Pathogenesis of Otitis Media:

The utilisation of fetal middle ear epithelial cells as a model for Non-typeable *Haemophilus influenzae*-induced acute otitis media

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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04/08/2023

I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not previously been presented for an award at this, or any other, university.

Acknowledgements

I would like to acknowledge and thank Dr Lynne Bingle and Prof. Colin Bingle for their supervision and the opportunity they have given me for conducting this research over the past 4 years. I would also like to thank the team of technicians, Mr Jason Heath, Mrs Brenka McCabe, Mrs Lijun Chang, and Dr Matt Worsley, for their unrelentless work and assistance in the development of this project. Finally, thank you to the University of Sheffield, for awarding me the doctoral scholarship that allowed me to carry out this work.

I would like to thank my friends and lab colleagues. To them, who have been on this crazy journey with me (I think calling it a rollercoaster would be the understatement of the year), I am so appreciative of every single moment we shared. You have guided, helped, and supported me through the lows of this wild research voyage, and I can only hope to repay the love, care and kindness you have shown me. Hollie, thank you for being so much more than a friend, for being found family, you have taught me new ways of being resilient, resourceful, and adventurous. Karolina, thank you for being the calming presence in an otherwise chaotic journey. Luna, thank you for being my greatest motivator, like a pocket-sized angel sitting on my shoulder, pushing me forward. Cathy, thank you for your constant positive energy, it may not have been contagious, but it was deeply appreciated. Shivani, Samie, Ben, Antonia, Cerys and Faisal, thank you for keeping things interesting in the office. To everyone in the Bingle group, Fatima, Irene, Vivian, thank you for your support.

I would like to thank my family. To my mum, for all your encouragement and unconditional support, for all the time you've dedicated into our family, for all the sacrifices you've made along the way; even when my life decisions kept taking me further and further away from home, you found it in your heart to understand it; your pride and love for me know no bounds, and for that I am forever thankful. To my dad, for sparking my passion in science and research, for teaching me to be hungry for knowledge and to stay curious about the world surrounding us, for always showing interest in my work, and reminding me that someone out there wants to read it. To my sister, who is brave and kind and smart, who shows me that facing adversity is challenging, but it can be done, who motivates me to be better (for the both of us). "Amo-vos mil milhões de infinitos". To my grandmas, vó Adelina and vovó Judite, who have been watching over me from far above, and to my grandpa, padrinho Manuel, who has been staying strong so far away. "Obrigada por tudo".

I would like to thank my partner. To Dan, you have been my biggest support from the day we met, you have celebrated every single victory of this journey, no matter how little or few there were; your selflessness and love carried me through the lows, and I don't know how I would have completed this journey without you. You remind me every day how strong I am, without realising so much of that strength comes from you. I love you, toots.

In the pursuit of great, don't let me fail to do good.

- Adapted from "Arcane, 2021"

Abstract

Otitis media (OM) is an inflammation of the middle ear (ME). 80% of children will experience a case of OM during their lifetime. The disease presents a significant health burden to both high and low-income countries. In high-income countries, OM is the leading cause for paediatric antibiotic prescription and administration of general anaesthesia and in low-income countries, lack of access to healthcare facilities leads to uncontrolled progression of disease, which commonly culminates in hearing loss, but can also lead to meningitis and death. This project aims to investigate 3 branches of the study of OM. 1) A better understanding of the ME embryonic development, will provide new insights into the ME cavity and its epithelial lining, and consequently the development of OM. To achieve this, immunohistochemistry staining for embryonic and epithelium differentiation markers of fetal ME tissue from 23CS to 17 post-conception weeks was performed. This part of the study presented significant challenges, particularly in the identification of very early embryonic tissues, however, new staining patterns were identified allowing novel insights into the early development of this part of a human embryo. 2) Finding sustainable alternatives to the current OM research tools is fundamental as currently, this is a significant limitation. A single human cell line is available (hMEEC-1), with most OM research being conducted in animal models. ME primary cells recovered from patients undergoing surgery also present limitations and few *in vitro* human 3D models have been established. Here, I report the recovery of ME epithelial cells from the fetal ME of early terminations of pregnancy (MEEC). MEEC present similar characteristics to hMEEC-1, while being primary cells. Furthermore, the cells naturally present extended lifespans when compared to previously published studies using primary cells recovered from paediatric patients for 3D model development. 3) OM is a disease of primarily bacterial sources, thus a strong knowledge of the interactions between otopathogens and the ME epithelium will inform decisions on future prevention and treatment methods. As NTHi emerges as a central otopathogen, replacing *Pneumococcal spp.*, it is fundamental to understand the ability of this bacteria to inhabit the ME epithelium and the cellular responses to infection. This study reports, for the first time, the ability of NTHi to intracellularly invade ME epithelium cells. Furthermore, the response of MEEC to infection by NTHi is proinflammatory and follows a chronological similar pattern to that seen in *in vivo* responses, according to the literature. Thus, MEEC are representative of OM disease and may prove to be an extremely useful tool in the research of OM pathogenesis.

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Abbreviations

AOM - Acute otitis media

ALI - Air-liquid interface

COM - Chronic otitis media

COME - Chronic otitis media with effusion

CSOM - Chronic suppurative otitis media

ET - Eustachian tube

hMEEC-1 - Human middle ear epithelial cells - 1 (human cell line)

ME - Middle ear

MEEC - Middle ear epithelial cells (human fetal primary cells)

NTHi- Non-typeable *Haemophilus influenzae*

OM - Otitis media

OME - Otitis media with effusion

PAFr - Platelet activating factor receptor

PCho - Phosphorycholine

rAOM - Recurrent acute otitis media

1. Chapter 1 - Literature Review

1.1. Sound and the importance of hearing

Sound perception is a requirement for the survival of most animals. The ability to hear brings individuals' awareness to their surrounding environments, facilitating navigation and providing safety. The comprehension of sounds has evolved greatly, allowing for various populations to communicate effectively, for example through languages, used by humans. Thus, whether an animal's nature is to live within a pack or alone, reliance on sound is fundamental for endurance. Humans rely on sound to connect with others and their surroundings. For most of us the ability to hear is accepted as the norm, an essential component of our day-to-day life, however, we do need to consider how an individual is affected when they are born unable to hear or when they lose this sense.

