

The Genetics of Cholesteatoma

A thesis submitted by

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Acknowledgement

To my teachers, to my colleagues, to my trainees and above all to my patients, who have been my inspiration.

I record my enormous gratitude to my supervisors, Dr Barbara Jennings and Professor Carl Philpott, who have been a constant source of wisdom and encouragement.

Dedication

This thesis is dedicated to my family, to my father, who wrote his MD about Bornholm disease in 1948, the year that the NHS started, and who is a professor of geriatric medicine , age 97, in Melbourne and especially to my wife, Marian Prinsley D Ed Psych, who have been the greatest supporters.

Abstract

Introduction A cholesteatoma is a mass of keratinizing epithelium in the middle ear. It is a rare disorder, associated with significant morbidity, especially deafness. There is little evidence for Mendelian inheritance, but an original observation by the author of affected families in Norfolk, including individuals with bilateral disease, suggests a genetic component for its aetiology.

Methods A systematic literature review to identify studies about the genetics of cholesteatoma has been performed and a biobank for subsequent whole exome sequencing studies of familial disease has been established. A pilot sequencing study to identify candidate variants that segregate with the disease phenotype, using NimbleGen® library construction and exome capture and the Illumina HiSeq4000® platform, has been completed.

Results The literature review identified several case-series with multiply-affected families and associations with congenital malformation syndromes.

DNA and clinical data has been collected from 66 participants from 13 multiply affected Norfolk families. The pilot whole exome sequencing [WES] study of 16 participants from four families identified 95,437 variants. In one family all five recruited individuals have been sequenced. Variant filtering, using pedigree analysis, has identified several mutations of potential significance.

Conclusion A systematic review has been completed and a unique biobank to explore the genetics of cholesteatoma is established. A WES strategy and bioinformatics pipeline have been developed in the

pilot study and preliminary filtering has identified candidate variants that could have an impact on relevant biological pathways. There are no other published descriptions of a WES strategy to investigate the genetics of familial cholesteatoma. The potential impact of an understanding of the genetic basis of cholesteatoma is discussed.

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List of abbreviations

Chole	Cholesteatoma
CINAHL	Cumulative Index of Nursing and Allied Health Literature
CLAP/CL	Cleft lip and palate/cleft lip
CRN	Clinical research network
CSOM	Chronic suppurative otitis media
CT	Computer tomography
dB	Decibel
DNA	Deoxyribose nucleic acid
EBSCO	Elton B Stephens Company
EDTA	Ethylenediamine tetra-acetic acid
EGF(R)	Epidermal growth factor (receptor)
EMBASE	Excerpta Medica dataBASE
ENT	Ear Nose and Throat
FDH	Focal dermal hypoplasia
FU	Follow up
GOC	Genetics of Cholesteatoma
HR	Hazard ratio
HUGO	Human Genome Organization Nomenclature Committee
IFN γ	Interferon gamma
IL-1	Interleukin 1
IMD	Index of multiple deprivation

K	Keratins or cytokeratins
Medline	Medical Literature Analysis and Retrieval System Online
NGS	Next generation sequencing
NHS	National Health Service
NIHR	National Institute of Health Research
OME	Otitis media with effusion
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase chain reaction
PRISMA	Preferred reporting items for systematic reviews and meta analyses
RNA	Ribose nucleic acid
rs	Reference number for SNP
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
STMEC	Surgical treatment of middle ear cholesteatoma
STR	Short tandem repeat
STREGA	Strengthening Reporting of Genetic Association Studies
STROBE	Strengthening reporting of observational studies
TGF(α)	Transforming growth factors (alpha)
TNF	Tissue necrosis factor
VCF	Variant call files
VEP	Variant effect predictor
VNTR	Variable number tandem repeats

VTI	Ventilation tube insertion
WES	Whole exome sequencing

Note about Gene nomenclature

Each gene has a unique internationally agreed identifier consisting of italic letters and numbers. HUGO or the Human Genome Organization Nomenclature Committee is responsible for this system.

<https://varnomen.hgvs.org/>

Chapter 1 The Genetics of Cholesteatoma

An introduction to an MD Thesis by Peter Prinsley FRCS

Introduction

The old Norfolk and Norwich Hospital is mostly demolished. The remains of the Victorian buildings have become expensive flats and there is a brand new Norfolk and Norwich University Hospital outside the city. I started work at the old hospital as a Consultant ENT Surgeon, with what the BMJ had advertised as a “responsibility to provide an otology service to the people of Norfolk”, in the summer of 1996.

In one of my first clinics, a mother walked in with three children, identical twin sisters and an older brother. A note from the GP had asked me to see the twins about their ears.

“What is the matter with the girls’ ears?” I asked.

“Cholesteatoma” says mum.

“How on earth do you know that?” I said.

“Well, their brother here had the same and he’s had his ear operated by another doctor”.

Sure enough, one of the girls had pus discharging from one ear and her sister was discharging pus from both ears. Mum was quite correct. I couldn’t resist looking into their brother’s ear and observed a beautifully healed mastoid cavity operated some years earlier by my colleague. Four cholesteatomas in three siblings!

A little enquiry revealed a cousin with a cholesteatoma and an account of a great uncle who had died as a small child in the 1920s as a result of an ear infection. In the family pedigree drawings the solid symbols represent affected individuals and the asterisk refers to a recruit to the Genetics of Cholesteatoma Project.

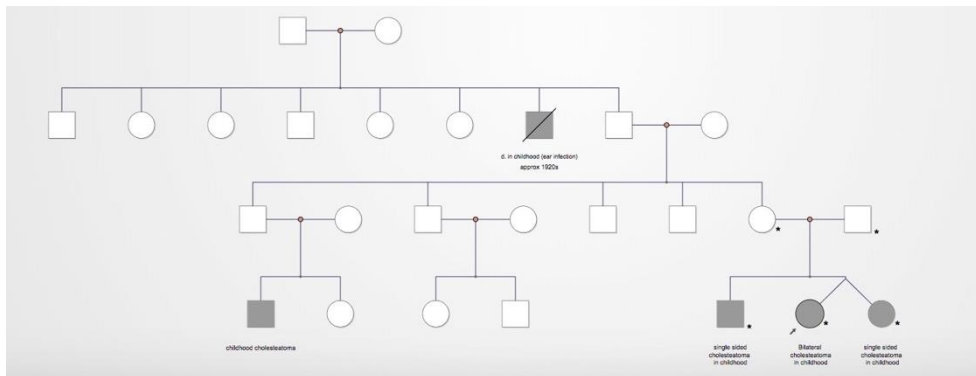


Figure 1-1 The twins' extended family. There is an affected cousin and a family story that a great uncle had died in childhood from an ear infection.

Cholesteatoma, which is a chronic destructive disease of the ear causing discharge and deafness, is not traditionally considered to be a genetic disorder but I could not fail to notice that there was something rather unusual about this family.

Before too long several more families came to light. One day, during a lull in the clinic, a nurse called K, asked me to look in her ear.

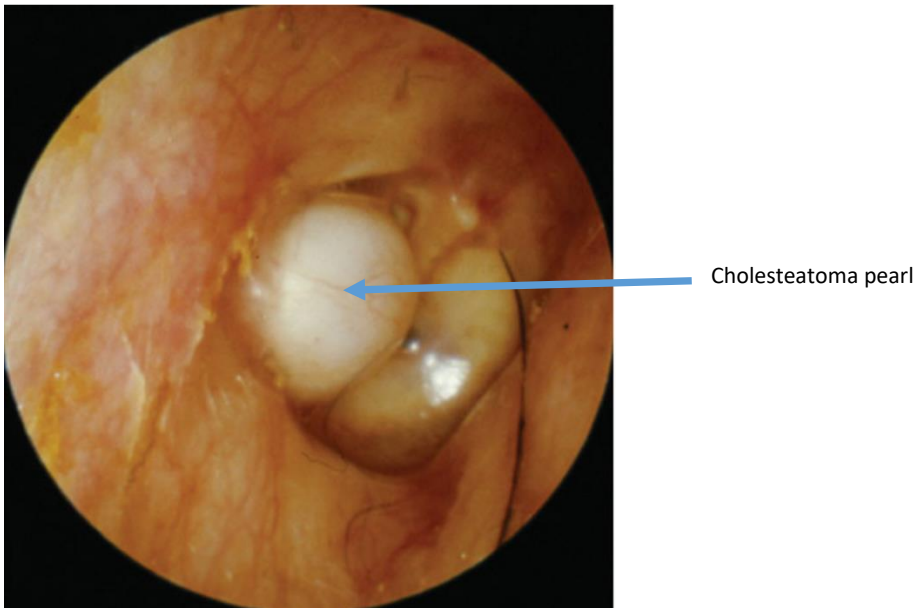


Figure 1-2 Endoscopic view of K's ear

K had been previously operated for a perforated ear drum but felt something blocking her ear. There was an obvious cholesteatoma “pearl” behind an intact drum. It turned out there were plenty of people in K's family with cholesteatoma.

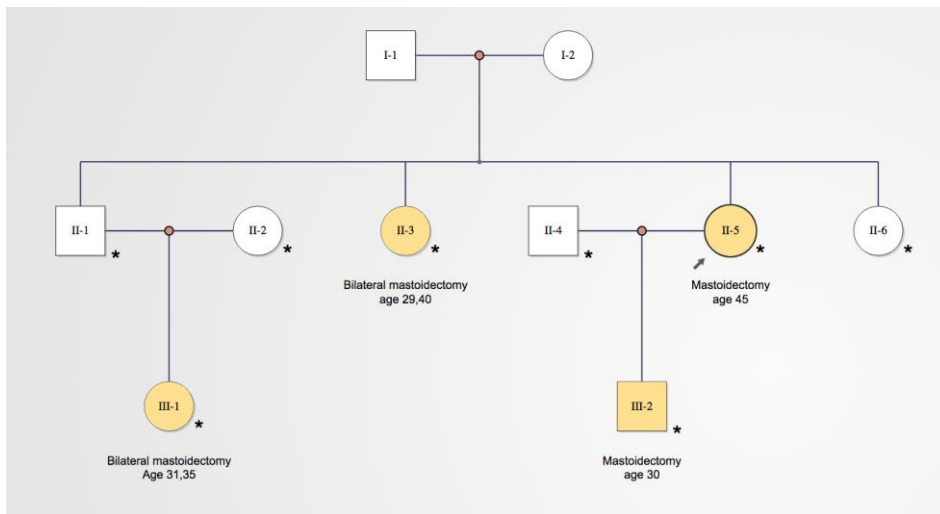


Figure 1-3 K's family. K and her sister C both have bilateral cholesteatoma and other family members who are affected are shown.

Over the next decade a substantial number of families in which there were two or more affected individuals were collected and this was published as a report in in 2009 (Prinsley, 2009a).

One of the most remarkable families was D's family [Figure 1.4].

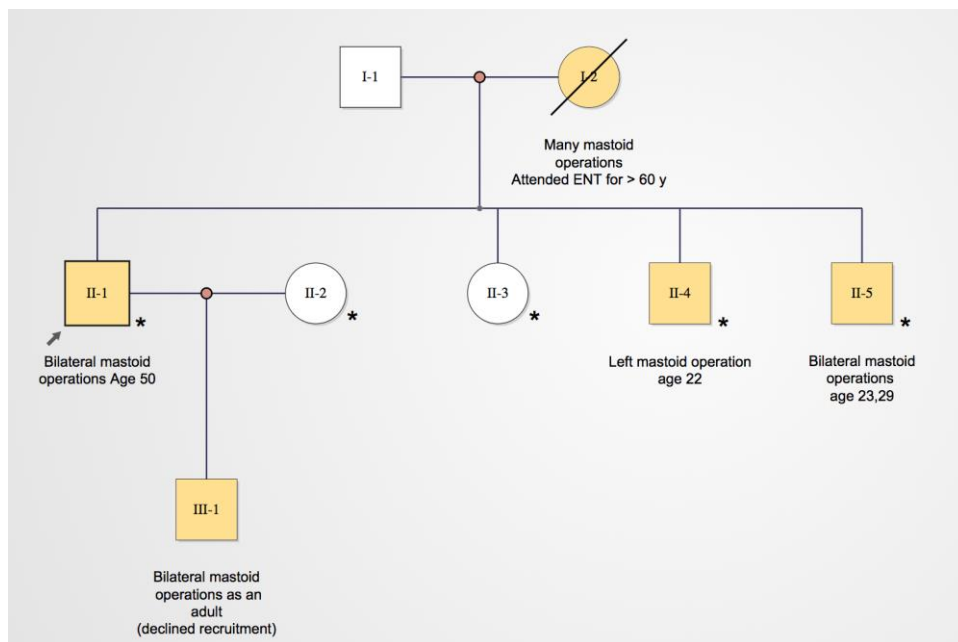


Figure 1-4 D's family. Missing from the diagram is III-1's daughter who has been subsequently diagnosed with a cholesteatoma.

This introduction presents some observations about cholesteatoma based on my own clinical experience and uses data from the Norfolk patients under my care recorded in the web based international otology audit. The Norfolk Genetics of Cholesteatoma Project is a scientific enquiry into the possible genetic basis of the condition based on these familial observations.

The ear drum

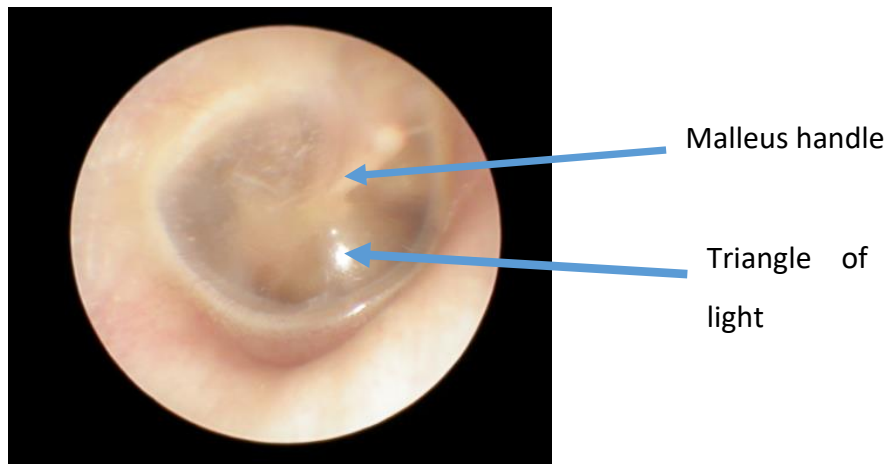


Figure 1-5 Normal ear drum. Photograph of a normal human right ear drum showing the malleus handle and the triangle of light which is created by the concavity of the anterior drum and is sign of a healthy ear drum.

The ear drum consists of modified skin which forms the lateral aspect of the air-filled middle ear. It is part of an exquisite biological mechanism which has evolved to convert mechanical energy of sound waves into electrical signals within the cochlea of the inner ear via a delicate chain of tiny bones called the malleus, the incus and the stapes [figure1-6]. In health, the middle ear is filled with gas at atmospheric pressure which equilibrates with the partial gas pressures of the surrounding blood and is connected intermittently to the air of the nasopharynx via the Eustachian tube. Posteriorly the middle ear space is contiguous with a complex of open spaces within the mastoid bone termed the “air cells”.

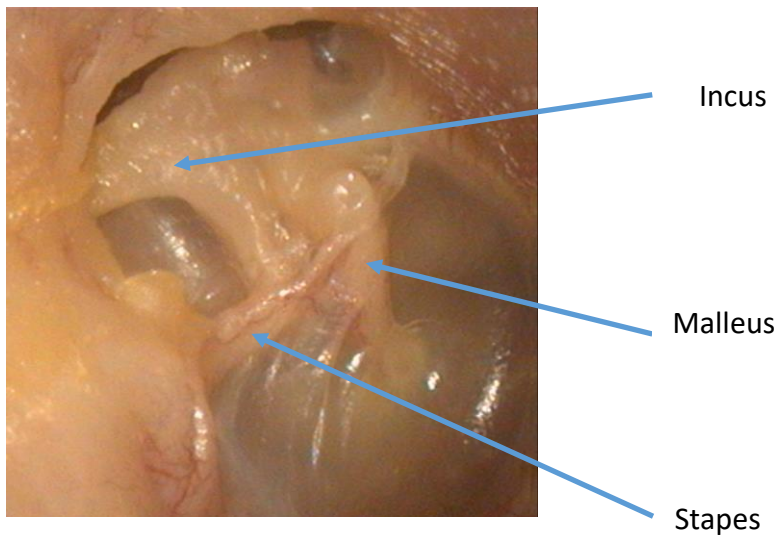


Figure 1-6 In this patient the outer attic wall or scutum has been resected to reveal the ossicular chain which has been preserved.

What is a cholesteatoma

Cholesteatoma is a chronic disorder of the ear which is an important cause of acquired deafness in children and adults. It occurs throughout the world and can only definitively be treated by complex microsurgery to try and remove the disease from the ear. There are over 7000 operations for this disease in the UK NHS each year and the majority of patients suffer lifelong hearing loss as a consequence of the disease and the surgery. Perhaps a million people are afflicted annually worldwide.

Pathologically cholesteatoma is a well-demarcated non neoplastic lesion in the temporal bone that arises from an abnormal growth of keratinizing squamous epithelium (Semaan and Megerian, 2006) (Bhutta et al., 2011).

Abnormality of drum skin migration and desquamation is thought to predispose to the accumulation of cholesteatoma within the middle ear which may be locally invasive and capable of causing bone destruction.

A patient with a cholesteatoma might notice a blocked sensation in the ear and complain of a painless foul smelling discharge. The diagnosis is made on otoscopy which reveals a number of different characteristic drum abnormalities.

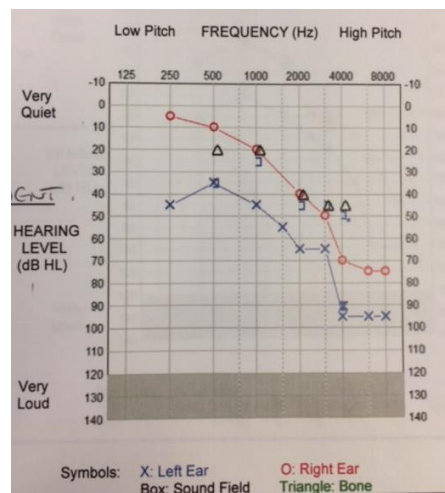
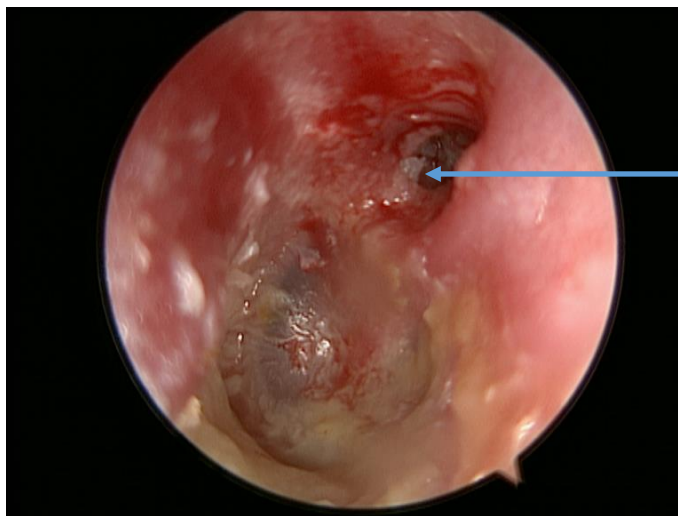


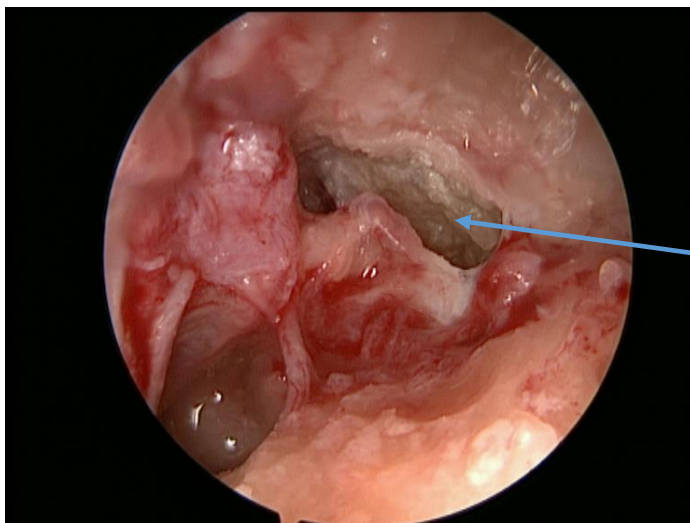
Figure 1-7. An attic crust and the audiogram showing a hearing difference of 20 to 30 dB between the right and left ear.

The elderly patient in figure 1-7 has an “attic crust”. When this was removed a collection of white squamous debris was observed to have accumulated within a drum defect and to have filled the middle ear. There was destruction of the incus long process and conductive deafness.

Operative photographs taken through a 30 degree endoscope of a 38 y old patient with a history of deafness and otorrhoea demonstrate the extent of the disease [Figure 1-8].



Attic defect
revealing the neck of
a cholesteatoma



Extent of the
cholesteatoma
exposed by bone
removal

Figure 1-8 Operative photographs showing the extent of the cholesteatoma

The first picture shows the view at the start of the operation and the second picture shows the extent of the cholesteatoma in the mastoid as the overlying bone has been removed with an operating drill.

Many patients like these present to the ENT clinics and a great deal of my own work as an otologist in Norfolk has been spent trying to help people with the condition. The only curative treatment for cholesteatoma is surgical excision, although by no means all patients with the condition are operated. Hospital Episode Statistics from NHS Digital show that about 7000 people are admitted for surgical treatment of middle ear cholesteatoma [STMEC] annually in England and Wales. Personal data shows approximately 33 patients admitted annually for STMEC in the Norfolk and Norwich and James Paget University Hospitals. Surgical data for cholesteatoma has been prospectively collected for 10 years between 2007 and 2017 using the International Otology Database (www.ear-audit.net) (Van Rompaey et al., 2010).

Cholesteatoma is usually unilateral. In my own operated patients, between 2007 and 2017, 293 patients underwent 332 operations for middle ear cholesteatoma and 17 of the patients had bilateral surgery which 5.8%. The true incidence of bilateral disease is higher than this because some patients with cholesteatoma were not operated and some patients had the first ear operated elsewhere and are not included in the audit.

Cholesteatoma progresses to bone destruction and deafness or runs an indolent course with little in the way of symptoms, and therefore requires no treatment other than periodic observation and cleaning of the ear in the

out patients department. Sometimes a cholesteatoma will expel itself into the ear canal as the bone margins of the tympanic membrane are eroded to create an appearance referred to as an auto-mastoidectomy.

Symptoms of cholesteatoma

Figure 1-9 shows that the predominating presenting complaints are otorrhoea and hearing loss in the 332 operations recorded in the audit of the Norfolk patients treated for cholesteatoma.

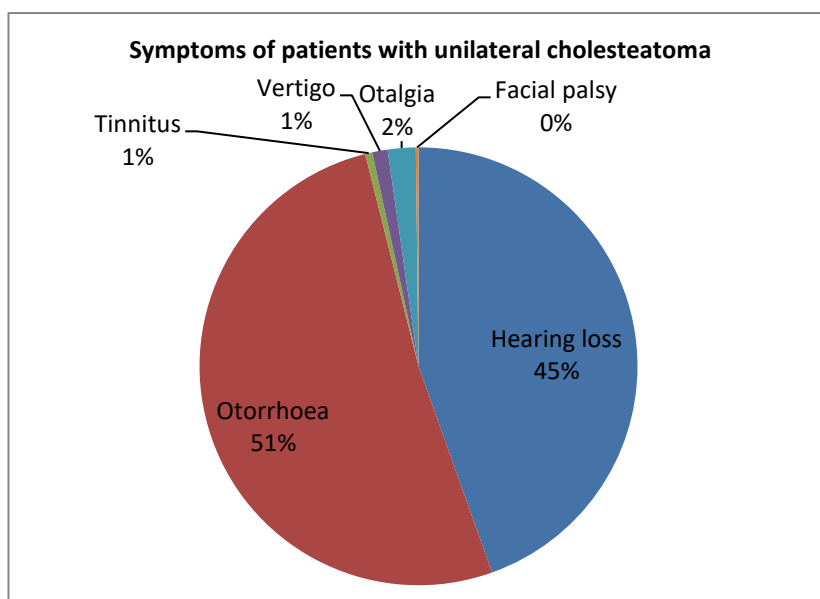


Figure 1-9. Chart showing the predominating presenting symptoms in cholesteatoma.

Cholesteatoma is associated with osteolytic bone reabsorption and superimposed bacterial infection. Erosion of the ossicular bones, most often of the long process of the incus results in a conductive deafness.

The audit of 293 patients operated for cholesteatoma over 10 years in Norfolk recorded ossicular erosion in 67.4% of unilateral cases and 83.3% of bilateral cases, with incus erosion in 56.9% of unilateral cases and 80.6% of bilateral cases. This is the main cause of the conductive deafness in cholesteatoma.

The average presenting conductive element of hearing loss attributed to the cholesteatoma was approximately 20dB. In table 1-1, this is the difference between the operated and non-operated ear in the unilateral cases. The bilateral cases in the chart show bilateral hearing loss. Complete disconnection of the ossicular chain results in a 60dB air bone gap but if the disconnection is partial or the cholesteatoma itself acts as a conducting mechanism to the stapes superstructure or footplate then the hearing is better. This is called “hearing through disease”.

Pure tone Audiogram	Unilateral	Bilateral	p Value [Student T – Test]
Mean Air-Bone gap – Operated Ear	27.5	28.7	0.518
Mean Air-Bone gap - Non-operated ear	7.17	21.2	0.005

Table 1-1. Table to show the presenting dB hearing loss in the unilateral and bilateral cases.

There are numerous reports of personal and institutional series of hearing results for cholesteatoma surgery. There is bias in the sense that experts are more likely to report than others. Children are reported as having less good hearing results.

Ossicular damage correlates with hearing loss and revision cases seem to do worse in keeping with the Norfolk series. There are differences between the

unilateral and bilateral operative findings, perhaps suggesting that patients who have bilateral cholesteatoma have a constitutional factors predisposing to more aggressive disease. The p value needs to be interpreted with caution however in the absence of a Bonferroni adjustment.

Operative Findings	Unilateral [%]	Bilateral [%]	p Value
Anterior retraction pocket	5.1	5.5	0.572
Posterior retraction pocket	18.5	30.6	0.015
Attic Retraction	23.2	27.8	0.169
Sinus Tympani Cholesteatoma	18.5	19.4	0.519
Antrum Cholesteatoma	29.3	30.6	0.509
Attic Cholesteatoma	69.6	80.6	0.112
Mastoid Air cell cholesteatoma	19.2	27.8	0.047
Ossicle Erosion	67.4	83.3	0.035
Erosion of Incus	56.9	80.6	0.006
Erosion of Malleus	15.2	18.9	0.327
Erosion of Stapes	21.7	16.7	0.324
Erosion of more than one ossicle	27.2	30.6	0.401

Table 1-2. Table to show the operative findings of patients with unilateral and bilateral cholesteatoma.

Tympano-mastoidectomy is often successful in removing cholesteatoma but the restoration of hearing, even with the use of ossicular prostheses, is less assured. Only 60-70% of patients with cholesteatoma will recover functional hearing after tympano-mastoidectomy, and hearing is a major determinant of quality of life after such surgery (Nadol et al., 2000, Louw, 2013).

Erosion of the cochlea or labyrinth results in a sensorineural deafness. Conductive deafness also results from surgery to treat cholesteatoma if it is necessary to remove ossicular bones, and in 2.7% of cases cochlear deafness as a consequence of bone erosion of the inner ear (Prinsley, 2013). Loss of

bone overlying the vestibular fluid chambers of the inner ear, most commonly the lateral semicircular canal, results in vertigo. Gentle pressure of a finger in the ear canal displaces cholesteatoma overlying the labyrinth and causes dizziness and nystagmus. This is called a positive fistula sign. Such erosion may be well seen on axial CT scans as in Figure 1-10 and reported by the author (Prinsley, 2013). A loud sound may induce vertigo as a result of displacement of the fluid in areas of exposed membranous labyrinth known as Tullio phenomenon.

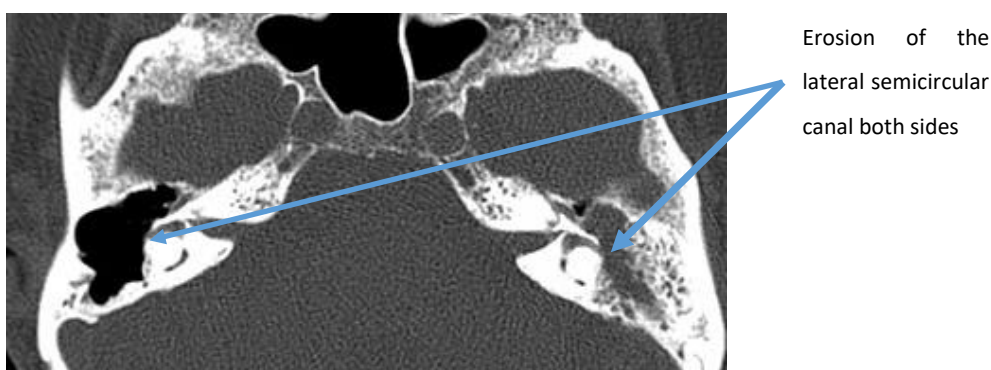


Figure 1-10. Axial CT scan showing labyrinthine fistulas.

This CT scan is taken from a female patient of 83 years. There was a history of previous cholesteatoma surgery on the right side with hearing loss. The scan shows a large clean cavity with an eroded labyrinth on the right side and a cholesteatoma on the left side with erosion of the lateral semicircular canal.

Erosion of the temporal bone may progress to cause intracranial disease [Figure 1.11]. Oscar Wilde probably succumbed to this condition. Although there is controversy, Robins and Sellars suggest that he died of encephalitic meningitis secondary to chronic ear suppuration, most likely cholesteatoma (Robins and Sellars, 2000). There is a sad irony in the fact that Oscar Wilde's father was Sir William Wilde, one of the first and one of the most celebrated ear surgeons in Ireland.

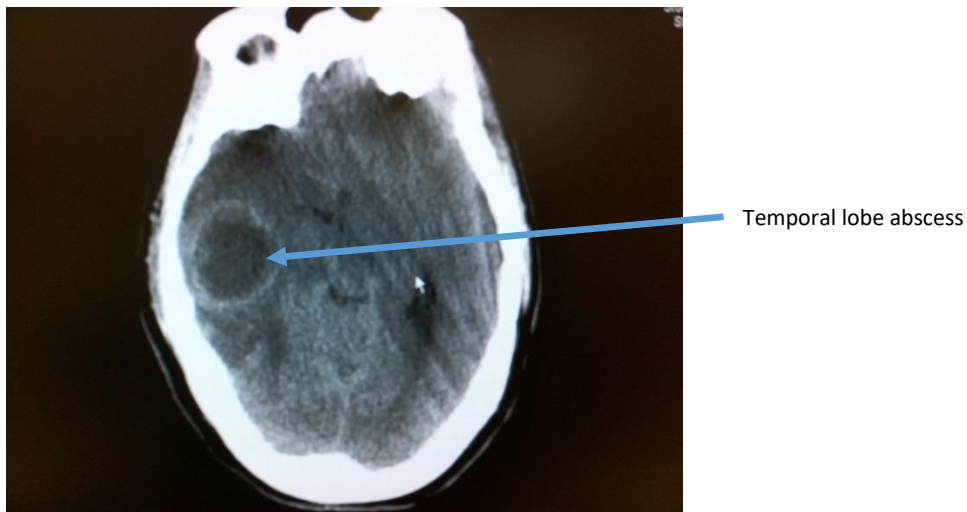


Figure 1-11. The author performed a mastoidectomy on this patient who had been treated at a regional neurosurgical centre for cerebral abscess.

The Cause of Cholesteatoma

The cause of this disease, so relentlessly destructive of the anatomy and function of the ear, is elusive. The epidemiology and pathogenesis of the condition are the subject of separate chapters in this thesis.

Familial clustering , or the aggregation of a certain biological trait within a family, which has been observed in the patients reported in this study, may be environmental or genetic in origin. Some of the families reported here have no doubt shared “environments” but the multi- generational family trees presented for this rare disorder is compelling for a consideration of genetic factors and the observation of familial clustering is one of the first lines of evidence that a disease may have a genetic etiology.

Chronic otitis media in childhood predisposes to development of cholesteatoma (Djurhuus et al., 2015a) , but only a small proportion of those with chronic otitis media will develop cholesteatoma. Animal models confirm the role of chronic mucosal inflammation in inducing cholesteatoma

(Huang et al., 1988, Vassalli et al., 1988, Masaki et al., 1989), but have also failed to illuminate how or why this occurs. There is mounting evidence that predisposition to cholesteatoma has a genetic basis. Despite this being a rare disorder with an incidence of 1:10,000 per year, those who develop cholesteatoma have at least a 5% chance of developing disease in the contralateral ear. In addition to my own report, others have described familial clustering of cholesteatoma (Prinsley, 2009a, Podoshin et al., 1986b). Because cholesteatoma is rare, perhaps genetic predisposition is determined by only a handful of genes, making it a polygenic rather than multigenic disorder.

The Genetics of Cholesteatoma Project

The pilot project presented in this thesis developed from the original observation about familial cholesteatoma 22 years ago. The science of genetics has advanced at an astonishing pace since then. In 2000, the draft sequence of the Human Genome Project was announced. It has become feasible to search for a possible genetic basis of cholesteatoma and the cost and speed of DNA sequencing are now at the point where this is practical.

In the intervening two decades much has also changed in Norwich. There is a new Medical School with an Academic Department of Genetics. My former trainee has returned as a Consultant colleague and has been appointed as Professor of ENT Surgery. The Earlham Institute for Genome Analysis has sprung up next to the new hospital and all the resources are now present in Norfolk for such an enterprise.

