATTTC	TTTGGAAATCGAGACGCGAGGCTTT	A	C
	rs10936575	3:168865151	GAGACAGTTCATGAAATAGAAACCCG	AGAGACAGTTCATGAAATAGAAACCCA	ACGGGATCGTCCCCTTTCGCAA	C	T
	rs16853239	3:168860665	TAACCCAATGGTTTCCTAAATCAAATG	CTTAACCCAATGGTTTCCTAAATCAAATA	CAAGCAACCACTTCAATATGGTCTTTGAA	C	T
	rs11718015	3:168820726	AACTTGGCATGTGCCTTGTAAGAC	AACTTGGCATGTGCCTTGTAAGAG	GAATGTTTCTCGAAAAGTGTGGTTACATAA	C	G

	rs11721038	3:168849576	CAAATCCTACTTCACAAATCAAATTTTCTC	CCAAATCCTACTTCACAAATCAAATTTTCTT	TATGTGACGCAGCTAGCCTTGCATT	C	T
	rs12634348	3:168810980	AATATTCATAGGGCATGTTTTAAGAAACAG	AATATTCATAGGGCATGTTTTAAGAAACAA	CTAGCAACTTATTTGGAAACTCACTCCTT	C	T
TGIF1	rs7229546	18:3414008	GTTATTATCCTAATGTGTAGATGAAAAAACAAA	ATTATCCTAATGTGTAGATGAAAAAACAAG	ACTCTGTGACCTTAGGCAAATTATGTCAT	A	G
	rs7235674	18:3433425	AAAATCACTTTAAGATTAAAAATTACAAGACA	AAAATCACTTTAAGATTAAAAATTACAAGACG	CCTTCGGCCATGTTATAGAATATATCAGA	A	G
	rs7234567	18:3420806	AGGAGGGGTGTTTAAGAAATATCAATA	AGGAGGGGTGTTTAAGAAATATCAATG	GGCTAAAAGCAGCAAAAAGCAGTCAAAAT	A	G
	rs12954964	18:3460915	CATTTTCACTTAAAACAAGCGTCATTTAAAG	ATCATTTTCACTTAAAACAAGCGTCATTTAAAT	GAACATCAGAAATGGTCAATGTTTGGCAA	G	T
	rs238134	18:3449286	TGACCGAAACAGACGAGCAGG	GACCGAAACAGACGAGCAGC	GGCAGCGGCCACTTTTACAGCTA	C	G
	rs1020301	18:3426724	CAGAACAACTTCCTAAAAGTGTGATTT	CAGAACAACTTCCTAAAAGTGTGATTC	GGCAATATCTAACAAAGCCACACGTTTAT	A	G
	rs4797112	18:3410242	AATGATTGTTGAAATATTATGACTTTCCATGT	AATGATTGTTGAAATATTATGACTTTCCATGA	AGTTCTGACCAGGAGGCTGAATGTT	A	T
	rs881835	18:3449310	GCGTGGTCACCCCTTAGCTG	GGCGTGGTCACCCCTTAGCTA	CCCCGGCAGCGGCCACTTT	C	T
	rs1962914	18:3426898	GGGTACTATGCAGCGGTAAAAAAG	GGGGTACTATGCAGCGGTAAAAAAT	CTACAAGAGTGAAACTCCGTCTCCAA	G	T
	rs8095532	18:3425778	GGTGATGACTGTCTCCTGCGTA	GTGATGACTGTCTCCTGCGTG	AGTCAAGAAAGAGTTCAGTTGACACGAT	A	G
	rs238135	18:3448979	CACACAATGAGACTTCCCCCG	CCACACAATGAGACTTCCCCCA	TGCTAGGCTTATTTACGTTTAGGGCTTTT	C	T
	rs238541	18:3444603	ATGATGTGTTAGGCACTGTATTAAGTG	CTATGATGTGTTAGGCACTGTATTAAGTA	CGTCTCAAAAACAACAACAACAAAAAACAA	C	T
	rs7229123	18:3459378	CCTAATTTTCTTACGCCTCTTGGTAG	ACCTAATTTTCTTACGCCTCTTGGTAA	CTCAAAGACTTTCCTTGTAAGTGAAAGAAA	C	T
	rs2238536	18:3452978	GGAAAATCCACTAAGTCTTTTTGCTCT	GAAAATCCACTAAGTCTTTTTGCTCC	CAATCCCAAAGCAGGAATTCAACAGTTAA	A	G
	rs387462	18:3458997	GTTTATTAGGTGAGTATTTTCCAAGGTCA	GTTTATTAGGTGAGTATTTTCCAAGGTCT	ACAAACTCCACTTGTACATAGCACCATTT	A	T
	rs2020436	18:3448762	CGGAGCGTCTGTTTCAGGAAC	CGGAGCGTCTGTTTCAGGAAT	TAAACTCTGGCCTCCACGCATTCTA	C	T
	rs8082866	18:3425211	AAGATGGAGTTAGCCATGTCACG	CAAGATGGAGTTAGCCATGTCACA	GCCTTTGCAAAATTATGACAGCAAGAGAA	C	T
	rs151472	18:3447871	TTATAGCTTGAAAACAAACTTTCCAGGA	ATAGCTTGAAAACAAACTTTCCAGGG	TCTCCGCCCCTCCCCCCTT	A	G
	rs238533	18:3451762	TAAACTTGAAACTCGGATCAACTGGT	AAACTTGAAACTCGGATCAACTGGC	CCGCGGGGAGGGGTCCTTA	A	G
	rs16973789	18:3445826	AAGAGAGCTTCTTGTAAAACTGGAGA	GAGAGCTTCTTGTAAAACTGGAGG	TGGGGGGTTGTGCTCTGAAATCTA	A	G
	rs8087619	18:3441167	AAGCCCAATTGACTTAATTCATGAAGG	AAGCCCAATTGACTTAATTCATGAAGC	CACAGAGTTTTTCTTTTCTACGGTTTGTAT	C	G
	rs11661340	18:3457185	AGAAGGCTTATGACAGGTGGG	AACTAGAAGGCTTATGACAGGTGGT	TATCTGAGCTCCGCCAAGAAAGCAA	G	T
	rs11664784	18:3419463	CACAAAAATAAAAAGCATTCTCCTGACCT	CAAAAATAAAAAGCATTCTCCTGACCC	CACATATAGAAGACACCACGCTTGTATT	A	G
	rs8082964	18:3426587	TTCTCCACAGTATTAGCAAACAAAATAT	TCTCCACAGTATTAGCAAACAAAATAC	CTGTACAGATAACCTATGGGCCTAATATA	A	G
SMAD2	rs1792658	18:45382605	AGGGAGATTATAGGTCAAAGGGTATAA	GGGAGATTATAGGTCAAAGGGTATAC	ATCTCTGGGCATATCTTACATATCTGCAA	A	C
	rs1787177	18:45394919	TTAAAGTTTTCAACAAGAAGAGAAGTTGC	TTTTAAAGTTTTCAACAAGAAGAGAAGTTGT	CGAGTAGGTGGACCCTAGCTTTAAA	C	T

	rs4940086	18:45446307	GATAGAAAATTTAACAATATGTGGTTTGCG	CTGATAGAAAATTTAACAATATGTGGTTTGCA	TTCTTTAGACCAGTGCTATCCAACAGAAA	C	T
	rs17814648	18:45413714	GCTATTTCAGAACCGGGAGG	CCTGCTATTTCAGAACCGGGAGA	CCTCTTCCAGTACTCTCCAGCTATT	C	T
	rs1792689	18:45368587	GATCTTATTATTCACCATCATCCAAAATG	GTGATCTTATTATTCACCATCATCCAAAATA	TAATAGTATCTACATTCTCTCTCAGGTGTT	C	T
	rs1792668	18:45410742	CCCCATTTGACAAATGAGGTAATGG	CCCCATTTGACAAATGAGGTAATGA	TAGGACCTTAGACGTGTTACTTTGTGTTT	C	T
	rs8098413	18:45362721	GAAAGCTCTGCCATATACCCCG	AAAGAAAGCTCTGCCATATACCCCA	CATCCCAGAATCTGCTGGATGTATAATAA	C	T
	rs1787199	18:45404603	GATTAGGAAGACTTGGTATTAGTTTATTTTTTA	AGGAAGACTTGGTATTAGTTTATTTTTTT	CACACAATGGTGTGGTACTGTTATTTGTA	A	T
SMAD4	rs12958604	18:48560325	CATAATCTAAGCCTTGATGAGTTGCTA	ATAATCTAAGCCTTGATGAGTTGCTG	CAACTGGTCTTTGGTACTTATCTGATCTA	A	G
	rs948589	18:48586184	ATATTTAAGTAAGATTTACTGAAAGTTTTAGCG	ATATTTAAGTAAGATTTACTGAAAGTTTTAGCA	GAACAGGCCCTAAAGAAAAGACAACAAAA	C	T
	rs10502913	18:48568271	AGACTAGCCAATCCTGACTGATACA	ACTAGCCAATCCTGACTGATACG	CATAGGTAACCTAGGTCTTGGCACAA	A	G

For genotyping in part 1 of the analysis, two pairs of sequencing primers had to be redesigned. Primers for rs3136367 in *FBXO11* were redesigned due to mispriming, and those for rs16853191 in *EVI1* were redesigned due to poor genotyping success. Experimentally derived optimum PCR temperatures to maximise genotyping success are detailed in table 2 (where acceptable genotyping success is defined as >80% automated calls per run). Several hundred genotyping results were obtained using pyrosequencing (data not shown), before switching to part 2 of the analysis. A sample pyrogram is shown in figure 7.

For genotyping in part 2, mean genotyping success across the 61 SNPs analysed was 97.0% (range 95.6-98.1%). Cluster plots for selected SNPs are shown in figure 8.

Discussion and Conclusion

The mean DNA yields in this study are 35-75% lower than yields from saliva reported in other studies[16-19]. I have been unable to identify reasons for this. Regardless, the high genotyping success demonstrates that the DNA yield is sufficient for downstream applications.

The derivation of tag SNPs using the latest Phase 3 HapMap data enables a more detailed analysis of candidate susceptibility loci for association with otitis media. Such analysis is the subject of the following chapter.

Figure 7: Sample of light emission from pyrosequencing (pyrogram) in part 1 of the study. Data are for SNP rs17323768 in *FBXO11* (see table 2), and for family 113 of the study. Data visualized using manufacturer's software.

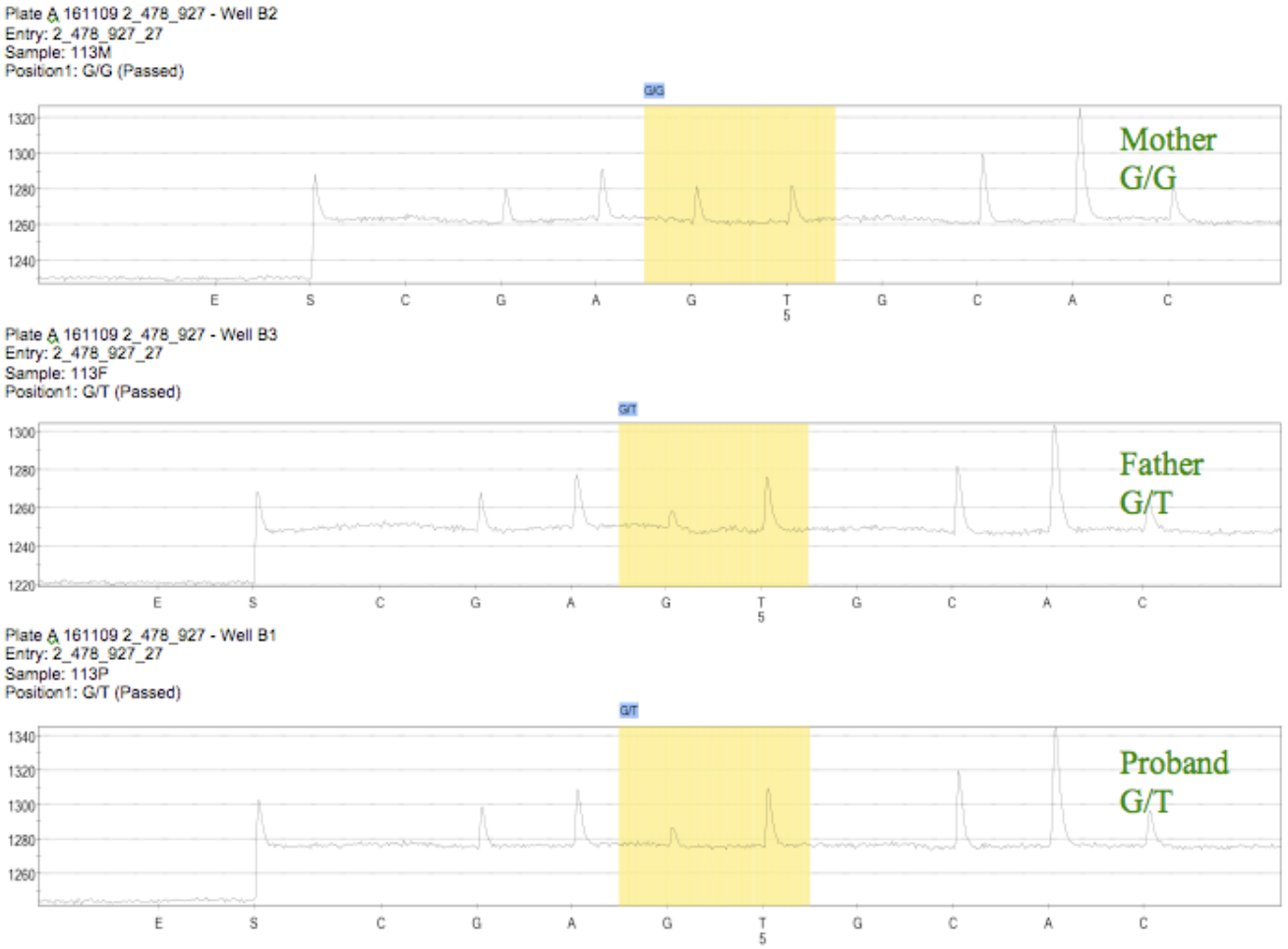
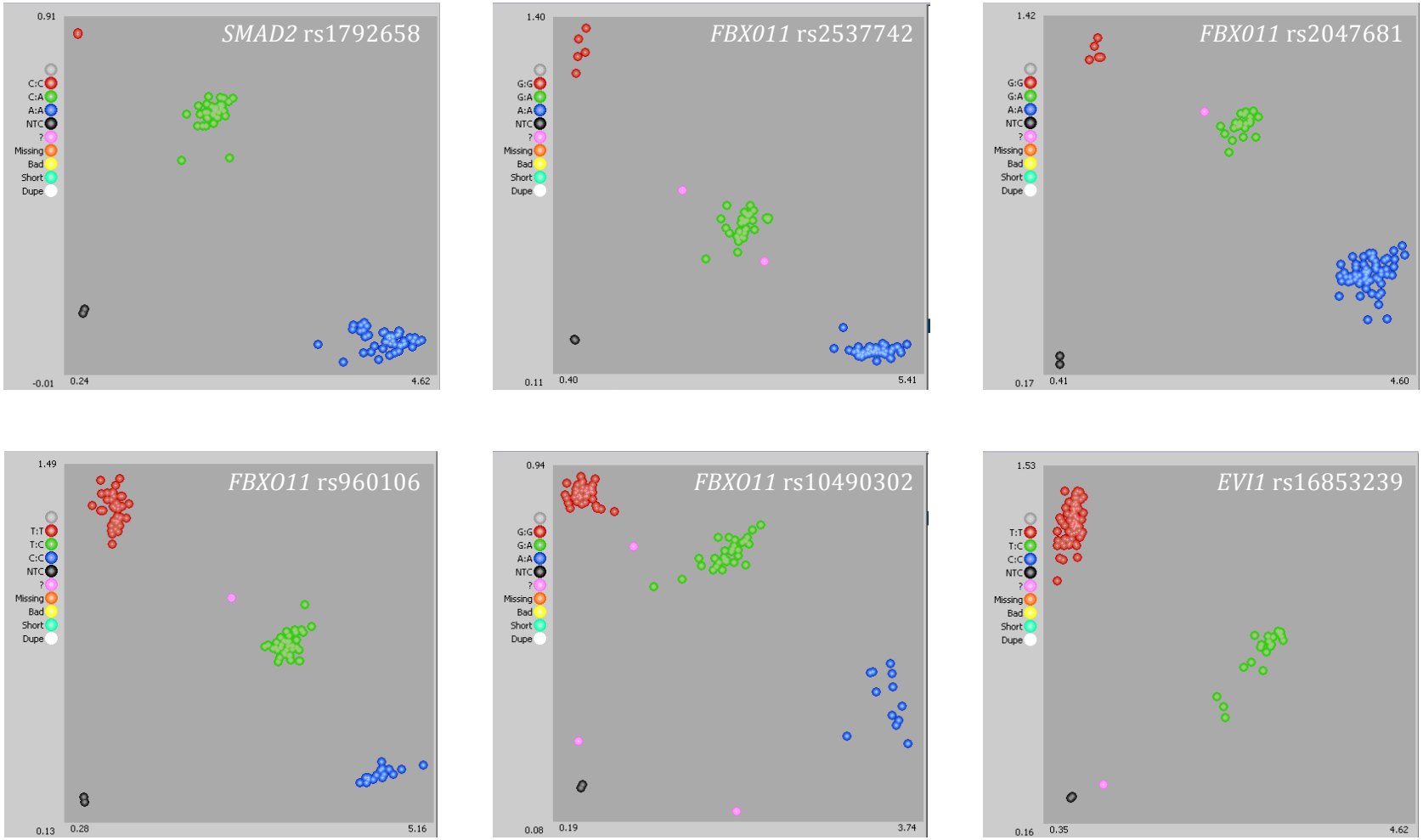