Ear infections, such as otitis media, are a common feature in childhood. They present a substantial threat for affected patients whose immune systems cannot resolve the infection or for those individuals who do not have access to quality healthcare systems, as otitis media can lead to hearing loss.

This project is of major interest in the current medical climate; most otitis media (OM) cases are caused by bacteria, and thus antibiotics are a common form of treatment, but as the number of antibiotic-resistant bacteria increases, so does the need for alternative therapies. Furthermore, treatment of severe cases of OM often involves small invasive surgeries with associated complications. A better understanding of the disease mechanisms and the use of immunotherapies could prevent the need for such procedures. In high-income countries, OM presents a massive burden to healthcare systems, as children and parents may require multiple doctor visits and follow-up appointments, administration of different forms of treatments, and invasive surgeries which increase stress levels. In low-income countries, however, the disease presents as a burden to society, as individuals who do not have access to treatment may face the complications associated with the unhampered progression of the disease, e.g., hearing loss or meningitis, which hinders their growth and development.

1.2. The ear

1.2.1. Sound transmission and the ear anatomy

In humans, the auditory system represents a sophisticated signal processing mechanism responsible for a variety of functions, spanning from sound localisation, body balance, perception of language and emotional prosody. These functions are supported by the crosstalk amongst chemical, neural and mechanical components. [1]

The ear is segmented into 3 areas, the outer (or external) ear, the middle ear and the inner ear (Figure 1). In humans, the sound perception commences in the external auditory meatus (or ear canal), where the sound waves travel through the outer ear, hit the tympanic membrane, also known as the eardrum, causing it to vibrate. The resulting vibrations perturbate the auditory ossicles in the middle ear, which in turn cause the fluid in the cochlea, located in the inner ear, to move. Upon amplification and transduction into electrical signals by the organ of Corti, the vibrations are sent to the brain, where sound is decoded and comprehended. [2]

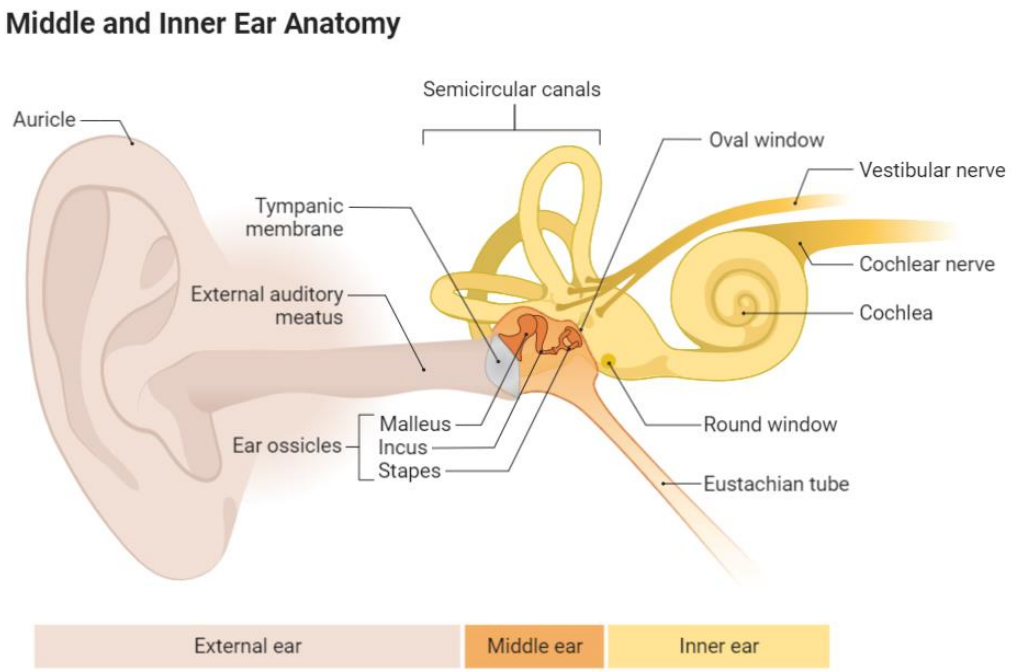


Figure 1 - Diagram of the anatomy of the ear. Adapted from https://app.biorender.com/.

1.3. The middle ear

The middle ear (ME) comprises diversified and specialised epithelial linings: a pseudostratified epithelium, with basal, goblet, and ciliated cells, and a simple squamous epithelium. The ME acts as a sealed bridge between the outer and inner ear. Although sealed, the ME is connected to the outer ear through the tympanic membrane and to the inner ear through the round window, which is also sealed by a secondary tympanic membrane. The ME cleft encompasses the ME cavity, the mastoid air cells and the Eustachian tube (ET), which provides a form of contact with the external environment. The ME is continuous with the respiratory airways and is also lined with respiratory membranes, thus able to secrete mucus. The ME cavity houses the auditory ossicles: malleus, incus and stapes. On the outer limit of the ME is the tympanic membrane and on the inner limit is the cochlea. The upper limit, or attic, is beneath the brain's middle lobe and the lower limit lies above the great vein and carotid artery. Towards the anterior of the ME is the opening to the ET, and at the posterior is the opening to the mastoid air cells. Both these structures are a vital component of the maintenance of pressure equilibrium within the ME (Figure 2). [3]