A research team has assembled to identify genetic pathways predisposing to cholesteatoma. The study of families in which cholesteatoma is segregating involves genome sequencing coupled to a linkage analysis of DNA collected from affected and non-affected individuals. In conjunction

with pedigree mapping, there is a unique opportunity to identify genetic polymorphisms associated with the formation of cholesteatoma, and by using multiple affected families, to identify recurrent pathways or genes by this method.

Research Team and Funding

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Gavin Willis ,PhD
Principal Clinical Scientist, Norfolk and Norwich University Hospital, Norfolk UK.

Jane Woods, RN,
Research Nurse, James Paget University Hospital, Norfolk UK.

The project has been approved by the Regional Research Ethics Committee and has been adopted by the NIHR Portfolio. The Norfolk families described above have been enrolled in this study in the early part of 2017 and the project was opened to recruitment around the UK in July 2017 to recruit families in which there are two or more affected cholesteatoma patients.

Grants applications have resulted in the following funds being obtained for the pilot study:

Royal College of Surgeons of England Modi Pump Priming Grant: £5000,

Rosetrees Trust: £20,000,

Bernice Bibby Fund: £10,000.

Chapter 2 The Surgical Pathology of Cholesteatoma

A case report of a child with bilateral cholesteatoma with observations on surgical pathology and theories of causation.

Introduction

Cholesteatoma is not a good name since there is neither cholesterol nor neoplasia and the name should really be changed. The first use of the term is attributed to the German physiologist Johannes Muller in 1838 who described a pearly fatty tumour of the ear (Olszewska et al., 2004). **Eroding keratinocyst of the ear** would be a better term. Joseph Toynbee, who is widely regarded as the father of British otology, and who was the first surgeon to be described as an aural surgeon did not use the term at all in his famous book Diseases of the Ear published in 1860 (Toynbee, 1860). Many patients died as a result of untreatable ear infections at this time and his book contains a number of detailed accounts of patients succumbing to the intracranial infections which resulted from what were described as bone replaced by a “soft cheesy mass” which must have been cholesteatoma.

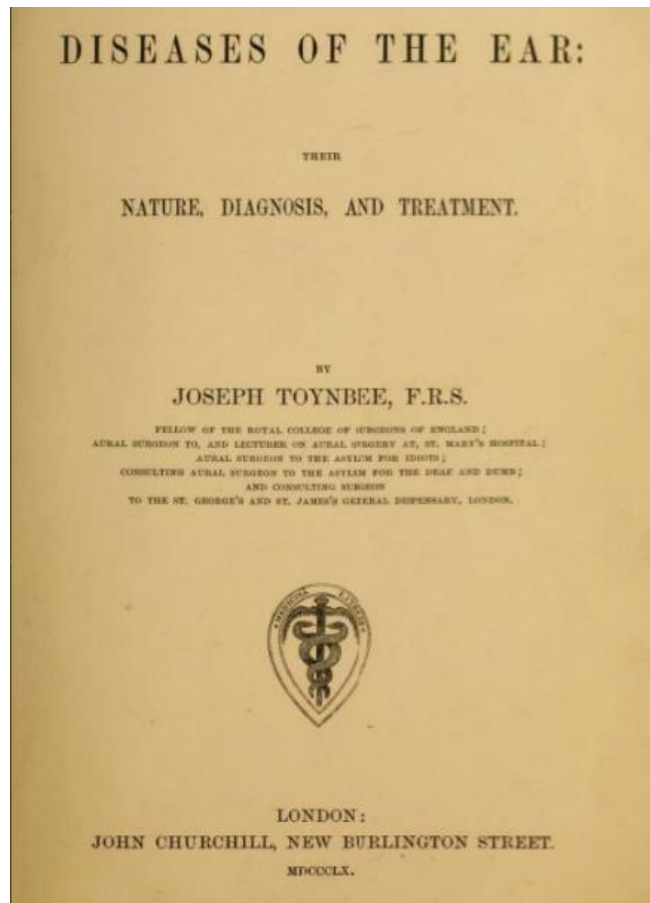


Figure 2-1 Front piece from Joseph Toynbee 1860.

The disease is characterized by an accumulation of keratinizing squamous epithelium within the middle ear which is locally destructive and often associated with infection. The patients generally present when there is either hearing loss or otorrhoea and the diagnosis can usually be made by otoscopy.

The photograph in figure 2-2 shows the typical otoscopic view of an attic cholesteatoma in a patient presenting with hearing loss and otorrhoea.



Figure 2-2 Attic cholesteatoma.

Classification of Cholesteatoma

Traditionally cholesteatoma is classified into congenital and acquired. Congenital cholesteatoma, which is rare, occurs behind an intact tympanic membrane and acquired cholesteatoma which is much more common is generally associated with tympanic membrane defects.

A useful classification might also describe the surgical extent of the disease, any complications and recidivistic properties.

A recent classification in figure 2-3 is the result of an international collaboration of otologists and was the subject of a consensus meeting in Edinburgh in 2016 (Yung et al., 2017).

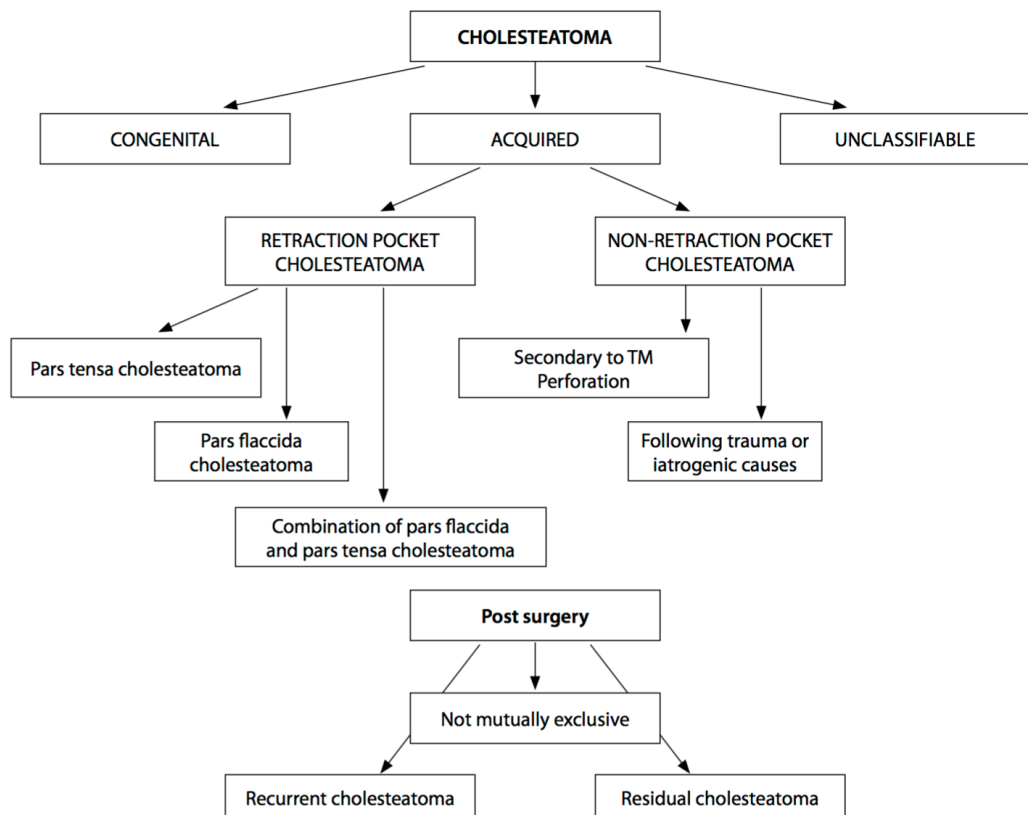


Figure 2-3 Classification of cholesteatoma from Cholesteatoma 2016 Edinburgh.

The anatomical spaces affected by cholesteatoma are divided into T for tympanic cavity, A for attic and M for mastoid. S1 and S2 refer to the surgically awkward epitympanic space and sinus tympani which is difficult to access since it is medial to the facial nerve.

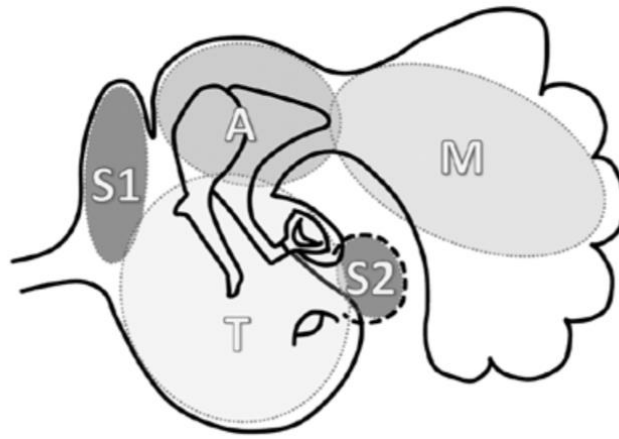


Figure 2-4 Anatomical regions for cholesteatoma classification.

The cholesteatoma is staged according to the number of anatomical sites. Stage 1 is localized at the primary site and stage 2 refers to spread to an adjacent sites. Stage 3 refers to complications such as labyrinthine fistula, facial palsy, post auricular abscess and other extracranial complications. Stage 4 refers to intracranial complications such as meningitis, brain abscess and sinus thrombosis.

Figure 2-5 shows the operative findings in 293 patients operated by the author. Almost all of the cases would be classified as stage 1 or 2 according to the classification. Ossicular erosion is common and important for hearing loss yet ossicular erosion and hearing loss are not part of the Edinburgh classification.

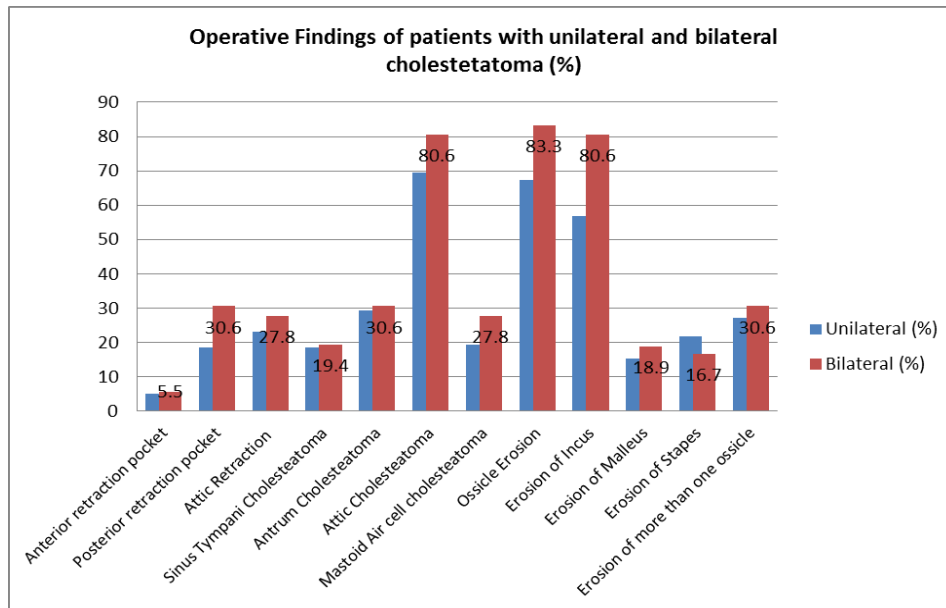


Figure 2-5 Operative findings in cholesteatoma from the author's series of patients.

A case of recurring bilateral cholesteatoma in a child.

Cholesteatoma in children presents a particular challenge. The disease spreads widely within the temporal bone and is especially troublesome if it occurs bilaterally as in the case described here.

The case note entries are summarized over a period of 8 years between September 2010 and January 2018.

23/09/2010:

- DM, a boy age 7, presented to the ENT clinic with a 2 year history of foul smelling discharge from the left ear. Grommets had been inserted at age 4 for bilateral glue ear.
- Hearing was normal. Clinical examination revealed a left attic cholesteatoma.

- The mother reported that both a maternal and a fraternal uncle were known to have cholesteatoma. The patient and his mother have recently been recruited to the Genetics of Cholesteatoma project.

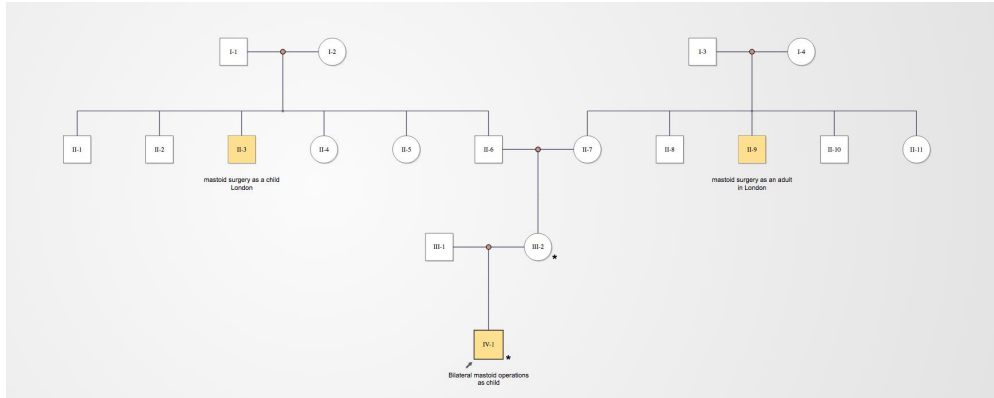
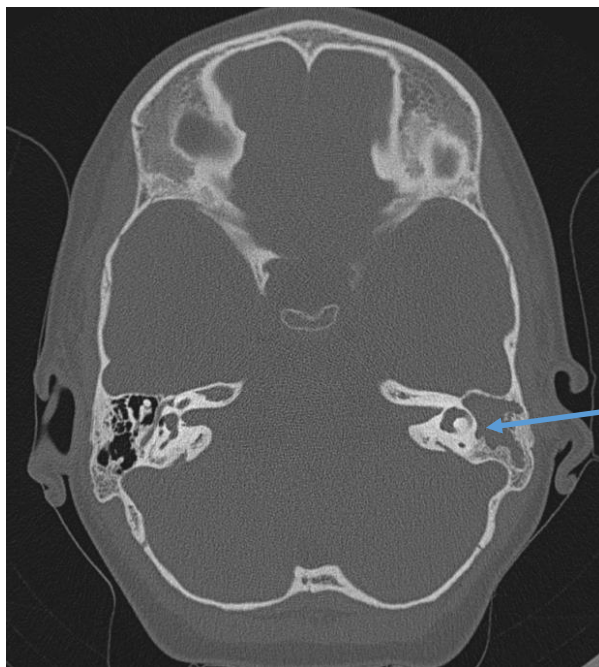


Figure 2-6 Pedigree of DM family. Two maternal uncles are reportedly affected.



Opaque mastoid and eroded air cells

Figure 2-7 A CT scan showed opacity throughout the middle ear and mastoid with bone erosion of the ossicles. The right ear was normal.

1/11/2010:

- A left closed cavity mastoidectomy was carried out to remove a large cholesteatoma in the mastoid and middle ear. The roof of the external ear canal had been eroded by the disease and was repaired. The incus and the head of the malleus were removed.

11/07/2011:

- A planned revision or "2nd look" of the mastoidectomy was carried out to reveal and remove a small recurrent pearl of cholesteatoma in the attic. The stapes was seen to be clear of disease and the post-operative hearing gradually returned to normal by the hearing test of 03/09/2012.
- The patient was seen annually for review and audiology. There was a slight decline in the hearing on the left side but the hearing on the right remained normal.

28/09/2015:

- The patient developed a discharge in the right ear and there was evidence of an attic pit in the drum.

6/2016:

- A CT scan showed opacity and bone erosion on the right side which had been completely normal on the CT scan from 5 years earlier.
- There was also opacity seen on the left mastoid cavity suggestive of recurrent disease.

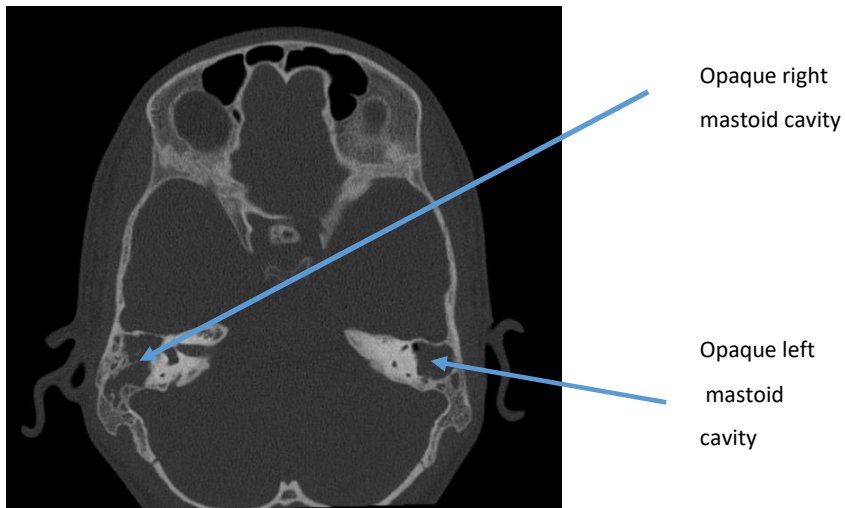


Figure 2-8 CT scan showing disease in both sides.

9/2016:

- A right closed cavity mastoidectomy was done to remove a large mastoid and middle ear cholesteatoma. The incus and malleus head were removed and there was a postoperative conductive deafness in what had been the better hearing ear.

9/2017:

- A left mastoidectomy revealed a massive recurrence of the cholesteatoma and an open cavity mastoid operation was therefore performed. This had recurred despite an apparently complete clearance of the disease in 2011, near normal hearing and a trouble free ear with no discharge. The photograph shows a large cholesteatoma being removed from the left cavity.

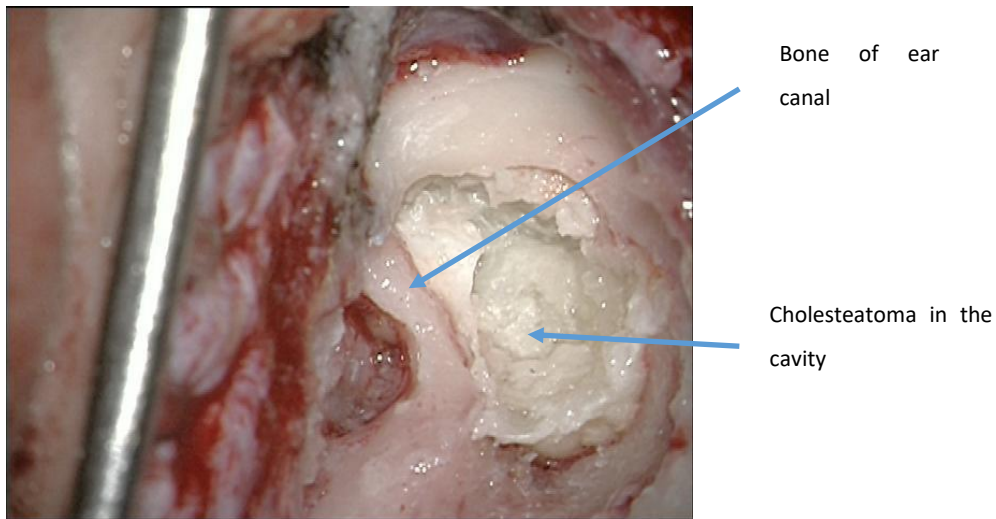


Figure 2-9 Operative photograph showing disease in the cavity.

1/2018:

- The photograph in figure 2-10 was taken of the patient having a revision, or planned 2nd look, mastoid operation on the right side.
- The disease can be seen advancing around the bone of the ear canal and “dripping” very slowly into the cavity. The disease has eroded the arch of the stapes and there was a large conductive hearing loss.

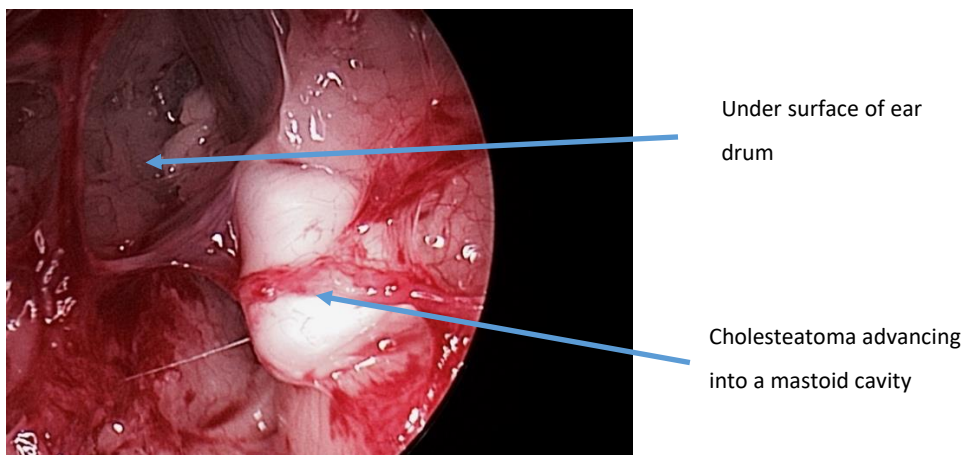


Figure 2-10 Cholesteatoma can be seen creeping around from the middle ear to the mastoid cavity.

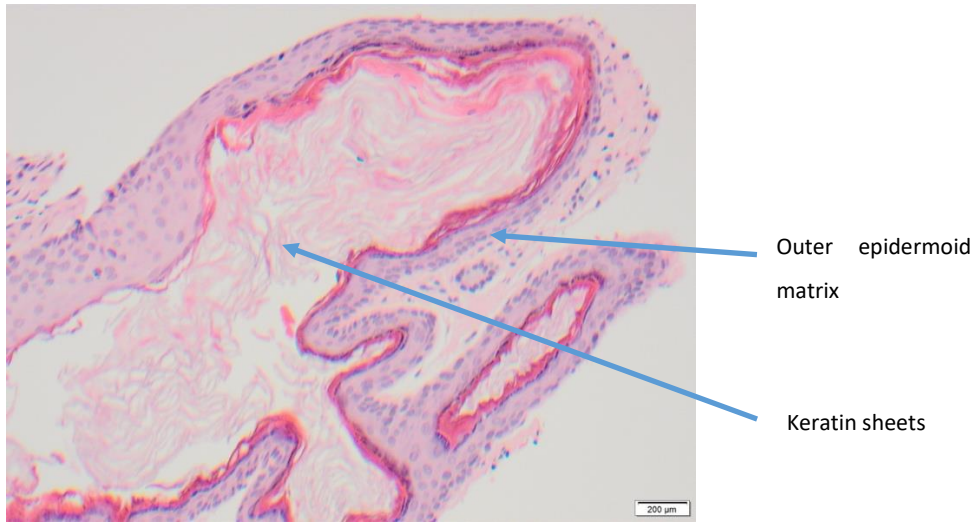


Figure 2-11 Histology of the cholesteatoma.

The histology slide prepared from a sample of the cholesteatoma shows the typical appearance of a keratinizing squamous epithelium with and an underlying perimatrix which is inflamed.

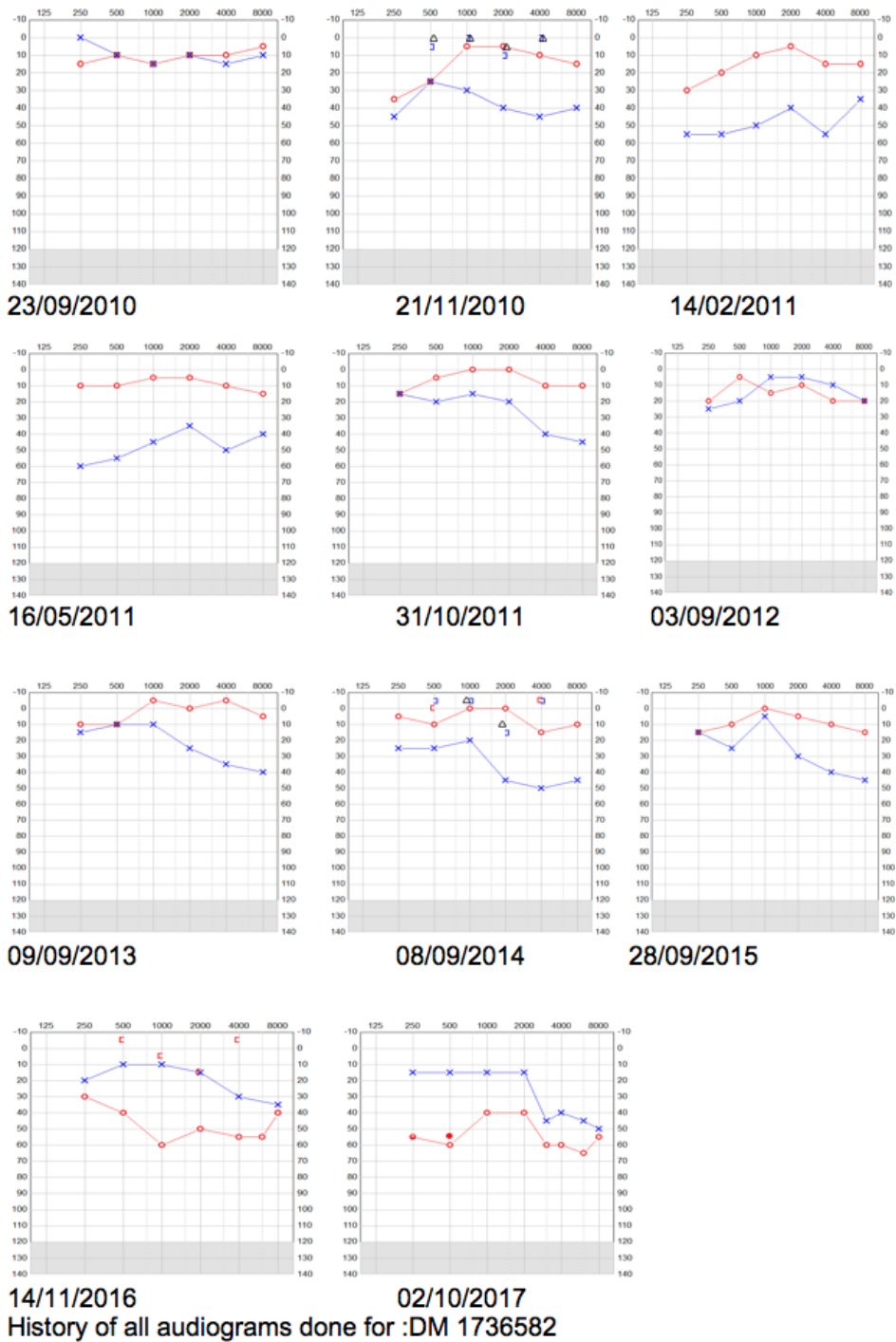


Figure 2-12 Audiograms showing the change in the hearing over 7 years in both ears.

The hearing has fluctuated over a period of 7 years. At the beginning the patient had normal hearing. In the most recent test the patient had good hearing in the low tones on the left because there is direct contact of the drum with the stapes superstructure. There is a large conductive loss on the right as a result of loss of the stapes superstructure. This can be repaired by means of an ossiculoplasty.

In summary this is a child with bilateral destructive cholesteatoma and a family history on both sides of his mother's family. The disease shows recidivism and developed in the 2nd ear "hidden in full sight" of the otologist who carried out regular reviews.

The fact that cholesteatoma may be so destructive in children is supported by data from my own series (tables 2-1,2-2) which has compared the site of the cholesteatoma and the state of the ossicular chain in adults and children. In the Norfolk series there were 71 operation in 58 children and 281 operation in 260 adults.(Jackson et al., 2018)

Cholesteatoma was found to be more extensive and more likely to have resulted in ossicular erosion.

Cholesteatoma site	Children (n (%))	Adults (n (%))	p-value*
Under tympanic membrane	20 (28.2)	38 (13.5)	0.007
Sinus tympani	26 (36.6)	41 (14.6)	0.001
Mastoid antrum	31 (43.7)	78 (27.8)	0.014
Mastoid air cells	25 (35.2)	51 (18.1)	0.003
Attic	53 (74.6)	199 (70.8)	0.559

*Fisher's exact test

Table 2-1 Site of cholesteatoma in children and adults.

Ossicle eroded	Children (n (%))	Adults (n (%))	p-value*
Malleus	17 (23.9)	37 (13.2)	0.041
Incus	50 (70.4)	159 (56.6)	0.042
Stapes superstructure	25 (35.2)	48 (17.1)	0.0016

*Fisher's exact test

Table 2-2 Ossicular erosion in children and adults.

Pathological theories

The cells of the tympanic membrane, uniquely within the body, exhibit centrifugal migratory properties (Broekaert, 1990). Experiments in which small ink dots painted on the drum are observed by serial drum photography have been used to investigate the migratory pattern of the drum cells which originate around the umbo at the tip of the malleus and migrate radially. Thus the drum regenerates by migration of new cells from the centre rather than by superficial desquamation. Probably this is the basis of a self-cleaning mechanism which fails when a cholesteatoma develops.

Although squamous metaplasia of middle epithelium has been proposed as a cause of cholesteatoma (Sadé, 1971) most otologists believe that cholesteatoma arises from drum epithelium either as a result of invagination and pocket formation or as an error of drum healing.

Migration of cholesteatoma from the edge of a drum perforation is familiar to otologists and is surely the mechanism of causation in some cases.[Fig 2.13]

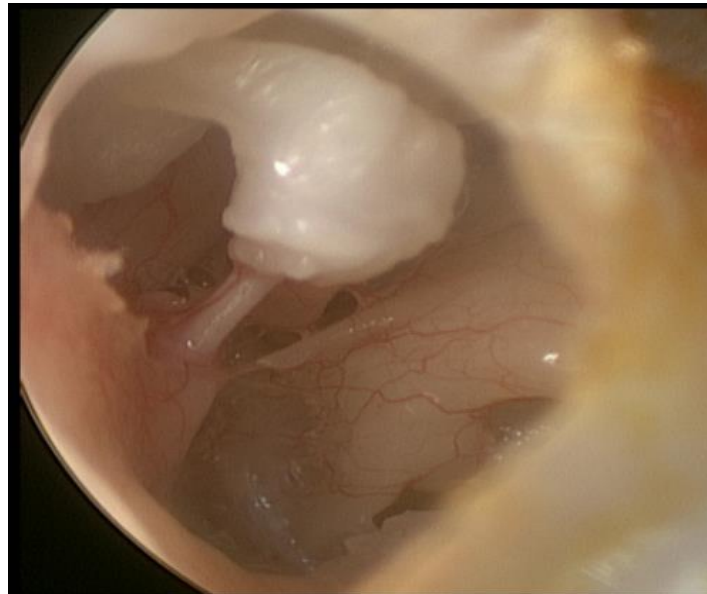
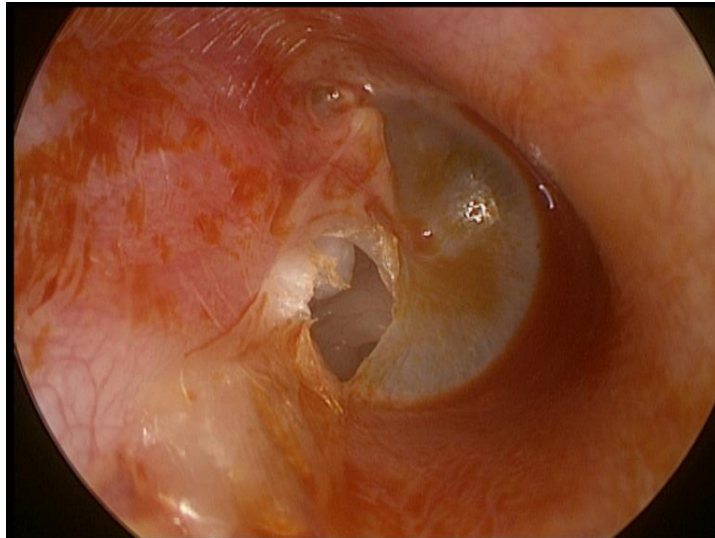


Figure 2-13 Operative photographs which show a cholesteatoma apparently arising at the margin of a perforation.

The magnified image shows cholesteatoma enveloping the incudo- stapedial joint. There was erosion of the incus long process and a 60dB conductive hearing loss.

Cholesteatoma most commonly starts within a drum retraction, perhaps as a result of negative pressure within the middle ear. The pars flaccida, which lacks the strong fibrous layer of the pars tensa, is most susceptible to this which explains why most cholesteatoma arises in the attic region. Focal disruption of the basal epithelial layers of the pars flaccida in particular permits the ingress of keratinocytes resulting in the formation of keratinizing inclusion cysts (Sudhoff et al., 1996).

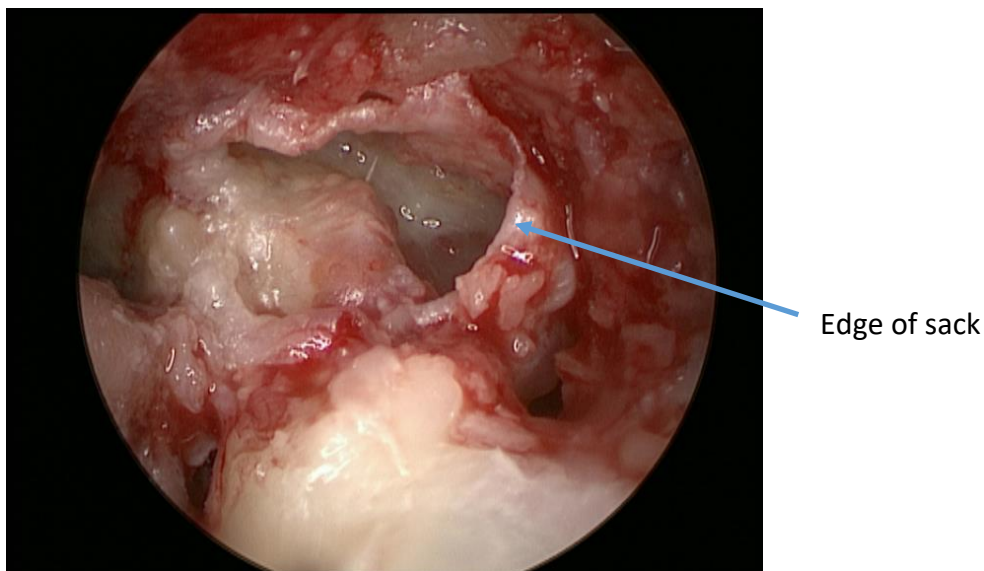


Figure 2-14 Operative photograph showing the sack of the cholesteatoma

The photograph shows how a sack of invaginated epithelium is revealed after some of the overlying bone has been drilled away.