Figure 8: Sample fluorescence cluster plots for a single 96 well plate from KASPar sequencing in part 2 of the analysis (all data for a single SNP cannot be shown in a single plot because fluorescence readers differ between plates and are individually calibrated). Clusters are well demarcated (red, green, and blue clusters). Note that the six SNPs shown here are the top six hits in part A of the statistical analysis in chapter 4.3. Data visualized using SNPViewer2 software[20].



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Chapter 4.3
Association testing for *FBXO11*, *EVI1*, *SMAD2*, *SMAD4*, & *TGIF1*

I undertook association testing in collaboration with Martin Farrall and Anuj Goel at the Wellcome Trust Centre for Human Genetics. We used the PLINK[1] [2] tool and PedCheck[3] [4] program for data quality control, and the TRANSMIT[5] [6] test for association testing.

TRANSMIT is a variant of the Transmission Disequilibrium Test (TDT). The TDT test utilizes case-parent triads and so, unlike the case-control approach, is immune to confounding by population stratification or admixture[7]. In addition, TRANSMIT deals efficiently with missing genotypes, and handles families with multiple affected individuals correctly.

Materials and Methods

We performed association testing in two parts. In part 1 we tested all individuals genotyped by 1st November 2011, which includes proband phenotypes of both COME and rAOM. In part 2 we repeated testing in the subset with a proband phenotype of COME.

Genotyping data were coded into .map and .ped format (Simon Greenaway, MRC Harwell) for use with PLINK v1.07. Our data quality control excluded individuals with a genotyping success rate below 80%, and SNPs with a genotyping success rate below 95%. We tested remaining SNPs for departure from Hardy-Weinberg Equilibrium, with $p<1e^{4}$ as the criterion for exclusion. Any minor alleles with a frequency of less than 1% in our sample were also excluded.

Remaining data were imported into PedCheck v1.0 and analysed for Level 0-2 errors. If one or two SNPs were discordant within a nuclear family, discordant genotypes in the parent or sibling were reassigned as unknown. If three or more SNPs were discordant, all data for the discordant

individual were excluded from subsequent analyses, on the assumption that this is evidence of high levels of genotype miscall in this individual, or evidence of a non-biological relationship.

Association testing was performed using TRANSMIT v2.5.4 for the loci *FBXO11*, *EVI1*, *SMAD2*, *SMAD4*, and *TGIF1* (chapter 4.2). Any individual who had grommet surgery in the past was classified as affected, all others' affectation status was coded as unknown.

Results

Part 1

500 nuclear families (1944 individuals) were included in the analysis of the combined COME/rAOM phenotype. 78 individuals (4.7%) were excluded due to low genotyping success (figure 1). All SNPs had a genotyping success rate of greater than 95%. The SNP rs7235674 in the *TGIF1* gene was excluded due to departure from Hardy Weinberg equilibrium ($p<9.41^{e-174}$), which presumably represent artefact from genotyping miscall. No minor alleles had a frequency of less than 1%. On Pedcheck analysis 74 individuals (9 mothers and 63 fathers) were excluded due to significant genotyping discordance within families.

Results of TRANSMIT testing are shown in table 1, and a Q-Q plot of expected and observed chi squared values in figure 2.

Figure 1: Plot of genotyping success rate at the 61 SNPs for the 1944 individuals in part 1 of the analysis. Only 2.3% of individuals had a genotyping success rate of less than 80% (the majority of these were low DNA yield samples from young siblings).

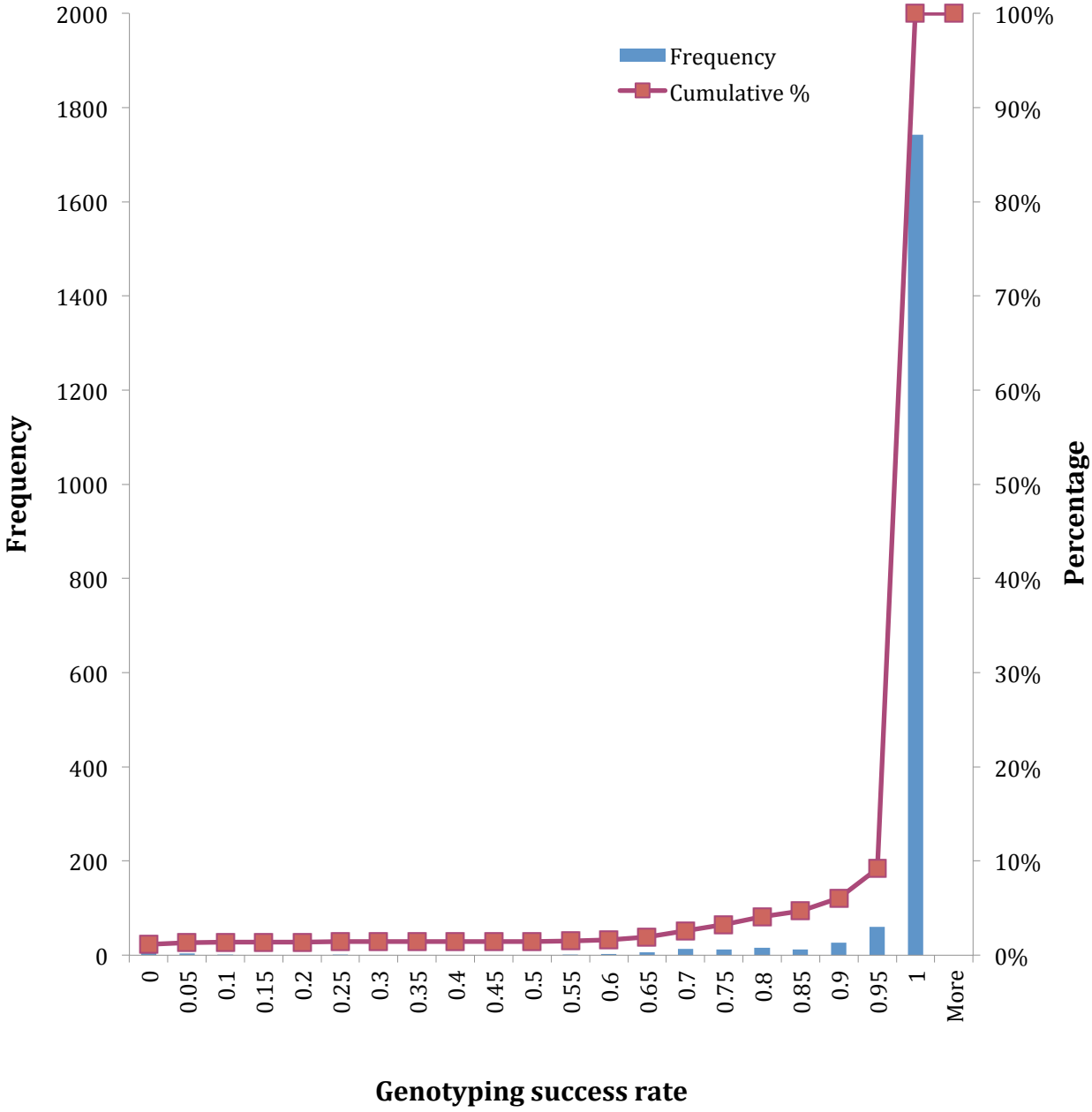
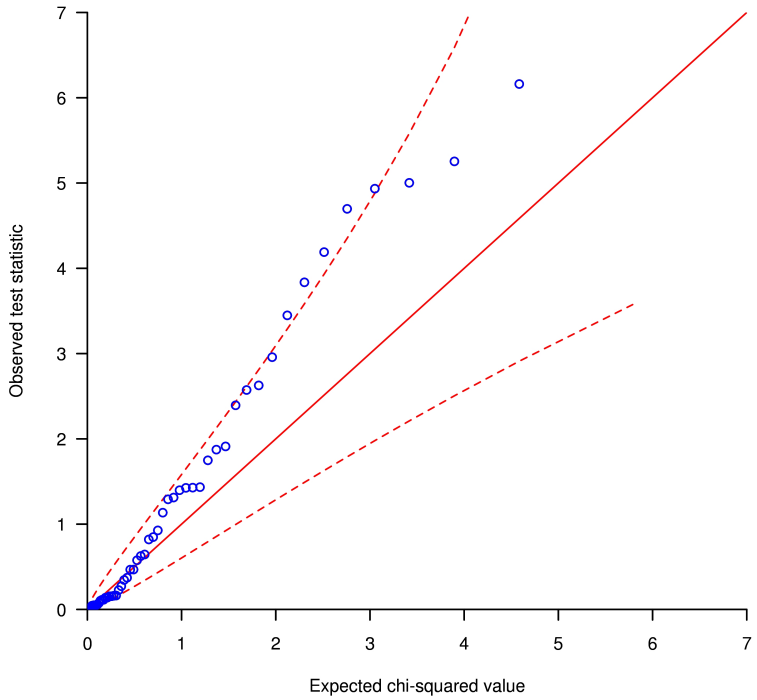


Table 1: Part 1 analysis (rAOM and COME phenotypes). SNPs associated with p<0.05 are shown in red. For each SNP there were at least 461 informative families (mean 471).

Locus	SNP	Chi Square	p value	Odds ratio (95% C.I.)	Overtransmitted allele
SMAD2	rs1792658	9.0126	0.0026	1.402 (1.127, 1.760)	C
FBXO11	rs2537742	6.161	0.0130	1.306 (1.059, 1.619)	G
FBXO11	rs2047681	5.2538	0.0218	1.342 (1.046, 1.738)	G
FBXO11	rs960106	5.0024	0.0253	1.233 (1.027, 1.485)	C
FBXO11	rs10490302	4.9338	0.0263	1.249 (1.028, 1.525)	A
EVI1	rs16853239	4.6973	0.0302	1.472 (1.044, 2.126)	T
TGIF1	rs12954964	4.19	0.0406	1.274 (1.012, 1.615)	G
FBXO11	_rs3732191	3.8357	0.0501	1.361 (1.003, 1.874)	A
TGIF1	rs387462	3.4482	0.0633	1.207 (0.991, 1.476)	T
TGIF1	rs881835	2.9579	0.0854	1.381 (0.961, 2.031)	T
TGIF1	rs238135	2.6269	0.1050	1.178 (0.967, 1.440)	C
SMAD2	rs8098413	2.5731	0.1086	1.321 (0.944, 1.880)	T
FBXO11	rs874869	2.3934	0.1218	1.156 (0.962, 1.393)	G
TGIF1	rs2020436	1.9114	0.1668	1.165 (0.939, 1.451)	T
FBXO11	rs13430439	1.8737	0.1710	1.169 (0.936, 1.465)	T
TGIF1	rs151472	1.7485	0.1860	1.140 (0.939, 1.386)	A
TGIF1	rs8095532	1.4339	0.2311	1.255 (0.868, 1.846)	A
FBXO11	rs10182633	1.428	0.2320	1.131 (0.925, 1.386)	A
TGIF1	rs1962914	1.4255	0.2325	1.331 (0.837, 2.189)	G
SMAD2	rs1792689	1.3978	0.2370	1.168 (0.904, 1.518)	T
EVI1	s7615880	1.3119	0.2520	1.172 (0.894, 1.544)	C
SMAD4	rs948589	1.291	0.2558	1.198 (0.879, 1.647)	T
TGIF1	rs238134	1.1346	0.2867	1.124 (0.907, 1.396)	G
SMAD2	rs4940086	0.92665	0.3357	1.103 (0.904, 1.348)	T
EVI1	rs13090810	0.8481	0.3570	1.106 (0.893, 1.372)	G
TGIF1	rs11664784	0.82026	0.3651	1.097 (0.898, 1.342)	G
FBXO11	rs12712997	0.64425	0.4221	1.080 (0.895, 1.305)	C
FBXO11	rs330787	0.62598	0.4288	1.076 (0.897, 1.293)	T
SMAD4	rs12958604	0.57659	0.4476	1.077 (0.890, 1.305)	A
TGIF1	rs8087619	0.46749	0.4941	1.069 (0.883, 1.296)	C
SMAD2	rs1792668	0.46647	0.4946	1.064 (0.891, 1.271)	T
EVI1	rs11718015	0.34038	0.5420	1.063 (0.873, 1.297)	G
TGIF1	rs7229123	0.27989	0.5567	1.055 (0.883, 1.260)	C
EVI1	rs10936575	0.24641	0.6017	1.048 (0.878, 1.252)	T
TGIF1	rs238541	0.20774	0.6339	1.138 (0.666, 1.980)	T
TGIF1	rs16973789	0.2068	0.6865	1.083 (0.735, 1.605)	G
FBXO11	rs7562048	0.16232	0.6892	1.037 (0.867, 1.241)	G
TGIF1	rs238533	0.15001	0.6940	1.060 (0.793, 1.419)	G
TGIF1	rs11661340	0.14487	0.6951	1.069 (0.765, 1.499)	T
TGIF1	rs7234567	0.13999	0.7095	1.040 (0.847, 1.278)	G
FBXO11	rs2134056	0.13032	0.7108	1.045 (0.829, 1.318)	T
SMAD2	rs1787177	0.096243	0.7362	1.060 (0.753, 1.499)	C