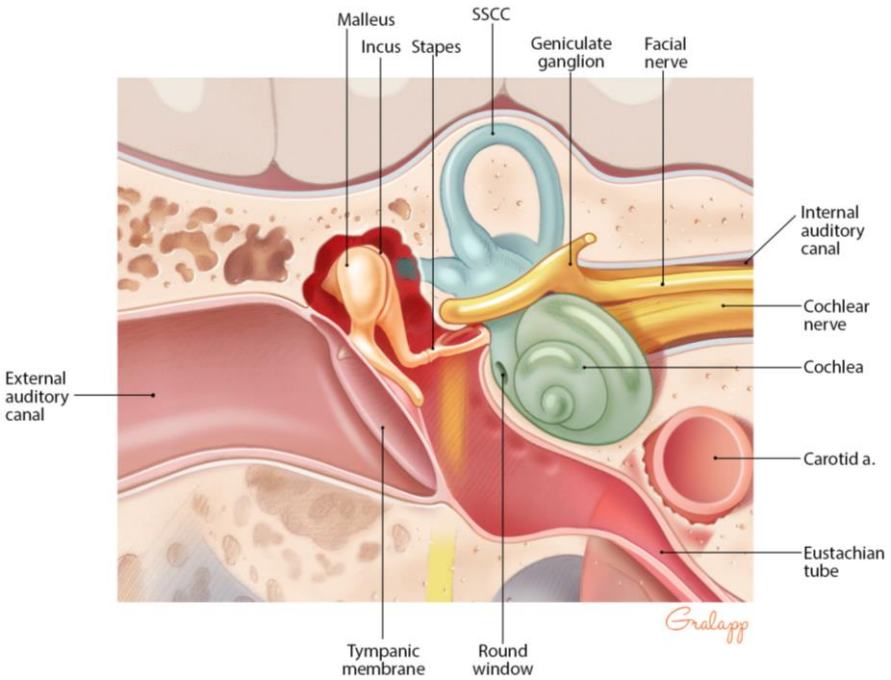


Figure 2 - Diagram of the ear, highlighting middle and inner ear structures, such as the auditory ossicles (malleus, incus and stapes) housed in the ME, and the cochlea located in the inner ear. The movement of the ME ossicles oscillations, caused by the impact of sound waves in the tympanic membrane, is sensed and transduced within the cochlea into electric impulses that are subsequently transmitted and interpreted by the brain.

Adapted from https://otosurgeryatlas.stanford.edu/otologic-surgery-atlas/surgical-anatomy-of-the-ear/middle-ear-mastoid/#.

The ET is responsible for connecting the ME to the nasopharynx. As the tympanic membrane forms a tight seal, pressure differences between the middle and outer ear can be significant and, when excessive, can lead to membrane perforation. However, the connection to the nasopharynx through the ET allows for normalisation of pressure in the ME. Due to its vertical angle and the presence of ciliated cells, the ET also allows for drainage of ME secretions into the nasopharynx, and it protects the ME from nasopharyngeal content reflux (Figure 3). Conversely, the mastoid air cells act as a "buffer system" to pressure changes, releasing air into the tympanic cavity when the pressure is too low (Figure 4). [4]

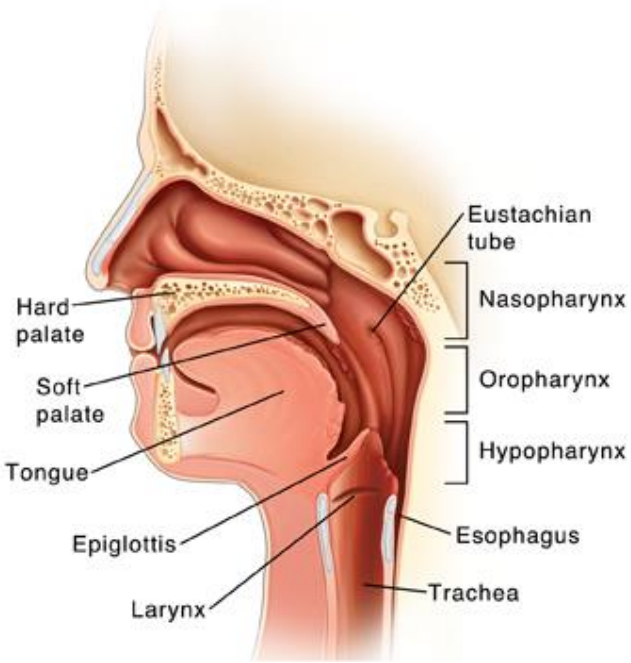


Figure 3 - Diagram of nasopharynx, highlighting the eustachian tube. The eustachian tube is the ear's connection to the nasopharynx, essential for pressure maintenance within the ME. However, this connection can also act as a bridge for virus and commensal or pathogenic bacteria into the ME. Adapted from: https://www.saintlukeskc.org/health-library/parts-throat-and-neck.

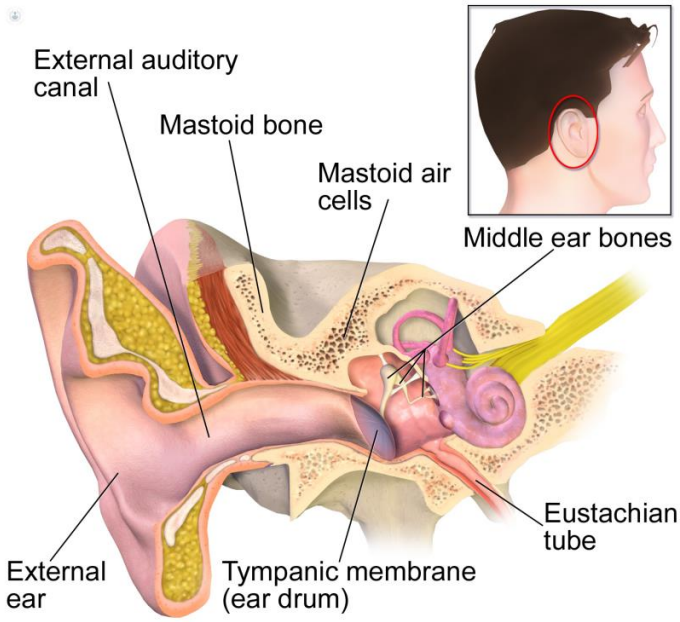


Figure 4 - Diagram of the location of the ear, and its insertion within the mastoid bone. The mastoid bone possesses a honeycomb-like structure, containing air-filled spaces known as the mastoid cells. Adapted from https://commons.wikimedia.org/wiki/File:Ear_Anatomy_and_Mastoid_Bone.png