Essentially cholesteatoma can be regarded as a situation in which there is unregulated control of drum epithelium growth usually with invasion and hyperproliferation of the cells. Why does this occur?

Investigative approaches

Investigators have regarded cholesteatoma as a disorder of growth control, not neoplasia, but rather invasion and hyperproliferation of keratinizing squamous epithelium.

Studies have compared the molecular chemistry of cholesteatoma cells with post auricular skin taken at the time of operation. Immunohistochemistry and microarray analysis has been used in many reports of this type to try and understand the apparently disordered growth regulation (Olszewska et al., 2004, Kwon et al., 2006) (Macias et al., 2013b, Friedland et al., 2009, Kuo, 2015). These sort of studies investigate gene expression in tissue after the biological change in the cells associated with the cholesteatoma has developed and no distinction can be made between somatic mutations which are not inherited and germ line mutations which are the fundamental cause of a genetic trait.

A variety of cytokines and other molecular mechanisms have been studied by this technique:

- Cytokeratins 13/16 and proliferating cell nuclear antigen: (Jin et al., 2011)
- Transforming Growth Factor Alpha: (Ergün et al., 1996)
- c-Jun and P53: (Shinoda and Huang, 1995)
- c-Myc: (Holly et al., 1995)
- Gap junction protein expression: (Klenke et al., 2012a)

Animal models of cholesteatoma have also been described (Yamamoto-Fukuda et al., 2011). Ligation of the ear canal in Mongolian gerbils can be

used to experimentally create cholesteatoma (McGinn et al., 1982). Mutated mice models deficient in Transforming Growth Factors [TGF] (Ergün et al.) have been studied (Wright et al., 1996).

Such approaches have suggested a number of relevant molecular pathways in which hyperproliferation of keratinocytes associated with inflammatory reactions are triggered by complex immune and cytokine messenger systems (Macias et al., 2013b).

Cytokines

These are small protein signalling molecules involved with cell to cell communication particularly in immune responses. The growth of cholesteatoma is associated with an immune infiltration and the release of cytokines and growth factors. Keratinocyte proliferation is associated with expression of transforming growth factor alpha [TGF α], epidermal growth factor [EGF] and its receptor EGFR (Bujía et al., 1996). DNA microarray chip analysis studies such as that of Macias et al comparing the cholesteatoma cells to the postauricular skin have shown that genes involved in cytokine mediated inflammation have altered expression (Macias et al., 2013b).

Cytokeratins

Cytokeratins or keratins [K] are filamentous proteins [number 1 to 20] present in epithelial cells. K expression varies with stages of differentiation and as such K expression has been used to better understand cell dynamics. The K expression demonstrated by such studies reflects the hyperkeratinisation of the developing cholesteatoma but does not explain why this occurs (Kim et al., 2001, Olszewska and Sudhoff, 2007).

A cycle of keratinocyte activation in wound healing and other conditions has been postulated which is controlled by the activation of growth factors and

cytokines. Resting keratinocytes either differentiate or activate in response to chemical signals which affect the production of various keratin K proteins within the cell. A disruption of such a cell cycle might have importance in cholesteatoma pathology and certainly an accumulation of excessive keratin and migration of keratinocytes are hallmarks of the disease.

In the cycle presented in the diagram 2-15 basal keratinocytes producing K5 and K14, either differentiate to produce K1 and K10 or receive a signal from IL-1 and become activated to express K6 and K16. TNF- α and TGF- α keep the keratinocyte activated until a contracting signal from IFN- γ induces K17 and causes keratinocyte contraction. TGF- β is a signal for the cell to revert to the resting state (Freedberg et al., 2001).

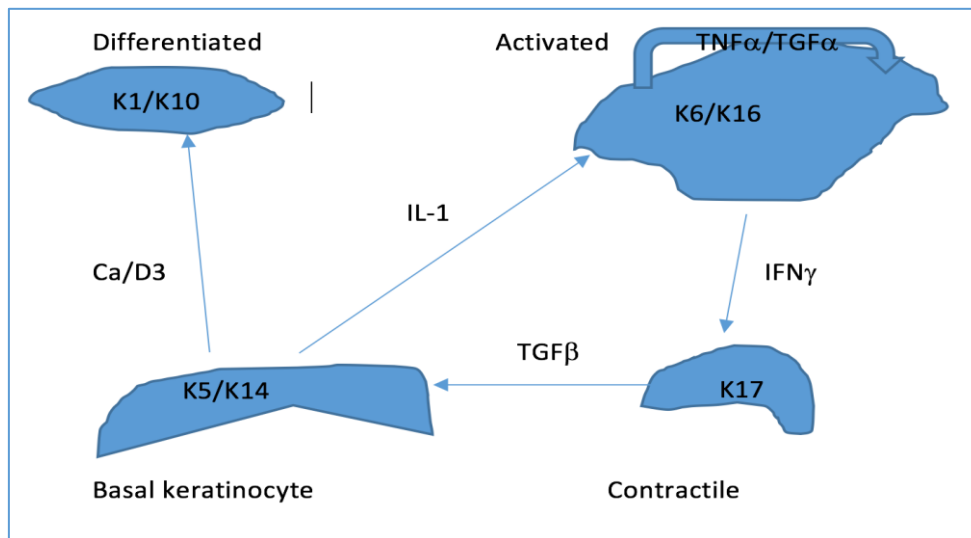


Figure 2-15 Drawing of a keratinocyte cycle

The role of infection

Many cholesteatomas are associated with bacterial infection and otorrhoea. Might bacterial infection induce immune mechanisms important in cholesteatoma proliferation? The anaerobic bacteria *Pseudomonas aeruginosa* creates a biofilm lipopolysaccharide that has been shown to induce keratinocyte hyperproliferation (Preciado, 2012). The destructive nature of the proliferating keratinocytes is increased in the presence of infection. However many cholesteatomas develop insidiously in the apparent absence of active bacterial infection and infection is probably not the cause of cholesteatoma per se.

Conclusion

Cholesteatoma has defied explanation since it was first described. Surgical pathology and histopathological examination have suggested a number of pathogenic processes and molecular studies using immunohistochemistry and microarray analysis in particular to compare cholesteatoma with matched post auricular skin have unravelled some of aspects of protein signalling and expression at a cellular level.

The Genetics of Cholesteatoma whole exome sequencing pilot study described in this thesis has adopted a completely different approach. It makes use of the observations about a familial tendency and seeks to identify mutations that co segregate with the phenotype. Work which has identified biochemical pathways of interest informs this genome study of the molecular factors and biological pathways that have a role in cholesteatoma formation.

Chapter 3 The Epidemiology of Cholesteatoma

A literature review and a presentation of ear surgery data from Norfolk and England.

Abstract for chapter 3

Background

Epidemiological information about cholesteatoma might inform theories of genetic causation that are the subject of this specific investigation. Basic data about age of onset and gender can be extracted from published case series and national health statistical databases. There is much less information about other commonly reported epidemiological characteristics such as social class and comorbidity, for example the association with childhood ear infection.

Objectives

- 1 To complete a systematic literature review of epidemiology studies about cholesteatoma.
- 2 To contribute data about the age and sex and anatomical distribution of the disease in defined UK populations within Norfolk and England.

Data sources

1. Online databases: Medline, EMBASE, CINAHL.

2. Contact Centre Team NHS Digital that collates Hospital Episode Statistics for England.
3. The International Otology Database: www.ear-audit.net .

Search criteria terms

Cholesteatoma, middle ear and epidemiology.

Study eligibility for literature review

The search criteria terms *cholesteatoma, middle ear, epidemiology* were used.

No restrictions on date.

English language papers.

Appraisal and synthesis of literature review

A total of 154 references were revealed by the search strategy. Abstracts were hand scrutinized for content of interest. Studies were sorted into observational population studies and studies reporting institutional or personal case series with epidemiological observations.

Studies reporting comorbidity associations and syndromal associations and also other factors such as social deprivation were all assessed for relevance.

Results

Summary findings from 21 articles about the epidemiology of cholesteatoma are tabulated. Epidemiological observations and associations are described.

Conclusions

Epidemiological aspects of cholesteatoma have attracted rather little interest in the literature. What is known about the epidemiology is derived largely from a number of case series and from identifiable disease in cases with syndromal associations. It is known that the condition is slightly more frequent in males and that there is a peak age of incidence in the second decade of life. Association with childhood ear infection is well described but by no means universal.

Causation remains enigmatic but epidemiology can inform futures avenues of research including research into the genetic basis of the disease that is the subject of this thesis.

Introduction

Rationale

Cholesteatoma is an enigmatic condition of the middle ear of unknown causation. Observations reported by the author of a familial tendency in East Anglia suggest a role for genetic factors (Prinsley, 2009a). Epidemiological observations have the potential to inform studies into the possible genetic basis of this condition. A systematic epidemiological literature review has been undertaken and observations collated from a personal database of cases and from NHS Digital that collates cases nationally are also presented.

Objectives

1. To complete a systematic literature review of epidemiology studies about cholesteatoma.
2. To contribute data about the age and sex and anatomical distribution of the disease in defined UK populations within Norfolk and England.

Background

Cholesteatoma, although not exceptionally rare, can hardly be considered an epidemic in the usually understood meaning of the word. The Oxford English Dictionary describes an epidemic as a disease prevalent in a community at a particular time and produced by special causes not generally present in the affected locality. It is this concept of special cause that is being sought by a review of disease association presented in this chapter.

Methods

Information sources

Medline, EMBASE, CINAHL. No restrictions on date or language.

Search history

The search criteria terms *cholesteatoma*, *middle ear*, *epidemiology* were used as follows:

1. Medline; *CHOLESTEATOMA/ OR *CHOLESTEATOMA, MIDDLE EAR/; 4100 results.
2. Medline; *EPIDEMIOLOGY/; 657533 results.
3. Medline; 1 AND 2; 70 results.
4. EMBASE; *CHOLESTEATOMA/; 4165 results.
5. EMBASE; *EPIDEMIOLOGY/; 42852 results.
6. EMBASE; 4 AND 5; 1 results.
7. EMBASE; exp CHOLESTEATOMA/ep [ep=Epidemiology]; 95 results.
8. CINAHL; *CHOLESTEATOMA/; 427 results.
9. CINAHL; exp EPIDEMIOLOGY/; 318880 results.
10. CINAHL; 8 AND 9; 20 results.

Eligibility for inclusion in the literature review

Abstracts of 154 different articles identified using the search strategy above were scrutinized; full text was obtained for clarity where necessary.

Inclusion criteria

Articles describing populations or case series containing clear epidemiological data were identified.

Exclusion criteria

- Small retrospective series of operated cases and operative technique.
- Anatomical reports about disease distribution and the facial nerve.
- Radiological reports about disease extent.
- Articles about pathological theories of causation.
- Bacteriology articles.
- “Epidemiological” reports with no information about denominator populations.
- General cholesteatoma reviews with no original observations.

Data collection and synthesis of results

Epidemiological data such as incidence, age, gender, syndrome and disease association have been tabulated for 21 articles together with a note of the main observation made in the report.

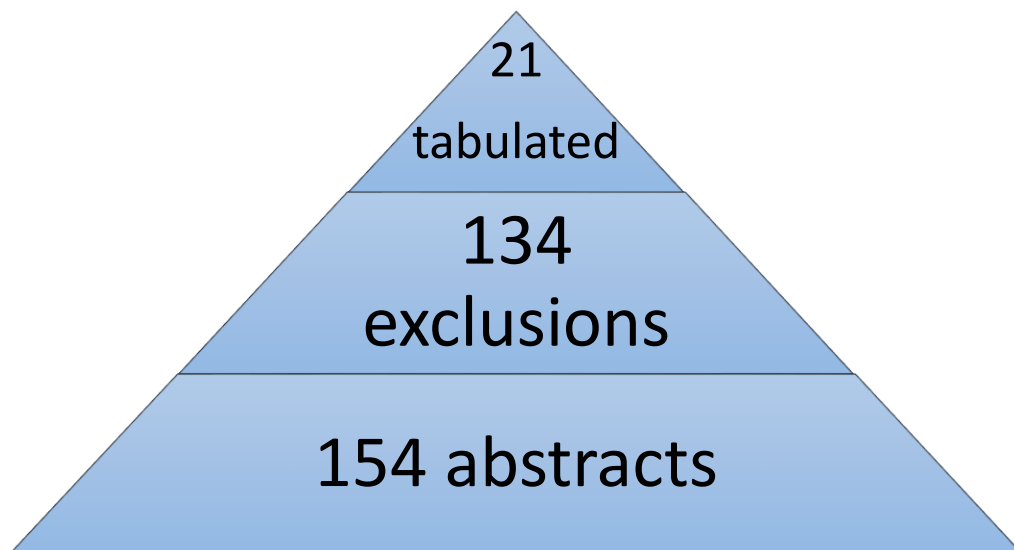


Figure 3-1 Filtering of the epidemiology literature review.

Risk of bias

Retrospective population based cohort studies that use routinely collected hospital data and statistics are of great value because almost all cases of cholesteatoma that require surgery require admission to hospital. Personal and institutional reports that comprise much of the literature are subject to reporting bias and are hard to verify.

Results

Study selection

The electronic library search revealed 154 references. Abstracts were screened and assessed for eligibility. The summary data for 21 articles of particular interest are tabulated.

Study characteristics identified

The author and country of origin together with date of the study/publication were identified. The study population was described. Some institutional reports describe cholesteatoma case series with little definite information about denominator populations of unaffected patients. Other cohort reports look at cholesteatoma in the context of whole populations. Incidence and gender are tabulated where this information is available. Some reports identify a social class effect. The summary observations in each of the reports are tabulated.

Table of results

1 st Author Country	Dates	Study population	Incidence Gender	Social Class	Main observations
Bergamaschi Italy (Bergamaschi et al., 2008a)		173 people with Turner Syndrome	94 had middle ear disease 15% had chole		1 st and 2 nd arch growth disturbance associated with ear disease
Djurhuus Denmark (Djurhuus et al., 2010)	1977 to 2007	13,606 STMEC Approx. 5 million pop	14.3/9.1/m/f/100,000 in 1982 8.5/5.4/m/f/100,000 in 2007	No affect	Decrease in incidence of surgery for chole in 30 y period
Djurhuus Denmark (Djurhuus et al., 2015a)	1997 to 2011	In 217,206 children who had VTI 374 had STMEC 36,981 children had no VTI 5 had STMEC	1.71% of VTI patients 0.013% of non VTI patients		Prolonged otitis media requiring VTI makes chole much more likely. Increasing number of VTI in an individual increases risk for chole
Djurhuus Denmark (Djurhuus et al., 2015c)	1936 to 2009	8,593 cleft patients and 6989 non syndromic siblings 249,708 controls and 175,724 siblings of controls	201 and 21 STMEC 485 and 332 STMEC 5% sample of Danish population 249,708 people 485 chole 0.2%, 175,724 siblings 322 chole 0.2%		HR CLAP 14, HR CL 20 HR siblings of CP 2.1

Fiedler(Fiedler et al., 2013) Germany	2005	All ENT depts in 1 German state 1037 pts	Ear surgery numbers for various disorders		15/100,000 pop STMEC
Harris Canada(Harris et al., 2013)	1981 to 2005	Toronto Sick Children Hospital	2737 palatoplasty children developed 44 chole age 5 to 18 which is 2.2%. 200 x the base rate CLAP 3 x risk of CP alone		Strong CLAP association
Hasegawa Japan(Hasegawa et al., 2006)		171 chole Tohoku University Hospital	m/f = 171/100		Sniffing habit related to hearing and air bone gap in chole patients
Kemppainn Finland(Kemppainen et al., 1999)	1982 to 1991	500 chole patients in 2 hospitals in Finland	Incidence of 9.2/100,000 Previous VTI in 10.2% 8% of CLAP patients	No class effect	Typical personal/institution report over 10 y
Khaiid Raja UK(Khalid-Raja et al., 2015)	4 y of NHS data	UK Health statistic for 4 separate years	See Fig 3-7	Yes	Chole operations more common in areas of higher multiple deprivation
Kinsella Ireland(Kinsella, 1996)	1965 to 1988	Clinical notes of patients having ear surgery in 2 hospitals	Chole varied from 0.01 to 0.16/1000 population No change first 5 y to last 5 y despite 8 x increase in VTI		No reduction chole by VTI
Mason(Mason and Wabnitz, 2002) Australia	1998 to 2001	Case review at Royal Darwin Hospital	5/100,000 aboriginal and 6/100,000		No increase in chole in aboriginal patients

			non aboriginal		despite greater incidence of CSOM
Maw(Maw et al., 2011) England	2001	Avon Study of Parents and Children	Video otoscopy of 6908 children		15 /6908 had overt or suspected chole
Migirov Russia(Migirov, 2012)	2009 to 2012	237 right handed patients 31 left handed	m/f = 158/79 6 to 81 y		59.5% right handed patients had right chole 83.9% left handed patients had left chole
Padgham Scotland (Padgham et al., 1989)	1966 to 1986	Tayside	Chole incidence 0.94/10,000 to 1.88/10,000 Mean 1.32/10,000 VTI increased 60 x		No reduction chole by VTI
Prescott S Africa(Prescott, 1999)	1988 to 1996	96 chole in 81 children in S African hospital	m/f = 44/37 age 2 to 12 24/96 presented with mastoiditis		Late presentation Extensive disease and poor FU typical in “developing” country
Rakover Israel(Rakover et al., 2000)	1961 to 1998	Israeli hospital	413 chole op 1961 to 1970 228 chole op 1989 to 1998 30y gap in which VTI was introduced 20 to 6.6 /10,000 population		Belief that VTI reduces long term chole incidence
Roland England(Roland et al., 1992)	1963 to 1990	Paediatric population in Liverpool	Decline in mastoid operations and		Increasing VTI not correlated with reduced

			increased use of VTI But no correlation		mastoid surgery
Shibata Japan(Shibata et al., 2015)		Fukuoka City, Japan	Incidence 6.8 to 10/100,000		PMH of otitis media and habitual sniffing relevant
Spilsbury Australia (Spilsbury et al., 2010)	1980 to 2004	45,980 children who had VTI in W Australia	460 developed chole Increasing number of VTI increased chance of chole 1 VTI 0.9%,2 VTI 2.1%,3VTI 3.8%,4VTI 5.2%	Rural effect	Approx 1% of children who have had a VTI get chole
Wang(Wang et al., 2015) Taiwan	1997 to 2008	37,124 cohort osteoporosis	See Fig 3-8		1.31 HR for chole in osteoporosis
Zakzouk(Van Rompaey et al.) Saudi Arabia	2000	Epidem study in Central Province	9/9540 children had chole in 1982 6/6421 children had chole in 1991		Part of a study showing decrease in CSOM but no change in chole

Table 3-1 Summary of literature table

STMEC	Surgically treated middle ear cholesteatoma
Chole	Cholesteatoma
CSOM	Chronic suppurative otitis media
VTI	Ventilation tube insertion
HR	Hazard ratio
CLAP	Cleft lip and palate
CP/CL	Cleft palate/lip
FU	Follow up

Epidemiological Statistics for England and for Norfolk

The NHS Hospital Episode Statistic for England [Fig 3.2] shows that there were 5,201 operations for patients who were coded as having a diagnosis of cholesteatoma of the middle ear [H71.X] in the year 2014/15. This gives an indication of the annual incidence of surgical approaches to cholesteatoma in England. The peak age group is 10 to 14 years and there was an almost 3 to 2 male to female ratio. If the population of England is approximately 50,000,000 then this is approximately 10 cholesteatoma operations per 100,000 population per year.

Cholesteatoma of the middle ear 2014/15: Health and Social Care Information Centre English NHS. This chart was devised by the author using data from NHS Digital.

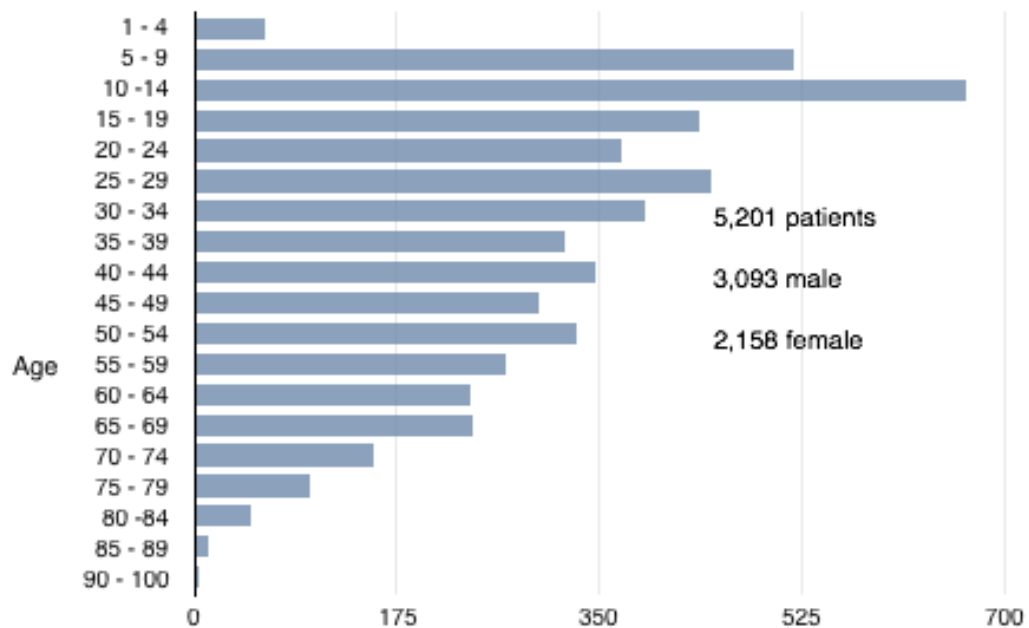


Figure 3-2 Cholesteatoma of the middle ear 2014/2015 English NHS

Figure 3-3 shows that there are 406 operations for cholesteatoma in the series of patients recorded by the author over 10 years. The peak age group is also 10 to 14 years. There is another peak in the age group 40 to 49 but broadly the distribution of age is similar to the national picture. The sex distribution is somewhat more equal in the Norfolk cases; 52.7% male in Norfolk v 59.4% male nationally.

This is approximately 40 cases each year suggesting a population of about 400,000 people, which is approximately 10 operations per 100,000 population per year. The two Norfolk hospitals serve a population of about 750,000 people but there are several other otological surgeons.

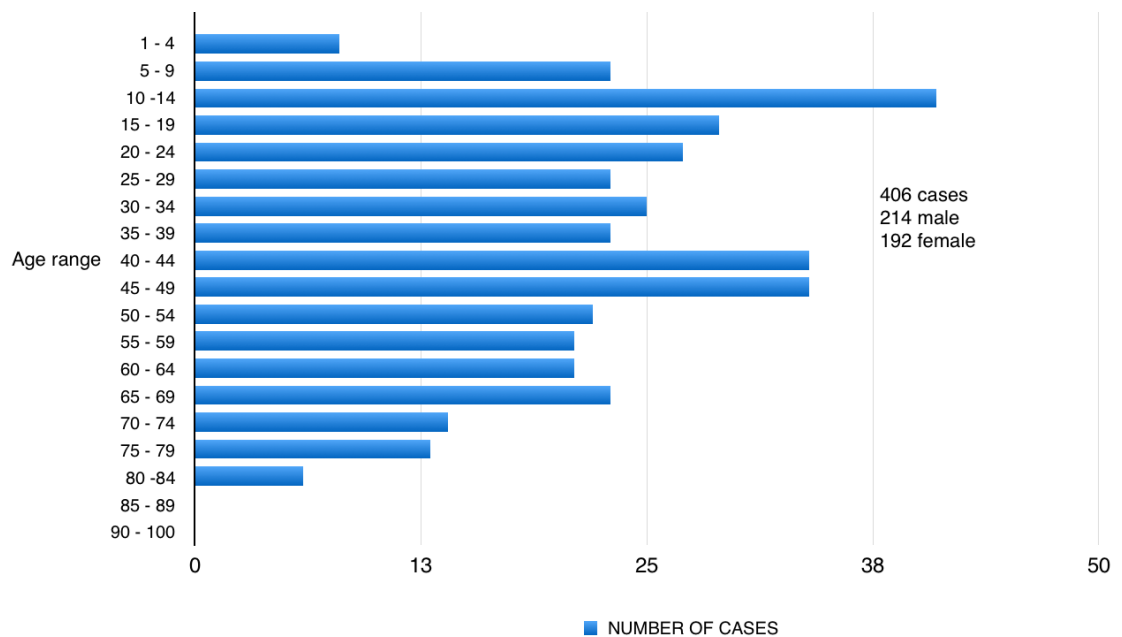


Figure 3-3 Cholesteatoma surgery data from www.ear-audit.net for the author's cases 2006 – 2016.

Discussion

Incidence

Djurhuus (Djurhuus et al., 2010) has published an analysis of Danish national health statistics that has studied 13,606 cases of cholesteatoma [Figure 3.4]. The incidence of cholesteatoma was a maximum of 14.3/100,000 in males in 1982 falling to 8.5/100,000 in 2007. There was a similar fall in female cases. Age specific incidence per 100,000 person ears is also presented. The figures are taken from the Djurhuus' study.

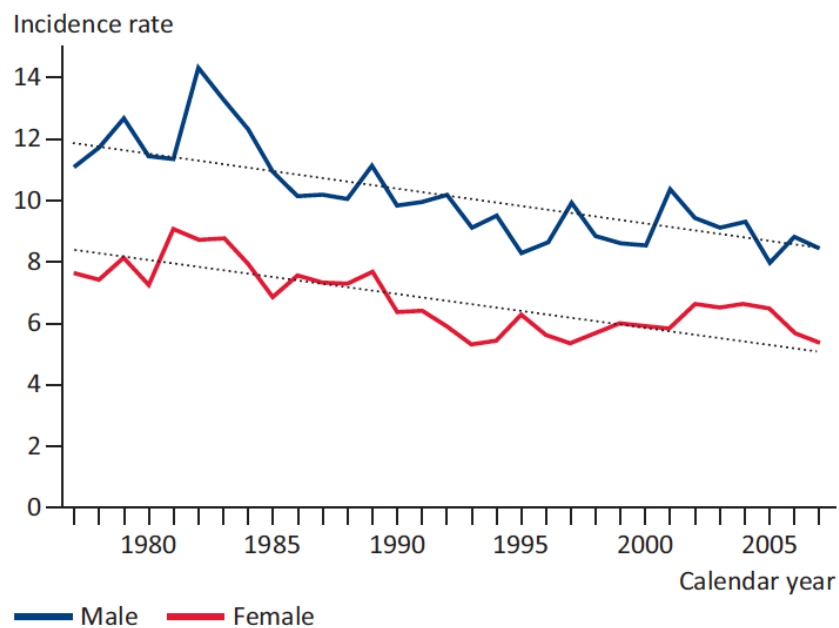


Figure 3-4 The incidence rate of cholesteatoma in Denmark.

The age-specific incidence rate per 100,000 person-years for surgically treated middle ear cholesteatoma in Denmark, calculated as the first occurrence of a cholesteatoma diagnosis combined with relevant surgery in the Danish National Hospital register. The graph was smoothed using the moving 5-year average.

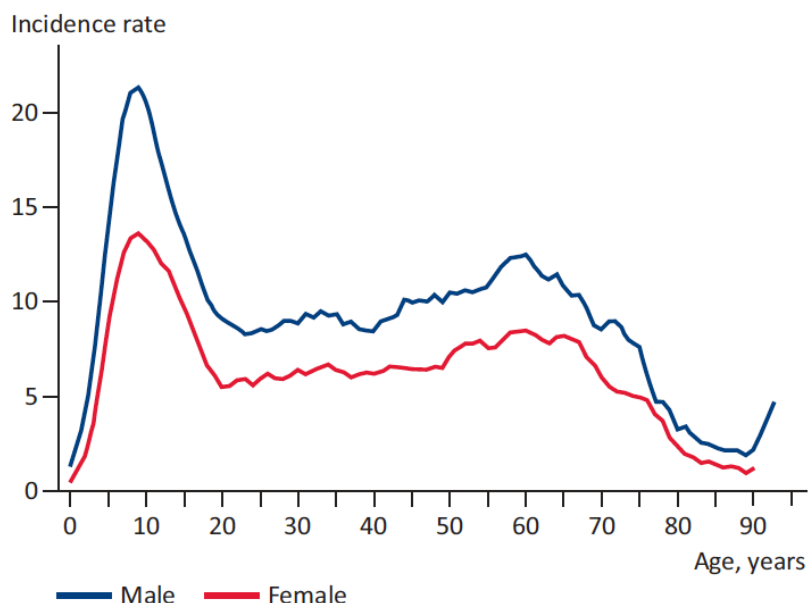


Figure 3-5 Cholesteatoma in Denmark. Age at surgery.

The reported incidence per 100,000 in Scotland of 13.2, Finland of 9.2 and Japan of 6.8 to 10 look broadly similar to the Danish figures. In Germany the incidence of STMEC was 15 per 100,000 in one federal state in 2005 (Fiedler et al., 2013) and the figures presented from Norfolk and from NHS Digital also support this. Maw identified 15/6908 children screened by video-otoscopy as having overt or suspected cholesteatoma that seems a remarkably high number and may reflect a subjective opinion of suspected cholesteatoma on video images (Maw et al., 2011).

Gender

All reports of gender data suggest that the condition is more common in males and this is also the direct observation from the national and local

databases. The English statistic for 2014/15 was 59.4% male and the Norfolk statistic for 2006 to 2016 was 52.7% male.

There is an association with Turner syndrome, which is a condition in which there is no Y chromosome. Reports emphasize the anatomical changes in the skull base associated with Turner syndrome as a possible explanation but a fascinating possibility is that there might also be an X linked disease association to explain the male to female ratio? The author has several patients with Turner syndrome and chronic middle ear disease.

Age of onset

The databases show that the peak age for surgical intervention is in early teenage years but that there is a wide distribution with some patients operated in the 7th and 8th decade of life. The age distribution in Denmark from Djurhuus shown in figure 3-5 closely matches the pattern of the Norfolk patients with a bulge in later middle age.

Childhood otitis media with effusion [OME] and ventilation tube insertion [VTI]

Spilsbury (Spilsbury et al., 2010) reports a huge study from W Australia of 45,980 children who had a VTI between 1980 and 2004 [figure 3-6]. 460 patients went on to develop cholesteatoma, which is almost exactly 1%. Repeated tube insertions increased the likelihood of cholesteatoma although early intervention seemed to reduce it. Adenoidectomy was associated with a 27% reduction in the chance of developing cholesteatoma.

Spilsbury et al. show that the chance of developing a cholesteatoma increases with increasing numbers of VTI operations [with permission]

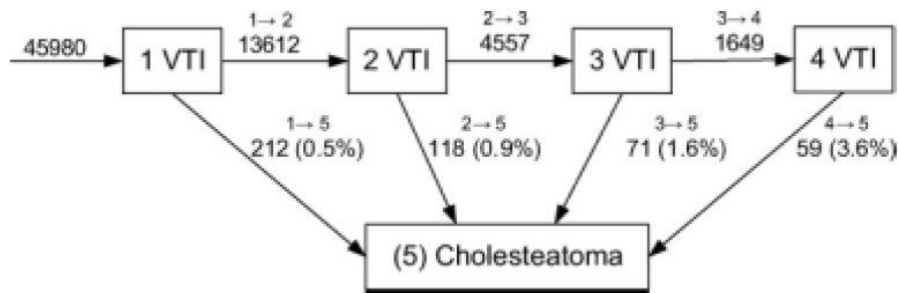


Figure 3-6 Spilsbury et al Western Australia showing that the chance of a cholesteatoma increases with additional VTI operations.

Djurhuus (Djurhuus et al., 2010) studied 217,206 children from the Danish National Patient Register born between 1996 and 2011 who had a VTI and found that 374 of these children went on to have STMEC which is 1.7% of the patients. Later age of first VTI and greater intervals between successive VTI operations increased the chance of STMEC. A matched group of 36,981 children from the Danish National Patient Register without VTI was scrutinized and only five of these people had STMEC [0.013% of the non-VTI sample].

It is therefore clear that there is an association between childhood OME, VTI and cholesteatoma. The question of whether the widespread introduction of VTI for childhood glue ear affected the amount of cholesteatoma surgery has been studied in two similar papers from Scotland and Northern Ireland reporting that the great increase in the use of VTI between the 1960s and the 1980s was associated with no change in the amount of STMEC. Rackover however makes an opposite interpretation of data from Israel in a study that identified 413 operations in the 1961 to 1970 and 228 operations between 1989 and 1998 which represented a fall for 20 per 10,000 population per

decade to 6.6 per 10,000 population. The demographic makeup of the State of Israel changed markedly as a result of mass immigration in the intervening 30 years and this may be relevant.

Social class

A social deprivation effect is explored in the paper by Khalid Raja. The Index of Multiple Deprivation [IMD] deciles in figure 3-7 show that the numbers of operations for cholesteatoma was greater in areas of the country with a higher IMD (Khalid-Raja et al., 2015). IMD ranks 7 domains including income deprivation, employment deprivation and health deprivation and disability. This paper used data from the UK office for national statistics and calculated the number of cholesteatoma operations in each UK local government authority for 4 separate years. The paper from Finland however found no social class effect but there are few details about how social class was estimated in this paper.