SMAD4	rs10502913	0.088694	0.7371	1.037 (0.839, 1.283)	G
SMAD2	rs1787199	0.075192	0.7486	1.029 (0.863, 1.227)	T
EVI1	rs12631447	0.068807	0.7826	1.025 (0.862, 1.219)	T
FBXO11	rs17395881	0.059038	0.8083	1.038 (0.769, 1.401)	C
TGIF1	rs7229546	0.040089	0.8221	1.023 (0.838, 1.249)	A
TGIF1	rs2238536	0.03158	0.8236	1.041 (0.731, 1.485)	G
EVI1	rs12634348	0.027671	0.8241	1.022 (0.844, 1.239)	T
EVI1	rs11721038	0.020715	0.8312	1.035 (0.751, 1.430)	T
FBXO11	rs2881863	0.016997	0.8336	1.037 (0.735, 1.466)	C
FBXO11	rs13035558	0.016734	0.8555	1.019 (0.830, 1.253)	T
FBXO11	rs2072447	0.00889	0.8930	1.015 (0.822, 1.253)	C
TGIF1	rs8082964	0.006849	0.9145	1.016 (0.757, 1.365)	G
TGIF1	rs1020301	0.004016	0.9281	1.012 (0.781, 1.312)	G
FBXO11	rs17395820	0.001744	0.9519	1.009 (0.753, 1.352)	C
TGIF1	rs4797112	0.001489	0.9781	1.003 (0.838, 1.200)	A
SMAD2	rs17814648	0.001274	0.9817	1.005 (0.664, 1.520)	T
TGIF1	rs8082866	0.001112	0.9828	1.002 (0.818, 1.228)	C
EVI1	rs6804720	0.000360	0.9887	1.001 (0.832, 1.205)	C
EVI1	rs7633965	2.18e-05	0.9955	1.001 (0.696, 1.440)	C

Figure 2: Q-Q plot of the expected and observed chi-square distribution from testing 61 SNPs using the TRANSMIT test. Solid line = expected distribution, dotted line = 95% confidence intervals. Blue dots = observed values from analysis. Data are for part 1 of the analysis (rAOM and COME phenotypes).



Because several SNPs in *FBXO11* demonstrated association, we went on to repeat association testing with haplotype blocks of the top three SNPs at this locus (table 3). In this analysis the lowest p-value was 0.004. Because the hit at *SMAD2* is in the same pathway as *FBXO11*, we wondered if this allelic variant may have a synergistic effect with a variant at *FBXO11* in disease susceptibility, and so we repeated this haplotype analysis but also incorporating the allelic variant at rs1792658 in *SMAD2*. Here the lowest p-value was 0.019.

Table 3: Association testing of haplotype blocks incorporating the top three hits at *FBXO11* in part1 of the analysis. The first nucleotide refers to the allele at rs2537742, the second to the allele at rs2047681, and the third to the allele at rs960106. All tests are undertaken using the TRANSMIT test, with one degree of freedom, except for the global chi-squared which is for six degrees of freedom. Values with p<0.05 are shown in red

Haplotype	Chi square	p value
A/A/C	0.012	0.912
G/A/C	2.025	0.1548
A/G/C	0.998	0.3177
G/G/C	8.302	0.004
A/A/T	3.676	0.552
G/A/T	0.016	0.901
A/G/T	0.585	0.444
Global	12.584	0.050

Table 4: Association testing of haplotype blocks incorporating the top three hits at *FBXO11* and the top hit at *SMAD2* in part1 of the analysis. The first nucleotide refers to the allele at rs2537742, the second to the allele at rs2047681, the third to the allele at rs960106, and the final to the allele at rs1792658 in *SMAD2*. All tests are undertaken using the TRANSMIT test, with one degree of freedom, except for the global chi-squared which is for 12 degrees of freedom. Values with p<0.05 are shown in red

Haplotype	Chi square	p value
A/A/C/A	0.039	0.843
G/A/C/A	0.770	0.781
G/G/C/A	2.321	0.128
A/A/T/A	5.522	0.019
G/A/T/A	0.620	0.431
A/G/T/A	0.948	0.330
A/A/C/C	3.061	0.080
G/A/C/C	1.572	0.210
A/G/C/C	1.005	0.316
G/G/C/C	4.961	0.026
A/A/T/C	0.339	0.561
G/A/T/C	0.825	0.364
A/G/T/C	1.069	0.301
Global	22.127	0.036

Part 2

404 nuclear families (1571 individuals) were included in the analysis of the COME phenotype. 93 individuals (4.4%) were excluded due to low genotyping success (data not shown). All SNPs had a genotyping success rate of greater than 95%. Again the SNP rs7235674 in the *TGIF1* gene was excluded due to departure from Hardy Weinberg equilibrium ($p<2.061^{e-138}$). No minor alleles had a frequency of less than 1%. On Pedcheck analysis 53 individuals (6 mothers and 47 fathers) were excluded due to significant genotyping discordance within families.

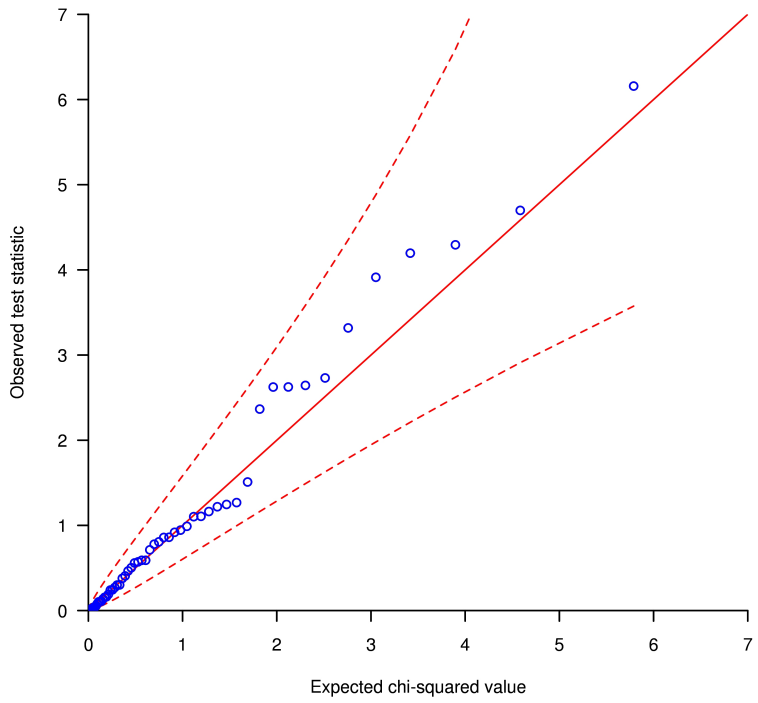
Results of TRANSMIT testing are shown in table 2, and a Q-Q plot of expected and observed chi squared values in figure 3.

Table 2: Part 2 analysis (COME phenotype only). SNPs associated with p<0.05 are shown in red. For each SNP there were at least 375 informative families (mean 383).

Locus	SNP	Chi Square	p value	Odds ratio (95% C.I.)	Overtransmitted allele
TGIF1	rs881835	6.1581	0.0130	1.695 (1.130, 2.666)	T
FBXO11	rs10490302	4.6984	0.0301	1.264 (1.024, 1.568)	A
FBXO11	rs960106	4.2941	0.0382	1.241 (1.013, 1.527)	C
FBXO11	rs2537742	4.1963	0.0405	1.266 (1.012, 1.594)	G
SMAD2	rs1792658	3.913	0.0479	1.284 (1.004, 1.655)	C
FBXO11	rs3732191	3.3185	0.0685	1.360 (0.981, 1.919)	A
FBXO11	rs2047681	2.731	0.0984	1.261 (0.960, 1.670)	G
TGIF1	rs387462	2.6435	0.1039	1.201 (0.964, 1.503)	T
TGIF1	rs1962914	2.6253	0.1051	1.527 (0.926, 2.672)	G
EVI1	rs16853239	2.6243	0.1052	1.384 (0.939, 2.092)	T
TGIF1	rs12954964	2.3655	0.1240	1.220 (0.948, 1.581)	G
FBXO11	rs874869	1.5095	0.2192	1.136 (0.927, 1.397)	G
TGIF1	rs8095532	1.2677	0.2601	1.266 (0.842, 1.944)	A
EVI1	rs7615880	1.2458	0.2643	1.190 (0.878, 1.626)	C
TGIF1	rs151472	1.2189	0.2695	1.129 (0.911, 1.404)	A
FBXO11	rs13430439	1.1627	0.2809	1.142 (0.898, 1.458)	T
FBXO11	rs7562048	1.1062	0.2929	1.111 (0.913, 1.355)	G
TGIF1	rs11664784	1.1024	0.2937	1.123 (0.905, 1.396)	G
TGIF1	rs238135	0.98919	0.3199	1.118 (0.898, 1.396)	C
TGIF1	rs8087619	0.94438	0.3311	1.111 (0.898, 1.391)	C
FBXO11	rs2134056	0.91863	0.3378	1.129 (0.828, 1.452)	T
EVI1	rs10936575	0.86035	0.3536	1.096 (0.903, 1.332)	T
FBXO11	rs10182633	0.8583	0.3542	1.111 (0.890, 1.391)	A
EVI1	rs11721038	0.80631	0.3692	1.174 (0.828, 1.680)	T
SMAD2	rs1792689	0.77945	0.3773	1.143 (0.850, 1.547)	T
SMAD4	rs12958604	0.71284	0.3985	1.096 (0.887, 1.357)	A
SMAD2	rs17814648	0.59016	0.4423	1.188 (0.766, 1.876)	T
TGIF1	rs2020436	0.58985	0.4424	1.097 (0.866, 1.395)	T
FBXO11	rs2881863	0.57169	0.4495	1.147 (0.804, 1.650)	C
EVI1	rs13090810	0.55893	0.4546	1.092 (0.866, 1.381)	G
TGIF1	rs238541	0.50246	0.4784	1.229 (0.695, 2.254)	T
SMAD2	rs8098413	0.46507	0.4952	1.148 (0.772, 1.726)	T
*SMAD4_*	rs10502913	0.4062	0.5239	1.081 (0.850, 1.378)	G
EVI1	rs6804720	0.37808	0.5386	1.065 (0.871, 1.305)	A
TGIF1	rs7234567	0.30117	0.5831	1.064 (0.853, 1.329)	G
TGIF1	rs16973789	0.29938	0.5842	1.120 (0.746, 1.696)	G
SMAD2	rs4940086	0.27111	0.6025	1.060 (0.850, 1.324)	T
TGIF1	rs238134	0.24357	0.6216	1.060 (0.840, 1.340)	G
SMAD2	rs1792668	0.2407	0.6237	1.051 (0.862, 1.283)	T
TGIF1	rs7229123	0.19049	0.6625	1.045 (0.858, 1.272)	C
SMAD4	rs948589	0.16184	0.6874	1.073 (0.759, 1.524)	T
FBXO11	rs13035558	0.15566	0.6931	1.047 (0.833, 1.317)	C

SMAD2	rs1787199	0.1362	0.7120	1.039 (0.849, 1.271)	T
TGIF1	rs238533	0.10981	0.7403	1.055 (0.768, 1.452)	G
FBXO11	rs2072447	0.10457	0.7464	1.039 (0.822, 1.315)	C
FBXO11	rs17395820	0.10165	0.7498	1.053 (0.764, 1.455)	C
TGIF1	rs4797112	0.079961	0.7773	1.030 (0.840, 1.262)	T
EVI1	rs12634348	0.056417	0.8122	1.026 (0.832, 1.265)	C
EVI1	rs12631447	0.041428	0.8387	1.020 (0.846, 1.230)	T
TGIF1	rs11661340	0.040945	0.8396	1.040 (0.711, 1.525)	T
FBXO11	rs17395881	0.028715	0.8654	1.030 (1.730, 1.455)	C
TGIF1	rs7229546	0.02443	0.8757	1.017 (0.821, 1.261)	A
TGIF1	rs1020301	0.022483	0.8808	1.022 (0.765, 1.368)	A
FBXO11	rs12712997	0.012681	0.9103	1.012 (0.824, 1.243)	A
SMAD2	rs1787177	0.010284	0.9192	1.020 (0.688. 1.516)	T
FBXO11	rs330787	0.010261	0.9193	1.010 (0.828, 1.233)	C
EVI1	rs7633965	0.007898	0.9291	1.018 (0.678, 1.533)	C
TGIF1	rs8082866	0.007747	0.9298	1.010 (0.809, 1.261)	C
TGIF1	rs2238536	0.006958	0.9335	1.017 (0.682, 1.517)	A
TGIF1	rs8082964	0.001033	0.9743	1.005 (0.726, 1.392)	A
EVI1	rs11718015	0.000085	0.9926	1.001 (0.807, 1.241)	G

Figure 3: Q-Q plot of the expected and observed chi-square distribution from testing 61 SNPs using the TRANSMIT test. Solid line = expected distribution, dotted line = 95% confidence intervals. Blue dots = observed values from analysis. Data are for part 2 of the analysis (COME phenotype only).