1.3.1. The development of the middle ear

The ear first develops on embryonic days 22 or 23, through a thickening of the ectoderm, on both sides of the rhombencephalic region, forming the otic placode. During week 4, the otic placode invaginates into the underlying mesenchyme, forming the otic pit, eventually fusing and forming the otic vesicle. The ventral portion of the otic vesicle later results in the saccule and cochlear duct, whereas the dorsal portion gives rise to the semicircular canals and endolymphatic duct (Figure 5). The inner ear fully develops before the other two ear sections. During week 7, the cochlear duct epithelial cells differentiate into the Organ of Corti, the specialised structure that allows sound vibrations to be translated into neural signals. In week 9, the otic vesicle condenses and differentiates into the otic capsule, which between weeks 16 and 23 ossifies, to form the bony labyrinth, encapsulating the membranous labyrinth. The inner ear reaches its adult size and morphology by weeks 20 and 22. [5]

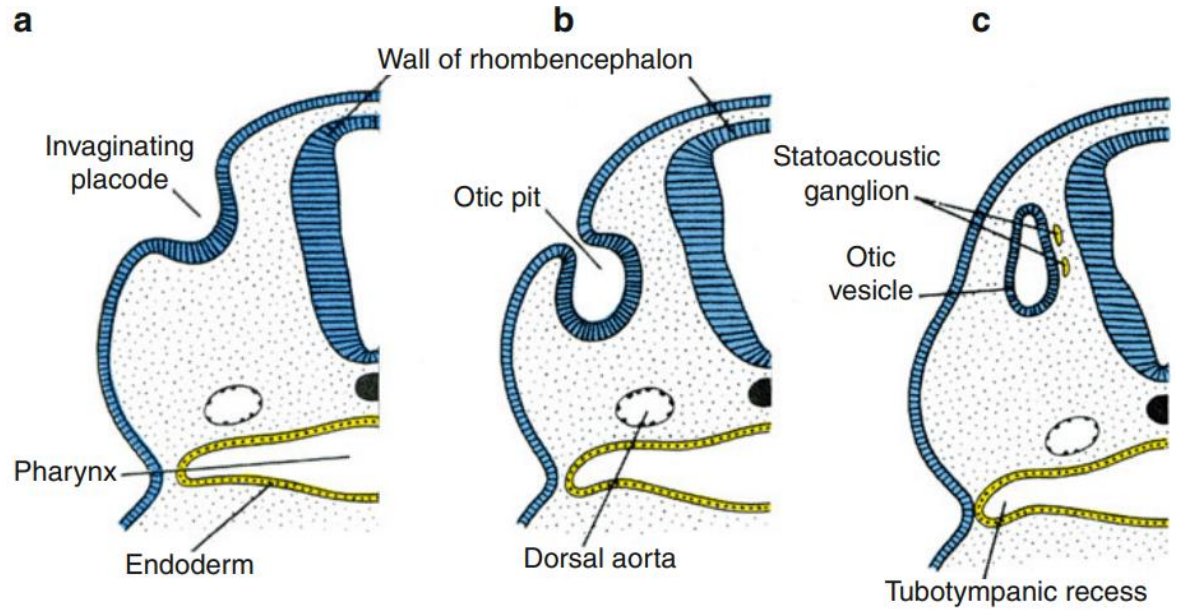


Figure 5 - Diagram of the embryology of the ear: formation of the otic vesicles; (a) 24 days (b) 27 days (c) 4.5 weeks. Adapted from https://link.springer.com/referenceworkentry/10.1007/978-3-642-23499-6_531

The ME development begins around week 5 of embryonic development, with the invagination and lateral extension of the first pharyngeal endodermic pouch, towards the developing inner and middle ear structures, forming the tubotympanic recess. Progressively, the dorsal portion of the tubotympanic recess continues to expand, encapsulating the ME ossicles, and forming the tympanic cavity at its distal end, which becomes filled with loose mesenchymal tissue. The ventral portion of the tubotympanic recess develops into the ET with a layer of endodermal epithelium lining the inner surface of the developing tympanic membrane and the ventral regions of the cavity. Alongside the formation of the ME cavity, there is the development of the ME ossicles, which are at early stages of development and undergoing condensation. During week 7, the cartilaginous precursors of the auditory ossicles condense in the mesenchyme of the branchial arches near the tympanic cavity (Figure 6). The ossicles remain enveloped until month 8, when the cavitation process begins, and the mesenchyme recedes; the endodermal epithelial lining of the tympanic cavity extends to the top of the cavity and connects the ossicles to the wall of the cavity. During month 9, in a process known as cavitation, the mesenchyme that is filling the ME cavity regresses and, finally, the suspended ossicles take their permanent locations and link to adjacent structures, such as the tympanic membrane and the oval window. [6]