A chart showing the number of operations in a health district in relation to the deciles of index of multiple deprivation [from Khalid Raja et al with permission]

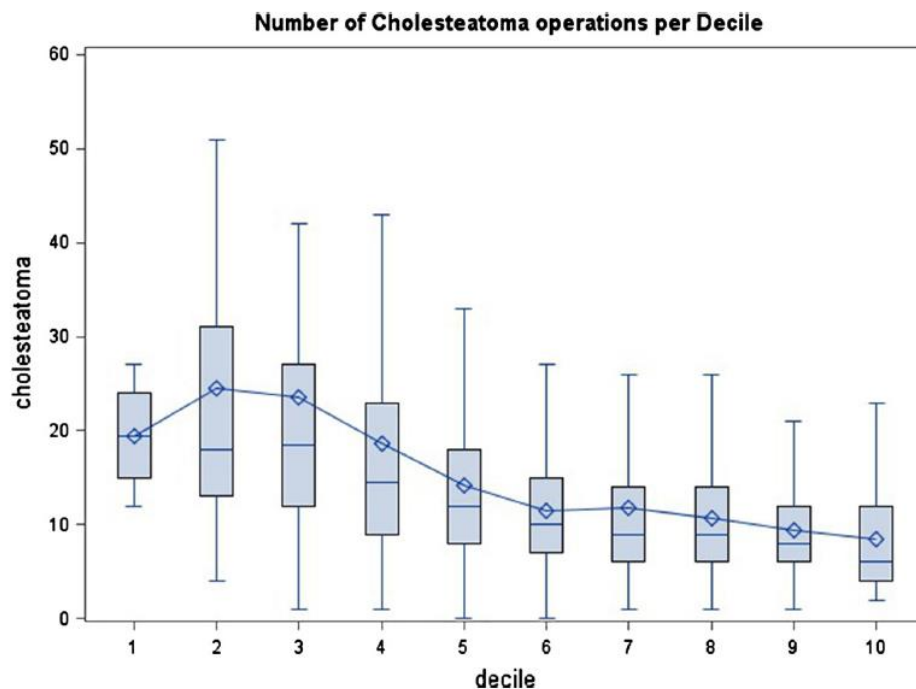


Figure 3-7 Effect of social class. Each decile represents an index of social deprivation.

Cleft lip and palate and other syndromes

There is a much-increased incidence of glue ear in children with CLAP or CP alone. A large study from Toronto over 25 years showed that there was a 200-fold increase in the cholesteatoma rate over baseline in palatoplasty patients [2.2% of 2737 patients]. A Danish whole population study showed a similar rate of 2.34% cholesteatomas in palatoplasty patients. Intriguingly non-CLAP siblings of such patients were found to have a more than doubled risk of cholesteatoma which suggests a genetic factor.

An Italian study of hearing problems in Turner syndrome showed that 15% of 173 patients with Turner syndrome had a cholesteatoma (Bergamaschi et al., 2008a). Turner syndrome, which is caused by the deletion of one of the X chromosomes, is one of the commonest major chromosomal disorders occurring in 50 per 100,000 live female births. Turner syndrome is associated with short stature and various dysmorphic features. The association with chronic otitis media was reported in 1963 in Sweden. The Italian report refers to literature suggesting that anatomical features of Turner Syndrome may predispose to middle ear disease and middle ear ventilation difficulties, although it is unclear if there is a discrete increase in the risk of a cholesteatoma.

Other factors of interest

A curious association of cholesteatoma with habitual sniffing is described in two papers from Japan (Shibata et al., 2015, Hasegawa et al., 2006). The premise is that habitual sucking causes movement of a floppy segment of ear drum leading to chronic retraction and a predisposition to cholesteatoma. Another enigmatic association was the association of handedness with laterality of unilateral cholesteatoma described in a paper by Migirov. Right ears were more often affected in right-handed people and left ears in left handed people.

Osteoporosis

A study from Taiwan using the National Health Statistic service showed that there was a small increase in the incidence of cholesteatoma in patients with a diagnosis of osteoporosis. Some speculation as to the mechanism of bone loss in osteoporosis is included in this paper together with a bizarre

suggestion that otolaryngologists evaluate the middle ear in patients with osteoporosis.

Chart showing the cumulative incidence of cholesteatoma in patients with and without osteoporosis (Wang et al., 2015).

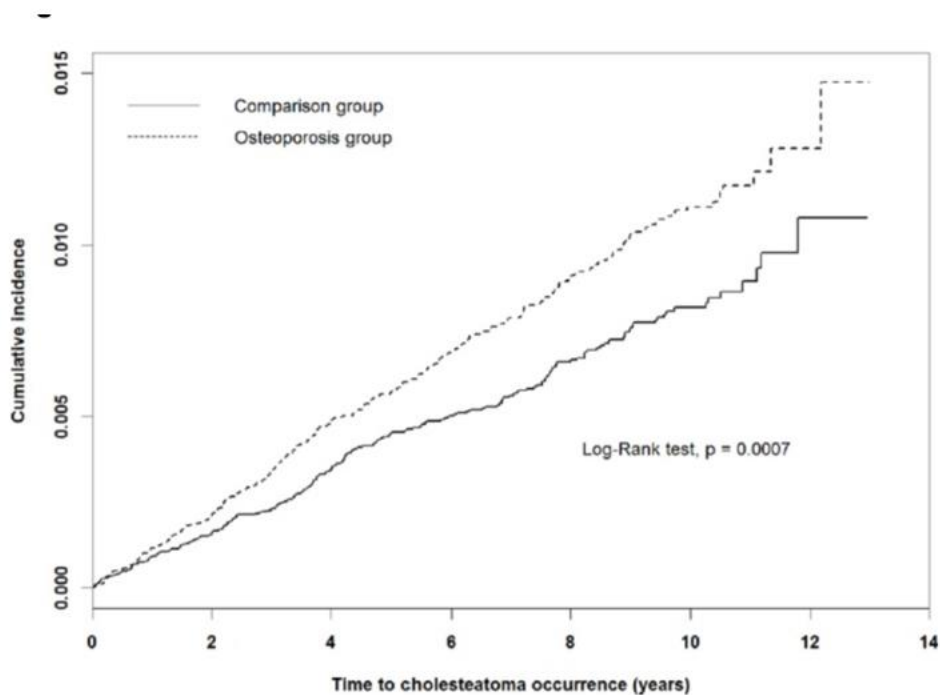


Figure 3-8 Effect of osteoporosis.

Ethnicity

Little useful data about this has been identified. In the Northern Territory of Australia chronic ear infection is common in the aboriginal population. The hospital records from the Royal Darwin Hospital showed that there was however no greater incidence of cholesteatoma in the aboriginal people as compared to the non-indigenous Australians (Mason and Wabnitz, 2002). In

Saudi Arabia a survey of children showed a cholesteatoma incidence in children of about 1 in 1000 and is thus much higher than in other population groups reported above (Zakzouk and Hajjaj, 2002).

A 2004 WHO report about Chronic Suppurative Otitis Media presents data about the incidence of CSOM and although no clear distinction about the diagnosis of cholesteatoma is usefully made in this report the disease does occur worldwide.

Conclusion

Cholesteatoma is an enigmatic condition of unknown aetiology. Epidemiological approaches can identify associations but since this condition is rare conventional epidemiological investigations of pathogenesis or causation are problematic. Cholesteatoma is seen to affect all age groups with a main peak in teenagers and a small male predominance. Sometimes cholesteatoma presents for the first time in old age. The Norfolk patients have a somewhat different sex and age distribution from the UK average in the year 2014/15. Many others around the world have reported series of patients with cholesteatoma and shown that it causes serious morbidity and mortality.

The literature suggests an association with CSOM and with a history of ventilation tube insertion although association and causation are quite different. There are reports of syndrome associations most notably with cleft palate. Some have suggested an association with social class and economic disadvantage but this may simply reflect the greater incidence of CSOM in such populations.

Genetic approaches to the aetiology of cholesteatoma are the subject of this thesis and the literature about this is reviewed in a separate chapter.

Chapter 4 The Genetics of Cholesteatoma Literature Review

Acknowledgement

Norwich Medical School librarian, William Jones, and the inter-library-loans team for assistance with the identification and sourcing of the cited literature, and to Alison Willis and Dietmar Steverding for translations of French and German case reports.

This chapter has been published separately (Jennings et al., 2018) and the author acknowledges the contribution of the principal reviewers, Dr Barbara Jennings and Dr Gavin Willis. The tables which summarize the literature review are incorporated in the appended supplementary publication.

Abstract of chapter 4

Objective

A cholesteatoma is a mass of keratinizing epithelium in the middle ear. It is a rare disorder that is associated with significant morbidity, and its causative risk factors are poorly understood; on a global scale up to a million people are affected by this each year. A systematic literature review to identify reports about the heritability of cholesteatoma or any constitutional genetic factors that may be associated with its aetiology has been conducted.

Data sources

A systematic search of MEDLINE [EBSCO] and 2 databases of curated genetic research [OMIM and Phenopedia] was conducted.

Study selection

The participants and populations of interest for this review were people treated for cholesteatoma and their family members. The studies of interest reported evidence of heritability for the trait, or any association with congenital syndromes and particular genetic variants.

Data extraction

The searches identified 449 unique studies, of which 35 were included in the final narrative synthesis.

Data synthesis

A narrative synthesis was conducted and data were tabulated to record characteristics, including study design, genetic data and author conclusions. In a few case-reports, congenital and acquired cholesteatoma have been shown to segregate within families in the pattern typical of a monogenic or oligogenic disorder with incomplete penetrance. Evidence from syndromic cases suggest that genes controlling ear morphology may be risk factors for cholesteatoma formation.

Conclusion

This systematic review about the genetics of cholesteatoma identified a small body of relevant literature that provides evidence of a heritable component for its aetiology. Cholesteatoma is a complex and heterogeneous clinical phenotype, often associated with chronic otitis media and with some rare congenital syndromes known to affect ear morphology and related pathologies.

Introduction

A cholesteatoma is a self-perpetuating erosive mass of stratified keratinising squamous epithelium in the middle ear (Bhutta et al., 2011). Cholesteatoma has both an acquired and a congenital form. It activates osteoclasts and so will erode through bone, which may include the endocranium, with an attendant risk of life-threatening intracranial infection.

The acquired form of cholesteatoma originates as an inward growth from the lateral epithelium of the tympanic membrane. A typical sequence of events in the onset of the disease includes a history of chronic otitis media [COM] in childhood, subsequent development of retraction of the tympanic membrane, and then a cholesteatoma developing within and perforating through this retraction. This seems to particularly occur if the retraction is located in the superior tympanic membrane [pars flaccida] (Caye-Thomasen et al., 2008, Schilder et al., 1995, Maw and Bawden, 1994). In children with a history of chronic otitis media with effusion [COME], 15-35% will develop a retraction of the pars flaccida [at up to 25 years follow up], but only 0.1-2% will develop a cholesteatoma [at up to 8 years follow up] (Schilder et al., 1995, Tos and Poulsen, 1976, Bonding and Lorenzen, 1974, MacKinnon, 1971). Both presence and duration of COME are predictive of tympanic membrane retraction (Maw and Bawden, 1994, Schilder et al., 1995), but tympanic retraction has been documented to occur in the absence of preceding COME (Schilder et al., 1995). However histological studies suggest that in such cases there is nevertheless chronic middle ear inflammation, it is just not clinically apparent (Yoon et al., 1990). Thus cholesteatoma is predisposed to by COM, but only a small proportion of those with COM will develop cholesteatoma. What determines the transition from COM to cholesteatoma is not known, but could be due to environmental factors, heritable factors, or random effects. But those who develop cholesteatoma

have been reported to have between a 7% and a 20 % chance of developing disease in the contralateral ear (Rosenfeld et al., 1992a, Rosenfeld et al., 1992b, Aquino et al., 2011), highlighting the importance of shared genes and shared environments.

Cholesteatoma can also be found behind an intact tympanic membrane (Kazahaya and Potsic, 2004). This form is thought to be congenital, and may result from persistence of the foetal epidermoid formation, a small collection of squamous epithelial cells in the middle ear that normally undergoes apoptosis before or shortly after birth. Congenital cholesteatoma can grow laterally and erode through the tympanic membrane, and at that point it can be difficult to differentiate congenital from acquired disease.

Cholesteatoma is a rare disorder [1:10,000 per year] (Bhutta et al., 2011), and therefore epidemiological studies are difficult to conduct, and causative risk factors are still poorly understood. The citations about cholesteatoma in the definitive catalogue of genes and genetic diseases, *Online Mendelian Inheritance in Man*, documents minimal evidence for the Mendelian inheritance of this disorder (Graham and Allanson, 1999). However, reports of familial clustering of disease and of association with genetic syndromes [reviewed here] suggest underlying, but as yet unidentified genetic risk factors. Identifying these could enhance our understanding of disease biology, and open up pathways for diagnostic screening, and therapeutic interventions.

One way to identify candidate genetic factors is through analysis of products of gene expression in pathological specimens. There are two published large-scale analyses comparing RNA transcript expression in cholesteatoma to that in skin of the external auditory canal skin. These have shown several hundred genes are differentially regulated in cholesteatoma samples,

including genes with products involved in growth, differentiation, signal transduction, cell communication, protein metabolism, and cytoskeleton formation (Klenke et al., 2012a, Macias et al., 2013a). However, the results from these studies are inconsistent, and are measuring gene expression once cholesteatoma has formed, and so have failed to significantly further our understanding of constitutional risk.

Here described are the findings from a systematic review of the genetics of congenital and acquired cholesteatoma. The aims from this review were to describe how susceptibility is transmitted within families showing disease clustering, to better understand the genetic architecture of disease, and to document any genotypes shown to co-segregate with the cholesteatoma phenotype. It was also the aim of the review to classify genetic syndromes associated with increased risk of cholesteatoma, which may implicate candidate genetic loci for further investigation.

Materials and methods

Objectives

To synthesize published evidence that addresses the following questions:

(1) Can the development of a cholesteatoma be described as a heritable trait, or is there a genetic predisposition to cholesteatoma within some families?

(2) Have any genetic alterations or congenital syndromes been associated with cholesteatoma?

Registration of the method

The protocol was registered with the Prospero international prospective register of systematic reviews database in June 2015 (Jennings, 2015).

Search strategy

The MEDLINE [EBSCO], OMIM (<http://www.ncbi.nlm.nih.gov/omim>), and Public Health genomics Knowledge Base were interrogated (<https://phgkb.cdc.gov/HuGENavigator/startPagePhenoPedia.do>) from 1980 to July 2015 using the terms “Cholesteatoma” AND “famil* [OR Gene* OR hered* OR inherit* OR syndrom* OR kindred OR pedigree OR oncogene* OR tumour suppressor OR tumor suppressor OR epigenetic* OR mutat* OR somatic OR homeobox]”. We supplemented the search with relevant references identified in the citation lists at the article review stage.

Inclusion and exclusion criteria

Studies were identified from the titles and abstracts using the following inclusion criteria:

Primary studies of kindreds that provide information about familial clustering.

Primary epidemiological studies that provide evidence of heritability including ethnic differences.

Relevant systematic reviews that provide information about genetics or heredity for cholesteatoma.

Case Reports that refer to familial clustering of the cholesteatoma phenotype [> 1 family member affected].

Case Reports or epidemiological studies that provide evidence of association between cholesteatoma and syndromes.

Studies were excluded if they were general narrative reviews or opinion pieces; about non-human or experimental disease models; or described pathologies other than cholesteatoma.

Study selection and data extraction

Full reports of potentially relevant articles were retrieved and data were extracted. The study design, patient characteristics, and nature of the outcomes were collated and coded red for exclusion; green for inclusion; and amber to indicate uncertainty ['RAG review']. When there were uncertainties about inclusion or data interpretation, the articles were discussed by the reviewers to reach consensus. All studies that met the

inclusion criteria were included regardless of quality, which was subsequently appraised.

Data synthesis

A narrative synthesis was conducted to explore the review questions about heritability and genetic associations reported for the cholesteatoma phenotype. The date of the study, first author, study design, number of subjects, sub-type of cholesteatoma, genetic investigations [including family history], associated congenital syndromes, gene nomenclature, and direct quotations from discussion or conclusions were tabulated.

Risk bias and quality assessment

Appraisal of the quality of epidemiological studies by reference to the Strengthening Reporting of Observational Studies STROBE guidelines and the Strengthening Reporting of Genetic Association Studies (STREGA) guidelines (Little et al., 2009) was performed. The evidence for each study was mapped to the five levels described by the Oxford Centre for Evidence Based Medicine (*Oxford Centre for Evidence-based Medicine – Levels of Evidence (March 2009)*).

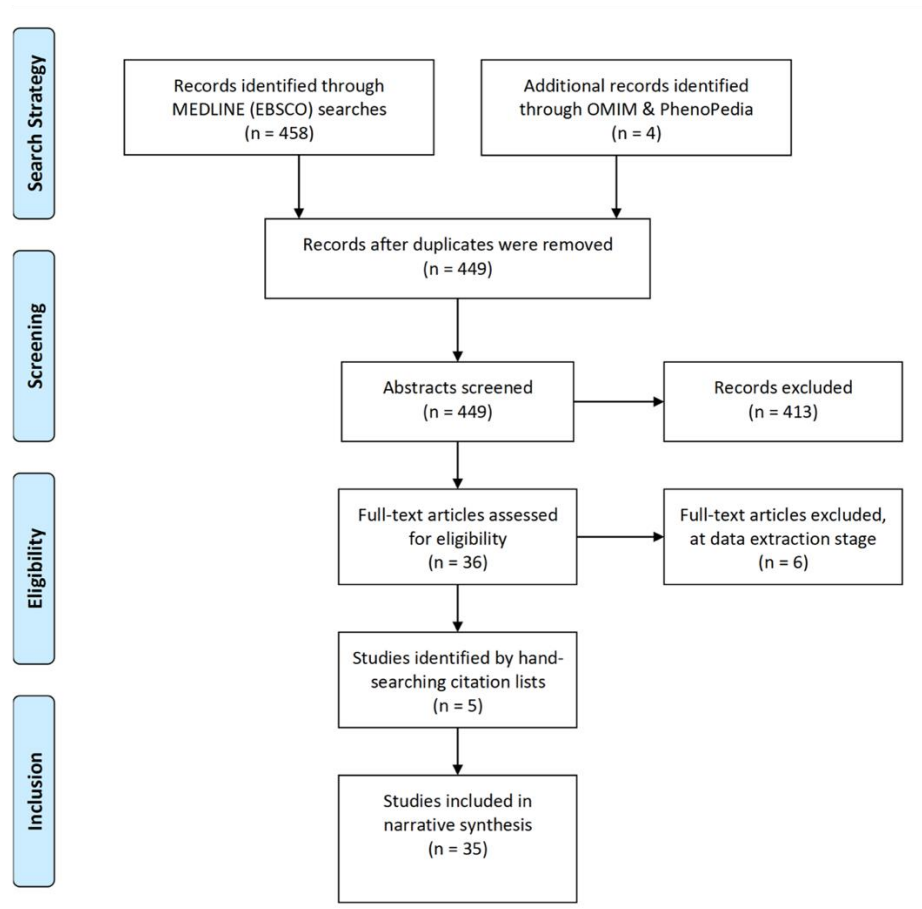


Figure 4-1 Flow chart showing selection of articles for review The identification and screening of studies for inclusion in the narrative synthesis is illustrated in this PRISMA flow diagram.

OMIM: Online Mendelian Inheritance in Man (NCBI)

Phenopedia is an information database curated by the US Center for Disease Control

Results

Study selection and data extraction

The search identified 449 unique studies, of which 36 met the initial inclusion criteria. Most studies were excluded at the abstract or primary manuscript review stage; but six manuscripts were excluded at the data extraction stage because there were no relevant primary data identified about cholesteatoma or genetic phenomena (Serra et al., 2003, Blaser et al., 2006, Plester, 1980, Tange et al., 2000), or because the paper described external auditory canal cholesteatoma (Gadre and Gadre, 1989). The studies identified in the initial search were supplemented by five additional reports identified by hand-searching citation lists (Ray, 1973, Saito et al., 1983, Al Balushi et al., 2013, Naito, 1986, Reber et al., 1987). Thirty-five studies were finally included in this narrative synthesis. Figure 4.1 summarizes these steps.

Familial clustering

Nine studies (classified as case reports, case series, and epidemiological studies) present evidence for familial clustering of cholesteatoma (Ray, 1973, Lipkin et al., 1986, Podoshin et al., 1986a, Naito, 1986, Homøe and Rosborg, 2007, Prinsley, 2009b, Al Balushi et al., 2013, Landegger and Cohen, 2013, Djurhuus et al., 2015b).

The author describes clustering of cholesteatoma in 15 families in East Anglia (Prinsley, 2009b), with more than one generation affected in some families. It seems likely that all or most of these cases were of acquired cholesteatoma, and the vertical pattern of transmission suggests an

autosomal dominant inheritance pattern, with incomplete penetrance. Homøe describes a nuclear family with several members affected by acquired cholesteatoma (Homøe and Rosborg, 2007) and Naito describes dizygotic twins who are both affected; these findings are also consistent with an autosomal dominant inheritance pattern with incomplete penetrance (Naito, 1986). (Landegger and Cohen, 2013, Lipkin et al., 1986, Ray, 1973) all describe parents and siblings within a single nuclear family with congenital cholesteatoma and (Al Balushi et al., 2013) describes monozygotic twins affected by cholesteatoma, suggesting that risk of congenital disease may also be transmitted in an autosomal dominant manner. The identification of affected siblings in a single generation [horizontal transmission] could also be interpreted as a recessive pattern of inheritance (Naito, 1986, Ray, 1973, Al Balushi et al., 2013, Landegger and Cohen, 2013) in some families.

Two epidemiological studies, a cross-sectional study conducted in a Kibbutz (Podoshin et al., 1986a) and a recent Danish cohort study of orofacial cleft (Djurhuus et al., 2015b), present further evidence of a role for heritable factors in the development of cholesteatoma.

No studies reporting familial clustering have undertaken DNA analysis of affected members. The study presented in this thesis is probably the first attempt anywhere in the world to do this.

In addition to these eight studies, there was a cross-sectional survey of cholesteatoma incidence in 5637 patients undergoing treatment for chronic suppurative otitis media (CSOM) in Nepal (Thornton et al., 2011), of whom nearly 20 % had cholesteatomas. One of the study aims was to determine whether ethnicity affects the frequency of cholesteatoma, and surname data were used to categorise participants as Indo-Caucasian (n = 4875) or

Tibeto-Mongolian (n = 765). The authors concluded that ethnicity was not a determinant of cholesteatoma pathogenesis in this population.

Syndromes and cholesteatoma

22 case reports and epidemiological studies describe the occurrence of cholesteatoma in patients affected by congenital and malformation syndromes (Daugbjerg and Everberg, 1984, Graham and Allanson, 1999, Lipkin et al., 1986, Worley et al., 1999, Djurhuus et al., 2015b, Lau et al., 1988, Bacciu et al., 2005, Saito et al., 1983, Suzuki and Ohtani, 2004, Büchner and Itin, 1992, Herrmann et al., 2005, Jin et al., 2010, el-Sayed et al., 1997, Colnaghi et al., 2006, Mann et al., 2014, Bergamaschi et al., 2008b, Hall et al., 2009, Lim et al., 2014, Kornblut et al., 1982, Iino et al., 1987, Vaglio et al., 2008); several of which have a known underlying genetic aetiology.

Some of these reports are of cholesteatoma occurrence in a single case of a particular syndrome, for example Beckwith-Wiedemann syndrome, Granulomatosis with Polyangiitis, Nager syndrome, primary ciliary dyskinesia, Tolosa-Hunt syndrome, Treacher Collins Syndrome, and Wolf-Hirschhorn Syndrome. Single occurrences of a disease, whether associated with a syndrome or not, are susceptible to publication bias and so do not add to understanding of disease risk in isolation. Also Tolosa Hunt syndrome and Granulomatosis with Polyangiitis are not generally regarded as inherited conditions, rather acquired inflammatory conditions of unknown cause.

There are three reports of congenital cholesteatoma in Branchio-oto-renal syndrome, an autosomal dominant disease characterised by malformation of the outer, middle and inner ear, the pharyngeal arches, and sometimes the kidneys. The author has as patients two sisters with this syndrome who each have cholesteatoma.

Case series document an increased risk of acquired cholesteatoma in microtia, a malformation of the pinna that has poorly understood aetiology, but can be associated with other craniofacial malformation. Acquired cholesteatoma is also described in series of patients with Down syndrome [trisomy 21], Turner syndrome [45X] and cleft palate [multiple and complex genetic aetiology]. It should be noted that each of these latter syndromes is also known to be associated with an increased risk of COME (Bhutta, 2013a), and these studies have not evaluated whether the increased risk of cholesteatoma actually relates to the higher incidence of COME in these syndromes which is in itself a risk factor for cholesteatoma, or whether the increased risk is over and above this.

There were three reports of other otological pathology in association with cholesteatoma. One case report describes a 69 year old male diagnosed with a putatively congenital cholesteatoma, co-existent with a vestibular schwannoma in the contralateral ear (Ogungbo et al., 2002). A second case reports bilateral congenital cholesteatoma associated with anomalies of both ossicular chains in a 15 year old male (Suetake et al., 1991). The third is a case control study of radiological anatomy of the cochleovestibular apparatus in 31 patients with congenital or acquired cholesteatoma (Propst et al., 2008), with findings of abnormal vestibular anatomy, including dilated endolymphatic fossa, large vestibular aqueduct, and hypoplastic vestibule.

Candidate genes and variants

Just two published studies of DNA-based laboratory investigations of particular gene sequences considered in association with the cholesteatoma phenotype were identified in the review. One is a case report of a 6 year old boy with a congenital cholesteatoma who was shown to have a deletion in the *APC* tumour suppressor gene (Shaoul et al., 1999). A single case report

is of limited relevance. The other is a candidate-gene association study of polymorphisms of the *GJB2* and *GJB6* loci that encode connexins (James et al., 2010) in a cohort of 98 children undergoing surgery for cholesteatoma. *GJB2* “may be more common” in a minority of children with cholesteatoma and there is a known association with neural deafness. This is much too small a study for useful gene association conclusions to be drawn with essentially negative findings{14/96 children}. Neither study was considered useful in the design of the Genetics of Cholesteatoma study.

Risk of bias and quality assessment

Only a small body of literature that was relevant to questions about a heritable component for cholesteatoma aetiology was identified. Many of the studies provide some indirect evidence only, given that the authors’ objectives were to describe cholesteatoma management or associated environmental factors.

Most of the studies identified in the literature search, and described here, are case reports and so represent the lowest level of evidence. Case reports were automatically categorized as level 5. The remaining observational studies include case series, cross-sectional surveys, case-control studies and cohort studies; each of these manuscripts was reviewed to define the level of evidence presented; STROBE and STREGA guidelines were referred to in classifying the quality of the methodology used in the case-control and cohort studies. The level of evidence ranged from 4 [for low quality case control studies, surveys and case series] and 2b for a high quality cohort study (Djurhuus et al., 2015b, Lim et al., 2014) [see tables in the published article which is in the Appendix]

Discussion

This is a systematic review to explore the constitutional genetics of individuals affected by cholesteatoma. Data from 35 published studies about familial aggregation of disease; its association with congenital syndromes; and genes that were directly analysed in patients with cholesteatoma has been synthesised.

Heritability

The published evidence about the heritability of acquired and congenital cholesteatoma is summarised. Although there are few case reports and case series that show two or more affected first degree relatives, there are nevertheless some compelling individual observations, including affected monogenic (Al Balushi et al., 2013) and digenic twins (Naito, 1986, Prinsley, 2009b), a vertical pattern of inheritance within families (Lipkin et al., 1986, Homøe and Rosborg, 2007, Prinsley, 2009b), and high rates of bilateral disease in affected families (Naito, 1986, Prinsley, 2009b). It is possible that for such families cholesteatoma arises because of causative alleles of major phenotypic effect.

To explore any genetic architecture for sporadic cholesteatoma cases, genome-wide-complex-trait-analysis could be used, but large cohorts of study participants would be needed to provide sufficient power to identify candidate alleles (Yang et al., 2011). As outlined in the introduction, acquired cholesteatoma often arises against a background persistent mucosal inflammation after a period of chronic otitis media with effusion (COME), and therefore any genetic study for inherited risk factors might be expected to identify markers already associated with COME pathophysiology. In childhood, COME has an estimated heritability of 71%

(Casselbrant et al., 1999) and Bhutta *et al.* have previously reviewed candidate genetic variants that may be risk factors for that condition (Bhutta, 2013b).

Observations about the familial aggregation of phenotypes are often followed by more discriminating epidemiological methods to distinguish the influence of heritability from shared environments. Because cholesteatoma is so rare, a classical twin-study has not been conducted and is not feasible. However, a study or register to collect data about the incidence of bilateral disease in an individual in comparison to the general disease incidence and the coincidence of disease in dizygotic twins might be able to provide information about a genetic component to the aetiology of cholesteatoma in a manner analogous to studies of monozygotic versus dizygotic twins.

Congenital syndromes

Several lines of evidence suggest that variants in genes regulating ear embryogenesis and tissue architecture also increase the risk of cholesteatoma. For example congenital cholesteatoma is more common in the malformed ears of people with branchio-oto-renal syndrome. The most common cause of this syndrome is a mutation in the gene *EYA1*, which is thought to play a role in transcriptional regulation during embryogenesis. Acquired cholesteatoma occurs more often in individuals with microtia. Radiological evidence of vestibular malformation has been described in some of those with congenital or acquired cholesteatoma. Hence, some of the genes regulating ear formation may also be candidates for association with non-syndromic congenital or acquired cholesteatoma.

Two studies independently discussed associations for cholesteatoma with Focal Dermal Hypoplasia [FDH] (Büchner and Itin, 1992, Reber et al., 1987),

which is also multisystem disorder that can be associated with facial asymmetry and dysmorphology. Mutations in the X-linked, *PORCN* gene have recently been identified as causative genetic variants for FDH; the *PORCN* locus encodes a regulator of Wnt cell-signalling (Grzeschik et al., 2007).

The association of acquired cholesteatoma with Down Syndrome, Turner Syndrome and cleft palate is more difficult to disentangle, as these syndromes also place individuals at increased risk of COME, which often precedes development of cholesteatoma. Whether these syndromes are in themselves associated with increased risk of cholesteatoma is difficult to say. In contrast, Djurhuus *et al.* (Djurhuus et al., 2015b) showed a doubling of risk of cholesteatoma in siblings of patients with cleft palate. This finding should nevertheless be taken with some caution, the associated p-value was 0.026, which would be considered insignificant if it had been subject to Bonferroni adjustment due to the multiple hypothesis testing present in this study. A sub clinical effect on muscular activity around the Eustachian tube was the suggested explanation in the siblings.

Gene associations

Little evidence is presented in the literature about the role of particular genes in cholesteatoma biology because only two studies reported the analysis of gene sequences: a case report and a small candidate gene association study. The case report describes a 6 year-old boy affected by familial adenomatous polyposis who had cholesteatoma, and an inherited deletion in the tumour suppressor gene *APC*. The *APC* protein is expressed in many tissue types; influencing cell migration, adhesion and morphogenesis. Loss of *APC* expression in the colonic epithelium leads to an imbalance of cell growth over cell death (Kinzler and Vogelstein, 1996),

but whether this is relevant to cholesteatoma biology is not known (Shaoul et al., 1999). The second study was a candidate-gene association study of 98 children with cholesteatoma for variants in the connexin gap-junction encoding genes, *GJB2* and *GJB6* (James et al., 2010); some mutations of these loci are known to lead to recessive congenital deafness. Although the authors suggest a high frequency for some *GJB2* gene variants associated with cholesteatoma, no conclusions can be safely drawn from this study, because it lacked a control population and had a small sample size, placing it at risk of false discovery.

Limitations

Non-English manuscripts and studies published before 1980 were excluded from the initial search [the earlier and/or non-English articles were subsequently included in the narrative synthesis because they were identified by hand searching citation lists]; it is therefore possible that relevant publications have been omitted.

The over-representation of case reports, case series and historical epidemiological studies is unsurprising given that cholesteatoma is a rare disease, but such studies provide low-level evidence in the research hierarchy because they are usually retrospective with incomplete data collection or follow up, and are subject to author bias, ascertainment bias and publication bias. In addition such findings may not be generalizable, and should be interpreted with caution, particularly with respect to theories about the underlying aetiology of cholesteatoma.

Conclusion

Cholesteatoma is a complex and heterogeneous clinical phenotype. In a handful of case-reports or case-series, congenital and acquired cholesteatoma has been shown to segregate within families in the pattern typical of a monogenic or oligogenic disorder with incomplete penetrance. The liability threshold for the observed cholesteatoma phenotype could therefore depend on a combination of environmental and genetic factors of variable penetrance. Evidence from syndromic cases suggest that genes controlling ear morphology may be risk factors for congenital or acquired cholesteatoma formation.

Chapter 5 The Genetics of Cholesteatoma: Experimental Methods, Results and Discussion

Summary of contents

This chapter describes the method by which families with several affected individuals were recruited to the pilot study. The method of collecting and storing the family history and drawing the family trees is explained. The method for extracting DNA from blood samples at the molecular genetics laboratory is described together with the sequencing method used at the Earlham Institute. The bioinformatics strategy used to identify genetic variants of interest is described.

The pedigrees of the recruited families are described and the results of the pilot sequencing and bioinformatics experiments are presented and discussed.

Introduction

The human genome and its variants

The human genome consists of 3 billion or so nucleotides that make up the DNA in all 46 chromosomes. Exons [derived from EXpressed RegiON] make up only about 1.5% of genomic DNA which are expressed as amino acid sequences to form proteins. The exome is the name given to all of the exons. Introns refer to the large sections of DNA base sequences that lie between the exons.

Each gene has a unique internationally agreed identifier consisting of letters and numbers. HUGO or the Human Genome Organization Nomenclature Committee is responsible for this system. : <https://varnomen.hgvs.org/>

An rs number for Reference Single Nucleotide Polymorphism [SNP] is a unique number applied to each SNP. dbSNP is a resource which acts as a single database to identify genetic variation.