Discussion

The results presented here are interim, as they include only around half of the target sample size for this study. However, it is interesting that the top hits from the interim association analysis do suggest that *FBXO11* and *SMAD2* confer disease susceptibility, and possibly also *TGIF1*.

The results from the part 1 analysis (table 1) include four hits with p-values of <0.05 at *FBXO11*. Disease susceptibility is associated with presence of the minor G allele at rs2537742 (p=0.0130), the minor G allele at rs2047681 (p=0.0218), the minor C allele at rs960106 (p=0.0253), and the minor A allele at rs10490302 (p=0.0253). The statistical significance of these results can be interpreted under a frequentist or Bayesian philosophy.

The frequentist statistical approach suggests results should be subject to Bonferroni adjustment for multiple hypothesis testing. Bonferonni adjustment for 61 SNPs tested would imply that p > 0.05/61 = 0.0008 is a non-significant result, and as a consequence all results presented here are not significant. There are good arguments for Bonferroni adjustment in post-hoc analyses, but for prospective testing, such adjustment may unnecessarily increase the risk of type II error[8]. The Q-Q plot (figure 2) suggests that the top hits in table 1 deviate from the expected chi squared distribution, and so the associations for the top hits probably are true positives.

Increasingly a Bayesian statistical approach is being advocated for association studies[9 10]. This integrates factors such as biological plausibility and noteworthiness into findings, to reduce the risk of type 1 and type 2 errors. Given that polymorphism at *FBX011* is known to cause chronic OM in the *Jeff* mouse (chapter 1.3), and that previous human studies have reported association

of disease with this locus (chapter 1.4), the *FBXO11* locus has a high pre-test probability of association. Under Bayes theorem, the discovery of four hits at this locus would be considered significant.

One method to formalize the Bayesian approach is the false positive report probability (FPRP) calculator, developed by Gormli and Wacholder[11]. The FPRP calculator integrates prior probability of association and a range of potential and actual odds ratio of disease susceptibility to ascertain noteworthiness of findings. I have applied this method to calculate FPRP for the top hit at *FBXO11* in rs2537742, using the online tool[12]. The prior probability of association for this SNP (given previous research findings) I estimate at 0.05. The odds ratio of disease in this study was 1.306, with a 95% confidence interval of 1.059-1.619. Entering these data into the FPRP calculator means that the result at *FBXO11* rs2537742 is "noteworthy" at the 0.5 level if the actual odds ratio of disease associated with polymorphism at this SNP is at least 1.3 (note that previous SNP associations with common diseases have shown a median odds ratio of 1.3[13 14]). Because some values need to be estimated, the Bayesian approach may appear less satisfactory that traditional frequentist analyses, but I believe it is a better representation of the real odds of the hypothesis being tested.

Haplotype block analysis incorporating the top three hits at *FBXO11* showed that a haplotype block incorporating all of the associated SNPs (i.e. the G allele at rs2537742, the G allele at rs2047681, and the C allele at rs960106) gave an even stronger association with disease (p=0.004). This suggests that this haplotype block more often tags a causal variant(s) than a G polymorphism at rs2537742 by itself (the highest single SNP associated with disease).

The analysis in part 2 uses a majority of the families in part 1 (80.5% of recruits suffered from COME), and so unsurprisingly shows a very similar pattern of hits, but with slightly less

significant p-values (table 2). The less significant p-values may reflect a smaller sample size, rather than evidence that the *FBXO11* hits reported are not specific to the chronic OM phenotype. However, there is also a hit at rs881835 in *TGIF1*. This latter result needs verification and replication, but as it stands, suggests that this locus may be associated specifically with susceptibility to chronic OM. No previous studies have looked at this locus for disease susceptibility. At the time of writing, I am working with our collaborators in Western Australia (members of the Otigen consortium) to replicate these findings in *TGIF1* in their cohort (with a predominantly rAOM phenotype).

I did not perform analyses based upon only the rAOM phenotype, as the number of probands (96 families) is too small to give reliable results.

It is important to note that my interim results have not replicated the results reported by Segade et al[15] or Rye et al[16] (chapter 1.4), specifically I found no strength of association at the SNPs rs2134056 (part 1 p=0.7108), rs12712997 (part 1 p=0.4221), or rs330787 (part 1 p=0.4288). However Rye et al focused on a rAOM phenotype, and Segade et al had a more broad definition of OM, whereas my focus has largely been on chronic disease. The location of the SNPs at the *FBXO11* locus that have been reported to be associated with OM in previous studies and in my study here do not demonstrate clustering at a region coding for a particular functional domain of the FBXO11 protein (figure 4).

The hit at *SMAD2* rs1792658 (p=0.0026) is also noteworthy, where carriage of the minor C allele is associated with increased risk of OM. This SNP was also associated with risk of rAOM in the Raine study (p=0.038), but here disease association was with the major A allele, and the finding was not replicated in an independent cohort[17]. Regardless it is interesting that two studies have highlighted the same SNP as being associated with different phenotypes of OM

(figure 5).

Overall, these interim results do provide evidence that polymorphism at *FBXO11* is associated with risk of chronic OM. There is also some evidence that polymorphism at rs1792658 in SMAD2 is important, and possibly also rs881835 in *TGIF1* (figure 6). Although my results have replicated findings at the same loci as the Raine study, they have not replicated the same polymorphism. It is possible that haplotype blocks are significantly discordant in white UK versus white Australian populations, but given their shared recent ancestry this seems unlikely. Another possibility, given that the group in the Raine study was predominantly a rAOM phenotype and the group here is predominantly COME, is that these SNPs could be tagging haplotype blocks with alternate biological function. I have suggested (chapter 1.2) that rAOM and COME may be opposing phenotypes. It is interesting to conjecture that the associated SNPs in the Raine study are perhaps tagging a hypomorph haplotype of *FBXO11* or *SMAD2*, whereas the SNPs in my study are tagging a hypermorph haplotype (or vice versa).

Clearly these results need to be confirmed with a larger dataset once the target recruitment has been achieved. There would also be value in replicating the results within the Otigen consortium (chapter 4.1), although none of the replication cohorts is robustly phenotyped for chronic otitis media. Once results are confirmed, further analysis within haplotype blocks may enable imputation of causal variants, and *in silico*, *in vitro*, or *in vivo* analysis of potential functional effects. It is also possible that lack of association at the loci *EVI1* or *SMAD4* are false negative results. A larger sample size will give more confidence.

Conclusion

On interim analysis, the dataset presented here gives good evidence of a functional role of TGF-β signaling in conveying susceptibility to persistent chronic otitis media with effusion in children, through polymorphism at *FBXO11*, and possibly also *SMAD2* and *TGIF1*.

Figure 4: A: Structure of the *FBXO11* locus annotated with location of SNP hits in my study (blue), those reported by Rye et al[16] (green), and by Segade et al[15] (grey). Diagram created using Fancygene 1.4[18 19] using UCSC data, corrected to GrCh37 coordinates. B: Hypothesised protein domains of FBXO11. The F-box domain binds SKP1, CULLIN1, and ROC1 to form the SCF ubiquitin ligase complex[20 21] for protein degradation. The function of the other domains is unclear. CASH domains bind carbohydrates[22] and zinc finger domains (ZnF) typically interact with nucleic acids, proteins or small molecules[23]. Diagram created using DOG 2.0[24 25] with data from Ensembl.

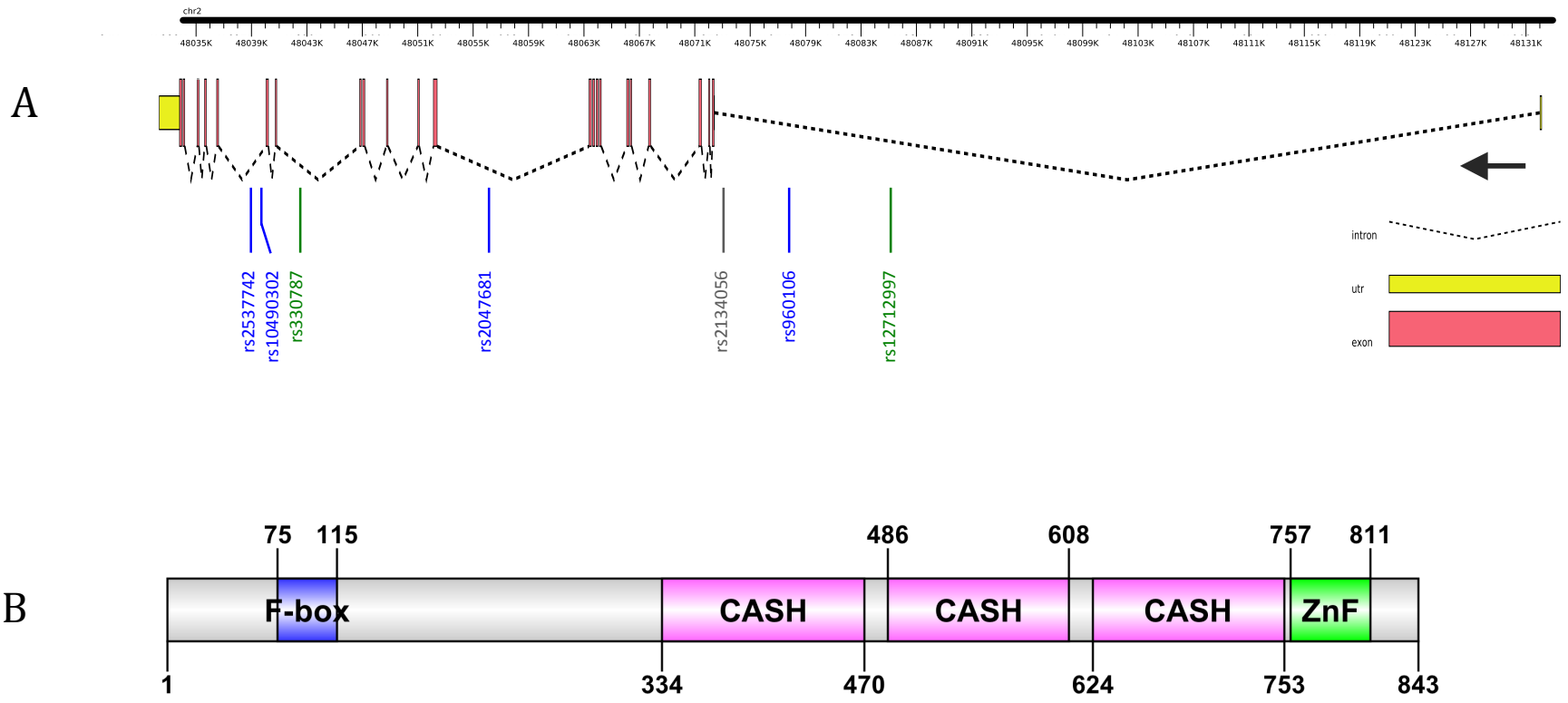


Figure 5: A: Structure of the *SMAD2* locus annotated with location of SNP hit at rs1792658 in my study (also approaching significance in the study by Rye et al[16]). Diagram created using Fancygene 1.4[18 19] using UCSC data, corrected to GrCh37 coordinates. B: Hypothesised protein domains of SMAD2. MAD / SMAD domains are involved in DNA binding and transcriptional control[26]. Diagram created using DOG 2.0[24 25] with data from Ensembl.