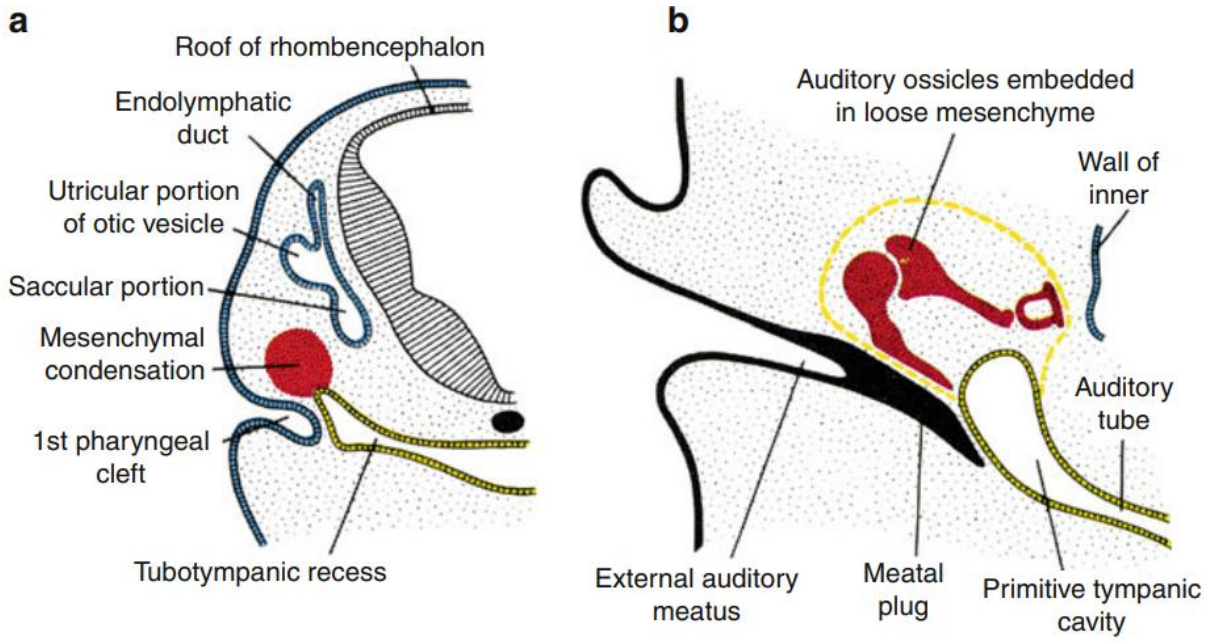


Figure 6 - Diagram of the embryology of the ear: extension of the tubotympanic recess. (a) 7 weeks (b) ME highlight of the cartilaginous precursors of the auditory ossicles, enclosed in loose mesenchyme. Adapted from https://link.springer.com/referenceworkentry/10.1007/978-3-642-23499-6_531

The tympanic membrane forms from the ventral aspect of the pharyngeal membrane, and it separates the first pharyngeal cleft from the first pharyngeal pouch. The first pharyngeal cleft originates from the external auditory meatus, whereas the first pharyngeal pouch develops into the tubotympanic recess. As development of the embryo continues, the mesenchyme between the two areas intercedes; the tympanic membrane then comprises an outer ectoderm-lining, an intermedial mesodermal layer and an inner endoderm-lining (Figure 7). [7]

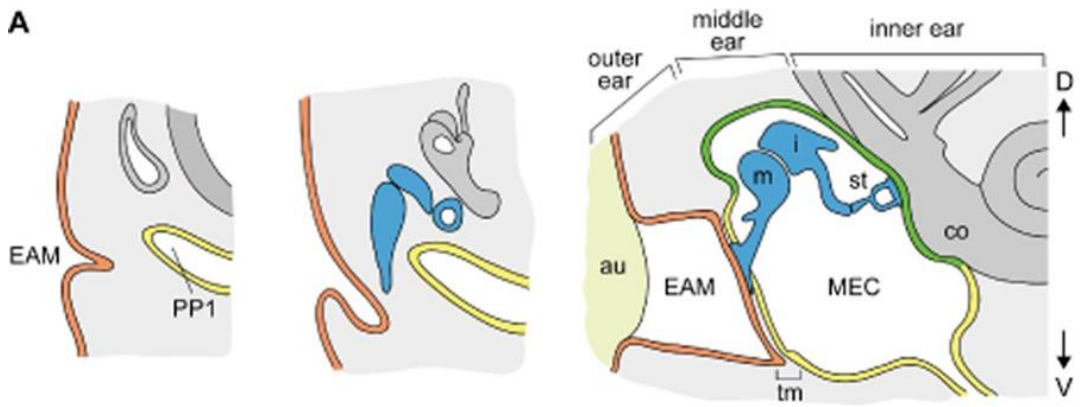


Figure 7 - Development of the tympanic membrane. Adapted from https://doi.org/10.1111/cga.12132

Although the antrum is close to full size at the time of birth, newborns usually do not present mastoid air cells, as the pneumatisation process only occurs after birth, when the epithelium of the cavity extends to the mastoid bone and lines it with respiratory epithelium. [5]