GeneCards®The Human Gene Database is a free to use comprehensive website that provides information on human genes by integrating genomic and clinical information and can be used to interrogate the genetic variants that are revealed: <https://www.genecards.org/>

Essentially three features of code variation are sought from analysis of the DNA sequence.

1. Variants that have a known impact on gene expression such as:

- A duplication called a short tandem repeat [STR]. If these occur in regulatory or coding regions they can have a large effect but if they occur in intergenic regions they are less likely to important.
- A deletion or a change of a DNA base at a single location [in/dels]. If this occurs in a coding region the whole sequence may be shifted known as a frame shift. This can have a big effect on the amino acid sequence.
- Copy number variants occur if there is a duplication or loss of an entire section of DNA or even a chromosome[trisomy] Exon sequencing does not detect this and is not relevant to this study.

- Complex structural chromosome variants with loss or translocation of large sections of DNA potentially causing large phenotype effects.
- Single nucleotide variants [SNV]. These are the commonest variants. This is change of a single base and the effect will depend on whether there is an effect on an amino acid. If it is a common variation it is called a single nucleotide polymorphism. Because the code contains 64 possible codons for only 21 amino acids there is redundancy in the code and many SNV have no effect on the amino acid sequence.

2. Variants that are observed to co segregate with the phenotype are of interest. That is to say they are present in the affected individuals and absent in the unaffected relatives within a family. Non penetrance is a confounding factor which means that the gene variant may be present but is apparently not expressed in the individual. Variants that co segregate with the phenotype across several families are of particular interest.

3. Variants that are rare in the general population studied are variants of interest. For example if 50 % of a population would be expected to exhibit the variation that is not of interest but if <1 % of the population would be expected to have this variant then that is very interesting.

Genetic mapping

Before whole genome and whole exome sequencing was feasible at scale, the mapping and elucidation of pathogenic disease variants was a painstaking process.

Traditionally identification of a disease causing mutation started with linkage analysis in a sufficient number of families. Linkage to a particular chromosome involves identification of DNA polymorphic markers with known positions on genetic maps and observations about associations with the disease. Such markers may, for example, be single nucleotide polymorphisms [SNP] or clusters of known repeat sequences called variable number tandem repeats [VNTR]. This is then further narrowed with finer more densely packed markers to narrow the region of the chromosome under investigation. The closer the mutation to the marker the greater the chance of them appearing together. Markers such as SNP are detected by hybridization to microarrays and since the completion of the Human Genome Project and the production of reference sequence maps it has been possible to directly interrogate the candidate region.

Other methods of gene mapping include functional cloning if the nature of the molecular protein change is known and candidate gene approaches which use knowledge of the function of previously isolated genes.

In families with evidence that a trait is inherited as a monogenic or oligogenic disorder, Next-Generation-Sequencing (NGS) studies (Ott et al., 2015) can now be used to identify functionally significant genetic variants that co-segregate with the phenotype. An example from ENT surgery of the successful use of this technique is described in a study using NGS of patients

with congenital anosmia by Alkelai et al in which WES of 8 multiply affected families is described and in which 548 rare segregating variants were identified (Alkelai et al., 2017). NGS studies of rare familial phenotypes often reveal private [kindred-specific] mutations, but such findings, derived from a small number of individuals within a single family, can also be generalizable if they identify the genes and biological pathways that are also perturbed in other cholesteatoma patients.

Whole genome sequencing WGS compared to whole exome sequencing WES

WGS	WES
Entire 3 billion base pair genome is sequenced; includes regulatory elements and introns	Only 30 million bases of the genome sequenced but the exome codes all the proteins. Cheaper and quicker laboratory and bioinformatic steps
All variants detected including large structural changes missed by WES	Good for rapid detection of important snv and in/dels
Costly data storage and sequencing	Cheaper
Could identify variants outside the exome	Most pathological variants for genetic disease are snv or in/dels that lie in the coding regions
Typically 5 million variants to the reference genome	Typically 30,000 variants to the reference genome. Only a few hundred are rare and may or may not have importance

Table 5-1 WGS v WES

Whilst whole genome sequencing is the most comprehensive method of gene analysis it is also the most expensive, especially in relation to sequencing costs and data storage, and since most pathogenic mutations are thought to occur either within exons or near to exons WES was considered an appropriate strategy to use in the Genetics of Cholesteatoma project. Changes in protein expression regulating ear keratinocytes are thought likely to be revealed by WES strategy with a relatively small chance of missing significant mutations.

Study objectives

To identify genetic pathways predisposing to cholesteatoma.

1 The first goal was to establish a database of multiply-affected families; to record their family histories [for otology and genetics]; and to collect biological samples from participants for DNA extraction and storage in a biobank.

2 The second goal was to undertake whole exome sequencing of affected and unaffected individuals in the recruited families and to complete bioinformatics studies to identify candidate genetic variants that co-segregate with cholesteatoma.

Materials and Methods

Study Population

A simple enquiry about a family history in a patient presenting with a cholesteatoma has been the means by which the author has created a database of 38 families with 2 or more members in whom a diagnosis of cholesteatoma has been made over a period of 22 years of otology practice at 2 hospitals in Norfolk UK. The diagnosis of cholesteatoma was based on the author's personal clinical records in many cases and also by inspection of the hospital case records.

This formed the population group studied in the first pilot sequencing experiments described in this thesis. The British Society of Otolaryngology, which is a network of otologists working in the UK, and other informal personal networks have been used to recruit other promising families of affected patients throughout the country for future inclusion. The study is adopted by the UK National Institute for Health Research which facilitates and supports medical research in multiple recruitment sites.

Families who have been identified were contacted by letter or telephone and enrolled and consented by face to face contact. Arrangements were made to see patients and their families either in their own homes or at the local hospital research facility/ENT clinic as appropriate and agreed.

Eligibility for recruitment to the study

Inclusion criteria

1 Patients with at least one ear affected ear by cholesteatoma and who have a family history of cholesteatoma.

2 Families of patients in which there are one or more other affected individuals who agree to participate in the study by donating a biological sample for DNA extraction.

Exclusion criteria

1 Only one affected individual with a confirmed case of cholesteatoma in the family.

2 Families unwilling to consent to study participation.

Consent and participant information

The study was explained in detail and informed consent obtained. The participant personally signed and dated the informed consent form which was countersigned by a delegated member of the research team. The participants were presented with age appropriate information sheets explaining the rationale for the study, the constraints of the protocol and the safeguards. It was made clear to the participants that they were free to decline or withdraw at any time without explanation and without prejudice to their medical care. Copies of the signed consent and assent forms were retained by the participants and by the research team.

See appendix for consent /assent forms / patient information sheets.

Participant Numbers for Database

The numbering system to track participants incorporated the hospital site; & then the kindred/family; and then the individual within the family.

The recruiting research nurse/clinician allocated these numbers.

These **site/family/individual** codes were added to the **Sample Number** field at the bottom of the DNA extraction request form.

Family History

A family history was collected from the participant. The primary phenotype of interest was the diagnosis of cholesteatoma. Secondary phenotypes of interest were other otology disease including chronic otitis media; and

diagnosis of genetic disease or congenital disorders for example Down syndrome.

For each index case/relative in the 3 or 4 generation kindred the following data on a proforma was collected: relationship to index case; name; date of birth; alive or dead (and date or year of death if applicable); surgical treatment for cholesteatoma, age at diagnosis and age at time of surgery if applicable, with dates of all surgical treatments unilateral or bilateral disease, indicating which ear/s affected; secondary otology & genetic phenotypes of interest.

Sketches of family history or formal pedigree diagram were recorded and the Phenotips software tool was used to create family tree diagrams. (<https://phenotips.org/> (Girdea et al., 2013))

See appendix for the phenotype proforma, DNA extraction form and postal instructions.

The computer data base recording the recruits and the code is securely held in the clinical research office in the hospital and can only be accessed by members of the research team.

Biological samples and DNA extraction

Blood samples from participants were collected in 3ml EDTA tubes by the research clinician and DNA extraction completed using the QIAamp DNA Blood Mini Kit [Qiagen, UK]. All biological samples (blood and/or DNA) are stored in a biobank by the Department of Molecular Genetics at the Norfolk and Norwich University Hospital.

Whole Exome Sequencing

Library preparation, target capture and sequencing method used for the pilot study

The library construction and subsequent captures were performed using an amended v5.1 protocol from NimbleGen [NimbleGen 2015]. 1µg of DNA was sheared to 300bp using a Covaris LE220 and libraries were constructed on a PerkinElmer silicone automation platform using KAPA HTP DNA library preparation kit and a bead based size selection step. 5 cycles of PCR were carried out and the 12 samples were combined equimolarly into 4-plex pools at a concentration of 1.2µg.

The hybridisations were set up using NimbleGen SeqCap EZ_ Human Exome V3.0 bait set [Design Name: 110823_HG19_Bex_L2R_D03_EZ_HX1]. This bait set was designed to target selected regions using annotations as in using the human genome reference sequence [version GRCh37/hg19].

Each pool of DNA libraries was hybridized at 47°C for 72 hours in a verti PCR machine with a lid heated to 57°C. The reaction was optimized and the amount of universal blocking oligos was elevated from 1µl to 2.4µl, 12µl Cot-1 DNA was added and finally 1.2µl of pooled blocking oligos were used. The pull down and washes were performed on the bench and the captured DNA received a final 9 cycles of PCR. The resulting libraries were tested using the Agilent BioAnalyser to check the quality or by a qubit fluorometer or q-PCR to check the quantity. Each of the 4-plex hybridization pools were then pooled together to form a 16-plex pool for WES on an Illumina HiSeq4000 platform with a 75bp paired end read metric.

Next-generation sequencing, library construction and production of files for bioinformatics analysis was delivered for the pilot experiments by the BBSRC

National Capability in Genomics [BB/J010375/1] at Earlham Institute by members of the Genomics Pipelines Group.

Bioinformatics

Variant calling, annotating, and filtering, used a bespoke pipeline or in other words a specially designed software programme designed to identify variants of interest.

The WES (whole exome sequencing) computer generated readings were aligned to the latest human reference genome sequence. This uses an alignment algorithm with the variants being identified using software called FreeBayes [a genetic variant detector]. This software uses Bayesian theory to make predictions of probability based on incomplete data. *VCF-tools* were used to intersect the genotype files to identify common mutations between the family groups. Finally functional information about the identified variants is available from a VEP (variant effect predictor) software programme. (McLaren et al., 2016)

Variants that segregate with cholesteatoma across sequenced trios or sub-groups [within each family] were determined using VCF-isec software (Danecek et al., 2011). These variants were stratified by their minor allele frequency and compared with data from the Exome Aggregation Consortium (Lek et al., 2016) in order to exclude common variations that likely to be non-pathogenic. Candidate variant lists were compiled for further analysis with reference to genes and gene families identified in our systematic literature review (Jennings et al., 2017).

The bioinformatics analysis of the VCF files was completed by Dr Dan Swan, formerly head of the Genomics Pipelines Group at the Earlham Institute,

Norwich; and currently Bioinformatics Delivery Manager at NCIMB, Aberdeen.

Ethics & Research Governance

The study was granted ethical approval by East of England Cambridge Research Ethic Committee REC 16/EE/01311 after application using the Integrated Research Application System IRAS ID 186786.

See appendix

All members of the research team are trained in Good Clinical Practice NIHR CRN and are familiar with standard research governance protocols.

Flow chart illustrating outline of study protocol

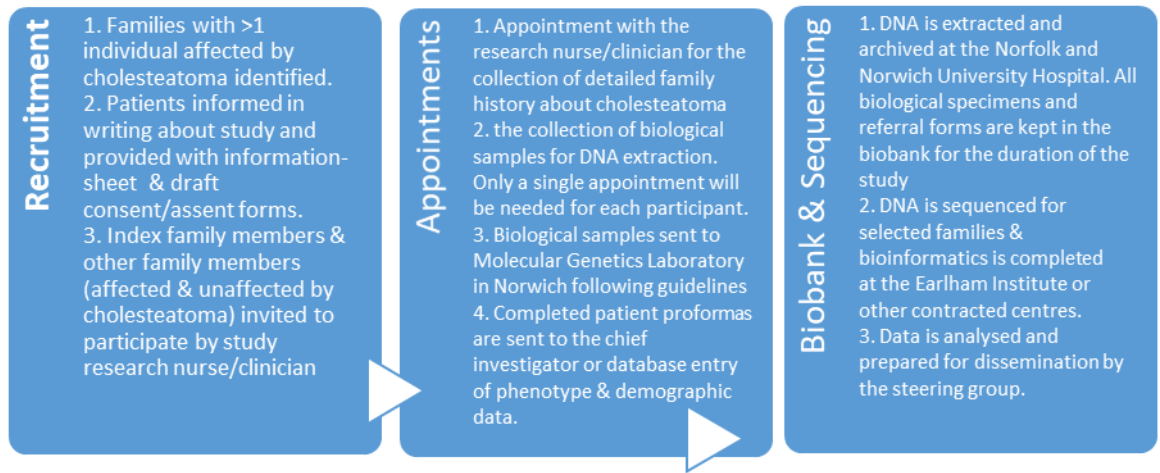


Figure 5-1 flow chart for GoC protocol

RESULTS OF THE PILOT STUDY

In phase 1 of the pilot study a biobank with linked database has been established. A whole exome sequencing strategy and bioinformatics pipeline have been developed to investigate the genetic architecture of familial cholesteatoma. Preliminary filtering has identified candidate variants that could have an impact on the disease process.

Recruited individuals and families

66 individuals from 13 East Anglian families have been recruited to the Genetics of Cholesteatoma study between November 2016 and April 2018. 31 are definitely affected with cholesteatoma mostly operated by the author.

The table shows the recruited individuals and families. Each colour represents a recruited family.

Index/relationship	Study no	Uni, Bilateral, N/A
Index case	GY0101	Unilateral
Sister	GY0102	Bilateral
Sister	GY0103	N/A
Brother	GY0104	N/A
Husband	GY0105	N/A
Neice	GY0106	Bilateral
Sister-in-law	GY0107	N/A
Son	GY0108	Unilateral
Index case	GY0201	Bilateral
Wife	GY0202	N/A
Sister	GY0203	N/A
Brother	GY0204	Unilateral

Brother	GY0205	Bilateral
Son	GY0206	Bilateral
Index case	GY0301	Bilateral
Brother	GY0302	N/A
Brother	GY0303	Bilateral
Mother	GY0304	N/A
Father	GY0305	N/A
Brother	GY0306	Unilateral
Brother	GY0307	N/A
Index case	NN0401	Bilateral
Sister (twin)	NN0402	Unilateral
Brother	NN0403	Unilateral
Mother	GY0404	N/A
Father	GY0405	N/A
Index case	GY0501	Unilateral
Daughter	GY0502	Unilateral
Father	GY0503	N/A
Husband	GY0504	N/A
Sister	GY0505	Bilateral
Index case	GY0601	Bilateral
Wife	GY0602	N/A
Daughter	GY0603	Unilateral
Daughter	GY0604	N/A
Daughter	GY0605	N/A
Index case	GY0701	Unilateral
Husband	GY0702	N/A
Daughter	GY0703	Unilateral
Index case	GY0801	Bilateral
Daughter	GY0802	N/A
Brother	GY0803	Unilateral
Wife	GY0804	N/A
Index case	GY0901	Bilateral

Husband	GY0902	N/A
Daughter	GY0903	N/A
Son	GY0904	Unilateral
daughter	GY0905	N/A
Index case	GY1001	Unilateral
Mother	GY1002	N/A
Auntie/Uncle	GY1003	Unilateral
Grandmother	GY1004	N/A
Index pt	NN5001	Bilateral
Patients mother	NN5002	N/A
Index pt	NN5101	Unilateral
Daughter	NN5102	N/A
Son	NN5103	N/A
Daughter	NN5104	Unilateral
Father	NN5105	N/A
Mother	NN5106	N/A
Cousin	NN5107	N/A
Index pt	NN5201	Bilateral
Twin	NN5202	Unilateral
Mother	NN5203	N/A
Sister	NN5204	N/A
Sister	NN5205	N/A
Sister	NN5206	N/A

Table 5-2 List of recruits to the Genetics of Cholesteatoma Study

The pedigree diagrams illustrate the recruits

The shaded symbols represent individuals affected with cholesteatoma and have all been treated surgically. The arrow is the index case. The asterisk represents an individual who has been recruited to the study. Only one individual affected with cholesteatoma from family GY02 declined recruitment.

The history of mastoid surgery for cholesteatoma is included with the age at operation if known. The disease is recorded as unilateral or bilateral.

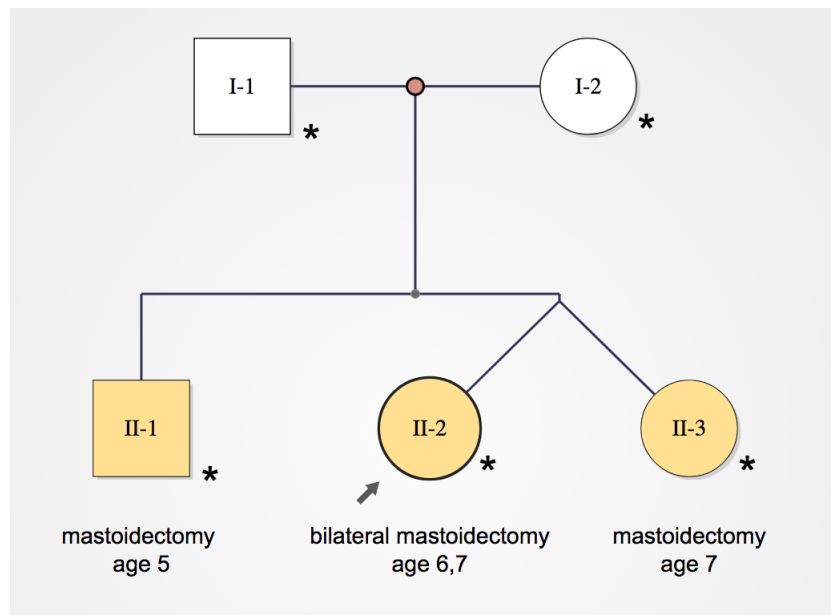


Figure 5-2 Family NN04. Almost certainly a dominant trait. All 3 children are affected and 1 of the identical twins has bilateral disease. This family has been subjected to detailed bioinformatics since all of the individuals are recruited to the study.

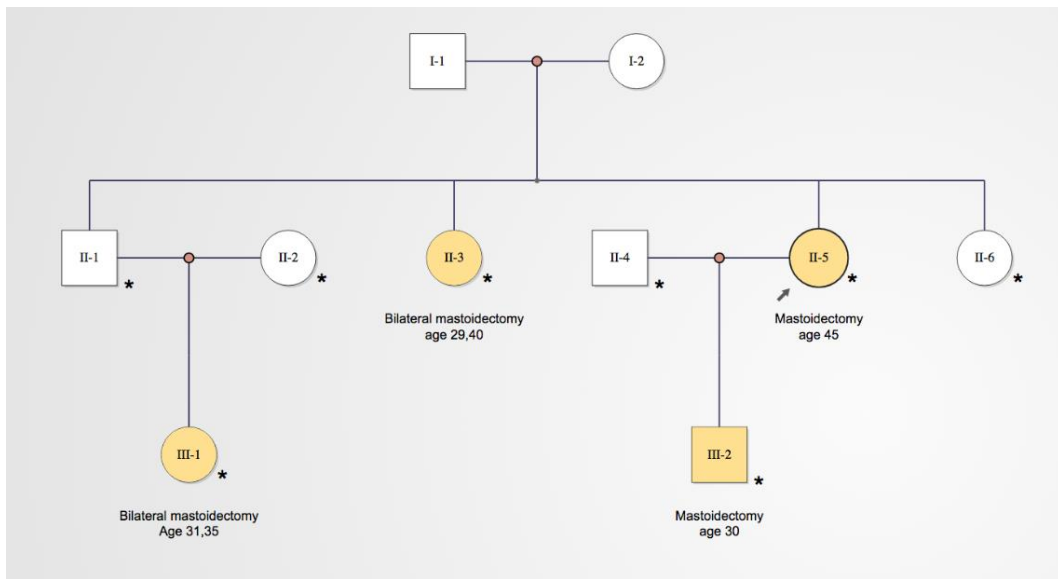


Figure 5-3 Family GY01. There are 4 affected individuals with 2 bilateral cases and an affected niece.

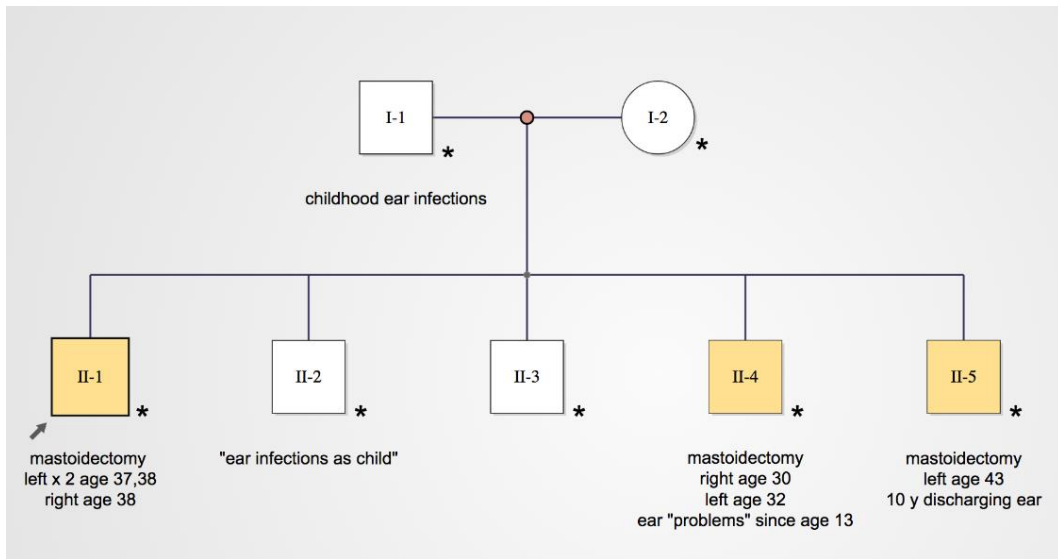


Figure 5-4 Family GY03. There are 3 affected brothers, 2 of whom have bilateral disease.

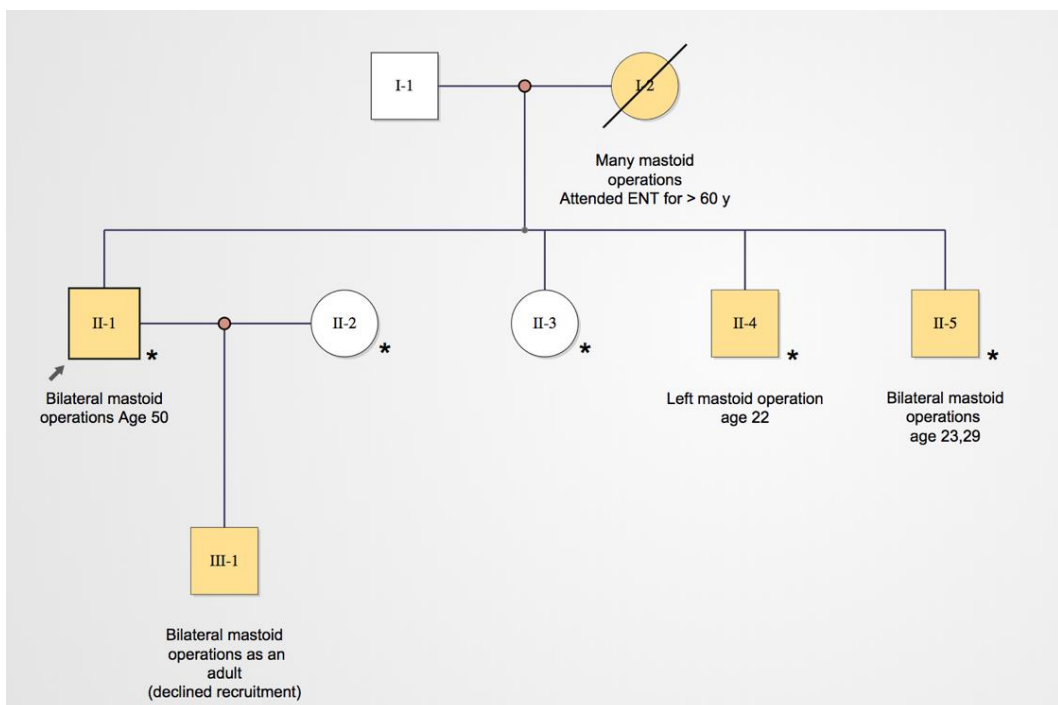


Figure 5-5 Family GY02. Here are 3 generations with several bilateral cases. Sadly case I-2 is deceased.

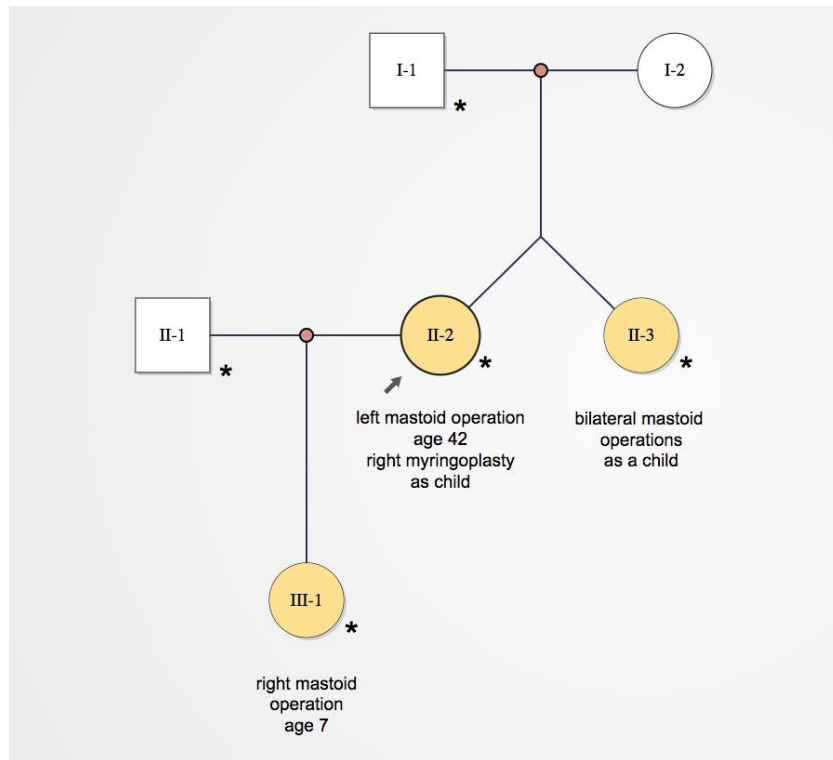


Figure 5-6 Family GY05. Twins with bilateral ear disease and 1 affected daughter.

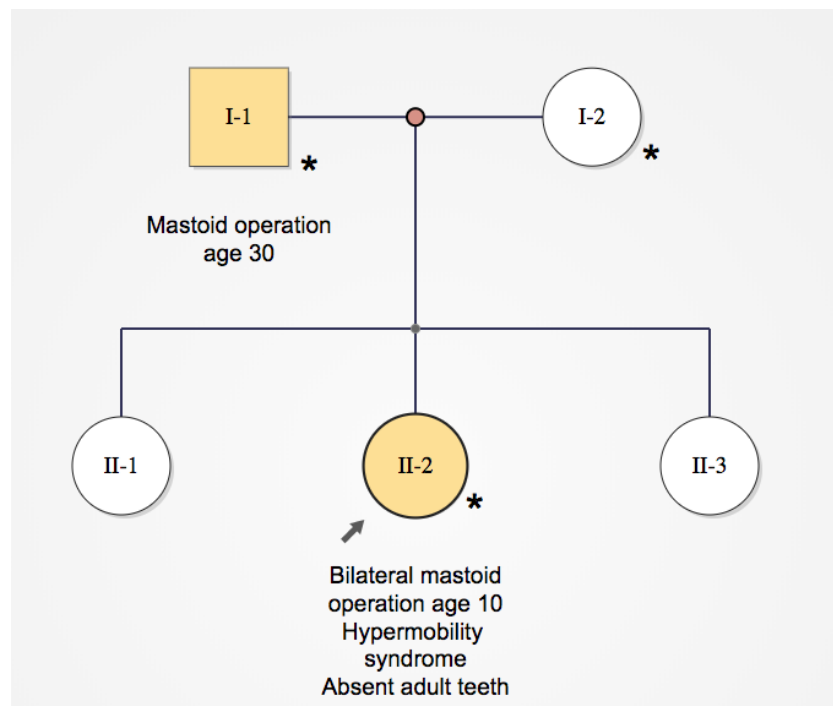


Figure 5-7 Family GY06. Case II-2 has unusual inherited traits.

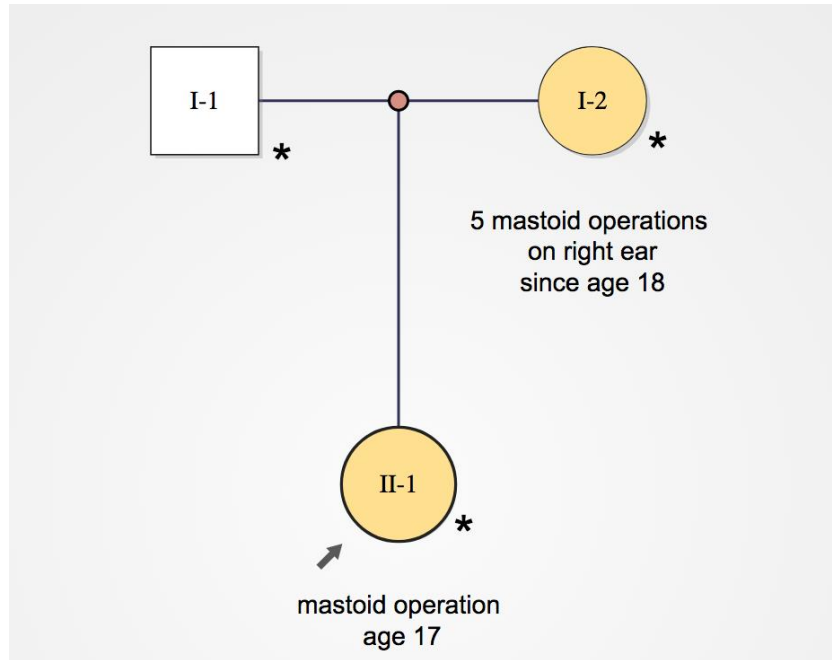


Figure 5-8 Family GY07. All 3 members of this pedigree are recruited. The mother has recidivistic disease.

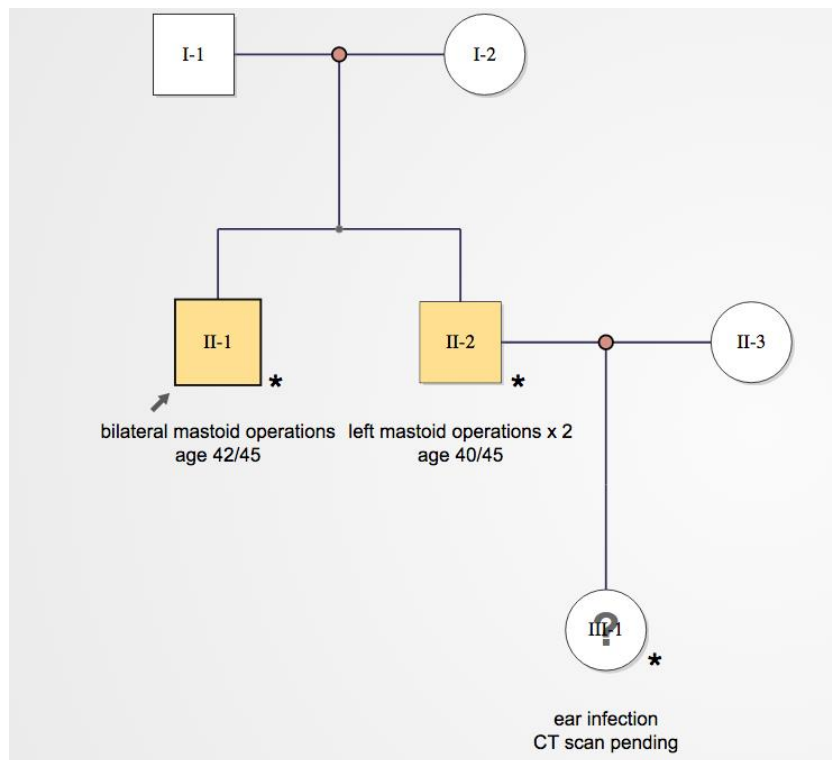


Figure 5-9 Family GY08. Case 111-1 looks to be at high genetic risk of cholesteatoma.

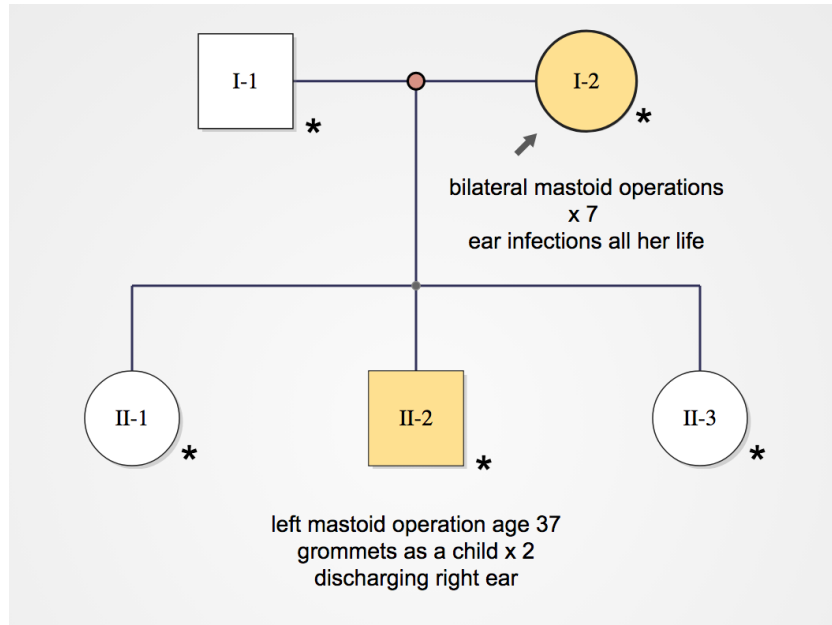


Figure 5-10 Family GY09 All 5 members of this family are recruited to the study.