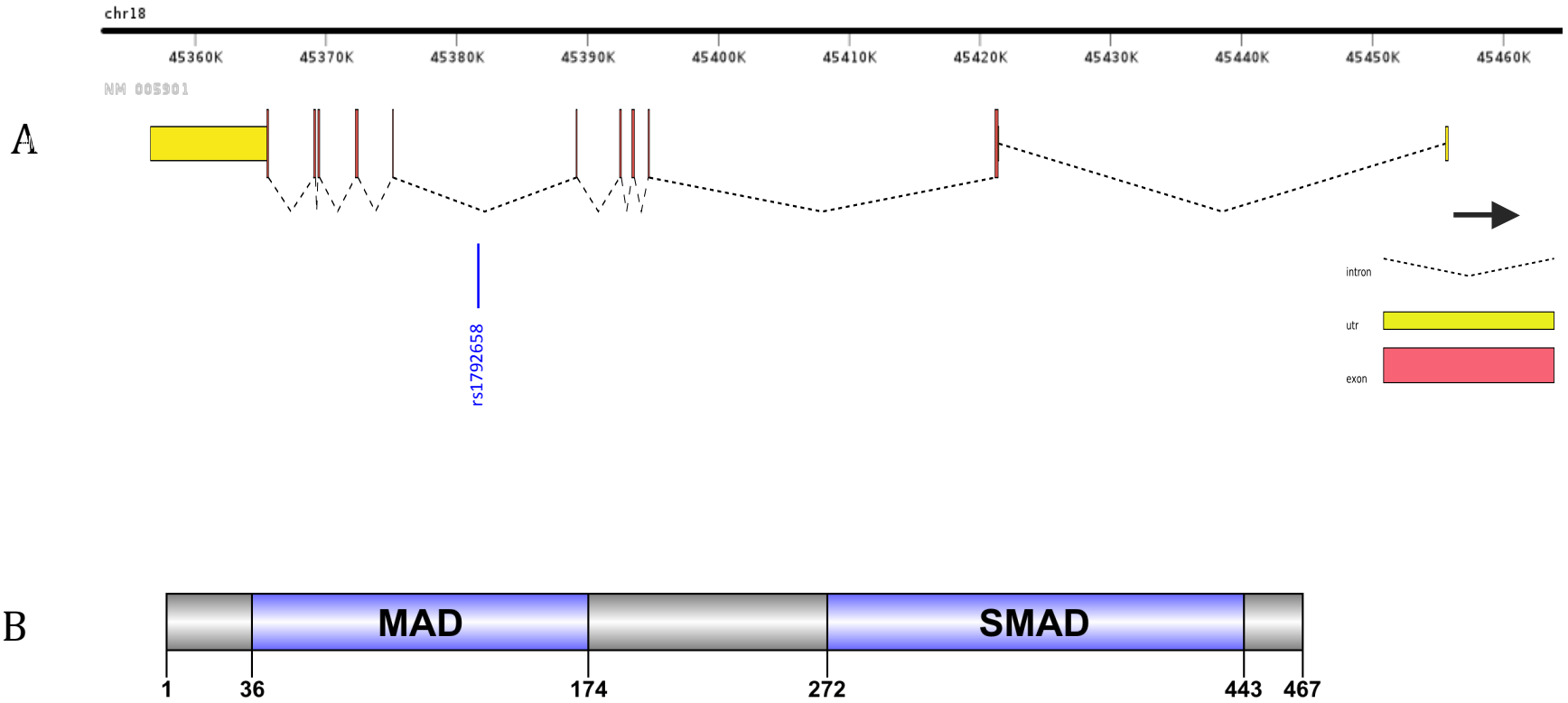
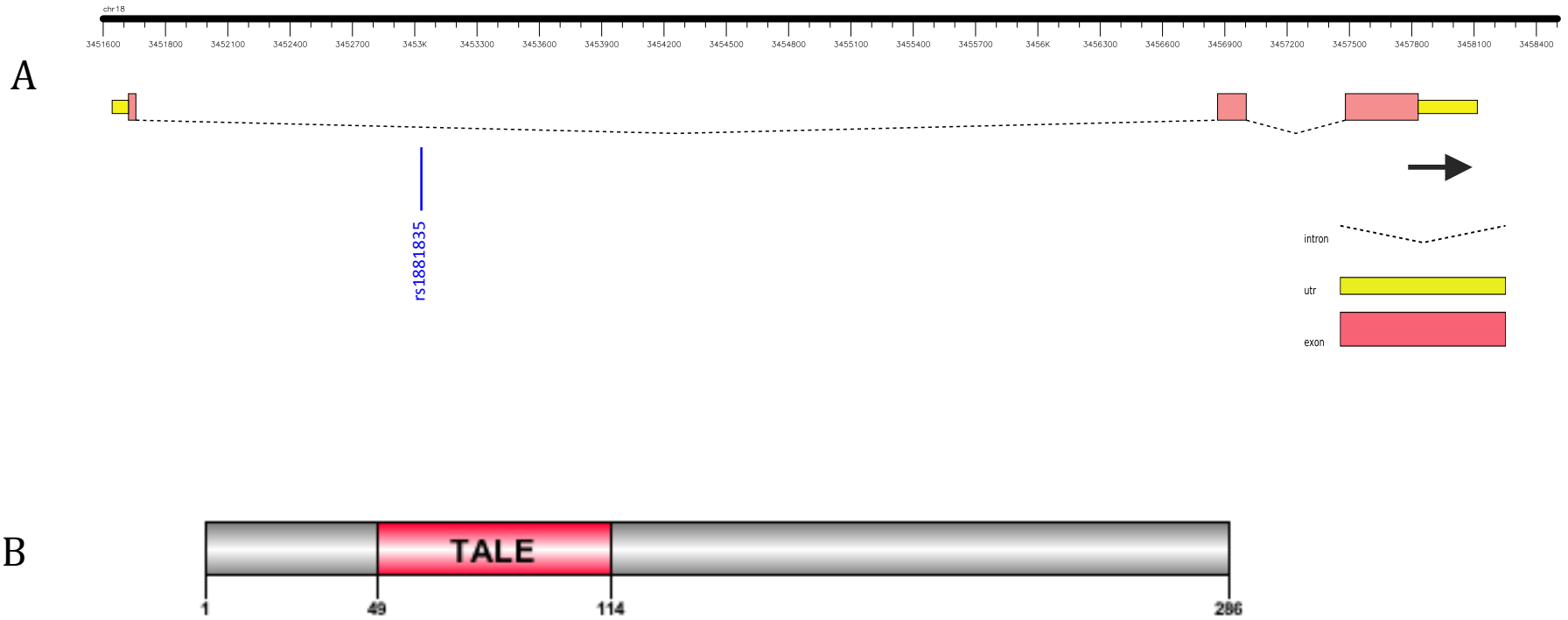


Figure 6: A: Structure of the *TGIF1* locus annotated with location of SNP hit at rs881835 in Part 2 of my study. Diagram created using Fancygene 1.4[18 19] using UCSC data, corrected to GrCh37 coordinates. B: Hypothesised protein domains of TGIF1. The TALE homeodomain is a transcriptional regulator, known to inhibit retinoic acid transcriptional activation and to co-repress SMAD2 transcription. Diagram created using DOG 2.0[24 25] with data from Ensembl.



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Conclusion

In this thesis I have used mouse models to explore the genetic and molecular basis of chronic otitis media in man. The finding from the main part of this thesis, a human association study, is confirmation that polymorphism at the *FBXO11* locus (and possibly also *SMAD2*and *TGIF1*) is associated with susceptibility to chronic otitis media with effusion in childhood (chapter 4.3). In comparison to previous human association studies (chapter 1.4), this is the first study to focus on a well-defined chronic otitis media phenotype.

The finding at *FBXO11* epitomises the value of the mouse to man approach to study the genetic basis of human disease. Through a systematic review of all reported mouse models of otitis media (chapter 1.3), I reasoned pathways, loci, and polymorphisms that were the most compelling candidates genes for human association (chapter 4.2). The *FBXO11* locus is the homologue of the site of mutation in the *Jeff* mouse model, and so my results demonstrate that a hypothesis-driven candidate-gene association study can be rewarding, and that, in spite of their limitations, mouse models can be a successful way to identify loci for such association testing.

More pertinent to the aim of understanding the biology underlying persistent inflammation of the middle ear, my results propose that *FBXO11* is a component of pathways activated in chronic otitis media. Given previous analyses from our unit on the function of FBXO11 in the *Jeff* mouse, this implicates a pertubation of TGF-β signaling in chronic otitis media.

Middle ear inflammation reflects a number of inter-related phenotypes (chapter 1.1), and a complex aetiology incorporating a number of susceptibility factors, both environmental and genetic (chapter 1.2). The work in this thesis has clearly only scratched the surface of the genetic architecture of chronic otitis media, and many more

susceptibility loci remain to be found. As the next stage of my investigations I hope to undertake a genome-wide association study, which may discover some of what's missing, but admittedly so far such studies have made somewhat disappointing progress in the study of complex diseases. Probably this is because genetic susceptibility to complex disease is distributed across many quantitative trait loci, each with a relatively small relative risk, meaning such loci are difficult to identify in population based association studies. Analysis of severe phenodeviants, or the more recent interest in whole exome sequencing, offer methods to circumnavigate this problem. But at present, I believe there continues to be a role for animal models in discovering susceptibility loci, and also in undertaking functional analyses of candidate genes.

In this thesis I have added two potential techniques to discover more mouse models. First, the incorporation of oto-endoscopy as a phenotyping tool for otitis media in mutagenesis programmes (chapter 2.1). Second, the interrogation of aneuploid mouse models to identify loci that may lead to chronic otitis media in copy number variation: here using Down Syndrome mouse models (chapter 2.2). The ongoing recruitment of a cohort of families affected by chronic otitis media (chapter 4.1) should enable any compelling new loci in mice to be rapidly assessed for association with human disease.

I have also used the mouse-to-man approach to explore pathobiology. Specifically, previous work on the *Jeff* and *Junbo* mouse models has implicated tissue hypoxia as a common finding in chronic otitis media. Given that surgical ventilation is the most common and successful treatment for chronic otitis media in man, I have exploited the *Junbo* mouse model to explore the biological effects of this operation, showing that ventilation also reduces inflammation in the *Junbo* mouse model, possibly as a result of alleviation of tissue hypoxia (chapter 3.1). Early results from transcript analyses of leucocytes from effusions in human chronic otitis media also show upregulation of

hypoxia responsive transcripts (chapter 3.2). Hypoxia pathways present a new and exciting potential therapeutic target in chronic otitts media, and again mouse models will be critical in early trials of new therapeutic compounds.

Chronic otitis media is a significant health and economic burden in both developing world and developed world countries. We are at an exciting time in genetics where knowledge of the structure and variation of human DNA is expanding, and where sequencing technology is advancing at an unprecedented pace. Genetics is sure to play an increasingly important role in understanding disease, including chronic otitis media. This thesis offers some early progress in this field.

Appendix A.1
Otoendoscopy standard operating protocol

Oto-Endoscopy SOP

1.0 Purpose:

1.1 Evaluate the presence and severity of external or middle ear disease, especially inflammatory middle ear effusion (otitis media)

2.0 Scope:

2.1 Individuals who have been trained and are competent in performing the procedures described herein must follow this procedure.

2.2 Any queries, comments or suggestions, either relating to this SOP in general or to a specific problem encountered during a procedure, should be addressed to the Deafness Group Project Licence Holder

2.3 Any deviances from this protocol must be reported to the Deafness Group Project Licence Holder

3.0 Safety Requirements:

3.1 General laboratory procedures should be followed, which include: no eating, no chewing gum, no drinking, and no applying of cosmetics in the work area. Gloves must be worn at all times in the work area.

4.0 Notes

4.1 The tip of the oto-endoscope is fragile. Please take extreme care when handling and using the instrument to avoid snapping the end.

4.2 The endoscope is 0.9mm in diameter with a viewing field of 0-55^{o}.

6.0 Equipment:

6.1 Optimax endoscope

6.2 Light source (Inspection Optics) and power lead
Light lead

6.3 Camera and power lead
Y/C (S-Video) connection lead
Palmscope viewing screen and power lead

6.4 Heat pad

6.5 Heated cage

7.0 Supplies:

7.1 Diabetic syringes (with needle)

7.2 Anaesthetic containing: Ketamine (Vetalar V) - 1ml
Xylazine (Sedaxylan) - 0.5ml
Sterile water - 8.5ml
Administered at the rate of 0.1ml/10 grams

Anaesthetic reversal agent: Atipamezole (Antisedan) – 0.1ml
Sterile water – 9.9ml
Administered at the rate of 0.1ml / mouse

Viscotears

8.0 Procedure:

8.1 Plug in the light source. Connect the light lead to the output from the light source, and the other end to the side port of the endoscope.

8.2 Remove the lens cover from the endoscope and the lens cover from the camera. Push together the two black knobs on the camera (distal to the lens), and connect it to the endoscope. Plug in the camera.

8.3 Plug in the Palmscope viewer. Connect the Y/C output of the camera to the Y/C input of the viewer.

8.4 Turn on all equipment (light source, camera, viewer, heatpad). Turn up the illumination of the light source to maximum (the knob on the front of the light box).

8.5 Remove ambient lighting, including turning off lights and covering windows. This will help significantly with visualisation.

8.5 The light should be visible emanating from the end of the endoscope. The focal length is only a few millimetres, so check that you can visualise something at this distance (e.g some printed material)

8.6 Weigh the mouse and anaesthetise *intraperitoneally* with the appropriate quantity of anaesthetic solution.

8.7 Place mouse in heated cage until movement has ceased when handled.

8.8 Move mouse to heated pad.

8.9 Hold the endoscope in your dominant hand, as you would a pen, but with the weight of the camera resting on the first webspace of the hand (between thumb and first finger). Insert the endoscope into the external auditory canal of the mouse. Direct it carefully through the lumen of the auditory canal, and past the folds of skin, until you visualise the tympanic membrane. The insertion depth is only a few millimetres.

8.10 If necessary you can record static or dynamic images from the endoscope by placing a memory card into the Palmscope viewer.

8.11 Once assessment is complete, inject the mouse *subcutaneously* with the appropriate dose of Antisedan solution.

8.12 Place mouse in heated cage and monitor until recovered sufficiently to be returned to home cage.

8.13 Before turning off the light source, please turn down the light intensity to minimum to reduce the risk of the bulb blowing.

Appendix A.2
Participant information sheets and consent forms: LOOM Study

Oxford University Hospitals NHS Trust

Low Oxygen in Otitis Media (LOOM) Study

Information Sheet (Parent or Guardian)

Version 2, 12th August 2011

We are investigating the mechanisms that cause children to develop persistent inflammation of their middle ear. This inflammation is called "otitis media" by doctors and may be seen as fluid in the ear called "glue ear".

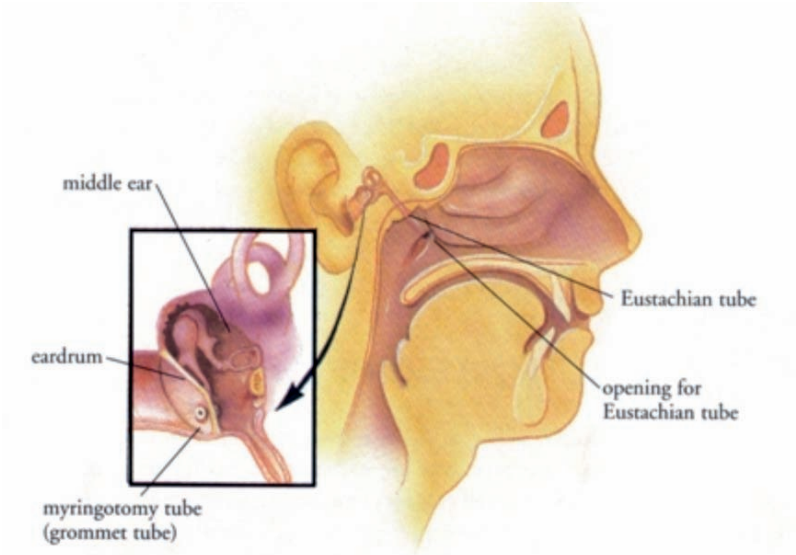
The research involves ENT surgeons in the NHS working with the University of Oxford and the Medical Research Council's genetics unit in Harwell.

Before you decide if you would like your child to take part you need to understand why the research is being done and what it would involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is this research about?

Glue ear is very common in children, and almost all will have had at least one episode of glue ear by the age of five. Usually the glue ear lasts for just a few days or weeks, but some children seem to get glue ear that just won't go away. If this happens, doctors often recommend a treatment called "grommets" which are little tubes surgeons put into the ear-drum to help dry up any fluid and stop the "glue" coming back.

Despite lots of research we still don't really understand why children get persistent glue ear. Recent research has shown that genes play a very important role in ear inflammation. Genes are bits of DNA, and DNA is the molecule that contains the blueprint for how our bodies develop and function. We don't know which genes are the cause of the problem, but we hope that this study may give us some idea. If we find out which genes make children more susceptible to persistent glue ear, it will help us tremendously in understanding why these problems occur.



In this study we are looking at genes that control the levels of oxygen in the middle ear.

There is evidence that the middle ear has low levels of oxygen when it is inflamed, and that this is part of the problem causing persistent glue ear. We will compare the activation of oxygen regulating genes in inflammatory blood cells from fluid in the ear, compared with blood.

What samples will you take and how?