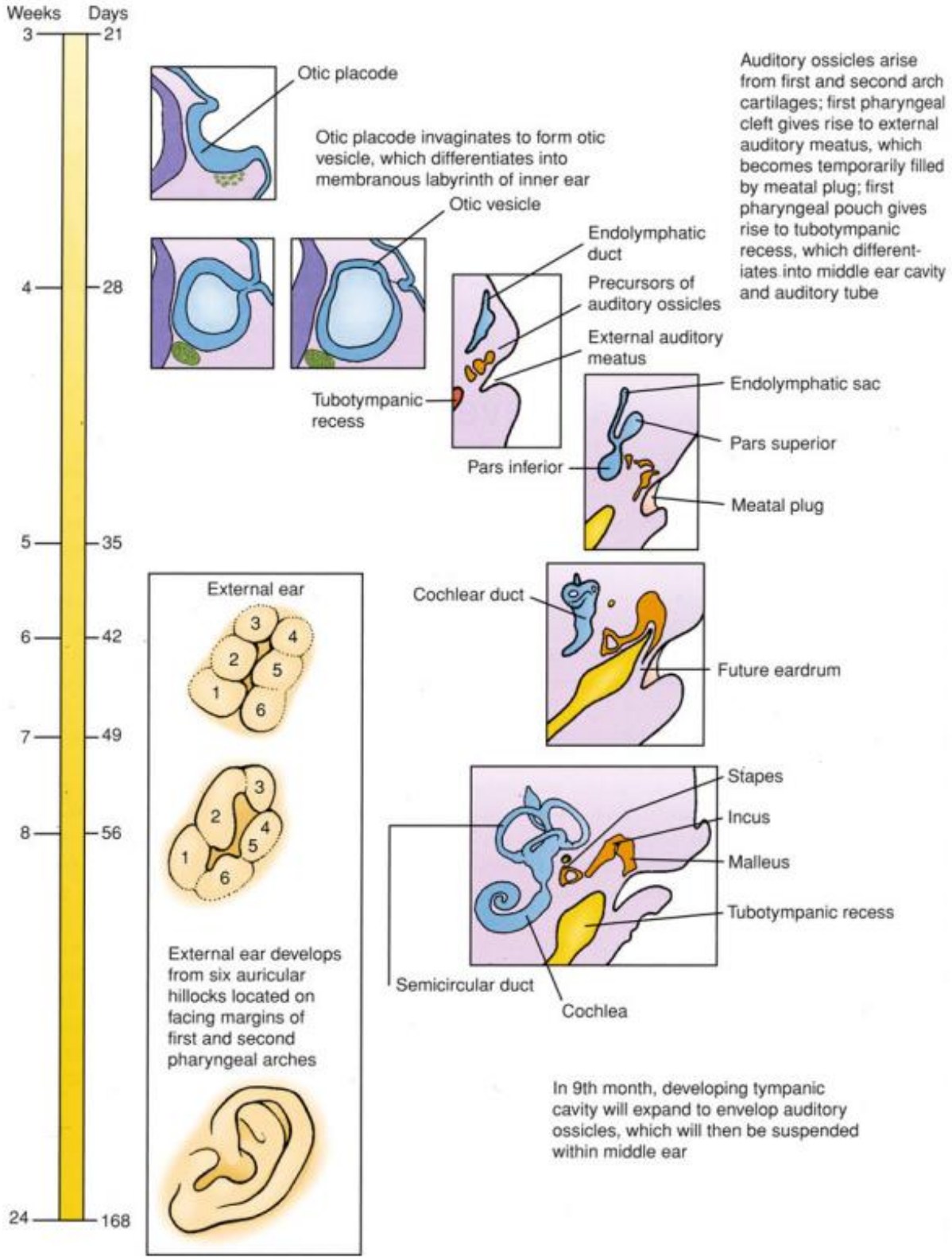


Figure 8 - Timeline of the development of the ear. Adapted from https://link.springer.com/referenceworkentry/10.1007/978-3-642-23499-6_531

1.3.1.1. The dual origin of the middle ear

Despite the general understanding of the formation of the ear, there are gaps in the details of its origins, as a consensus has not yet been reached regarding the development of the ME. Tucker and Thompson (2013) investigated the hypothesis of multiple origins of the tissues in the ear and demonstrated that the murine ME is lined with defined areas of cells that stain positively for endodermal cell markers and neural crest cell markers. This led to the proposition that during encapsulation of the ME ossicles, the first pharyngeal pouch ruptures and neural crest cells access the ME, adding to its endodermal lining. Furthermore, their data highlights two distinct regions. One contains densely ciliated epithelium and mucus-secreting goblet cells, and it is found close to the Eustachian tube, the ventral regions of the ME, and along the lateral edges of the cavity. This region overlaps with the areas that stained positively for endoderm. The other region is constituted by simple epithelium with low ciliation and can be found over the surface of the otic capsule and lines the attic. This region overlaps with the areas of neural crest origin (Figure 9). Given these observations, Tucker and Thompson (2013) suggested that neural crest cells undergo a mesenchyme-to-epithelium transformation after cavitation of the ME, an event previously reported in the corneal endothelium. [8]

Furthermore, while investigating the distribution of progenitor cells in the ME, Tucker *et al* (2017) found elevated numbers of proliferating cells in young mice MEs, suggesting that maturation and development of the ear and its mucosa is still ongoing 2 weeks post-birth. However, by 3 weeks post-birth, an adult morphology was evident, thus cell proliferation from this point onwards is likely for cell replacement, instead of growth. Despite Tucker and Thompson (2013) initial findings, suggesting that neural crest cells possessed reduced ability to differentiate into more complex epithelial cells, their recent study found multiple clusters of ciliated cells scattered within neural-crest derived regions, leading to the conclusion that some neural-crest cells can undertake a more complex epithelial morphology and some endoderm-derived cells can migrate to neural crest populated areas within the ME cavity. [9]

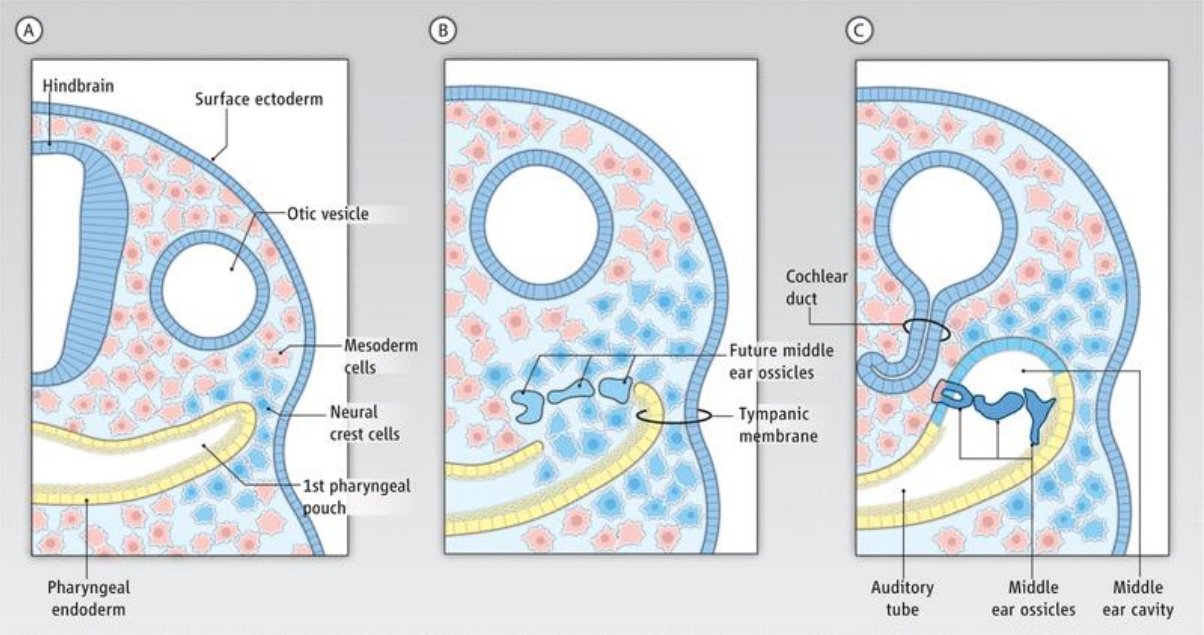


Figure 9 - Development of the ME, according the the dual origin hypothesis. Adapted from https://www.science.org/doi/10.1126/science.1236645.