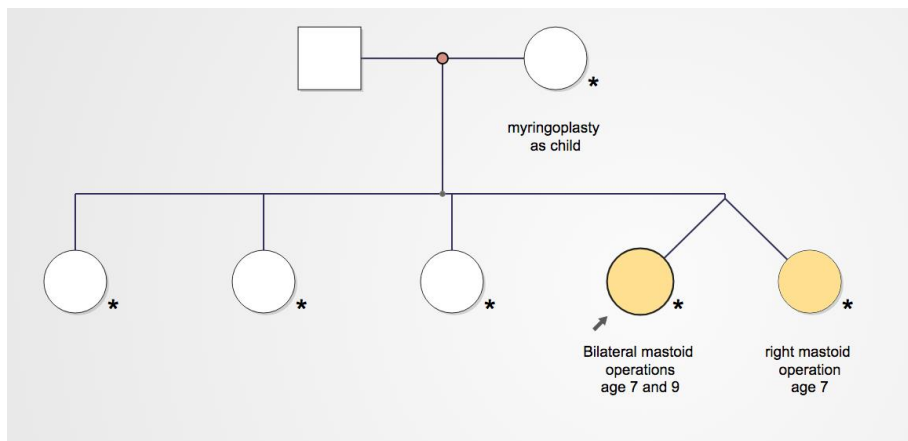


Figure 5-11 Family NN52. Identical twins only distinguished by the fact that one of them has bilateral disease. The mother had ear surgery as a child.

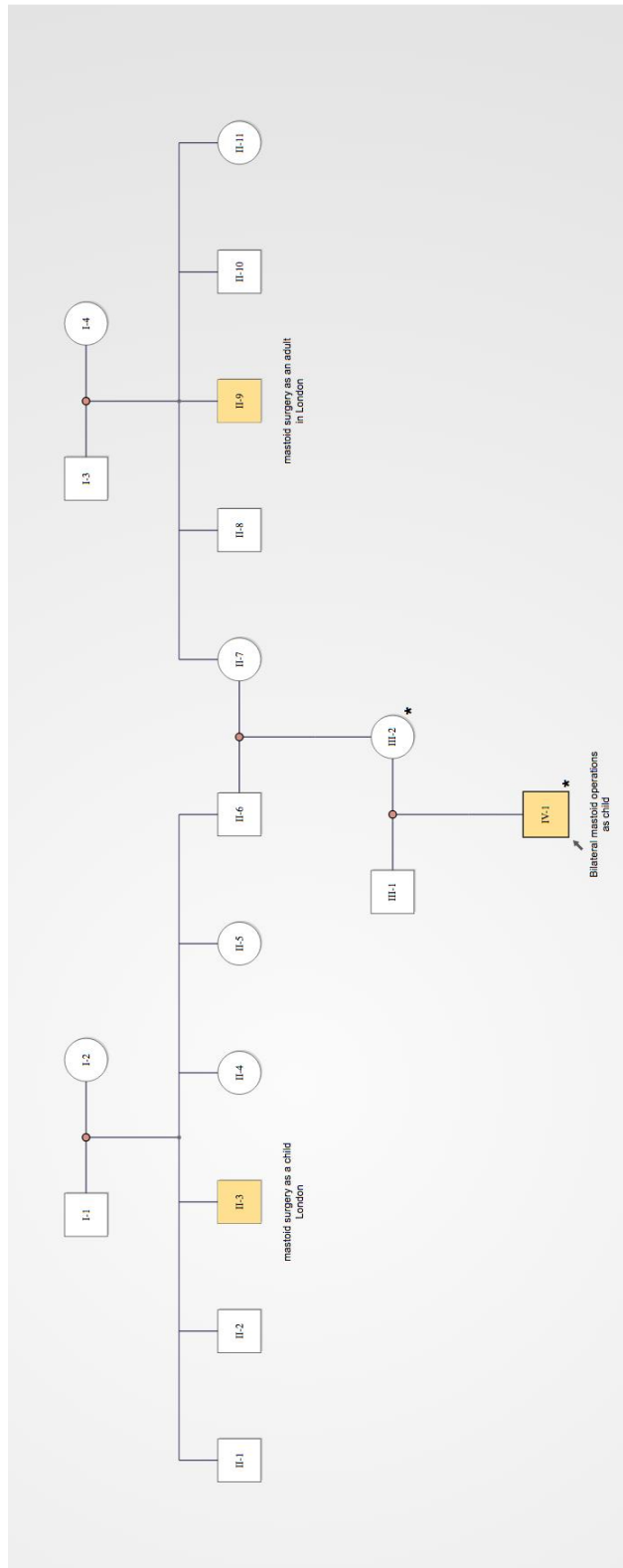


Figure 5-12 Family NN50. This is the pedigree of the patient discussed in chapter 2. There are maternal uncles on both sides of the family with a history of cholesteatoma. This is not a classical autosomal dominant pattern. The ancestry on both sides is suggestive of additive genetic factors.

Biobank

Blood samples from 66 participants were collected in 3ml EDTA tubes and DNA extraction was completed using the QIAamp DNA Blood Mini Kit [Qiagen, UK]. The protocol for DNA extraction was optimized to allow for the intermittent receipt of samples, the samples are now stored at -80°C on receipt and processed in batches. All biological samples [blood and/or DNA] have been stored in a biobank by the Department of Molecular Genetics at the Norfolk and Norwich University Hospital.

Sequencing and Analysis

The pilot analysis has considered primarily a dominant model of inheritance with variable penetrance. With a dominant mode of inheritance as our model, affected individuals must be heterozygous and unaffected individuals must be homozygous for the reference human genome sequence and the data are filtered accordingly. There is a need to be circumspect however because several generations can be affected for oligogenic and polygenic complex traits. There may be a threshold of genetic liability for affected individuals that may result from the accumulation of additive impacts from several or many key variants that are common within the families.

When full bioinformatics analysis is completed several models could be accommodated. The pilot study considers the dominant model because of the preliminary nature of the dataset and so this is a study limitation at present.

The pilot sequencing study identified 95,437 candidate variants that segregate with the disease phenotype [using NimbleGen exome capture; and the Illumina HiSeq4000 platform] in this experiment on 16 DNA samples collected from four families. Financial constraints have thus far restricted the WES to 16 of the 66 samples.

The performance of the exome capture on the samples was within expected tolerance. Mean target coverage for the exome sequence ranged from 24 fold to 56 fold. A minimum coverage of 30 fold is generally desired and only two samples were below 30x mean target coverage, due to lower read numbers for these samples.

A suggestion that there may be some common ancestry between the families recruited to the study was made, and although GY01 and GY02 look like they could be related on the basis of the principal component analysis, closer inspection of kinship metrics refuted this. There was therefore no genetic evidence of a common ancestry between the families and of a “founder effect”.

A number of gene variants co segregated with cholesteatoma patients in all of the four families.

There are six genes in coding regions of the exon sequences where a variant is in the same gene in all four of the families but in which the variants may not be for the same positions at the loci.

A Venn diagram reveals the overlap. Of particular note in this group are *CCHCR1* gene variants which have associations with psoriasis and a rare disorder called peeling skin syndrome and *DNAH9* variants associated with ciliary dyskinesia.

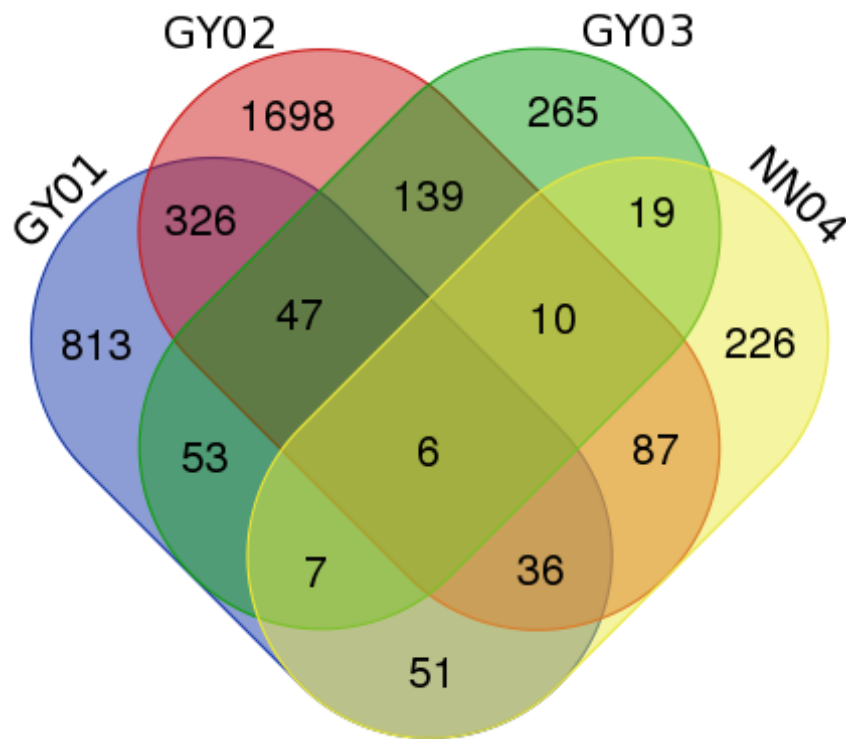


Figure 5-13 Venn diagram of overlapping variants.

The Venn diagram generated by the Ensembl Variant Effect Predictor bioinformatics programme shows the overlapping variants in the four families for genes predicted to have a high or moderate functional impact both within and outside of coding regions. The six that appear in all the families are within protein coding regions.

In addition three *non-coding* gene variants were seen to co-segregate with the phenotype in all of the families: Non coding DNA describes base sequences not translated into amino acids sequences but which may nevertheless have a role in gene regulation.

FAMILY NNO4

Family NN04 is the only family in which all the recruits have been sequenced. This family of three siblings and two parents has been subject to more detailed bioinformatics analysis. There is also an affected maternal cousin. In family NN04, based on the model of dominant inheritance with incomplete penetrance, we found variants in 442 genes that segregate with cholesteatoma.

Further filtering of this long list using Ensembl VEP to include only the rare co-segregating variants, with a rare allele frequency of ≤ 0.01 , has reduced the number of candidate genes to 32. A complete data set from these 4 multiply-affected families and a range of genetic models can be considered in the next phase of the pilot study when more sequencing data is available.

Missense variants that both software programmes (SIFT & POLYPHEN) predict to be benign or non-deleterious have been removed with the exception of the *DNAH6* variant rs192646174 which is predicted to be benign/tolerated but is in one of the genes mutated in all 4 affected families.

The remaining list of 32 variants is therefore of interest. Highlighted in red in table 5-2 are two single nucleotide variants of greater interest in view of the predicted large effect on gene expression which are further described in the discussion below.

Genes with co-segregating variants in coding DNA in all 4 families	Genes with co-segregating variants in non-coding DNA in all 4 families	Genes with co-segregating rare variants (allele frequency ≤ 0.01) predicted to have high/moderate functional impact in family NN04	Loss of function SNVs identified in family NN04 rs Id used = reference SNP number for the variant.
ZNF717	HYDIN	TMC6	EGFL8 stop gain variant rs141826798
PDE4DIP	EIF2A	TMEM150C	BTNL9 frameshift variant rs367635312
MKI67	SERP1	KTN1	
CTD-3088G3.8		HIST1H1B	
CCHCR1		DNAH6	
DNAH9		SORL1	
		CDH1	
		CCDC144NL	
		SYNRG	
		LAMA5	
		USP19	
		ZFAT	
		CSMD2	
		ATXN10	
		CLK4	
		ACTN4	
		HELZ2	
		RNF213	
		MFSD6	
		FKBP1C	
		SHANK2	
		EGFL8	
		STK31	
		IFIT2	
		FAM65A	
		TMPRSS3	
		CRYBG3	
		ZNF267	
		CCSER1	
		TNS3	
		ABCC12	
		BTNL9	

Table 5-3 List of Gene variants identified by the bioinformatic filters.

TGIF1

The long list of variants co segregating in family NN04 includes the gene *TGIF1* [no 226] on chromosome 18. This gene has been subject to a further analysis which illustrates the bioinformatics process which can be used to investigate variants of possible interest.

An analysis of the *TGIF1* gene is presented in figure 5-4 using images from the Broad Institute's Integrative Genomics Viewer of the variant call format file and scans for 5 members of NN04 and the *TGIF1* gene. Each individual in the family has been sequenced. The peaks represent the depth of the sequencing read overlaps and identifies the position of the variants along chromosome 18. The red arrow points to variants shared by Dad and the 3 siblings. The blue arrow points to variants inherited from Mum.

Variants of the Transforming Growth Interacting Factor [*TGIF1*] gene are of interest as a candidates for this study for several reasons:

- Knockout mice have conductive deafness; develop chronic otitis media with effusion (COME) and thickened middle ear mucosa (Tateossian et al., 2013).
- A Genome Wide Association Study of children affected by COME identified associations between *TGIF1* variants and the phenotype (Bhutta et al., 2017a).
- *TGIF1* variants have been associated with a second distinct phenotypic pathway associated with cholesteatoma: midfacial morphogenesis & developmental biology.
- Haploinsufficiency [mutations in the heterozygous state which fits with a dominant pattern of inheritance] for several genes including *TGIF1* is associated with the Holoprosencephaly disease phenotype.

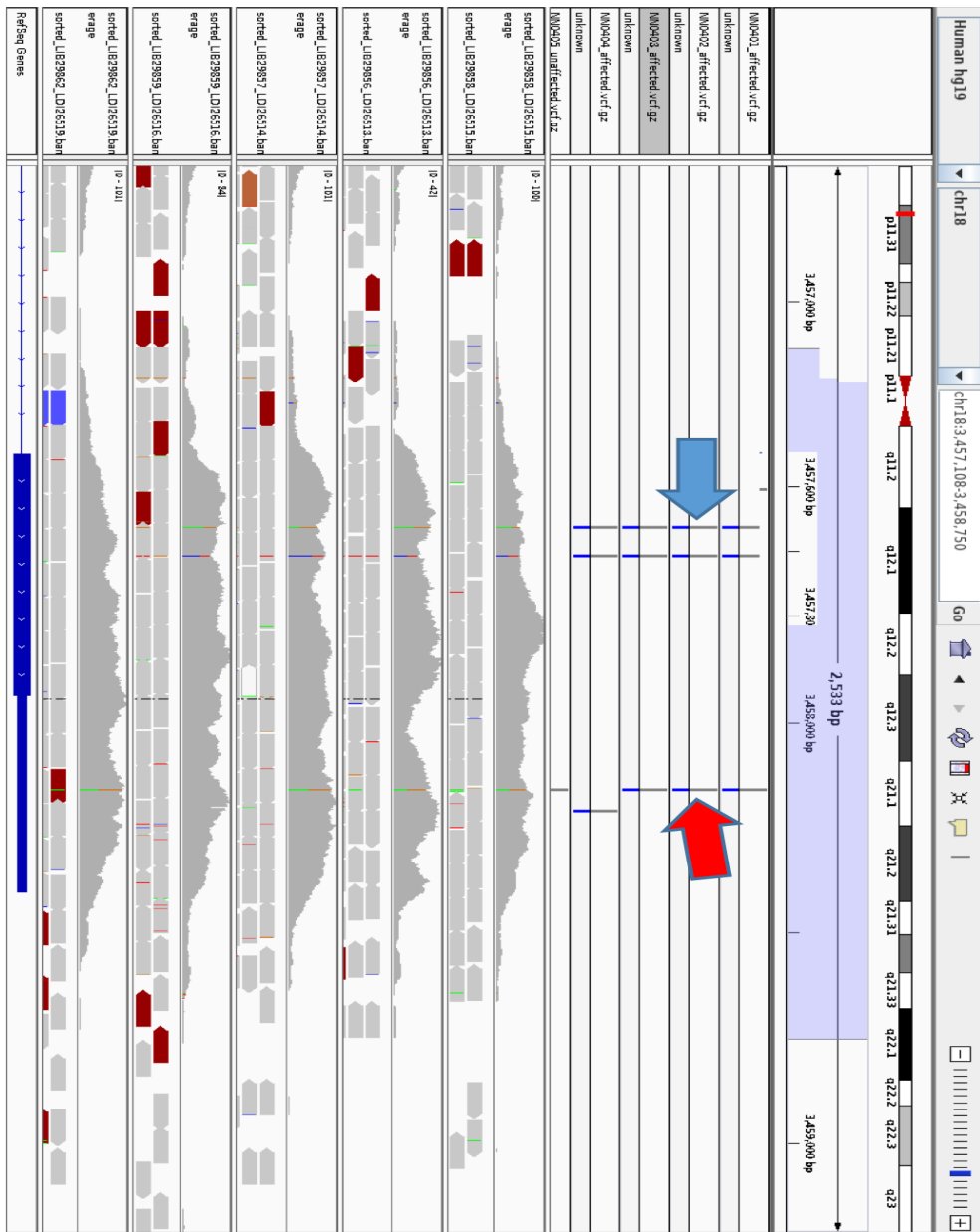


Figure 5-14 View of TG1F Exon

Homeodomain proteins derived from homeobox genes act as transcription factors binding to and controlling the activity of other genes. *TGIF1* is a homeodomain protein that has a role in 3 signalling pathways [RA, Retinoic Acid; TGF β ; Wnt/ β -catenin]. It acts as a transcriptional co-repressor. The first paper to describe its genomic structure; its alternate splicing regulation and the expression pattern of the gene/protein was in 2008 by Hamid et al. (Hamid et al., 2008).

Possibly some of those candidate variants have an impact on Transforming Growth Factor TGF β signalling and inflammatory processes known to be significant in chronic inflammatory and bacterial diseases of the ear? Transforming growth factors are also considered to be important cytokines in the keratinocyte cycle.

Three particular *TGIF1* mutations were identified by the bioinformatic programme but closer scrutiny of these variants makes it unlikely that they are of great importance in this family.

rs11571510

This frameshift mutation identified in the first bioinformatics is very common [allele frequency = 0.43] and it occurs in sequence upstream of the atg translation initiation site and is therefore not in the protein coding sequence. This means it is not expected to have any functional impact on gene expression. Two of the variants were carried by all three children and by Mum.

rs2229337

This variant definitely lies in the coding sequence. However it is a synonymous SNV and so not of interest functionally.

rs2229333

This is present in the undisputed coding region in the last [G8/H11] exon. European allele frequency 0.06, ~ 1/ 17 people carry it so it is not very rare but could be contributory given that it is a missense mutation resulting in a proline to leucine substitution [P163L].

Summary of sequencing findings

The aim of the pilot study was to develop a bioinformatics “pipeline” to:

- Distinguish gene variants from the reference human genome sequence.
- Interrogate and filter the variants with respect to predicted impact and disease associations.

95,437 variants were identified in 16 individuals from four families. Variant filtering, using pedigree analysis, identified 442 candidate genes. Variant effect prediction has narrowed this to a small number of genes of interest further identified as having possible disease associations.

Discussion

Introduction

The GOC project really began with a clinical observation made in 1996. A record of patients presenting with a family history of cholesteatoma was initiated. Over the years a series of families has been gathered which has formed the basis of the GOC pilot study.

The report in the *Journal of Otology and Laryngology* in 2009 (Prinsley, 2009a) stimulated interest and encouragement from colleagues and in time it became feasible to envisage DNA sequencing for such families. Until recently genomic technology made it possible to look for “causative mutations” in only one segment of a gene at a time which greatly limited the time and volume of the technique. Next generation sequencing makes it possible to sequence millions of fragments of DNA simultaneously and massively increases the scale and speed of genetic science. Whilst initial sequencing of the human genome took more than 10 years and cost £2bn, the genome can now be sequenced for £700 and takes a few days. (Turnbull et al., 2018)

In 1996 in Norwich there was no Medical School and no University Hospital. The Norwich Research Park was devoted largely to agricultural sciences at The John Innes Institute. Since then the situation has changed completely. There is a new University Hospital and a Norwich Medical School. The Earlham Institute (formerly called The Genome Analysis Centre or TGAC) has been constructed at the research park. Together with my supervisors, Dr Barbara Jennings and Professor Carl Philpott at the University of East Anglia, we have assembled a team capable of undertaking this project.

The project presented in this thesis is a pilot study to demonstrate the feasibility of the method that has been used to collect family information from individuals with cholesteatoma, extract blood for DNA analysis, carry out Whole Exome Sequencing and perform Bioinformatics. The study has attracted the attention of otologists throughout the UK and has been registered on the NIHR portfolio which facilitates and supports recruitment of patients and families for research in the NHS. The research team intends to expand the study and to recruit more patients and families for sequencing as the search for genetic variants widens.

Collection of pedigrees and blood samples

The scientific advances which have made the WES study possible are quite extraordinary but the fundamental task of the geneticist to collect family histories and recruit individuals for scientific study remains one of the most difficult tasks in a project of this sort.

Many of the patients had previously spoken about the possibility of a future DNA study and it was not a surprise when they were approached by the research nurse for recruitment. The recruitment of unaffected near relatives has been helped by the good relationships that have built up with many of the patients and their families and, in keeping with other genetic studies, there has been a genuine enthusiasm within the families to help with this study. The patients and their relatives are themselves as puzzled as anyone as to what has caused this ear disease which makes them deaf and for their ears to continuously weep. Having said that the diligence with which the research nurses have carried out this task is not to be underestimated and is hugely appreciated.

The patient information sheets and consent and assent forms were approved by the research ethics committee have been simple to use.

For small children, and for individuals reluctant to have a blood sample, salivary swabs are available but these have not been used in any of the recruits reported here and so far all the DNA has been extracted from blood. It is easier to extract reliable quality and quantity of DNA for analysis from blood than from saliva.

Database

A secure data base held in the research office of the James Paget University Hospital was used to reconcile the family history data with the samples sent to the molecular pathology laboratory at the Norfolk and Norwich University Hospital. As the project expands to incorporate other recruitment sites outside of Norfolk this is no longer practical. A web database using Research Electronic Data Capture (REDCap®) has been developed in collaboration with the Clinical Trials Unit at the Norwich Medical School to enable the family history and patient identifiable data to be securely entered at the recruitment site. This will then be accessible to the research team reconciling the samples for analysis with the individual family histories and patient data.

The family pedigree charts when presented as a group are quite striking. It is perhaps surprising that this observation has not been more often reported previously. A condition which occurs in about 1 in 10,000 individuals per year would not exhibit a random familial pattern and therefore the family trees are in themselves compelling supporting evidence of a genetic causation. The apparent pattern of inheritance guides the individuals selected for DNA

sequencing and also the bioinformatics strategy. In the pilot study only a dominant model for an allele with a major or moderate impact has been considered to date, coupled with incomplete penetrance. But an oligogenic or polygenic mode of inheritance, with the cumulative effect from many alleles, could be revealed by an experiment with the much larger number of individuals such is proposed by the study team.

Only a single affected individual declined to participate in the Genetics of Cholesteatoma Project and for the main part the families have supported the study into this disease. The families reported in this pilot study have been local to the Norfolk hospitals and one factor that has facilitated the study is the fact that in this part of England there is relatively little "migration". Norfolk families tend to stay in Norfolk and it is common for the author to have treated several generations of the same family. There are however several individuals of great interest who are not local and it is the intention of the research team to recruit these family members as the project expands.

Some of the families in the database which include several affected children. In the pilot study none of the children have so far been recruited. The protocol incorporates salivary sampling for DNA sequencing and an experiment to test the quantity and quality of the DNA obtained by this method is planned in the second phase of the study.

Sequencing

The aim in the pilot experiment was to sequence a batch of DNA samples from study participants to establish a sequencing and bioinformatics protocol. The DNAs were selected after ranking them for quality before

library construction; they came from affected & unaffected participants across four families. The DNA obtained from the blood samples was of sufficient quantity and quality for sequencing.

Gene variants co segregating with the cholesteatoma phenotype within and between the four families in the pilot experiment are variants of interest. Variants that are rare within a matched general population are also variants of interest. The impact of the variant is also significant since even a single nucleotide change may be biologically important. The bioinformatics software used in the experiment is able to sift the variants into those of high or moderate functional impact. The software also enables the samples to be filtered according to patterns of inheritance suggested by the family tree. With a dominant mode of inheritance as our model, affected individuals must be heterozygous and unaffected individuals must be homozygous for the reference human genome sequence and the data are filtered accordingly.

In the families that are reported in this pilot study cholesteatoma self-evidently exhibits a familial trend. The question of the contribution of genetics to this fact is the subject of this enquiry. Genetic architecture seeks to define the genetic contribution to human traits and diseases (Timpson et al., 2018). The genetic architecture refers to all of the genetic influences on a phenotype, the magnitude of the effect, the frequency of the effect and the interaction with the environment. In classical genetics a disease may be regarded as monogenic if caused by a single gene, oligogenic if caused by a few genes, or polygenic if caused by many genes. More recently an omnigenic concept has been developed by which is meant there are many possible small genetic effects within a diseased cell that are cumulative and sufficiently interconnected to result in the disease phenotype. In this theory mutations that are remote from the phenotypic biology might be

important.(Boyle et al., 2017). A pilot study such as this is designed to test if it is feasible to use a whole exome sequencing approach to search for genetic variants co segregating with the phenotype and this has been achieved in the population that has been recruited.

The cause of cholesteatoma

Despite the existence of many theories the cause of the disease remains unknown. A genetic component is probable and certainly the identification of a genetic basis for the disease could have a role in diagnosis, therapy and prognosis.

Much of the literature about the cause of the disease presents studies and audits of surgical series and of histopathological analyses (Louw, 2010). There are a number of well described histopathological mechanisms of abnormal epithelial migration but the fundamental cause is yet to be elucidated. DNA studies using microarrays of surgical specimens matched with post auricular skin as a control examine molecular pathways and gene expression that show altered regulation in cholesteatoma cells (Klenke et al., 2012b). Microarrays rely on hybridization probes of known sequence (Kahvejian et al., 2008) or in other words we can only look for mutations that are known to exist. Such studies have resulted in considerable conjecture about cholesteatoma causation. The Genetics of Cholesteatoma Project has adopted a completely different approach studying the whole exome sequence of affected and unaffected individuals in families in which the disease is segregating. This investigation seeks germ line rather than somatic mutations such as might be revealed by a study of the cholesteatoma tissue.

The literature review (Jennings et al., 2018) carried out as part of the project suggests a genetic component in causation although the published evidence is really not extensive.

The discovery of *DNAH9* variants overlapping within the four families was exciting because ciliary motility disorders are implicated in chronic sinus and middle ear disease and because *DNAH9* variants have a known association with ciliary dyskinesia. *HYDIN* variants identified in non-coding co-segregating genes may also be relevant in view of the role this gene is also known to play in ciliary motility disorders.

The tympanic membrane can be considered to be specialized skin. Might cholesteatoma have more in common with general dermatological disorders than is usually thought to be the case? Variants in *CCHCR1* were discovered co-segregating with cholesteatoma in all of the four families so far sequenced and these are known to be important in psoriasis which is very common and peeling disease of the skin which is very rare.

Within the group of 32 variants revealed by the bioinformatics in family NN04 are *EGFL8* which is a stop gain mutation with an allele frequency of 0.0026 and *BTNL9* which is a frameshift with an frequency of 0.0036. Both variants are predicted to be of high impact.

GeneCards® describes the location of Epidermal Growth Factor-Like Protein 8 *EGFL8* on chromosome 6 with a size of 3.703 bases on the cytogenic band 6p21.32. Associated diseases include ischaemic bone disease. *EGFL8* is highly expressed in the skin. Interestingly, the stop-gain variant in *EGFL8*, rs141826798, has recently been reported to be significantly associated with psoriasis in the one of the disease traits recorded for the UK Biobank

participants(Emdin et al., 2018). For both psoriasis and cholesteatoma, there is altered keratinocyte proliferation and differentiation. It is possible that *EGFL8* variants have pleiotropic effects and/ or that there is a common biological driver for these pathologies.

GeneCards® describes the location of the Butyrophilin Like 9 gene *BTNL9* on chromosome 5 with a size of 21,299 bases on the cytogenic band 5q35.3. It mediates pathways related to the innate immune system.

Guidelines for the analysis of sequencing variants associated with Mendelian traits in clinical settings are not directly applicable to research but they do present a useful variant classification system (Richards et al., 2015). Given the uncertainty inherent in filtering sequence data, individual variants are classified in one of five possible ways by clinical genetics laboratories:

1. Pathogenic
2. Likely pathogenic
3. Uncertain significance
4. Likely benign
5. Benign

The pilot genetics of cholesteatoma study has discovered two rare, loss-of-function variants in genes that co-segregate with the phenotype. These variants of the genes *EGFL8* and *BTNL9* have not been previously reported to be associated with cholesteatoma and there are no published studies that demonstrate that the variants are significant with respect to protein function. It is appropriate to refer to rs141826798 (g.32134395C>G) and rs367635312 (g.181050254delC) circumspectly as variants in genes of uncertain significance.

Epidemiological studies of cholesteatoma identify the association of chronic otitis media with effusion. The fact that *TG1F* variants were found in the pilot

study somewhat intriguingly fits with the genome wide association study recently published by Bhutta et al (Bhutta et al., 2017b). Although the variants found in *TGIF1* seem unlikely to be significant at this point, epidemiological associations with developmental disorders of the cranial development such as cleft lip and palate are well described (Spilsbury et al., 2013) and so the finding of co segregating *TGIF1* variants associated with skull dysmorphology is also notable.

Beyond the pilot study and next steps for the Genetics of Cholesteatoma Project

The pilot study was conceived to demonstrate that the research team could establish a protocol to collect family pedigrees, extract and sequence DNA and perform bioinformatic analysis. It was necessary to check that DNA of sufficient quality could be obtained to allow accurate sequencing to 30 x coverage. Funding constraints meant that only 16 WES were obtained to date but the results of the pilot will be used in future funding grant applications. The price of WES and bioinformatics is falling with recent quotes [November2018] at £290 for WES and £100 for associated bioinformatics.

The research team continues to recruit participants and extract DNA for storage in the Biobank. Additional resources will be sought to complete the WES on the first four families and to produce additional variant call files for subsequent bioinformatics analysis.

The Biobank will grow to become a research resource for future investigation. Additional recruitment sites around the UK are planned. The project needs a formal database and a REDCap® system has been developed for secure recording and storage of participant phenotypes and sample processing. The associated epidemiological questionnaire which has used social media is now the largest NIHR ENT study presently recruiting and is revealing fascinating data about family history in cholesteatoma patients worldwide.

A laboratory protocol for the non-invasive and remote collection of DNA samples using mouth swabs will be devised and the extracted DNA will be

tested for quality and quantity by comparing with at least one participant's blood DNA.

The research team plans to publish the findings of the pilot experiment in an open-access journal and to develop Research Councils UK applications for a project grant.

If a constitutional explanation for cholesteatoma is established then a number of research opportunities suggest themselves. Might recurrence be more likely in patients with a family history and is bilateral disease more common if there is a genetic predisposition? It is likely that such families are the best place to start looking for important variants with moderate and or major functional impacts. Some genes are known to be associated with skin disorders and chronic inflammatory conditions of the ear in children. What might be the association of these genes with "cholesteatoma genes"?

Personal reflections on the Genetics of Cholesteatoma project

This project has been a source of professional fascination for me and I have learned the way in which a medical research project of this type is established, organized and performed. The importance of team working has been very clear to me and I anticipate that the team will grow as the project expands and recruits more widely. I have learned about the need to develop distinct study protocols, the difficulties of grant applications and have understood the working of the research ethics system. Indeed one of the early challenges was to represent the team before a rather intimidating research ethics committee in Cambridge.

I have learned about basic molecular biology and the science of genetics and something also of the laboratory techniques, the sequencing and the bioinformatics that have been used. I was directed to the Future Learn initiative of the Open University which has been an excellent resource for a “mature” postgraduate student such as myself.

I have presented the project nationally and internationally and this too has been a new venture for me.

Chapter 6 Conclusion

This thesis has described the work which has been done to establish the Genetics of Cholesteatoma Project in Norfolk.

Cholesteatoma remains an enigma. Not a neoplasm, not an infection, not a simple inflammatory process but a rather singular pathology with features of all three. The surgical pathology and epidemiology of the Norfolk patients is broadly in keeping with the descriptions in the surgical literature and can be regarded as a representative group of patients, but the observation of familial clustering is really quite striking and it is surprising that this has not been previously more widely reported. These Norfolk families have formed the basis of the pilot investigation.

The protocol for the collection of family pedigrees and the extraction and sequencing of DNA has been defined by the research team. The bioinformatics which has been possible on the first 16 sequenced DNA samples has revealed a number of intriguing genetic variants, both between the affected individuals within the 4 families, and within the family NN04 which is the only completely recruited family so far sequenced.

It is likely that the genetic architecture of cholesteatoma will be further revealed as the Genetics of Cholesteatoma Project expands. The costs of DNA sequencing are continuing to fall and the speed and complexity of

bioinformatics computing is improving rapidly. The project which is adopted by the NIHR in the UK NHS is set to recruit additional families from several sites across the country as resources develop.

This project arose as a result of basic curiosity. What is the reason for the familial clustering of this rare ear disorder? If a genetic basis could be discovered then it is possible to imagine that the biochemical pathways and cell signal systems which are presumed to control tympanic membrane cell migration could also be better understood with considerable potential clinical relevance. A gene panel consisting of variants known to be associated with cholesteatoma could be constructed and then used for patients presenting with CSOM to predict cholesteatoma before it develops. Such a genetic screen would be inexpensive, simple to use and could have significant clinical application.