We need a sample of fluid from the ear and a small sample of blood. We normally remove the fluid from the ear as part of a child's operation, and so rather than throwing it away we are asking your permission to undertake some tests on this. The blood sample is about a teaspoon full, and can also be taken while your child is under anaesthetic. Your child will feel no ill effects from us taking these samples. It is important to realise that the operation will be performed in the normal manner and there is no additional risk of harm.

What genes are you testing for?

At the moment we do not know which genes might cause ear inflammation. We are testing for around 40 genes which we know to be involved in low oxygen levels. We suspect, but do not know, if they are involved in otitis media.

What will happen to the child's samples after the study is finished?

We will be testing the samples for genes regulating oxygen levels, but after we have done this we are asking if you would consider donating the remainder of your child's samples to the University of Oxford. This is because in the future we may find other genes that we think are important in causing glue ear and if so, we would like to test the samples for this as well. Any additional testing would be approved by a research ethics committee. When we have no further research we wish to perform on your child's samples we will destroy them.

Will you use the child's samples for anything else?

No. If you agree to us keeping your child's samples we will make sure that it is only ever used for research into ear inflammation and never for anything else. We will never pass your child's personal details on to anyone else.

Is your child's personal information kept secure?

Personal information is stored on a high security computer system at the University of Oxford. Access to this information is only allowed to the research team and to a selected few people at the University and in the hospital, whose job it is to check that the research is being undertaken correctly, including making sure the information is kept secure. Identifiable data will be kept no longer than a year after the study ends. At this point all identifiable data will be securely destroyed.

All the blood and ear fluid samples are stored at a separate site, at the Medical Research Council's genetics unit in Harwell. Your child's samples will be stored securely and anonymously, and are not linked to any of your personal information.

We take the security of your child's personal information very seriously.

Why has my child been chosen?

Your child has been chosen because they have developed glue ear that won't go away. We are asking all families whose child will have a grommet inserted if they would like to take part.

What happens if I agree for my child to take part?

If you agree to take part you will be asked to fill in a very short questionnaire about your child and sign a consent form.

Your child will then have their operation following our normal routines, except that we will collect some ear fluid and some blood whilst they are under anesthetic. They will return to the ward as normal after the operation.

Does my child have to take part?

No. If you decide not to take part it will not affect your child's care in any way. If you do decide to take part you are still free to withdraw at a later stage, at any time, and without giving a reason. The samples will then be destroyed.

What if there is a problem?

If you have a concern about any aspect of this study, or if you think something has gone wrong, you should ask to speak to the ENT doctors looking after you, or to the research coordinator. The research coordinator (Mr James Ramsden) can be contacted in the ENT department at the John Radcliffe hospital (telephone 01865 231062). Compensation for harm arising from an accidental injury and occurring as a consequence of your participation in the study will be covered by the NHS Trust. If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against the NHS Trust (in respect of any harm arising out of the participation in the Clinical Study).

What will the results of the study be used for?

The results of this study will be used to hopefully better understand the genes that cause ear inflammation, which may help us to prevent this problem in other children in the future. The results will be published in a scientific journal, but without identifying any of the people who took part.

Cont.

Has this study been approved?

This study has received a favourable ethical opinion for conduct within the NHS by the Oxfordshire Research Ethics Committee C. The Oxford Radcliffe Hospital's NHS Trust has also sponsored the study.

How can I find out more information?

You can ask any questions you have to the person who is taking consent from you to participate in this trial. If you have questions you would like to ask before or after this time please contact the research nurse or the research coordinator, both of whom will be happy to help. Their contact details can be found below

Research Nurse

Ms Jane Lambie
Lead Research Nurse
ENT Department,
John Radcliffe Hospital
Oxford OX3 9DU

Tel: 07590 355672
E-mail: omstudy@nds.ox.ac.uk

Research Coordinator

Mr James Ramsden
Consultant ENT Surgeon &
Honorary Senior Clinical Lecturer
ENT Department,
John Radcliffe Hospital
Oxford OX3 9DU

Low Oxygen in Otitis Media (LOOM) Study

Information Sheet (Child under 5)

Version 1, 13th July 2011

This information should be read to the child



We are asking you if you can help us.

You have a problem with your hearing.

Lots and lots of children get problems with their hearing, but doctors don't know why.

The doctor would like a little bit of fluid from your ear, while you have your operation. You won't feel anything as you will be asleep.

Ask the doctor or the person who looks after you if you want to know more. We hope you can help us, but you don't have to take part in this project.

Thank-you for listening!



Oxford University Hospitals NHS Trust

Low Oxygen in Otitis Media (LOOM) Study

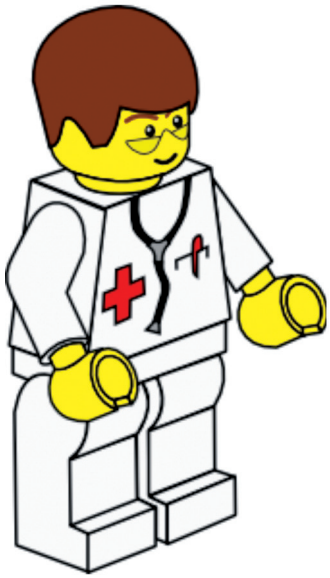
Information Sheet (Child 6-10)

Version 1, 13th July 2011

Why do some children get problems with hearing?
Why do some children have to have grommets put into their ears for "glue ear"?
Doctors aren't sure why these problems happen.



Doctors want to find out why you have a problem with your ear. We are asking you if we can have a sample of the fluid from your ear to test why this might happen.



We will collect a little bit of the fluid from your ear, and a very small amount of blood while you are asleep having your operation, to look for the reasons why you have your ear problem.

You don't have to take part in this research, but if you do it might help doctors to treat other children who have problems with their ears. If you don't want to take part you don't have to.

Before research happens, people have to make sure it is fair. The hospital has checked this research is OK and so has a group of people called a Research Ethics Committee.

Ask the person who looks after you, or the doctor if you want to know anything more.

Thank-you

Oxford University Hospitals NHS Trust

Low Oxygen in Otitis Media (LOOM) Study

Consent Form

Version 2, 12th August 2011

Please initial each box

Participant Identifier: ☐

☐ I confirm that I have read and understood the participant information sheet (version 2, dated 12th August 2011) and have had the opportunity to ask questions and have them answered satisfactorily.

☐ I understand that participation in this study is voluntary and I can withdraw my consent at any time without adverse effect on the care of my child.

☐ I understand that all identifiable data and samples will be treated as confidential and held securely. I understand that members of the University of Oxford and the John Radcliffe Hospital will have access to personal data for purposes of conducting and monitoring the research. I understand samples will be stored anonymously at the Medical Research Council's genetics unit.

☐ I agree to my child's anonymised data being stored on a secure University computer and Consent forms and Enrolment forms being stored in a secure University cabinet.

☐ I agree to the use of my child's ear fluid and blood samples for this study.

☐ I agree to my child's samples being stored securely and confidentially for future use in research into the cause of ear inflammation, as approved by an independent research ethics committee. I understand that my child's samples will not be used for any other purpose.

☐ I understand that my child's data will be cross-referenced to the database for the "Genetics of Otitis Media Study" if my family is also participating in this study.

☐ I understand that if I subsequently lose capacity to consent, the samples and data provided may still be used for the purpose consented to.

☐ I agree to enrol my child in the study.

Signature of parent .....

Name of parent ..... Date .....

Name of child .....

Name of person taking consent (if present) .....

Signature ..... Date .....

Oxford University Hospitals NHS Trust

Low Oxygen in Otitis Media (LOOM) Study

Enrolment Form

Version 1, 9th March 2011

CONFIDENTIAL

For completion by doctor/research nurse only

If the child is also enrolled in the "Genetics of Otitis Media Study" you may use the enrolment form from that study and enter only additional intra-operative information here

Questions? The research nurse can be contacted on 07590 355672

Enrolment date:

Participant identifier:

Genetics of Otitis Media Study identifier:

Contact phone number:

PARTICIPANT DETAILS

Participant **Date of Birth:**

Sex: M / F

Ethnic Code (overleaf):

Environment of child:

- ☐ Immunisation to Streptococcus pneumoniae (pneumovax)
- ☐ Predominantly home care (as opposed to use of nursery facilities)
- ☐ Exposure to household tobacco smoke
- ☐ Predominantly breast fed (for the first 6 months)
- ☐ History of atopy (asthma, atopic dermatitis, allergic rhinitis)

Ethnicity Codes

1	**White**		**2**	**Mixed**
1A	British		2D	White and Black Caribbean
1B	Irish		2E	White and Black African
1C	Any other White background		2F	White and Asian
			2G	Any other mixed background
3	**Asian or Asian British**		**4**	**Black or Black British**
3H	Indian		4M	Caribbean
3J	Pakistani		4N	African
3K	Bangladeshi		4P	Any other Black background
3L	Any other Asian background			
5	**Other Ethnic Groups**		**6**	**Not stated**
5R	Chinese		6Z	Not stated
5S	Any other ethnic group			

INTRA-OPERATIVE FINDINGS

Laterality of disease:

☐ Bilateral

☐ Unilateral

Left Ear

Consistency of Effusion:

☐ Mucoid

☐ Intermediate

☐ Serous

Right Ear

Consistency of Effusion:

☐ Mucoid

☐ Intermediate

☐ Serous

Appendix A.3
Participant information sheets, enrolment forms and consent forms: Genetics of Otitis Media Study

Genetics of Otitis Media Study

Information Sheet (Parent)

Version 3, 1st October 2008, Mahmood Bhutta

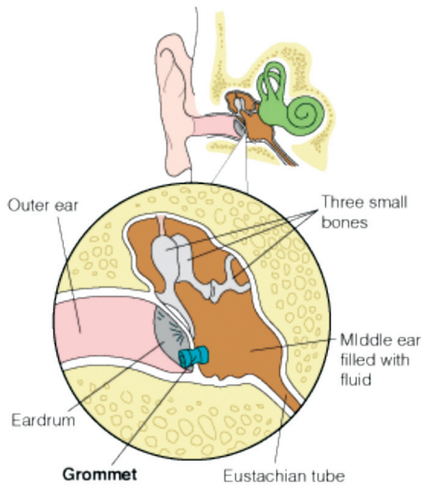
We are asking if you would like to participate in a research study. We are investigating the genes that may cause children to develop inflammation of their ear. This inflammation is called "otitis media" by doctors and may be seen as either repeated ear infections or as fluid in the ear called "glue ear". Your child has otitis media, and this is why we are asking if you would like to participate.

This study will be the largest study of its kind anywhere in the world. We are looking to recruit 1,000 children and their families into this study. The research involves ENT surgeons in several hospitals working with the University of Oxford, the Medical Research Council's genetics unit in Harwell, the Wellcome Trust in London and the Royal College of Surgeons of England.

Before you decide if you and your child would like to take part you need to understand why the research is being done and what it would involve for you and your child and family. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is this research about?

Inflammation of the ear ("otitis media") is very common in children. Two-thirds of children will have had at least one ear infection by their fifth birthday, and almost all will have had at least one episode of fluid in the ear called "glue ear". Usually children seem to grow out of these problems, but some children seem to get repeated ear infections or glue ear that just won't go away. If this happens, doctors often recommend a treatment called "grommets" which are little tubes surgeons put into the ear-drum to help dry up any fluid and stop infections or the "glue" coming back. This treatment has been recommended for your child, either because they have had lots of ear infections or because they can't hear well because the "glue ear" just won't go away.



Despite lots of research we still don't really understand why children get inflammation of their ears. There is a tube called the "Eustachian tube" that goes from the back of the nose to the ear and helps to keep the pressure in your ears at the right level (when you "pop" your ears, it is this tube that opens). Doctors think that this tube may be the cause of the problem, but they are not sure.

Recent research has shown that genes play a very important role in ear inflammation. Genes are bits of DNA, and DNA is the molecule that contains the blueprint for how our bodies develop and function. We know that ear problems seem to happen lots in some families and not at all in others. We don't know which genes are the cause of the problem, but we hope that this study may give us some idea. If we find out which genes make children more susceptible to ear inflammation, it will help us tremendously in understanding why these problems occur.

In this study we want to look at the DNA of your child and of your family from a sample of saliva (spit) to see if we can discover what might be the cause of ear inflammation. We will not be using the DNA for any other tests whatsoever, and will not give the information about your family or their DNA to anyone else.

What genes are you testing for?

At the moment we do not know which genes might cause ear inflammation. However, we have discovered some mice that seem to get the same problem as humans with their ears. We know from studying their DNA that there are problems with two genes called "Fbxo11" and "Evi1" that cause this problem. These genes seem to control how your ear reacts to levels of oxygen in the air. We want to find out if these genes cause the same problem in humans. We want to test the DNA of your child and of your family to see if there are problems with these genes.

What will happen to my family's DNA after the study is finished?

We would ask you whether you would consider giving your family's DNA samples as a 'gift' to us to allow for future research into the genes that cause ear inflammation. Collections of DNA already exist in the UK for other diseases, such as migraine, multiple sclerosis and heart disease, and the information from these DNA databases has been very helpful in UK scientists leading the world in understanding those problems. We want to create a collection of DNA for otitis media. This will be a public database of DNA, which means we will allow other researchers access to it for legitimate reasons. Other researchers will never have access to information on who the DNA came from.

You would not be contacted again in the future about using your family's DNA in this way, but approval for any new research would be obtained, and at all times your data would be kept anonymous. If in the future you lose capacity to consent, through mental or physical illness we would ask that we still be allowed to use your family's DNA and data, for the reason you have consented to.

Will you use my family's DNA for anything else?

No. If you agree to us keeping a small sample of your DNA we will make sure that it is only ever used for research into ear inflammation and never for anything else. We will never pass your personal details on to anyone else.

Is my personal information kept secure?

Personal information on your family is stored on a high security computer system at the University of Oxford. Access to this information is only allowed to the research team and to a selected few people at the University and in the hospital, whose job it is to check that the research is being undertaken correctly, including making sure your information is kept secure.

All the DNA samples you give us are stored at a separate site, at the Medical Research Council's genetics unit in Harwell. Your family's DNA will be stored securely and anonymously, and is not linked to any of your personal information. Even if people somehow got access to your family's DNA samples at Harwell (which is extremely unlikely) they would have no way of knowing who they came from.

We take the security of your family's personal information very seriously.

Why have I been chosen?

Your family has been chosen because your child has developed repeated ear infections or has glue ear that won't go away. We are asking all families whose child will have a grommet inserted if they would like to take part.

What happens if I agree to take part?