1.3.1.2. The single origin of the middle ear

Although Tucker and Thompson (2013) argue a dual origin of the ME, their research has only been conducted in the ME of mice. Waegeningh *et al* (2019) conducted the first human fetal ME immuno-histopathological study, in a 25-week-old fetus, where cavitation had initiated. Their findings contrast that of Tucker and Thompson (2013), and advocate for a single endodermal embryonic origin of the epithelium lining the human ME. The study by Waegeningh *et al* (2019) showed that, unlike the hypothesis for the dual origin theory, an endodermal rupture is not required for the epithelial-lined appearance of the human ME cavity. Instead, the pharyngeal pouch has the ability to invaginate the ME ossicles, that would otherwise cause a rupture, and be in functional continuity with the rest of the upper respiratory tract. [10] However, the discrepancies in findings between these two studies could be attributed to the different stages of epithelium differentiation - 25 weeks prenatal human vs 3 weeks postnatal mice. Milmoe and Tucker (2021) argue in their rejection of the single origin hypothesis that, even though ME cavitation had been initiated in the single origin study, it had not been completed in the attic region and the attic mucosa lining had not yet formed, thus an accurate study of the origin of the attic region of the ME could not be executed. It is important

to note that it is common in humans for the completion of the attic and mastoid aerated areas to occur up to the age of 4. [11,12]

In conclusion, the exact origins of the ME have yet to be defined and require further investigation.

1.3.2. The middle ear epithelium

1.3.2.1. Cell types and populations within the middle ear

Using single-cell transcriptomics, which allow for analysis of gene expression levels of individual cells within heterogeneous cell populations, Ryan *et al* (2020) characterised the cells of the normal, young adult (60-90 days old) murine ME. The results provide a molecular landscape of the cells lining the normal ME mucosa and define the roles of the ME cells. [13]

1.3.2.1.1. Epithelial Cells

The epithelial cells are responsible for detecting pathogens and responding accordingly, by secreting antimicrobials or recruiting and activating leukocytes. All clusters of epithelial cells found expressed *Bpifa1*, a gene resulting in the production of an upper respiratory airway antimicrobial. A total of six distinct clusters of epithelial cells were identified (See table 1).

Table 1 - List of epithelial cell clusters identified in the murine ME through single-cell transcriptomics.

Cell cluster type	**Proposed function/Expressed genes**
Basal cells	Tissue proliferation and expression of β-defensin 1
Epithelial cells	Aqp4 and Muc4

Mature high secretory epithelial cells	Antimicrobials and mucins
Low secretory epithelial cells	Surfactant D and extracellular matrix (ECM) genes
Epithelial cells	Innate immune genes
Transition cells	Transition between epithelial with innate immune gene expression and low secretory epithelial cells

Despite the findings of Thompson and Tucker (2013) on the dual origin of the ME, there were no recognised adult gene markers for cells of neural crest origin. The small number of melanocytes observed, which are assumed to be of neural crest origin, were scattered through the epithelial clusters and did not express epithelial markers. As predicted, the varied existing epithelial cell types were associated with maintenance of the epithelial population and defence functions, vital for sustaining homeostasis in the ME. The presence of highly specialised cells, such as mature high secretory epithelial cells, but also transition cells, likely contributes to a robust and easily adapted response to pathogens in the ME.

1.3.2.1.2. Stromal Cells

All stromal cells expressed genes involved in ECM generation and cell remodelling. Three distinct clusters were identified, including osteoblastic cells expressing genes related to bone maintenance and remodelling; cells expressing epithelial and stromal growth regulatory genes; and cells expressing complement factors, as well as negative immune regulators, pro-inflammatory genes, and inhibitory modulators of inflammation. As stromal cells are differentiating cells and typically not highly specialised, it is expected that they present a diverse number of functions, including in differentiation and replacement of cells. The presence of osteoblastic cells is also expected, as the mature ME is a bone encapsulated cavity,

harbouring the ME epithelium, and regulation of the formation and interactions with this region is essential.

1.3.2.1.3. Vascular Cells

Three cell clusters of vascular cells were identified, including vascular endothelial cells expressing genes related to vascular permeability and fluid transport, angiogenesis, recruitment of leukocytes and decoy-cytokine receptors; lymphatic endothelial cells demonstrating the presence of lymphatics in the ME and verifying that draining from the ME to neck lymph nodes occurs; and pericytes expressing genes with roles in hypoxia, potentially leading to activation of inflammatory pathways and T-cells. Antigen presenting cells, such as dendritic cells and monocytes, transport antigens to nearby lymph nodes, such as the ones in the neck, through the lymphatic system. In the lymph nodes, an adaptive immune response is initiated, which assists in the elimination of ME pathogens.

1.3.2.1.4. Leukocytes

All clusters of leukocytes expressed genes related to immune and inflammatory responses. A total of five cell clusters were identified. (See table 2)

Table 2 - List of leukocyte clusters identified in the murine ME through single-cell transcriptomics.

Cell cluster	**Function/Expressed genes**
Macrophages (including M2 type)	Maintenance of homeostasis and expression of anti-inflammatory factors
Dendritic cells and immature monocytes	Detection of pathogens, leukocyte chemotaxis, activation of the complement system and positive and negative regulation of inflammation

Killer phenotype (associated with dendritic cells)	Expression of cytotoxic genes (not previously identified in the ME)
Gamma delta T-cells and NK-cells	Detection of pathogens and expression of effector proteins
B-cells and ILC2s	Antibody production, T-cell regulation and induction of inflammation

The large number of negative regulators of innate immunity and inflammation observed in the transcriptomes could provide an interesting tool for the study of OM. Although the negative regulation process is essential in the maintenance of homeostasis, its consequences and the way pathogens can exploit it in OM requires further investigation.

However, this is the only study of its kind, and it focused on the mouse ME epithelium. A deeper investigation of the multiple cell types found in the human ME is crucial to our understanding of the development of inflammatory responses within the cavity, particularly in relation to remodelling of the ME and secretion of antimicrobials.