Experienced otologist know that there are some patients with cholesteatoma who seem to do badly and some patients who seem to do well irrespective of the operations which are performed. An early understanding of the constitutional nature of a patient presenting with a cholesteatoma might inform the timing and the nature of surgical intervention. Also as the project develops there are plans to investigate the genetic differences between single sided and bilateral cholesteatoma since patients with bilateral disease may have a constitutional risk of the disease with a genetic basis. Early identification of this has potential to prevent deafness.

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APPENDIX

Consent/assent/patient information sheets

 <p>Royal College of Surgeons ADVANCING SURGICAL CARE</p>	 <p>University of East Anglia</p>	 <p>Rosetrees Trust Supporting the best in medical research</p>
 <p>NHS National Institute for Health Research</p>	 <p>James Paget University Hospitals NHS</p>	 <p>NHS Foundation Trust</p>
		Lowestoft Road Gorleston Great Yarmouth Norfolk NR31 6LA
		Main switchboard: 01493 452452 Direct line: 01493 452832 E mail: brenda.peck@jpaget.nhs.uk www.jpaget.nhs.uk https://www.uea.ac.uk/rhinology-group/home
<h3>The Genetics of Cholesteatoma Study</h3> <p>Participant Information Form v1.2 10.02.2016</p>		
Project Sponsor:	University of East Anglia	
Chief Investigator:	Peter Prinsley	
Co-Investigators:	Barbara Jennings Carl Philpott Mahmood Bhutta	
Location:	University of East Anglia and its associated NHS Hospitals James Paget University and Norfolk and Norwich University NHS Foundation Trusts	
Dear		
<u>Introduction</u>		
You are invited to take part in this research project that is investigating the genetics of an ear disease called cholesteatoma (pronounced KOL-ES-TEA-ATOMA). This is because you or a member of your family has been identified as having a cholesteatoma. We will also seek your permission to contact any of your children to ask for their help with this project.		
Please take a few moments to read this document that will help you decide whether you wish to take part.		
What is genetic research?		
Genes are made of DNA – the chemical structure carrying your genetic information that determines many human characteristics such as the colour of your eyes and hair. Researchers study DNA to determine why some people might have a condition and some might not. Understanding a person's genes also may explain why some people respond to a treatment and some do not.		
What does participation in this research involve?		
You, or a family member, has been identified as a patient with a fairly rare ear condition called cholesteatoma. This chronic disease of the ear can cause hearing problems and a discharge from the ear amongst other symptoms. We have noticed that the condition sometimes runs in families.		
At first we would like to ask you to provide information about your family tree and, in particular, whether there are other members of the family who have been affected and how they are related to you.		
GoC Adult Information Sheet v1.2 10.02.2016		

This information will be secure and will not be shared with individuals not directly connected with this research - we will ask our research nurse to contact you directly to talk to you.

We will ask for your permission to collect a small blood sample to be used to do an analysis of your genetic code. We will also seek samples from affected and non affected members of your family, so that we can compare affected and unaffected people. In this way we hope to identify the gene or genes that might be the cause of the condition. All of this information will be kept secure and will be identifiable only by members of the research team. We expect that the interview with our research nurse to obtain your family history and to take a blood sample should take no more than an hour.

Genetic code testing has now advanced to the point where such a search for a gene in this rare disease is practical and affordable. We hope to identify the gene and try to find the way in which it causes the disease. If we succeed this will be an advance in our understanding of ear disease and has the potential to help us with many future patients.

We hope that you will be able to help us but we do of course understand if you do not wish to participate and will fully respect this decision.

What are the benefits to you of taking part?

There are no direct benefits to you or to your family of taking part in this project. This is scientific research aimed at understanding the cause of the condition so that we will better be able to diagnose and treat the condition in the future.

What are the disadvantages or risks of taking part?

There may be minor discomfort or bruising associated with taking a small blood sample and our research team will need a little bit of your time to ask about your family.

Our genetic analysis method will target genes that we believe are relevant to cholesteatoma. We will not return individual genetic data to you as a participant and will not identify individual genetic variants of clinical or unknown significance. Therefore we will not be able to provide you with any personal genetic information of direct significance to you.

How will we tell you what we have found?

We will establish a Genetics of Cholesteatoma website. We will use this to update participants on the progress of our research and to let you know of any of our findings. We hope to publish any findings of significance in scientific journals.

Further information

What will happen if I don't want to take part?

There will be no impact on any care that you may need.

How will my information be kept confidential?

The information about your family tree and any results of genetic testing will be kept in confidential hospital and university computers. The information will be coded so as to be only available to the research team.

Will my children be involved?

Cholesteatoma is a disease that affects all age groups. We will ask you permission to collect DNA from affected and unaffected children in your family. We have a kit available that lets us use a small swab of saliva instead of a blood sample in children if necessary.

Who is organising and funding this study?

This study is organised in Norfolk by a team of ENT surgeons and scientists. It is funded from scientific research grants that have been competitively awarded by the Royal College of Surgeons of England and by the Rosetrees Foundation Trust.

Will I be paid for taking part?

No. We are seeking volunteers to help us with this study. We may be in a position to refund reasonable travel expenses if these are necessarily incurred.

What will happen to the blood sample that I give?

The blood sample will be sent to a specialist laboratory at the Norfolk and Norwich Hospital where the DNA will be extracted and stored. The genetic testing will also take place in Norfolk at The Genome Analysis Centre at Norwich Research Park. The sample of DNA will be stored for 10 years so as to be available for future ethically approved research.

Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities has to be approved by an NHS Research Ethics Committee before it goes ahead. This study has been reviewed by the xx NHS Ethics Committee. Approval does not guarantee that you will not come to any harm if you take part, however approval means that the Committee is satisfied that your rights will be respected; that any risks have been reduced to a minimum; have been balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

What if I am harmed by the study?

Nothing in this study is expected to cause you any harm. In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs.

The normal National Health Service complaints mechanisms will still be available to you through:-

Tracey Noakes
Complaints Manager
James Paget University Hospitals NHS Foundation Trust
Lowestoft Road
Gorleston
Great Yarmouth
Norfolk
NR31 6LA
Tel: 01493 452019
complaints@jpaget.nhs.uk

Contact for further information

If you have any queries or concerns about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. In this situation please don't hesitate to contact:

Mr Peter Prinsley
ENT Consultant
James Paget University Hospitals NHS Foundation Trust
Lowestoft Road
GoC Adult Information Sheet v1.2

10.02.2016



James Paget University Hospitals 
NHS Foundation Trust

Lowestoft Road
Gorleston
Great Yarmouth
Norfolk
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Main switchboard: 01493 452452
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<https://www.uea.ac.uk/rhinology-group/home>

Assent/Consent Form

The Genetics of Cholesteatoma Study

Dear

I am writing to you to ask for your help in medical research.
You or somebody in your family has a fairly rare ear problem and I am helping some scientists at the University to see if we can find out what causes it.

I have sent you some information about this with this letter and can I ask you to have a look at it with your Mum or Dad and have a think about whether you would like to join in. If you don't want to join in you certainly don't have to.

What we will do is ask for a small sample of your blood or a small swab from inside your mouth which we will use to check the genes which are chemicals in the body that control how our bodies are put together and how they work. This will be done at a laboratory in Norwich. We hope that we might find the genes that cause the ear disease. If we do succeed then we think this will be a big help for people who have this problem with their ear.

Yours sincerely

Peter Prinsley (Doctor who treats patients with ear problems)

What is Medical Research?

This is science which is done to try and help people who have various diseases.

Why are you being asked to help?

You or a member of your family has a rare ear disease which we have noticed sometimes seems to run in families?

What are we planning to do?

We are planning to ask you and your family about any ear or hearing problems. We will then draw up a family tree to show how anybody who has an ear problem is related to other people in the family.

What else will we ask?

We will ask if we can take a small sample of your blood or a swab from your mouth using a little plastic spoon. This will allow us to collect some cells which we will send to a laboratory in Norwich.

What will happen to the sample?

We will ask our scientists to study the cells and find out about the genes inside them. Genes are made of special chemicals called DNA that tell the body how to grow. We hope to find the genes that might be causing ear problems in your family.

Please talk to your Mum or Dad about this.



Assent Form

I

Do agree to take part in the medical research about the ear disease which is explained in this letter

Signature

Date

Consent of parent or guardian

I

Do agree that I have spoken to my child about this research and we have jointly agreed to take part

Signature

Date

The Genetics of Cholesteatoma Study

To be read to very young children



Dear

Some people get sore ears and have a hearing problem.

Sometimes this problem runs in families and we are trying to find out why.

We have asked your Mum or Dad if they will help us but we want to tell you about it too.

The nurse would like to collect a little blood sample or a little spit from you. The scientists can check it to see what is in it.

Speak to your Mum or Dad if you want to know more but we really hope you will be able to help us



Yours sincerely

Peter Prinsley (ear doctor)



James Paget University Hospitals 
NHS Foundation Trust

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Signature

Date

Consent of parent or guardian

I

Do agree that I have spoken to my child about this research and we have jointly agreed to take part

Signature

Date

Proforma for collecting ENT history and family history

ENT-Focused Family History Questions & Proforma

Phenotypes of Interest = Diagnosis and surgical treatment for cholesteatoma. Secondary phenotypes: 1.Glue-ear treated with grommets
2.Hearing problems in childhood 3.Diagnosis of Genetic Disease/Congenital Disease.

For each relative in the 4 generation family tree we are seeking the following information/asking the following questions:

1. Name
2. Date* of Birth
3. Alive or Dead (+ date* of death if applicable)
4. Has he or she had surgical treatment for cholesteatoma
5. Age at diagnosis
6. Age at time of surgery (may be more than one episode; please record dates* of all surgical treatments)
7. Ask if one ear, or both ears, were affected. Indicate with a tick which ear (L and/or R) was/were affected.
8. Ask if he or she was affected by other phenotypes of interest (glue ear treated with grommets; hearing problems in childhood; or a diagnosed genetic/congenital disease) and make note of any details about disease management including the number of ear surgeries.

*date: use year of birth/death if more precise date is not known.

Index Case**
Q1
Q2
Q3
Q4
Q5
Q6

Q7. L	R
Q8 Notes	

Recording data for individuals in 4 generations:

**** Other titles for each table: Mother (of index case); Father; Mother's Mother; Mother's Father; Father's Mother; Father's Father; Brother/Sister; Child (of index case); Child of Brother; Child of Brother/Sister**

Q1	
Q2	
Q3	
Q4	
Q5	
Q6	
Q7 L	R
Q8 Notes	

GOC STUDY

Study of the Genetics of Cholesteatoma.

Send to Pathology Reception, NNUH.

Sample for Molecular Genetics at Norfolk and Norwich Hospital.

Contacts are Gavin Willis or Katy Smith ex. 3068 or 2420.

Note for Pathology Reception: Please don't book on to LabTrak; samples to go directly to Molecular Genetics.

Name.....

Hospital Number.....

Date of Birth.....

By adding my initials to the box below, I confirm that:

Participant consent form has been completed and archived for this study.

Name of Nurse/Doctor who collected sample

Signed _____ Date _____

Laboratory Use:

Date of receipt _____

Date of Storage _____

Date of DNA extraction _____

Sample number _____

Address to send samples to : FAO Dr Gavin Willis, Principal Clinical Molecular Geneticist, Department of Molecular Genetics, Norfolk and Norwich University Hospital, Norwich NR4 7UY.

The following advice has been developed using the standard operating procedure from the Norfolk & Norwich University Hospital for the postage of non-urgent pathology specimens. But the protocols recommended by other hospital trusts that are compliant with UN3373 regulations (<http://www.un3373.com/info/regulations/>) can also be followed.

Several companies produce bespoke packaging and postal systems if your department does not routinely use suitable polypropylene tubes, absorbent materials or postage boxes.

- a) Packing for despatch by courier /post (inland or overseas) must be the responsibility of a trained person. It should not be entrusted to an untrained member of the clerical staff.
- b) The packing to be used must meet the UN3373 regulations.

Pathological material **must be sent by First Class Letter Post only**. Post Office regulations specifically forbid sending such material by Second Class Letter or Parcel Post. The properly packed article is officially described as a 'packet'.

- (i) The specimen must be in a securely closed container, which must be robust and leak-proof. This is the Primary container.
- (ii) Each specimen container must be placed in a plastic bag and pad of absorbent material to prevent leakage in the event of damage to the container. There must be no glass-to-glass or plastic-to-plastic contact. This is the Secondary container.

- (iii) All letters and forms must NOT be placed inside with samples but if Marsupial bag used placed in the side pocket.
- (iv) The packed specimen must be placed in a box or case of suitably strong material such as fibreboard in such a way that it cannot move about.
- (v) The box or case must be securely closed with tape marked 'Pathological Specimen, Fragile Handle With Care' and labelled with name and address of the sender (to be contacted in case of damage or leakage).

If clip down container or polystyrene box is used they should finally be put in padded envelope and marked 'Pathological Specimen, Fragile Handle With Care' and senders name and address. In all cases a label with UN3373 on must be placed on package.

- (vi) The District Post Office must be notified at once if any infectious or potentially infectious material arrives in a damaged condition. The sender must be informed if an improperly packed specimen is received.



Mr Peter Prinsley
Lowestoft Road
Gorleston, Great Yarmouth
Norfolk
NR31 6LA

Email: hra.approval@nhs.uk

Letter of HRA Approval for a study processed through pre-HRA Approval systems

Study title: An Analysis of the Pedigrees and Genetic Profile of Patients and Families of Patients with Cholesteatoma

IRAS project ID: 186786

Sponsor University of East Anglia

Thank you for your request for HRA Approval to be issued for the above referenced study.

I am pleased to confirm that the study has been given **HRA Approval**. This has been issued on the basis that the study is compliant with the UK wide standards for research in the NHS.

The extension of HRA Approval to this study on this basis allows the sponsor and participating NHS organisations in England to set-up the study in accordance with HRA Approval processes, with decisions on study set-up being taken on the basis of capacity and capability alone.

If you have submitted an amendment to the HRA between 23 March 2016 and the date of this letter, this letter incorporates the HRA Approval for that amendment, which may be implemented in accordance with the amendment categorisation email (e.g. not prior to REC Favourable Opinion, MHRA Clinical Trial Authorisation etc., as applicable). If the submitted amendment included the addition of a new NHS organisation in England, the addition of the new NHS organisation is also approved and should be set up in accordance with HRA Approval processes (e.g. the organisation should be invited to assess and arrange its capacity and capability to deliver the study and confirm once it is ready to do so).

Long list of genes from family NNO4

– long candidate gene list (variants detected with high or moderate functional impact)

For family NN04, variants in protein coding regions of 442 genes co-segregate with cholesteatoma, for dominant model considered by Dan Swan.

- How many of these are variants in a European cohort with minor allele frequency of < 20 %

Het only VCF (protein coding only)

2133 variants (none novel)

http://grch37.ensembl.org/Homo_sapiens/Tools/VEP/Results?db=core;field1=IMPACT;from=1;operator1=is;size=792;tl=1n0cxdwOLGzhElaH-3899140;to=792;value1=MODERATE

< 10 %

Het only VCF

1478 variants (see Moderate impact variants)
--

http://grch37.ensembl.org/Homo_sapiens/Tools/VEP/Results?db=core;tl=xFEKEjPnE8AKdd8G-3899252

< 5 %

Het only VCF

1158 variants

http://grch37.ensembl.org/Homo_sapiens/Tools/VEP/Results?db=core;tl=t2A576y7jxPzPgWM-3899271

1. *FHAD1*
2. *CLCNKA*
3. *ATP13A2*
4. *ALPL*
5. *HSPG2*
6. *LACTBL1*
7. *MYOM3*

8. *CSMD2*
9. *EPHA10*
10. *MACF1*
11. *COL9A2*
12. *SPATA6*
13. *COA7*
14. *PODN*
15. *C8A*
16. *C8B*
17. *INADL*
18. *L1TD1*
19. *PDE4DIP*
20. *BCL9*
21. *KIAA1614*
22. *TSEN15*
23. *PPFIA4*
24. *PIK3C2B*
25. *EPRS*
26. *HLX*
27. *HHIPL2*
28. *CAPN8*
29. *CAPN2*
30. *DNAH14*
31. *TMEM63A*
32. *TRIM11*
33. *URB2*
34. *MCM10*
35. *FRMD4A*
36. *PIP4K2A*
37. *GAD2*
38. *ANKRD26*
39. *PTCHD3*
40. *ANXA11*
41. *AL359195.1*
42. *SH2D4B*
43. *IFIT2*
44. *PNLIPRP3*
45. *GRK5*
46. *BAG3*
47. *BTBD16*
48. *DMBT1*
49. *CHST15*
50. *MKI67*
51. *LGR4*
52. *TCP11L1*

53. KIAA1549L
54. SHANK2
55. PAAF1
56. SORL1
57. OR6T1
58. OR8B12
59. PANX3
60. CDON
61. A2ML1
62. PZP
63. CLEC2D
64. CLEC1B
65. ART4
66. MGP
67. CAS1
68. IFLTD1
69. OVCH1-AS1
70. OVCH1
71. OR9K2
72. OR6C74
73. OR6C1
74. GLS2
75. HELB
76. BEST3
77. MYRFL
78. KRR1
79. OTOGL
80. PTPRQ
81. RASSF9
82. CEP290
83. PLXNC1
84. CCDC41
85. VEZT
86. C12orf55
87. OAS3
88. GOLGA3
89. ZNF268
90. GTF3A
91. USPL1
92. ZAR1L
93. N4BP2L2
94. KL
95. SCEL
96. UGGT2
97. LIG4

98. *OR4N2*
99. *OR11H6*
100. *TEP1*
101. *RNASE4*
102. *OR10G3*
103. *TRAV12-2*
104. *OR6J1*
105. *HEATR5A*
106. *C14orf182*
107. *MAP4K5*
108. *FERMT2*
109. *KTN1*
110. *AL391152.1*
111. *SYNE2*
112. *PLEKHH1*
113. *SLC39A9*
114. *ABCD4*
115. *SAMD15*
116. *GALC*
117. *NRDE2*
118. *TRIP11*
119. *ATXN3*
120. *SERPINA6*
121. *NPAP1*
122. *EMC7*
123. *FSIP1*
124. *INO80*
125. *MGA*
126. *BP1*
127. *ZNF106*
128. *SEMA6D*
129. *SEMA6D*
130. *ADPGK*
131. *PML*
132. *PEAK1*
133. *CHRNA5*
134. *ADAMTS7*
135. *C15orf40*
136. *WDR73*
137. *ZNF592*
138. *ACAN*
139. *TICRR*
140. *WDR93*
141. *C15orf32*
142. *RGMA*

143. *TEKT5*
144. *PRM3*
145. *CTD-3088G3.8*
146. *KNOP1*
147. *POLR3E*
148. *USP31*
149. *PALB2*
150. *TNRC6A*
151. *PRR14*
152. *FBXL19*
153. *ZNF267*
154. *IGHV3OR16-13*
155. *ABCC12*
156. *NLRC5*
157. *CDH11*
158. *FAM65A*
159. *ACD*
160. *NRN1L*
161. *SLC12A4*
162. *CDH3*
163. *CDH1*
164. *NPIP15*
165. *GLG1*
166. *RFWD3*
167. *NUDT7*
168. *PKD1L2*
169. *CMIP*
170. *MTHFSD*
171. *MAP1LC3B*
172. *CTU2*
173. *GALNS*
174. *ZNF276*
175. *WDR81*
176. *SMG6*
177. *SGSM2*
178. *AC006435.1*
179. *OR1A2*
180. *TRPV3*
181. *TRPV1*
182. *SHPK*
183. *TRPV1*
184. *GGT6*
185. *VMO1*
186. *TMEM102*
187. *DNAH2*

188. *KDM6B*
189. *DNAH9*
190. *NCOR1*
191. *MPRIP*
192. *MYO15A*
193. *AKAP10*
194. *CCDC144NL*
195. *KIAA0100*
196. *ATAD5*
197. *SYNRG*
198. *PSMD3*
199. *KRT28*
200. *KRT39*
201. *KRT40*
202. *KRT33B*
203. *KRT14*
204. *ACLY*
205. *AOC3*
206. *B4GALNT2*
207. *ITGA3*
208. *MYCBPAP*
209. *COG1*
210. *C17orf80*
211. *TTYH2*
212. *LLGL2*
213. *MYO15B*
214. *Sep-09*
215. *TMC6*
216. *DNAH17*
217. *CANT1*
218. *RNF213*
219. *OGFOD3*
220. *HEXDC*
221. *C17orf62*
222. *RAB40B*
223. *USP14*
224. *C18orf56*
225. *NDC80*
226. *TGIF1*
227. *APCDD1*
228. *CIDEA*
229. *ZNF521*
230. *TAF4B*
231. *ASXL3*
232. *C18orf21*

233. *MOCOS*
234. *FHOD3*
235. *PCSK4*
236. *TMPRSS9*
237. *TBXA2R*
238. *PLIN4*
239. *RFX2*
240. *ACTN4*
241. *ECH1*
242. *AC104534.3*
243. *ECH1*
244. *AHSA2*
245. *VPS54*
246. *SLC1A4*
247. *APLF*
248. *ARHGAP25*
249. *GKN2*
250. *FIGLA*
251. *MPHOSPH10*
252. *MOGS*
253. *MRPL53*
254. *TTC31*
255. *LBX2*
256. *DNAH6*
257. *CD8B*
258. *ANKRD36C*
259. *STARD7*
260. *ITPRIPL1*
261. *NCAPH*
262. *FAM178B*
263. *VWA3B*
264. *IL1RL1*
265. *HOXD1*
266. *PDE11A*
267. *PRKRA*
268. *TTN*
269. *CCDC141*
270. *COL5A2*
271. *MFSD6*
272. *SPATS2L*
273. *AC079354.1*
274. *STK36*
275. *TTLL4*
276. *PRKAG3*
277. *CCDC108*

278. COL4A3
279. SPHKAP
280. SP110
281. SP110
282. ITM2C
283. SEL1L2
284. SCP2D1
285. CSTL1
286. BPIFB4
287. DHX35
288. ZHX3
289. PCK1
290. ZBP1
291. C20orf85
292. ZNF831
293. LAMA5
294. SRMS
295. HELZ2
296. RTEL1
297. TMPRSS3
298. KRTAP10-5
299. KRTAP10-12
300. PCNT
301. TRIOBP
302. KDEL3
303. NDUFA6
304. TCF20
305. PNPLA3
306. ATXN10
307. MOV10L1
308. TUBGCP6
309. KLHDC7B
310. IRAK2
311. PPARG
312. CAND2
313. C3orf20
314. FGD5
315. CX3CR1
316. ENTPD3
317. CCRL2
318. LRRC2
319. TDGF1
320. PRSS45
321. ZNF589
322. SPINK8

323. *USP19*
324. *FRMD4B*
325. *ZNF717*
326. *DHFRL1*
327. *CRYBG3*
328. *OR5H2*
329. *OR5K1*
330. *COMMD2*
331. *FAM194A*
332. *LEKR1*
333. *IFT80*
334. *RP11-432B6.3*
335. *IFT80*
336. *SPATA16*
337. *RTP4*
338. *ATP13A5*
339. *LSG1*
340. *XXYLT1*
341. *REST*
342. *UGT2B4*
343. *AMTN*
344. *C4orf26*
345. *TMEM150C*
346. *IBSP*
347. *HERC5*
348. *PYURF*
349. *CCSER1*
350. *SMARCAD1*
351. *CLDN24*
352. *ARHGEF28*
353. *SPZ1*
354. *VCAN*
355. *CCNH*
356. *KIAA0825*
357. *ANKRD32*
358. *RHOBTB3*
359. *ERAP1*
360. *LNPEP*
361. *SLCO6A1*
362. *ZRSR1*
363. *REEP5*
364. *YTHDC2*
365. *GABRP*
366. *SIMC1*
367. *CLK4*

368. *ZNF454*
369. *ZFP62*
370. *BTNL9*
371. *TRIM7*
372. *BTN3A3*
373. *HIST1H1B*
374. *CCHCR1*
375. *PRRC2A*
376. *PPT2*
377. *PPT2-EGFL8*
378. *EGFL8*
379. *NOTCH4*
380. *C6orf10*
381. *HLA-DOB*
382. *TAP2*
383. *PSMB8*
384. *BRD2*
385. *HLA-DOA*
386. *KIFC1*
387. *PNPLA1*
388. *KCTD20*
389. *CAPN11*
390. *TDRD6*
391. *EFHC1*
392. *FKBP1C*
393. *IRAK1BP1*
394. *PHIP*
395. *UBE3D*
396. *DOPEY1*
397. *PGM3*
398. *NT5E*
399. *GABRR1*
400. *MANEA*
401. *MMS22L*
402. *AKAP12*
403. *CCDC170*
404. *SYNE1*
405. *OPRM1*
406. *SCAF8*
407. *GPR146*
408. *GPBR1*
409. *IQCE*
410. *C1GALT1*
411. *VWDE*
412. *MEOX2*

413. *MACC1*
414. *ABCB5*
415. *STK31*
416. *CPVL*
417. *NPSR1*
418. *TRGC2*
419. *HECW1*
420. *ZMIZ2*
421. *TNS3*
422. *ABCA13*
423. *LANCL2*
424. *ZNF727*
425. *ZNF679*
426. *ZNF680*
427. *TYW1B*
428. *PCLO*
429. *LRRC61*
430. *KMT2C*
431. *ANK1*
432. *RB1CC1*
433. *TRPA1*
434. *SBSPON*
435. *STAU2*
436. *ZFAT*
437. *GPR144*
438. *AL590708.2*
439. *VAV2*
440. *C9orf141*
441. *CACNA1B*
442. *RBMXL3*

SUPPLEMENTARY PUBLICATIONS

Familial cholesteatoma in East Anglia, UK

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Main Article

Familial cholesteatoma in East Anglia, UK

P PRINSLEY

Abstract

Objective: To report a cluster of families affected by cholesteatoma in the East Anglia region of the UK.
Setting: Otolaryngology service for the population of Norfolk and North Suffolk, East Anglia, UK.

Method: Prospective and systematic collection of family history data for patients presenting with cholesteatoma over 10 years.

Result: Several families were identified with affected individuals over several generations.

Conclusion: There is likely to be a genetic propensity for cholesteatoma in some individuals.

Key words: Cholesteatoma; Genetic Diseases; Aetiology

Introduction

Since 1996, the author has worked as a consultant otolaryngologist, appointed with a 'specific role to assist in the provision of a specialist otology service to a population of 750 000 people in parts of Norfolk and Suffolk'.

Surgical otology has formed a major part of this work, with many cases being surgically treated for chronic otitis media and cholesteatoma. This paper presents the observation that a number of the patients diagnosed with and/or undergoing surgery for cholesteatoma, over a 10-year period, were either related to each other or related to other, previously operated upon patients.

The population of East Anglia, a region of east England, is mainly rural, with small towns and cities. It is a more settled, with stable population than those of the more urbanised and metropolitan areas of the country. It is not unusual to encounter extended families living fairly close together, with medical records at the same hospital stretching back several generations. This fact facilitated access to old records of previously operated upon patients.

Methods and results

The first family (family Ha; Figure 1) was detected following presentation of a set of non-identical twins, Ga and C, aged five years, who both presented with cholesteatoma, one bilaterally and one unilaterally. The mother volunteered the fact that the twins' elder brother and a cousin had undergone surgery for the condition. The brother happened to be present at the consultation, and on examination had a dry and healthy ear cavity (having undergone surgery in the

same hospital some years previously). The mother also reported that a brother of her father had died in childhood in the 1920s as a result of an 'ear infection'.

This family history prompted the author to begin to ask specifically about family history of cholesteatoma or surgery for ear problems, whenever a new patient with cholesteatoma was encountered.

Soon afterwards, two other affected families were discovered (families Sm and Ri), containing many affected individuals.

Family Sm came to light as a result of a local clinic nurse (K) requesting an ear examination, as she was having difficulties using her stethoscope. The nurse had undergone surgery some years previously for perforation of the ear drum. Endoscopic examination of the nurse's ear showed intratympanic cholesteatoma (Figure 2).

Nurse K was the sister of another patient with cholesteatoma, and the daughter of another. She said that cholesteatoma 'ran in the family'. Her son and two of her nieces had been diagnosed and operated upon for cholesteatoma.

Both K and her sister C had bilateral disease, as shown in Figure 3.

The strongest family pedigree was found in family Ri (Figure 4).

Patient D had been operated upon by the author for left-sided cholesteatoma in 1997, and then again in 1998 for right-sided disease. He reported that his elder brother T had been operated upon for right-sided cholesteatoma in 1974 and for left-sided cholesteatoma in 1981. This was readily confirmed by inspecting the hospital record. Patient D's younger brother Ji had also been operated upon for

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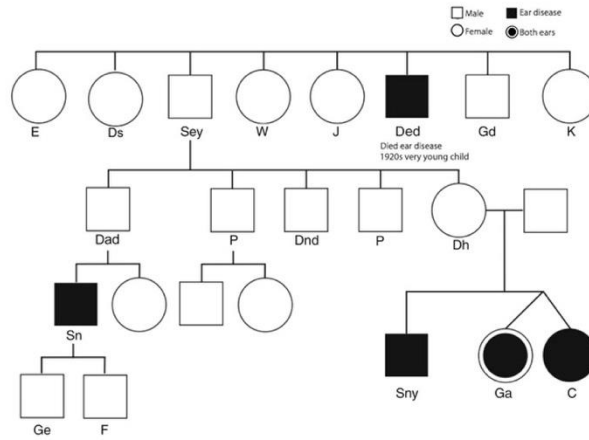


Fig. 1
Pedigree for family Ha.

cholesteatoma, as had both of D's parents, A (in both ears) and G (in one ear). Born in 1913, A had last been seen at the ear clinic in 1996 with bilateral, trouble-free ear cavities. The hospital records showed that she had been seen regularly at the Norfolk and Norwich Hospital ENT department for 60 years. In addition, D later reported that his son DJ was affected by cholesteatoma, and also his granddaughter (DJ's daughter) Ja. Although neither of these latter patients has been examined

by the author, if they were indeed affected by cholesteatoma then family Ri would contain four affected generations.

Simple enquiry about any family history of ear problems in all patients seen with cholesteatoma revealed four other individual families with three affected members and eight other individual families with two affected individuals, as shown in Table I.

Discussion

The aetiology of cholesteatoma is unknown, but genetic causes are not usually emphasised.

Conventionally, so-called congenital cholesteatoma arising behind an intact drum is considered distinct from acquired cholesteatoma associated with drum abnormalities. In this article, no formal



Fig. 2
Endoscopic view of K's ear (family Sm).

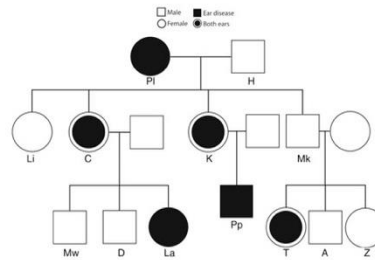


Fig. 3
Pedigree for family Sm.

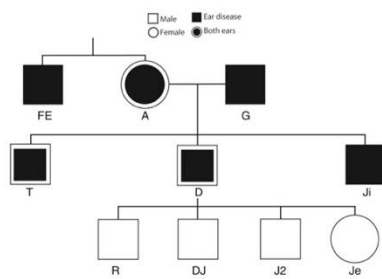


FIG. 4

Pedigree for family Ri. (Missing from the chart is DJ's cholesteatoma-affected daughter Ja.)

distinction is made between the two conditions, although the great majority of cases were associated with drum abnormalities and would not therefore normally be considered 'congenital'. Interestingly,

TABLE I
FAMILIES WITH CHOLESTEATOMA

Family	Affected individuals
Fx	CF, female, 8 y, cholesteatoma* LF, female, 8 y, identical twin of CF, CAT Holland* KF, mother of twins, mastoidectomy 1991 Sunderland
Al	MA, male, revision tympanoplasty 2000, 2nd revision 2005* AA, brother of MA, atticotomy 1994, revised 2003* MA, grandmother of brothers, mastoidectomy
Gl	VG, female, age ?, attic retraction* EL, brother of VG, mastoidectomy 1975 GY, maternal grandfather, died of ear abscess 1951 Great Yarmouth
Go	JG, female, 10 y, mastoidectomy 2003* MC, father of JG, cholesteatoma* JC, grandfather of JG, mastoidectomy
Ca	BC, mastoid obliteration 1998* PB, cousin of BC, mastoidectomy 1971, 1999*
Fa	DF, cholesteatoma 2006* PF, mother of DF, mastoidectomy 1954
Ni	GN, female, 8 y, tympanoplasty for cholesteatoma 2003* Grandmother had mastoidectomy 1950s Blackburn
Bu	MB, male, 30 y, mastoidectomy 2005* Father of MB had mastoidectomy Germany
We	TW, female, mastoidectomy 2005* MH, father of TW, mastoidectomy Northern England
Ph	SP, female, 62 y, mastoidectomy 2004* DS, brother of SP, mastoidectomy 1987
Do	DD, mastoidectomy 1978 DR, brother of DD, mastoidectomy 1994
Ba	JB, female, cholesteatoma 2006* LB, mother of JB, mastoidectomy 1984 Southend

*Treated by the author. Y = years; CAT = combined approach tympanoplasty

the endoscopic appearance of K's ear (Figure 2) might suggest a congenital cholesteatoma in the presence of an intact drum, but the history of previous surgery implies a possible secondary acquired mechanism. Lesinkas *et al.*,¹ writing in Lithuania in 2002, reviewed the theories of congenital cholesteatoma development, which include ectopic cell rests, ingrowth of meatal epidermis and metaplasia of refluxed amniotic cells. Theories of acquired cholesteatoma development include retraction pocket disease, basal cell proliferation, immigration of epithelium through a perforation, and squamous metaplasia of the middle-ear epithelium. A 2000 literature review by Tos² also suggested a variety of ways in which epithelial cell inclusions within the mesotympanum may develop in acquired cholesteatoma in children. These relate to an increase in the dynamics of the tympanic membrane, eustachian tube problems, otitis media and retractions, rather than congenital inclusions. A family history of the condition, however, is not usually considered to be of importance. However, an epidemiological study of kibbutz dwellers in Northern Israel in 1986 did identify a family history in 64 per cent of cholesteatoma patients.³ In 2006, Homoe and Rosborg⁴ claimed to publish the first report of an affected family, with a mother and three of seven children having cholesteatoma, in Greenland.