If you agree to take part you will be asked to fill in a very short questionnaire about your child and about the structure of your family by the doctor looking after you. We will need a sample of saliva (spit) from your child, from both parents, and from any brothers and sisters that your child has. The sample of saliva doesn't need to be very much, and you spit into a special container we will give you. If your child finds this difficult, we can take a painless swab to collect saliva from inside the mouth instead. Saliva contains DNA and we can get the DNA for this research from your saliva.

You can take the saliva containers home if not all members of your family can come in, and return these to the hospital or post them to the research coordinator at the address below. It is essential that the label that is put on the saliva container is kept with the correct sample. It is also essential that you return a signed consent form for each and every saliva sample.

As part of the questionnaire we will ask for your telephone number. This is only to contact you if there are problems with any of the information you have given or any of the saliva samples from your family. We will not be contacting you for any other reason.

What if you find that my family is not what you expected?

We know that sometimes when we test DNA, families are not what they seem. For example, the man who is the father of a child may not turn out to be the biological father. If we find this information we will not pass this on to anyone else, including yourself or your family. Nobody will know this information other than the researchers.

Do I have to take part?

No. If you decide not to take part it will not affect your care in any way. If you do decide to take part you are still free to withdraw at a later stage, at any time, and without giving a reason. The DNA will then be destroyed.

What if there is a problem?

If you have a concern about any aspect of this study, or if you think something has gone wrong, you should ask to speak to the ENT doctors looking after you, or to the research coordinator. The research coordinator can be contacted via the research nurse (07590 355672) or at the address below. Compensation for harm arising from an accidental injury and occurring as a consequence of your participation in the study will be covered by the University of Oxford. If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against the University of Oxford (in respect of any harm arising out of the participation in the Clinical Study).

What will the results of the study be used for?

The results of this study will be used to hopefully better understand the genes that cause ear inflammation, which may help us to prevent this problem in other children in the future. The results will be published in a scientific journal, but without identifying any of the people who took part. The database of DNA we will create will also help other scientists in the future to investigate this problem.

Has this study been approved?

This study has received a favourable ethical opinion for conduct within the NHS by the Oxfordshire Research Ethics Committee. It has also been approved by your local hospital's research committee.

How can I find out more information?

Please ask any questions you have to the person who is taking consent from you to participate in this trial. If you still have more questions contact the research nurse (see below) who will be happy to help.

Research Nurse

Telephone: 07590 355672

www.har.mrc.ac.uk/geneticsomstudy

Address for saliva samples:

Dr Mahmood Bhutta
Nuffield Dept of Surgery (Uni of Oxford)
Room 6607, Level 6
John Radcliffe Hospital
Headington
Oxford OX3 9DU

Genetics of Otitis Media Study

Information Sheet (Child under 5)

Version 1, 9th September 2008, Mahmood Bhutta

This information should be read to the child



We are asking you if you can help us.

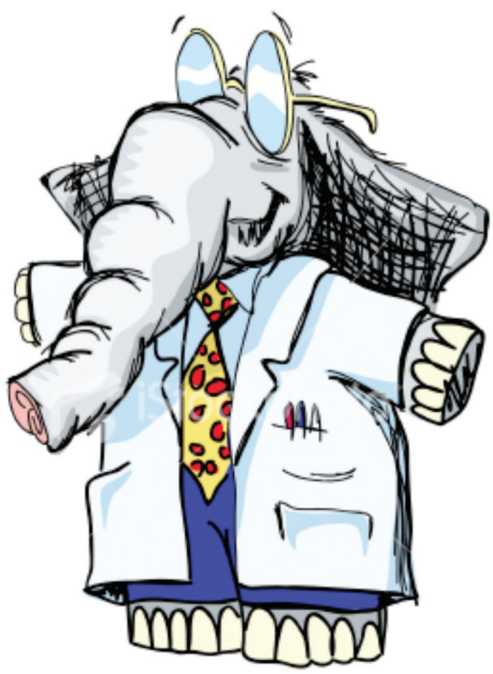
You have a problem with your ear.

Lots and lots of children get problems with their ears, but doctors don't know why.

The doctor would like a little bit of spit from you. They can do special tests on your spit that might tell them why you have a problem with your ear. Collecting your spit won't hurt at all.

Ask the doctor or your mum or dad if you want to know more. We hope you can help us, but you don't have to take part in this project.

Thank-you for your help!



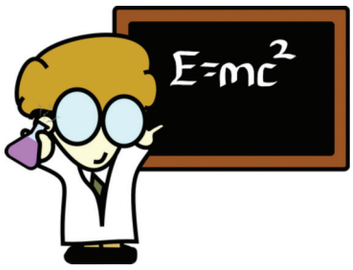
Genetics of Otitis Media Study

Information Sheet (Child 6-10)

Version 2, 9th September 2008, Mahmood Bhutta

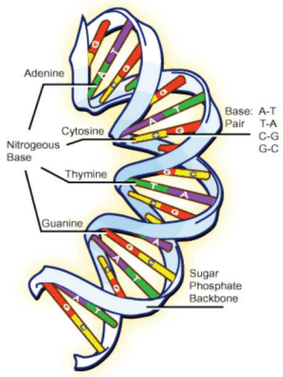
Why do some children get ear infections?
Why do some children get problems with hearing?
Why do some children have to have grommets put into their ears?
Doctors aren't sure why these problems happen.





Doctors want to find out why you, or your brother or sister, has a problem with their ear.
We are asking you if we can have a sample of your spit and that of your family to test why this might happen. Your mum or dad will give us permission to do this but we want you to know about it too.

We will ask you to spit into a little pot, or if you can't manage this we can put a little stick (like a lollipop) in your mouth to collect spit. It won't hurt you at all.



We can use your spit to collect something called DNA, which is a chemical that gives instructions to your body on how to grow. Nobody will know that the DNA has come from you. Doctors want to understand why some children's DNA makes them get ear problems, but other children's DNA doesn't.

You don't have to take part in this research, but if you do it might help doctors to treat other children who have problems with their ears. If you don't want to take part you don't have to and nobody will be cross with you.

Before research happens, people have to make sure it is fair. The hospital has checked this research is OK and so has a group of people called a Research Ethics Committee.

Ask mum or dad, or the doctor if you want to know anything more.

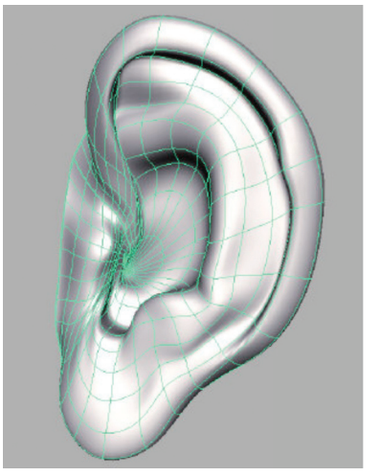
Thank-you for your help.

Genetics of Otitis Media Study

Information Sheet (Child/Young Person 11-15)

Version 1, 9th September 2008, Mahmood Bhutta

We are asking you if you would like to help us with some research. Your brother or sister has developed inflammation (swelling) in their ear, which doctors call "otitis media". Doctors treat this by putting a tiny tube (called a grommet) in your brother or sister's ear.



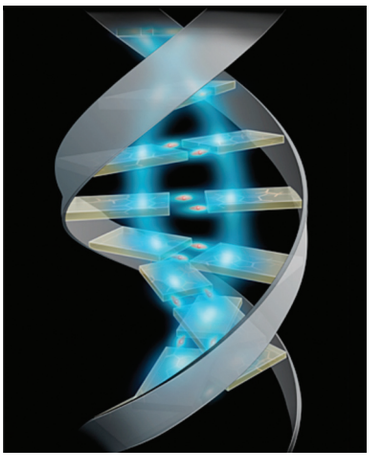
Doctors don't know why ear inflammation happens. We do know that this problem runs in families. This means that there must be something in the DNA of your family that makes your brother or sister develop ear inflammation. DNA is a chemical found in all the cells of your body that tells your body how to grow, or how to function. The doctor looking after your brother or sister wants to test the DNA of your family to see if they can find out why this has happened.



Before you decide if you want to join in it's important to understand why the research is being done and what it will involve for you. So please consider this leaflet carefully. Talk about it with your family, friends, or the doctor if you want to.

Why have I been chosen?

You have been chosen because your brother or sister has developed ear inflammation, which we will be treating. You have DNA that is similar, but not the same as your brother or sister. Finding out how your DNA is different to your brother or sister's, and how it is different to that of other people, might tell us why this problem has happened.



Doctors are testing 1,000 families in this way.

Do I have to take part?

No. It's up to you. If you do, we ask you to sign a form giving your consent or assent (your mum or dad will also be asked to sign). You are free to stop taking part at any time during the research without giving a reason.

What will happen if I take part?

If you do decide to take part we ask that you give us a little sample of saliva (spit) in a pot. This is really easy and painless. You can do it yourself. You can give the pot of spit to the doctor, or you can post it to us.

Doctors can get samples of your DNA from your spit and do tests on this. Doctors would also like to keep a sample of your spit to do more tests in the future. They will only test the DNA from your spit for problems that might cause ear inflammation and not for anything else.

What are the possible benefits of taking part?

There is not really any benefit to you in taking part. But, your sample of DNA will help us to understand why ear inflammation happens, and we might be able to do to stop other children getting this problem in the future.

What happens if something goes wrong?

It is very unlikely that anything serious will go wrong. If you think something has gone wrong you can talk to the doctors at the hospital or contact the research department at the hospital.

Will anyone else know I'm doing this?

All the information you give us is stored in a high security computer. Your saliva sample will be stored at a separate laboratory with its own security system. Nobody except the research team will have access to your data or your saliva sample. Nobody else will be told that you are taking part in this research.

Has anyone approved this research?

Before research happens it has to be approved. This research has been approved by the Oxfordshire Research Ethics Committee and by your local hospital's research department.

What if I want to know more?

If you want to know more, speak to the doctor looking after your brother or sister. You can also contact the nurse who is in charge of the research – your parents have the contact details.

Thank-you for your help

Genetics of Otitis Media Study

Consent Form 2 (Parent/Legal Guardian & Child/Young Person)

Version 3, 1st October 2008, Mahmood Bhutta

Please affix participant sticker here

Please initial each box

☐ I confirm that I have read the participant information sheet (version 3, dated 1st October 2008) and have had the opportunity to ask questions

☐ I understand that participation in this study is voluntary and I can withdraw my consent at any time without adverse effect on the care of my child

☐ I understand that all identifiable data and DNA will be treated as confidential and held securely. I understand that members of the University of Oxford and the hospital will have access to personal data for purposes of monitoring the conduct of the research. I understand DNA will be stored anonymously at the Medical Research Council's genetics unit.

☐ I agree to the use of my child's DNA for this study

☐ I agree to a sample of DNA being stored securely and confidentially in a public database for future use in research into the cause of ear inflammation. I understand that my DNA will not be used for any other purpose. I understand that if I subsequently lose capacity to consent, the DNA and data provided may still be used for the purpose consented to.

☐ I agree to enrol my child in the study

Signature .....

Name of participant ..... Date .....

Children/young people aged 11-16 should also sign overleaf

Name of person taking consent (if present) .....

Signature ..... Date .....

Child/Young Person Assent

We ask all children/young people aged 11-16 to sign below.
Younger children may also sign, but you may need to help them with this form.

Please ask your child/young person to circle all they agree with:

Have you read (or had read to you) about this project?	Yes/No
Do you understand what this project is about?	Yes/No
Have you asked all the questions you want?	Yes/No
Have you had your questions answered in a way you understand?	Yes/No
Do you understand it's OK to stop taking part at any time?	Yes/No
Do you understand that we will be storing a sample of your DNA, and do you agree to us doing this?	Yes/No
Are you happy to take part?	Yes/No

Your Name ……

Date ……..

Genetics of Otitis Media Study

Consent Form 1 (Adult parent, for use of their DNA)

Version 3, 1st October 2008, Mahmood Bhutta

Please affix participant sticker here

Please initial each box

☐ I confirm that I have read the participant information sheet (version 3, dated 1st October 2008) and have had the opportunity to ask questions

☐ I understand that participation in this study is voluntary and I can withdraw my consent at any time without adverse effect on the care of my child

☐ I understand that all identifiable data and DNA will be treated as confidential and held securely. I understand that members of the University of Oxford and the hospital will have access to personal data for purposes of monitoring the conduct of the research. I understand DNA will be stored anonymously at the Medical Research Council's genetics unit.

☐ I agree to the use of my DNA for this study

☐ I agree to a sample of DNA being stored securely and confidentially in a public database for future use in research into the cause of ear inflammation. I understand that my DNA will not be used for any other purpose. I understand that if I subsequently lose capacity to consent, the DNA and data provided may still be used for the purpose consented to.

☐ I agree to take part in the study

Signature .....

Name of participant ..... Date .....

Name of person taking consent (if present) .....

Signature ..... Date .....