The three East Anglian families described in the present study also support the theory of a genetic cause in some cases of cholesteatoma.

Bilateral cholesteatoma may be partly explained by genetic factors. Family Ri included a mother with bilateral disease and a father with unilateral disease who produced three children, all with cholesteatoma (two of whom had bilateral disease). If there is a strong genetic propensity, as there seems to be in this family, then it might be reasonable to suppose that the chance of bilateral disease would be greater.

- The aetiology of cholesteatoma is unknown, but genetic causes are not usually emphasised
- This observation of familial clustering of cholesteatoma patients in East Anglia is remarkable
- It supports the suggestion that a genetic predisposition exists for cholesteatoma

The mechanism of genetic influence, if one exists, is unknown. One proposal is that cholesteatoma may be due to altered genetic control of cellular proliferation. Recent molecular biology research suggests mechanisms by which genes might influence the behaviour of epithelial cells within the middle ear. Ectodermal remnants or cellular ingrowths are normally eliminated in mesenchymal tissue by apoptosis. This process may fail in cholesteatoma. Genes which control epithelial cell proliferation and differentiation have been studied. Albino *et al.*⁵ found increased expression of the nuclear phosphoprotein

p53 tumour suppressor gene in cholesteatoma, compared with postauricular skin. This gene is involved in the regulation of the cell cycle and apoptosis. Tokurika *et al.*⁶ used complementary deoxyribonucleic acid arrays to compare the cell biology of cholesteatoma tissue with that of skin taken from the retroauricular sulcus during surgery on the same patients. They described up-regulation of proliferation and differentiation genes in the cholesteatoma cells. A similar comparative study of cholesteatoma and retroauricular skin, by Kwon *et al.*,⁷ used microarrays and also identified a large number of up-regulated genes in the cholesteatoma tissue.

Conclusion

This observation of familial clustering of cholesteatoma patients in East Anglia is remarkable. It supports the suggestion that a genetic predisposition exists for this condition.

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The genetics of cholesteatoma. A systematic review using a narrative synthesis.

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ORIGINAL ARTICLE

WILEY

The genetics of cholesteatoma. A systematic review using narrative synthesis

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Objective: A cholesteatoma is a mass of keratinising epithelium in the middle ear. It is a rare disorder that is associated with significant morbidity, and its causative risk factors are poorly understood; on a global scale, up to a million people are affected by this each year. We have conducted a systematic literature review to identify reports about the heritability of cholesteatoma or any constitutional genetic factors that may be associated with its aetiology.

Data Sources: A systematic search of MEDLINE (EBSCO) and two databases of curated genetic research (OMIM and Phenopedia) was conducted.

Study Selection: The participants and populations of interest for this review were people treated for cholesteatoma and their family members. The studies of interest reported evidence of heritability for the trait, or any association with congenital syndromes and particular genetic variants.

Data Extraction: The searches identified 449 unique studies, of which 35 were included in the final narrative synthesis.

Data Synthesis: A narrative synthesis was conducted, and data were tabulated to record characteristics, including study design, genetic data and author conclusions. Most of the studies identified in the literature search, and described here, are case reports and so represent the lowest level of evidence. In a few case reports, congenital and acquired cholesteatomas have been shown to segregate within families in the pattern typical of a monogenic or oligogenic disorder with incomplete penetrance. Evidence from syndromic cases could suggest that genes controlling ear morphology may be risk factors for cholesteatoma formation.

Conclusions: This is the first systematic review about the genetics of cholesteatoma, and we have identified a small body of relevant literature that provides evidence of a heritable component for its aetiology. Cholesteatoma is a complex and heterogeneous clinical phenotype, and it is often associated with chronic otitis media and with some rare congenital syndromes known to affect ear morphology and related pathologies.

Barbara A. Jennings and Peter Prinsley contributed equally to the manuscript.

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1 | INTRODUCTION

A cholesteatoma is a self-perpetuating erosive lesion composed of stratified keratinising squamous epithelium in the middle ear.¹ Cholesteatoma has both an acquired and a congenital form. It activates osteoclasts and so will erode through bone, which may include the endocranium, with an attendant risk of life-threatening intracranial infection.

The acquired form of cholesteatoma originates as an inward growth from the lateral epithelium of the tympanic membrane. A typical sequence of events in the onset of the disease includes a history of chronic otitis media (COM) in childhood, subsequent development of retraction of the tympanic membrane and then a cholesteatoma developing within and perforating through this retraction. This seems to particularly occur if the retraction is located in the superior tympanic membrane (pars flaccida).²⁻⁴ In children with a history of chronic otitis media with effusion (COME), 15-35% will develop a retraction of the pars flaccida (at up to 25 years of follow-up), but only 0.1-2% will develop a cholesteatoma (at up to 8 years of follow-up).⁴⁻⁷ Both presence and duration of COME are predictive of tympanic membrane retraction,^{3,4} but tympanic retraction has been documented to occur in the absence of preceding COME.⁴ However, histological studies suggest that in such cases there is nevertheless chronic middle ear inflammation, it is just not clinically apparent.⁸ Thus, cholesteatoma is often preceded by COM, but only a small proportion of those with COM will develop cholesteatoma. What determines the transition from COM to cholesteatoma is not known, but could be due to environmental factors, heritable factors or random effects. But those who develop cholesteatoma have been reported to have between a 7% and a 20% chance of developing disease in the contralateral ear,^{9,10} highlighting the importance of shared genes and shared environments.

Cholesteatoma can also be found behind an intact tympanic membrane.¹¹ This form is thought to be congenital, and may result from persistence of the foetal epidermoid formation, a small collection of squamous epithelial cells in the middle ear that normally undergoes apoptosis before or shortly after birth. Congenital cholesteatoma can grow laterally and erode through the tympanic membrane, and at that point, it can be difficult to differentiate congenital from acquired disease.

Cholesteatoma is a rare disorder (1:10 000 per year),¹ and therefore, epidemiological studies are difficult to conduct, and causative risk factors are still poorly understood. The citations about cholesteatoma in the definitive catalogue of genes and genetic diseases, *Online Mendelian Inheritance in Man*,¹² document minimal evidence for the Mendelian inheritance of this disorder.¹³ However, reports of familial clustering of disease and of association with genetic syndromes (reviewed here) suggest underlying, but as yet unidentified genetic risk factors. Identifying these could enhance our understanding of disease biology, and open up pathways for diagnostic, screening and therapeutic interventions.

Keypoints

- We have synthesised data from 35 published studies in the first systematic review about the genetics of cholesteatoma.
- Only low quality evidence from case reports, case series, and small epidemiological studies was identified.
- Familial clustering suggests a possible genetic component to risk of cholesteatoma, and evidence from congenital syndromes suggests this could relate to loci regulating ear embryology.

One way to identify candidate genetic factors is through analysis of products of gene expression in pathological specimens. There are two published large-scale analyses comparing RNA transcript expression in cholesteatoma to that in skin of the external auditory canal. These have shown several hundred genes are differentially regulated in cholesteatoma samples, including genes with products involved in growth, differentiation, signal transduction, cell communication, protein metabolism and cytoskeleton formation.^{14,15} However, the results from these studies are inconsistent, and are measuring gene expression once cholesteatoma has formed, and so have failed to significantly further our understanding of constitutional risk.

Here, we describe findings from a systematic review of the genetics of congenital and acquired cholesteatoma. Our aims from this review were to describe how susceptibility is transmitted within families showing disease clustering, to better understand the genetic architecture of disease, and to document any genotypes shown to co-segregate with the cholesteatoma phenotype. We also aimed to classify genetic syndromes associated with increased risk of cholesteatoma, which may implicate candidate genetic loci for further investigation.

2 | MATERIALS AND METHODS

2.1 | Objectives

To synthesise published evidence that addresses the following questions:

1. Can the development of a cholesteatoma be described as a heritable trait, or is there a genetic predisposition to cholesteatoma within some families?
2. Have any genetic alterations or congenital syndromes been associated with cholesteatoma?

2.2 | Registration of systematic review method

The protocol was registered with the PROSPERO International prospective register of systematic reviews database in June 2015.¹⁶

2.3 | Search strategy

We searched the MEDLINE (EBSCO), OMIM (<http://www.ncbi.nlm.nih.gov/omim>) and Public Health genomics Knowledge Base (<https://phgkb.cdc.gov/HuGENavigator/startPagePhenoPedia.do>) from 1980 to July 2015 using the terms "Cholesteatoma" AND "famil*" [OR Gene* OR hered* OR inherit* OR syndrom* OR kindred OR pedigree OR oncogene* OR tumour suppressor OR tumor suppressor OR epigenetic* OR mutat* OR somatic OR homeobox].* We supplemented the search with relevant references identified in the citation lists at the article review stage.

2.4 | Inclusion and exclusion criteria

Studies were identified from the titles and abstracts by the primary reviewer (BAJ) and secondary reviewer (GW) using the following inclusion criteria:

1. Primary studies of kindreds that provide information about familial clustering.
2. Primary epidemiological studies that provide evidence of heritability including ethnic differences.
3. Relevant systematic reviews that provide information about genetics or heredity for cholesteatoma.
4. Case reports that refer to familial clustering of the cholesteatoma phenotype (>1 family member affected).
5. Case reports or epidemiological studies that provide evidence of association between cholesteatoma and syndromes

Studies were excluded if they were general narrative reviews or opinion pieces, about non-human or experimental disease models, or described pathologies other than cholesteatoma.

2.5 | Study selection & data extraction

Full reports of potentially relevant articles were retrieved, and data were extracted by the primary reviewer (BAJ). The study design, patient characteristics and nature of the outcomes were collated and coded red for exclusion, green for inclusion and amber to indicate uncertainty (RAG review). When there were uncertainties about inclusion or data interpretation, the articles were discussed by the reviewers (GW, MB and BAJ) to reach consensus. All studies that met the inclusion criteria were included regardless of quality, which was subsequently appraised (see Risk of bias and quality assessment below).

2.6 | Data synthesis

A narrative synthesis was conducted to explore the review questions about heritability and genetic associations reported for the cholesteatoma phenotype. We tabulated the date of the study, first author, study design, number of subjects, subtype of cholesteatoma, genetic investigations (including family history), associated congenital

syndromes, gene nomenclature and direct quotations from discussion or conclusions.

2.7 | Risk of bias and quality assessment

We appraised quality of epidemiological studies by reference to the Strengthening Reporting of Observational Studies (STROBE) guidelines¹⁷ and the Strengthening Reporting of Genetic association Studies (STREGA) guidelines.¹⁸ We mapped the evidence for each study to the five levels of evidence described by the Oxford Centre for Evidence Based Medicine.¹⁹

3 | RESULTS

3.1 | Study selection & data extraction

Our search identified 449 unique studies, of which 36 met the initial inclusion criteria. Most studies were excluded at the abstract or primary manuscript review stage, but six manuscripts were excluded at the data extraction stage because there were no relevant primary data identified about cholesteatoma or genetic phenomena,²⁰⁻²³ or because the study described external auditory canal cholesteatoma.²⁴ The studies identified in the initial search were supplemented by five additional reports identified by hand-searching citation lists.²⁵⁻²⁹ Thirty-five studies were finally included in this narrative synthesis (see Figure 1 for a flow chart which summarises these steps).

3.2 | Familial clustering

Nine studies (classified as case reports, case series and epidemiological studies) present evidence for familial clustering of cholesteatoma.^{25,27,28,30-35} The extracted study characteristics are described in Table 1.

3.3 | Congenital syndromes and cholesteatoma

Twenty-two case reports and epidemiological studies describe the occurrence of cholesteatoma in patients affected by congenital and malformation syndromes,^{13,26,30,35-52} several of which have a known underlying genetic aetiology. These are summarised in Table 2.

Some of these reports are of cholesteatoma occurrence in a single case of a particular syndrome, for example Beckwith-Wiedemann syndrome, granulomatosis with polyangiitis, Nager syndrome, primary ciliary dyskinesia, Tolosa-Hunt syndrome, Treacher Collins syndrome and Wolf-Hirschhorn syndrome. Single occurrences of a disease, whether associated with a syndrome or not, are susceptible to publication bias and so do not add to understanding of disease risk in isolation.

3.4 | Candidate genes and gene variants

We identified just two published studies of DNA-based laboratory investigations of particular gene sequences considered in association

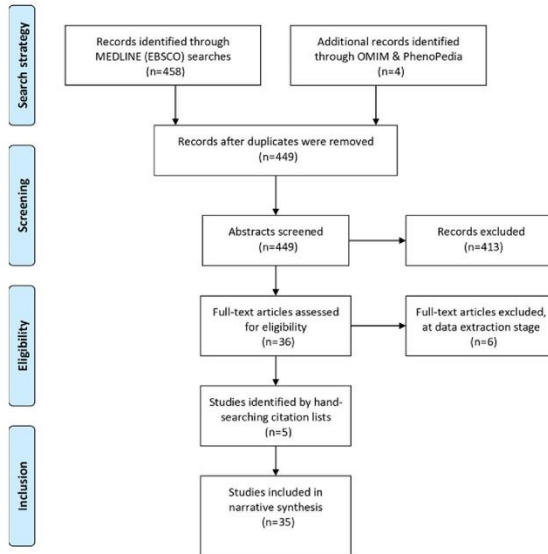


FIGURE 1 The identification and screening of studies for inclusion in the narrative synthesis is illustrated in this PRISMA flow diagram. OMIM: Online Mendelian Inheritance in Man,¹² Phenopedia is an information database curated by the US Center for Disease Control (CDC)

with the cholesteatoma phenotype. One is a case report of a 6-year-old boy with a congenital cholesteatoma who was shown to have a deletion in the *APC* tumour suppressor gene.⁵³ The other is a candidate gene association study of polymorphisms of the *GJB2* and *GJB6* loci that encode connexins⁵⁴ in a cohort of 98 children undergoing surgery for cholesteatoma. These studies are also described in Table 3.

3.5 | Risk of bias and quality assessment

We identified only a small body of literature that was relevant to our questions about a heritable component for cholesteatoma aetiology. Many of the studies provide some indirect evidence only, given that the authors' objectives were to describe cholesteatoma management or associated environmental factors.

Most of the studies identified in the literature search, and described here, are case reports and so represent the lowest level of evidence. Case reports were automatically categorised as level 5 (see Tables 1, 2 and 3). The remaining observational studies include case series, cross-sectional surveys, case-control studies and cohort studies; each of these manuscripts was reviewed by BAJ and GW to define the level of evidence presented; STROBE and STREGA guidelines were referred to in classifying the quality of the methodology used in the case-control and cohort studies. The level of evidence ranged from 4 (for low-quality case-control studies, surveys and case

series) and 2b for a high-quality cohort study^{35,49} (see Tables 1, 2 and 3).

4 | DISCUSSION

This is the first systematic review to explore the constitutional genetics of individuals affected by cholesteatoma. We have synthesised data from 35 published studies about familial aggregation of disease. The association of cholesteatoma with congenital syndromes and genes that were directly analysed in patients with cholesteatoma were also considered.

4.1 | Heritability

We have summarised the published evidence about the heritability of acquired and congenital cholesteatomas. We only identified a few case reports and case series that show two or more affected first-degree relatives; therefore, there is insufficient evidence to describe cholesteatoma as a heritable trait.

However, there are some compelling individual observations to consider, including affected monozygotic²⁷ and dizygotic twins,^{28,33} families with two or more affected generations,^{30,32,33} and high rates of bilateral disease in affected families.^{28,33} Such observations suggest rare genetic variants underlie the disease in some families.

TABLE 1 Familial clustering of the cholesteatoma phenotype

Year of publication	First author	Study design	Number of subjects	Phenotypes CC = congenital cholesteatoma AC = acquired cholesteatoma	Author conclusions extracted from manuscript (original language in parentheses)	Level of evidence
1973	Ray, J.	Case reports	2 siblings	Intramastoid CC in two brothers aged 3 years, and 8.5 months.	"First, and above all, the cholesteatomas are considered to be congenital. (D'abord et surtout parce qu'elles sont a inscrire au repertoire des cholesteatomes congenitaux.)"	5
1986	Lipkin, A.F.	Case reports	2	A mother and her daughter had CC treated at age 5.	"These two cases may represent a unique variant of branchio-oto dysplasia."	5
1986	Podoshin, L.	Prospective cross-sectional survey of Kibbutz population	3056 individuals; 12 (0.4%) had a cholesteatoma.	Unilateral cholesteatoma in 11 cases; bilateral disease in one case. No data presented on congenital/ acquired subtypes but histological examination of surgical tissue was carried out to confirm diagnoses.	"Among cholesteatoma patients a family history was found in 64%... One or more of their close family members had chronic otitis media or cholesteatoma."	4
1986	Naito, Y.	Case reports	2 siblings	Bilateral cholesteatoma in one dizygotic twin and unilateral cholesteatoma in other. Developed on a background of long-standing otitis media with effusion.	"More dizygotic and monozygotic twins with cholesteatoma should be studied to decide whether hereditary factors have any significant influence on the occurrence of this disease."	5a
2007	Homoe, P.	Case series	Family; two parents and seven siblings.	Mother and three siblings have AC; all surgically treated. All five family members seen had a dolicocephal appearance.	"to our knowledge this is the first report in the world literature of family clustering of AC."	4
2009	Prinsley, P.	Case series	15 families with ≥ 2 family members affected by cholesteatoma.	"no distinction is made but the majority had drum abnormalities and would not therefore normally be considered congenital."	"this observation of family clustering in East Anglia is remarkable." "It supports the suggestion that a genetic predisposition exists for cholesteatoma."	4
2013	Al Balushi, T. 2013	Case reports	2	CC	"we report congenital cholesteatoma in identical twins, a previously unreported occurrence."	5

(Continues)

TABLE 1 (Continued)

Year of publication	First author	Study design	Number of subjects	Phenotypes CC = congenital cholesteatoma AC = acquired cholesteatoma	Author conclusions extracted from manuscript (original language in parentheses)	Level of evidence
2013	Landegger, L.D.	Case report	2.	CC The index case was diagnosed at 34 months, his older brother was treated for CC at 2 years of age.	"This case joins a single, previous report describing congenital cholesteatoma in multiple family members, suggesting that in some cases, hereditary factors may play a role in the formation of the disease."	5
2015	Djurhuus, B.D.	Historic Cohort Study (Health Outcomes and demographic data extracted from Danish Cleft Lip and Palate database and the Danish National Patient Register)	441 014 individuals were included in the study, comprising 8593 cases of orofacial cleft and 6989 siblings and a random control group from the Danish population.	201 and 21 Surgically treated cholesteatomas in a population affected by orofacial cleft and their siblings, respectively. The authors note that "in Denmark, cholesteatomas are rarely histologically examined."	"We found a twofold increased risk of cholesteatoma in siblings of cleft palate cases." "A possible increased risk of cholesteatoma in siblings of CP cases may be explained by familial aggregation of subclinical muscular defect."	2b

Studies are presented in chronological order by year of publication.

The levels of evidence were mapped to those described by the Oxford Centre for Evidence Based Medicine.¹⁹

^aData from Naito et al. extracted from abstract only.

If a disease is inherited as a monogenic or oligogenic trait, next generation sequencing (NGS) studies⁵⁵ can now be used to identify DNA variants that are co-inherited with that trait. NGS studies of affected family members may reveal mutations that are unique to a single kindred (these are known as *private* mutations). But such findings can also be generalisable if they identify the genes and biological pathways that are altered in other cholesteatoma patients with more complex aetiologies.

Observations about the familial aggregation of phenotypes are often followed by more discriminating epidemiological methods to distinguish the influence of heritability from shared environments. But because cholesteatoma is rare, a classical twin study has not been conducted and is not feasible. However, a study or register to collect data about bilateral disease might provide information about a genetic component to the aetiology of cholesteatoma. The incidence of bilateral disease for individuals could be compared to the coincidence of disease in dizygotic twins, in a manner analogous to studies of monozygotic vs dizygotic twins.

4.2 | Congenital syndromes

Several lines of evidence suggest that variants in genes regulating ear embryogenesis and tissue architecture also increase the risk of

cholesteatoma. For example, congenital cholesteatoma is more common in the malformed ears of people with branchio-oto-renal syndrome.

The association of acquired cholesteatoma with Down's syndrome, Turner syndrome and cleft palate is more difficult to disentangle, as these syndromes also place individuals at increased risk of COME, which often precedes development of cholesteatoma. Whether these syndromes are in themselves associated with increased risk of cholesteatoma is difficult to say. In contrast, Djurhuus et al.³⁵ showed a doubling of risk of cholesteatoma in siblings of patients with cleft palate. This finding should nevertheless be taken with some caution, the associated *P*-value was .026, which would be considered insignificant if it had been subject to Bonferroni adjustment due to the multiple hypothesis testing present in this study.

4.3 | Gene associations

Little evidence is presented in our results about the role of particular genes in cholesteatoma biology because only two studies reported the analysis of gene sequences: a case report and a small candidate gene association study. The case report describes a 6-year-old boy affected by familial adenomatous polyposis (FAP) who had cholesteatoma, and an inherited deletion in the tumour suppressor gene

TABLE 2 Reports of cholessteatoma and congenital syndromes

Name of syndrome	First author and year of publication	Study design	Number of subjects	Subtype CC = congenital cholessteatoma AC = acquired cholessteatoma (original language in parentheses)	Genetic investigations and genetic loci noted	Author conclusions extracted from manuscript (original language in parentheses)	Level of Evidence
Beckwith-Wiedemann syndrome OMIM #130650	Daugbjerg, P. 1994	Case Report	7-year-old female	A small primary CC between the long process of incus and the handle of the malleus.	None	"The primary cholessteatoma might represent a fetal detachment of epidermis."	5
Branchio-otorenal syndrome OMIM #113650	Graham, G.E. 1999	Case Report	14-month-old female	Unilateral CC. Also, bilateral middle ear anomalies and abnormal inner ear morphology.	None	"Our index patient, represents the third individual reported to have congenital cholessteatoma in association with BOR syndrome."	5
	Lipkin, A.F. 1986 (also presented in Table 1)	Case Report	Mother and daughter. Both treated for cholessteatoma at 5 years.	CC	None	"These two cases may represent a unique variant of branchio-oto dysplasia."	5
	Worley, G.A. 1999	Case Report	8-year-old female	Bilateral CC	None	"An association between Branchio-otorenal syndrome and congenital cholessteatoma has never been documented."	5
Cleft palate OMIM #119530 1988	Djurhuus, B.D. 2015 (also presented in Table 1)	Historic Cohort Study	8592 cases of orofacial cleft; 201 of whom were treated for cholessteatoma.	Surgically treated cholessteatoma.	None	"A 20-fold increase in risk of cholessteatoma was found for individuals with cleft palate."	2b
	Lau, C.C. 1988	Cross-sectional study.	83 patients with cleft palate; two (6%) patients had cholessteatoma.	34 (23%) had middle ear disease including cholessteatoma.	None	"There is a need for early otological assessment of all patients with cleft palate at an early age."	4

(Continues)

TABLE 2 (Continued)

Name of syndrome	First author and year of publication	Study design	Number of subjects	Subtype CC = congenital cholesteatoma AC = acquired cholesteatoma (original language in parentheses)	Genetic investigations and genetic loci noted	Author conclusions extracted from manuscript (original language in parentheses)	Level of Evidence
Down Syndrome OMIM #190685	Bacchi, A. 2005	Retrospective observational study	Nine children with Down's syndrome who had been surgically treated for cholesteatoma	AC; two had bilateral disease.	Trisomy 21	"cholesteatoma is found to be more severe and extensive (in children with Down syndrome), probably related to a delay in diagnosis."	4
	Saito, R. 1983	Case Report	7-day-old girl	CC and congenital aural atresia	"Triomy of G group chromosome" (either -21 or -22) n.d. authors included this case report in the Down's syndrome subclassification because of cytogenetic findings but no confirmation in the manuscript.	"The formation of cholesteatoma was observed through the intermingling of the stratified squamous epithelium in the mucous epithelium of the hypotympanum."	5
	Suzuki, C. 2004	Case Reports	45-year-old female with Down's syndrome and 77-year-old male	Rupture of cholesteatoma.	None	"Infection of cholesteatoma matrix, rupture in particular, might have produced the easy transfer of endogenous substances from the cholesteatoma sac and epithelial debris that provoked the bone destruction in cholesteatoma."	5
Focal Dermal Hypoplasia (FDH)/Goltz syndrome, OMIM #305600	Reber, T. 1987	Case Report	21-year-old male.	"...partial cholesteatoma development in the upper part of the left tympanum ... (links Trommelfell subtotale randständige, im oberen Anteil polyose Cholesteatombildung) ..."	Cytogenetic analysis revealed a normal male karyotype, 46,XY.	"The detected cholesteatoma present in our patient is probably linked to GGS (Möglicheweise steht das bei unserem Patienten festgestellte Cholesteatom ebenfalls in Zusammenhang mit dem GGS.)"	5
	Buchner, S.A. 1992	Case Report	68-year-old male diagnosed with FDH; treated for cholesteatoma at 56 years.	Bilateral cholesteatoma	No genetic report but syndrome associated with X-linked dominant pattern of inheritance	"Of interest in this case is the development of bilateral cholesteatoma. To date, only one case of cholesteatoma associated with FDH has been reported in the literature."	5
Granulomatous Polyangitis [Wegener's Granulomatosis] OMIM #68720	Komblut, A.D. 1982	Case series	60 patients with Granulomatosis Polyangitis	Four case reports of aural disease, one case of cholesteatoma in a 77-year-old male.	None	"Only one patient...was found to have a cholesteatoma, and required radical mastoidectomy for management."	4

(Continues)

TABLE 2 (Continued)

Name of syndrome	First author and year of publication	Study design	Number of subjects	Subtype CC = congenital choisteatoma AC = acquired choisteatoma (original language in parentheses)	Genetic investigations and genetic loci noted	Author conclusions extracted from manuscript (original language in parentheses)	Level of Evidence
Nager Syndrome [Nager acrofacial dysostosis] OMIM #154400	Herrmann, B.W., 2005	Retrospective case series	10 patients with Nager acrofacial dysostosis.	Choisteatoma in one patient.	None	"pediatric patients with Nager acrofacial dysostosis exhibit conductive hearing loss due to middle and external ear pathology."	4
Microtia OMIM #610706 & #612290	Jin, L., 2010	Retrospective cross-sectional study	208 microtia patients (21 of 208 had a family history of microtia)	15 (7.2%) cases had middle ear choisteatoma	None	15 cases (7.2%) had middle ear choisteatoma. "Therefore we suggest that every patient with microtia undergo a temporal CT scan examination at an early age."	4
Primary Ciliary Dyskinesia	el-Sayed, Y., 1997	Case series	16 electron microscope-confirmed cases observed between 1971 and 95.	One adult case had bilateral choisteatoma treated with bilateral mastoidectomy.	None	"Otitis media is a prominent part of the primary ciliary dyskinesia syndrome."	4
Tolosa-Hunt Syndrome	Colnaghi, S., 2006	Case Report	40-year-old male	40-year-old male surgically treated for a collapsed right middle ear choisteatoma.	None	"There are no previous reports about a relationship between Tolosa-Hunt syndrome and choisteatoma. In the present case we only wish to point out the association between the two diseases, without any causal implications."	5
Treacher Collins Syndrome OMIM #154500 & #606847	Mann, W., 2014	Case Report	16-year-old male.	Hypotympanic choisteatoma.	None	"50% of TC patients have a congenital ...hearing loss resulting from major or minor ear anomaly."	5

(Continues)

TABLE 2 (Continued)

Name of syndrome	First author and year of publication	Study design	Number of subjects	Subtype CC = congenital cholestoma AC = acquired cholestoma (original language in parentheses)	Genetic investigations and genetic loci noted	Author conclusions extracted from manuscript (original language in parentheses)	Level of Evidence
Turner Syndrome OMIM references #312865 (X-linked loci)	Bergamaschi, R., 2008	Observational Case Series	173	10 cases of cholestoma (5.7%); and 9 of 10 had bilateral disease.	45:XO	"high prevalence of middle ear infections and CHL in TS probably due to growth disturbances of the structures from the 1st and 2nd branchial arches" "the incidence of cholestoma is higher in these children than in the general population."	4
	Hall, J.E. 2009	Retrospective case series	178	Six patients (3.4%) were found to have cholestoma	None	"recurrent and chronic otitis media is common in patients with Turner syndrome."	4
	Lim, D.B. 2014	Retrospective Cohort Study	179	Seven (3.9%) had cholestomas; two had bilateral disease	"risk factors include 45X and 46(Xq)"	"This study has confirmed a cholestoma prevalence approximately 1000 times higher than in the general population." "Our data support the observation that loss of one of the short arm pairs, for example through 45X monosomy or duplication of the q arm of X (DXq) is a risk factor for serious middle ear disease."	2b
Wolff-Hirschhorn Syndrome	Ilino, Y. 1987	Case Report	7-month-old female	"...two types of cholestomas in the middle ear cleft of the right temporal bone" Cholestatoma, which might be congenital in origin"	46,XX,4p- (deletion of the short arm of chromosome 4).	"the most interesting finding was the presence of two types of cholestoma in the middle ear cleft of the right temporal bone. It might have been derived from developmental migration of admixed epithelia in the middle ear cleft."	5
Unclassified Congenital Syndromes	Vaglio, A. 2008	Case Report	9-year-old female with dysmorphism and clinical features that included the absence of speech and poor mobility	Treated with surgery for cholestoma in her right ear.	46,XX,add(22)(q13.3) (pericentromeric bisatellited chromosome 22 associated with partial trisomy of chromosome 22). Duplication was paternal in origin.	"The serious clinical picture reported suggests that the proximal 22q region contains dosage-sensitive genes involved in development."	5

OMIM #: the numerical identifier used to catalogue entries about genes and traits in the Online Mendelian Inheritance in Man.¹²
The levels of evidence were mapped to those described by the Oxford Centre for Evidence Based Medicine.¹⁹

TABLE 3 Genetic association studies for choledochal cyst

First author and year of publication	Study design	Number of subjects	Subtype CC = congenital choledochal cyst AC = acquired choledochal cyst	Genetic investigations	Gene Loci & OMIM number	Author conclusions (extracted from manuscript)	Level of evidence
James, A.L. 2010	Candidate gene association study	98 children	Cholesteatoma cases include CC, AC and secondary acquired/implantation cholesteatoma.	Sequencing and MLPA analysis in candidate gene association study. But there was no control group data for comparison or HWE analysis.	GJB2 & GJB6 genes that encode connexions	"GJB2 gene variants are present in a minority of the sample of children with cholesteatoma (1.4%) but more commonly than in most normal populations."	4
Shaoul, R. 1999	Case report	6-year-old boy	CC	APC mutation analysis in mother and child (index case). A 5-bp deletion found in exon 15 of APC.	APC gene OMIM #175100	"This observation of a clinical association between FAP and cholesteatoma in this case may strengthen an association of the role of APC gene mutations with abnormal control of cell growth and spatial organisation."	5

All gene symbols are congruent with Human Genome Organisation (HUGO) nomenclature and linked data about each locus can be found at The Human Gene Database GeneCards® (<http://www.genecard.org/cgi-bin/candidsp.cgi?gene=GJB2>; <http://www.genecard.org/cgi-bin/candidsp.cgi?gene=GJB6>; and <http://www.genecard.org/cgi-bin/candidsp.cgi?gene=APC>). OMIM #: the numerical identifier used to catalogue entries about genes and traits in the Online Mendelian Inheritance in Man;¹² MLPA; multiplex ligation-dependent probe amplification; HWE: Hardy-Weinberg Equilibrium. The levels of evidence were mapped to those described by the Oxford Centre for Evidence Based Medicine.¹⁹

APC. The APC protein is expressed in many tissue types, influencing cell migration, adhesion and morphogenesis. Loss of APC expression in the colonic epithelium leads to an imbalance of cell growth over cell death,⁵⁶ but whether this is relevant to cholesteatoma biology is not known.⁵³ The second study was a candidate gene association study of 98 children with cholesteatoma for variants in the connexin gap-junction encoding genes, *GJB2* and *GJB6*,⁵⁴ some mutations of these loci are known to lead to recessive congenital deafness. Although the authors suggest a high frequency for some *GJB2* gene variants associated with cholesteatoma, no conclusions can be safely drawn from this study, because it lacked a control population and had a small sample size, placing it at risk of false discovery.

4.4 | Limitations

We excluded non-English manuscripts and studies published before 1980 from our initial search (the earlier and/or non-English articles were subsequently included in the narrative synthesis because they were identified by hand-searching citation lists); it is therefore possible that we have missed relevant publications.

The over-representation of case reports, case series and historical epidemiological studies is unsurprising given that cholesteatoma is a rare disease, but such studies provide low-level evidence in the research hierarchy because they are usually retrospective with incomplete data collection or follow-up, and are subject to author bias, ascertainment bias and publication bias. In addition, such findings may not be generalisable, and should be interpreted with caution, particularly with respect to theories about the underlying aetiology of cholesteatoma.

5 | CONCLUSION

Cholesteatoma is a complex and heterogeneous clinical phenotype. In a handful of case reports or case series, congenital and acquired cholesteatomas have been shown to segregate within families in the pattern typical of a monogenic or oligogenic disorder with incomplete penetrance. The liability threshold for the observed cholesteatoma phenotype could therefore depend on a combination of environmental and genetic factors of variable penetrance. Evidence from syndromic cases suggests that genes controlling ear morphology may be risk factors for congenital or acquired cholesteatoma formation.

We should accommodate the hypothesis that a range of aetiological pathways exist for cholesteatoma and that these may result in disease subtypes that differ in both severity and tractability.

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CONFLICT OF INTERESTS

There are no other conflict of interests to declare.

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