Genetics of Otitis Media Study

Enrolment Form

Version 1, 7th July 2008, Mahmood Bhutta

CONFIDENTIAL

For completion by doctor/research nurse only

UK CRN 5376

NRES 08/H0605/109

Questions? The research nurse can be contacted on 07590 355672

www.har.mrc.ac.uk/geneticsomstudy

Guidelines for completion

1. Use one form per enrolled family
2. Confirm eligibility: COME with an effusion **confirmed at operation**
   RAOM based upon **history**, with/without effusion at operation
   Exclusions: Down Syndrome, cleft palate, craniofacial malformation, ciliary abnormalities
3. Complete questions on suggested environmental risk factors for OM for the **proband** (proband = child having grommet insertion)
4. Only enrol parents/siblings who are **biologically related**. Do not enrol adopted or half-siblings. With large families there is no need to collect more than five siblings' DNA.
5. For each family member record where requested: date of birth, sex, ethnic code and whether they have ever had grommets inserted
6. **Provide one Oragene vial and one Consent Form for each participant.** Provide one saliva collection pack for any each participant unable to spit. Provide one mailing envelope per family and one set of participant instructions
7. Use supplied stickers: black sticker inside cover of proband's notes
   one sticker on the base of the Oragene saliva collection vial
   one sticker on the appropriate Consent Form (Consent Form 1, 2 or 3)
   Write the **participants initials** on each sticker so that the parent knows which Consent Form and which Oragene vial relates to which participant
8. After collection samples can be placed into the provided envelope and posted back by the patient, or left at the hospital to await collection by the research team.
9. Keep this form safe, for collection by the research team

Site

- ☐ High Wycombe
- ☐ Milton Keynes
- ☐ Oxford
- ☐ Swindon
- ☐ Kettering
- ☐ Northampton
- ☐ Stoke Mandeville
- ☐ Wexham Park

A1234

Contact Phone Number
(include full area code)

Indication of grommets:

- ☐ Chronic OME (> 3 months, with effusion confirmed at operation)
- ☐ RAOM (≥3 episodes ASOM in 6 months or ≥4 in 1 year)
  - ☐ Effusion at operation
  - ☐ No effusion at operation

Laterality disease:

- ☐ Bilateral
- ☐ Unilateral

Environment of proband (child having grommets):

- ☐ Immunisation to Streptococcus pneumoniae (e.g. PNEUMOVAX®)
- ☐ Predominantly home care (as opposed to use of nursery facilities)
- ☐ Exposure to household tobacco smoke
- ☐ Predominantly breast fed
- ☐ History of atopy (asthma, atopic dermatitis, allergic rhinitis)

Ethnicity Codes

1	**White**
1A	British
1B	Irish
1C	Any other White background
2	**Mixed**
2D	White and Black Caribbean
2E	White and Black African
2F	White and Asian
2G	Any other mixed background
3	**Asian or Asian British**
3H	Indian
3J	Pakistani
3K	Bangladeshi
3L	Any other Asian background
4	**Black or Black British**
4M	Caribbean
4N	African
4P	Any other Black background
5	**Other Ethnic Groups**
5R	Chinese
5S	Any other ethnic group
6	**Not stated**
6Z	Not stated

Child having grommets

Date of birth ☐☐☐☐☐☐

Sex: M / F Ethnic Code ☐☐

Biological Mother

Date of birth ☐☐☐☐☐☐

Grommets? ☐ Ethnic Code ☐☐

Biological Father

Date of birth ☐☐☐☐☐☐

Grommets? ☐ Ethnic Code ☐☐

Sibling 1

Date of birth ☐☐☐☐☐☐

Grommets? ☐ Ethnic Code ☐☐

Sibling 2

Date of birth ☐☐☐☐☐☐

Grommets? ☐ Ethnic Code ☐☐

Sibling 3

Date of birth ☐☐☐☐☐☐

Grommets? ☐ Ethnic Code ☐☐

Sibling 4

Date of birth ☐☐☐☐☐☐

Grommets? ☐ Ethnic Code ☐☐

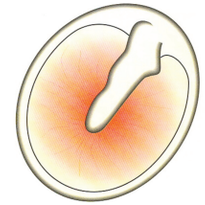
Sibling 5

Date of birth ☐☐☐☐☐☐

Grommets? ☐ Ethnic Code ☐☐

Genetics of Otitis Media Study





Thank-you for agreeing to participate in this study. Here are instructions on what you need to do now.

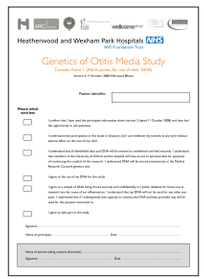
- Each member of your family has been coded. We use codes rather than people's names to keep your information secure. The code consists of a series of numbers followed by a letter. This letter tells us who the sample belongs to:
    - If the code ends in P the sample is for the child who is having grommets.
    - If the code ends in M the sample is for mother of that child.
    - If the code ends in F the sample is for the father of that child.
    - If the code end in S1, S2, S3 etc the sample is for the siblings (brothers and sisters) of that child – there will be initials written next to the code to tell you which sibling the sample is for.

- You should have
    - One coded collection vial and one coded consent form for **each** member of your family. (Do **NOT** worry about the expiry date printed on the vial).
    - One or more collection packs for young children who cannot spit (if required).
    - One mailing kit.
    - If you do not have everything you need contact the research nurse on the number below

Collection Vial



Consent Form

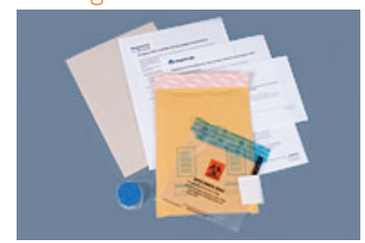

Heatherwood and Wexham Park Hospitals NHS
NHS Foundation Trust

Genetics of Otitis Media Study

Collection Pack for Young Children



Mailing Kit



- Collect saliva (spit) from each family member using the instructions provided inside the pack.
- If you have a child who cannot spit, then use the collection kit for young children and follow the instructions overleaf. We get a better sample from spitting than from using the sponges in the collection kit, so do encourage children who can spit to do so. If you are helping a child, try not to contaminate the sample with your own DNA – do not put your fingers inside the collection vial.
- Once the spit is collected, seal the sample with the plastic cap.
- We need a saliva sample and a signed consent form for **each** member of your family or else we cannot use the sample. If some members of your family do not want to participate that is OK, but the more that participate the better.
- Open the mailing kit you have been given. Place the closed collection vials in the plastic bag. Remove as much air as possible and seal the bag by removing the blue strip. Place the sealed plastic bag into the bubble envelope and seal this.
- Return the bubble envelope to your local hospital or place in the post (postage is paid).

Questions? Contact the research nurse Mon-Fri 9am-5pm on 07590 355672. The nurse can call you back **www.har.mrc.ac.uk/geneticsomstudy**

Instructions for collecting saliva from an adult or a child who can spit

- These can be found inside the oragene DNA collection vial packet

Instructions for collecting saliva from a child who cannot spit

- See opposite
- Caution should always be used when inserting anything into a child's mouth. Do not leave the child unattended when using the sponges. We recommend that you only use the sponges provided and that you do not substitute with other sponges or swabs.
- Although not required, the child may drink water or brush teeth with water before the collection. After rinsing or brushing, wait 10 minutes before collecting a saliva sample. If the child is nursing, wait 15-20 min after feeding before collecting a saliva sample.
- Some children find that the saliva sponge tickles their gums. Depending on the age of the child, it may be helpful to explain ahead of time that the collection will be a fun experience that will not take long and that the sponge may tickle.
- Try not to rub directly on the child's teeth to minimize the amount of bacteria transferred to the sponge.
- If a donor can provide some saliva, but not the full amount, through spitting, it is perfectly acceptable to combine saliva from spitting together with saliva sponges in the same Oragene•DNA kit.
- If the donor is unable to provide sufficient saliva within the recommended 10-15 min, the following procedure can be tried.
    - Securely cap the Oragene•DNA vial after 15 min – this will release the Oragene•DNA fluid.
    - Mix the contents of the vial gently by inversion 5 times.
    - When a second collection is to be attempted (this can be days later if convenient), place the vial on a flat surface and carefully open the vial, taking care to avoid spilling any of the liquid. Place the cap on a flat surface with the inside facing upwards. Proceed with further collection of saliva using the remaining sponges. Extra care should be taken when cutting off the sponge from the handle to avoid spillage.
- A maximum of 5 sponges should be used per Oragene•DNA vial.

ORAgene•DNA

Collecting Saliva

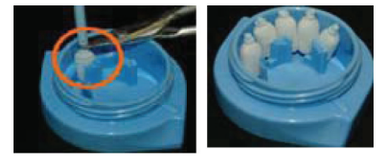


1. Place the saliva sponge into the child's mouth in the cheek pouch (the space between the gums and the inner cheek). Gently move the saliva sponge around the upper and lower cheek pouches on both sides of the mouth to soak up as much saliva as possible. There is no need to 'scrape' the inner cheek with saliva sponges – simply collect as much saliva as possible from the cheek pouches. The sponge will absorb more saliva if it is left in the child's mouth for a longer time (up to 60 seconds).



2. Once collected, cut the sponge into the blue base of the Oragene•DNA kit as follows. Place the sponge firmly against the bottom of the kit between the tooth and the kit wall (see picture below). This action will ensure that the sponge tip remains in the container during the cutting action. Using the scissors provided, cut the narrow part of the handle just above the sponge.

Recycle/discard the plastic handle.



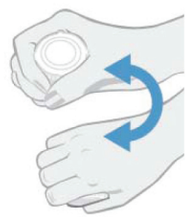
3. Collect up to 5 saliva sponge samples from the same child, repeat steps 1 and 2. Follow the sequence shown in the diagram below. A rest period of about 5 min between each collection of 2 sponges is helpful. To prevent the saliva samples from drying out, cap the vial (see step 4) within 15 min of the first colletion. If you have not had a chance to collect all 5 sponges within 15 minutes, you may carefully re-open the kit. If you remove the cap be sure that the inside is facing upwards when putting it on any surface. Do not spill the contents.

Follow these steps for collecting multiple sponges:





4. Carefully cap the kit and tighten it firmly. Once the Oragene•DNA liquid is released from the cap, it will preserve the DNA collected by the sponge(s).



5. Invert gently 5 times to mix the sample.

6. If the scissors are to be re-used, they should be rinsed with tap water and wiped dry between donors.

Appendix A.4
Presentations resulting from this work

Jun 2013
Hypoxia pathways in chronic otitis media
7th Extraordinary International Symposium on Recent Advances in Otitis Media, Stockholm, Sweden

Jun 2013
Sample sizes in genetic association studies
7th Extraordinary International Symposium on Recent Advances in Otitis Media, Stockholm, Sweden

Jun 2013
Mining the Down Syndrome mouse library identifies critical regions responsible for chronic otitis media
7th Extraordinary International Symposium on Recent Advances in Otitis Media, Stockholm, Sweden

Mar 2013
Surgical ventilation downregulates inflammation and cellular hypoxia in the Junbo mouse model of chronic otitis media
British Association of Audiological Physicians, Chesham, Buckinghamshire

Oct 2012
Chronic otitis media genetics
University of Western Australia, Perth

Oct 2012
Genetics of otitis media
VATES meeting, Wexham Park Hospital, Slough

Sep 2012
Mining the genetic mouse library identifies a critical region for the chronic otitis media phenotype in Down Syndrome
Otorhinolaryngology Research Society, Norfolk

June 2012
Genetics of otitis media
Anglo-Swedish otology meeting, Stockholm

May 2012
Susceptibility to chronic otitis media with effusion is associated with polymorphism at the loci *FBXO11* and *SMAD2*
European Society of Paediatric Otolaryngology, Amsterdam, Netherlands

May 2012
Otitis media: Definitions, aetiology, treatment
Regional Paediatrics Training Day, John Radcliffe Hospital, Oxford

Mar 2012
Surgical ventilation downregulates inflammation and cellular hypoxia in the *Junbo* mouse model of chronic otitis media
Otorhinolaryngology Research Society, London

Dec 2011
Genetics of otitis media
Klinikum Bielefeld, Germany

Sep 2011
Otitis media genetics
Glue Ear Seminar, Royal Surrey County Hospital

Sep 2011
Otitis media genetics
British Association of Paediatric Otolaryngology, St Hughs College, Oxford

Jul 2011
Genetics of Otitis Media: Mouse to Man
Universitat Autònoma de Barcelona, Spain

Jul 2011
8th Molecular Biology of Hearing and Deafness Conference (panel chair)
Hinxton, Cambridgeshire

Jul 2011
Congress of the Confederation of the European ORL-HNS (panel member)
Barcelona, Spain

Jun 2011
Oto-endoscopy: A reliable and validated technique for phenotyping otitis media in the mouse
10th International Symposium on Recent Advances in Otitis Media,
New Orleans, USA

Mar 2011
Genetics of otitis media
Recent Advances in the Molecular Biology of Otology, London

Dec 2009
The aetiology of cholesteatoma: Pleiotropic effects of cytokines involved in middle ear inflammation and embryology?
British Association of Clinical Anatomists Winter Scientific Meeting, Southampton

Appendix A.5
Publications arising from this work

Pending
Bhutta MF
Hypoxia pathways in chronic otitis media (book chapter)
Recent Advances in Otolaryngology 9 (Sudhoff & Bhutta eds). JP Medical Ltd, London.

Pending
Bhutta MF
Pathobiology of otitis media: advances from genetics (book chapter)
IAPO Handbook XI, Interamerican Association of Pediatric Otorhinolaryngology

Pending
Bhutta MF, Burton MJ
The genetics of chronic otitis media
The Otorhinolaryngologist

Jun 2013
Tyrer H, Crompton M, Bhutta MF
What have we learned from murine models of otitis media?
Current Allergy and Asthma Reports: June 18th [Epub ahead of print]

May 2013
Bhutta MF, Hobson L, Lambie J, Scaman ESH, Burton MJ, Giele H, Jamieson SE, Furniss, D.
Alternative recruitment strategies influence saliva sample return rates in community based genetic association studies 77(3): 244-50

Oct 2012
Bhutta MF
Mouse models of otitis media: Strengths and limitations
Otolaryngology Head and Neck Surgery 147(4):611-4

Nov 2011
Cheeseman MT, Tyrer HE, Williams D, Hough TA, Pathak P, Romero MR, Hilton H, Bali S, Parker A, Vizor L, Purnell T, Vowell K, Wells S, Bhutta MF, Potter PK, Brown SDM
HIF-VEGF pathways are critical for chronic otitis media in *Junbo* and *Jeff* mouse mutants
PLoS Genetics 7(10): e1002336

Feb 2011
Bhutta MF, Hedge EA, Parker A, Cheeseman MT, Brown SDM
Oto-endoscopy: A reliable and validated technique for phenotyping otitis media in the mouse
Hearing Research 272: 5-12

Feb 2011
Rye M, Bhutta MF, Cheeseman MT, Burgner D, Blackwell JM, Brown SDM, Jamieson SE
Unraveling the genetics of otitis media - from mouse to human and back again
Mammalian Genome 22(1-2): 66-82

Jan 2011
Bhutta MF
Chronic otitis media in mucopolysaccaridosis may not be due to Eustachian tube dysfunction
International Journal of Pediatric Otorhinolaryngology 75(1): 140-1

Sep 2010
Bhutta MF, Cheesman M, Burton M, Brown S.
The Genetics of Otitis Media
ENT & Audiology News 19(4): 104-106

Sep 2009
Bhutta MF
6th Extraordinary International Symposium on Recent Advances in Otitis Media
ENT & Audiology News 18(4): 23

At the time of writing, four additional papers are nearing journal submission, containing the work presented in chapters 1.1, 2.2, 3.1 and 3.2 respectively. Results presented in section 4 will also be published once the desired sample size is achieved.

Appendix A.6
Prizes and awards

2103
Hallpike Prize
British Association of Audiological Physicians

2012
Angell-James Prize
Otorhinolaryngology Research Society

2012
Philip Stell Prize
Otorhinolaryngology Research Society

2012
Parker Prize
Regional ENT Meeting, Oxford

2011
Sussana Leighton BAPO Travelling Fellowship
British Association of Paediatric Otolaryngology

2009-11
Phizackerley Senior Scholarship
Balliol College, Oxford

2009
Clinical Research Fellowship
Wellcome Trust

2009
Research Fellowship
Royal College of Surgeons of England