1.3.2.2. Middle ear epithelium similarities to the respiratory epithelium

The first in-depth analysis of the human ME and ET epithelium by Lim (1971) gathered histological, histochemical and ultrastructural information. The ET possesses a bony portion that blends with the bone of the ME, with epithelium composed of tall pseudostratified columnar cells, where 80% are ciliated and the remainder possess secretory functions. This epithelium contrasts with the epithelium of the cartilaginous section of the ET, towards the pharyngeal orifice, which is constituted by pseudostratified tall columnar ciliated epithelium with secretory and non-secretory cells, and regions of simple squamous epithelium, particularly near the orifice. The epithelium lining the ME cavity, near the ET and hypotympanum,

constitutes of tall columnar cells but near the promontory, and towards the attic, cuboidal cells and a simple squamous mucosal epithelium are seen. [14–16] Later, Lim (1974) and Hentzer (1976) found that the ME is lined by subepithelial connective tissue, where fibrocytes, mast cells, macrophages, and lymphocytes are present, as well as a basal epithelial layer. [17,18] Lim and Klainer (1971) suggested that, under pathological conditions, the basal mucosal epithelium had the potential to differentiate into ciliated or secretory cells, a cell morphology distribution that is commonly seen in the nasopharynx. [19,20]

The diversity of secretory cells and secreted products has also been previously investigated. Veltri and Sprinkle (1973) and Bernstein *et al* (1972) highlighted the production of lysozyme and lactoferrin in the ME epithelium, both of which can also be found in the nose and respiratory tract. [21,22] The presence of an active mucociliary system in the ME, similar to that seen in the nose and lungs, with ciliated and secretory cells and a mucus blanket has also been demonstrated by Sade (1967) and hypotheses of coordinated cilia beating have more recently been demonstrated by Luo *et al* (2017). [23,24]

A healthy ME has no associated lymphoid tissue and generally possesses very low numbers of resident lymphocytes, however, once inflammation is triggered, the site becomes heavily populated by immunoglobulin-producing cells, which arrive at the ME through lymph nodes and peripheral blood circulation. Furthermore, the ability of the ME to drain into the lymphatic system demonstrates that the ME mucosa can drain large molecules to local lymph nodes, but failure of this process can aggravate ear pathologies. [25] The presence of other immunocompetent cells such as mast cells, macrophages and neutrophils, and dendritic cells has also been reported by Freijd *et al* (1984), Palva *et al* (1991) and Suenaga *et al* (2001). Their presence within the ME, although in small numbers when compared to other mucosal organs, is essential for triggering an effective innate immune response, and providing a link to a robust adaptive immune response. [26–28]

The collective of these findings were fundamental in the understanding of the ME epithelium as a modified respiratory epithelium.

1.3.2.2.1. The middle ear and lungs

Similar to the ME, the lungs are lined by pseudostratified ciliated columnar cells, simple ciliated columnar epithelium, ciliated cuboidal cells, type I (simple squamous) and type II

(secretory) pneumocytes, macrophages and goblet cells. During embryonic development, the pulmonary primordium and the early ME cleft are both derivatives of the endoderm, though they later undertake different differentiation routes. [29]

Sadé and Ar (1997) have previously described the ME as a "miniature lung", as the ME, like the lung, requires an air-filled cavity to function optimally, and it relies on the ET for its ventilation in the same way as the large alveoli of the lung rely on the terminal bronchiole for air distribution. Furthermore, the ME mucosa, like the lung mucosa, reacts to alterations in air pressure, gas composition, and bacterial load in part due to the rapid action of goblet cells, which secrete mucins and proliferate upon stimulation. Finally, several studies have identified the presence of surfactant-like proteins in the ME mucosa, similar to those found in the pulmonary surface. [30]

The lungs and ME are also affected by a plethora of similar pathogens and their responses to infection follow similar stages: congestion, formation of effusion, suppuration, and resolution or further consequences. [31]

1.3.2.2.2. The middle ear and nose

The nose epithelium posterior to the limen nasi contains cells similar to those previously described in the ME: non-keratinised stratified squamous epithelium, respiratory pseudostratified ciliated epithelium, with columnar, cuboidal, goblet, ciliated and non-ciliated cells, but it also contains seromucous glands within the lamina propria. As with the respiratory tissues mentioned above, the nasal mucosa also reacts promptly to stimuli: pathogens and external factors, such as smoking, air pollution, inhalants and drugs. [32]

The nasal epithelium also acts as a barrier against pathogens, detecting these via pattern recognition receptors (PRRs) and producing antimicrobials to fight off infection. Cells involved in innate immune defence, such as macrophages and dendritic cells, are also found in the nasal epithelium. [33]

The epithelium is not the only similarity found between the nasopharynx and the ME. Due to their proximity, and access through the Eustachian tube, migration of microbial organisms between the two structures has been hypothesised. Jinhage *et al* (2021) analysed the nasopharyngeal and ME cultures of children with acute OM, in an attempt to link the

microbiome of the ME and the nose, as nasopharynx microbiology cultures are commonly requested methods to assist OM diagnosis in clinical settings, but its accuracy had not previously been established. Overall, the findings suggested that, despite not being a perfect correlation with the ME microbiome, nasopharyngeal cultures provide valuable insights into the pathology of acute OM. Jinhage *et al* (2021) reported that in some cases, the course of antibiotic treatment was altered, or extra appointments scheduled, and the cultures provided reliable information on what is and what is not growing in the ME, and their bacterial antibiotic resistance patterns. [34]

1.3.2.3. The cilia in the middle ear epithelial lining

As previously mentioned, the ME epithelium possesses specialised cells with secretory and ciliary functions. Motile cilia are key in maintaining the homeostasis of the ME and further understanding of their mechanism of action could improve our knowledge of how to treat diseased ears. Pathologies such as neonatal respiratory distress, chronic nasal congestion and chronic OM with effusion are common in individuals with cilia dysfunctions. Luo *et al* (2017) outlined the importance of motile cilia as agents in the clearance of the respiratory airways. This work provided knowledge regarding the previously uncharacterised cilia of the mouse ME, by identifying two ciliated cell populations: one that concentrates near the ET orifice, where the cilia are aligned towards the direction of the ET opening and express Vangl2 (a membrane protein involved in regulation of planar cell polarity), also found in the trachea; and a second population lining the ME cavity and parts of the attic. Though their data were consistent with previous reports of ciliated epithelium overlapping with the ventral region of endoderm origin, they also found sparse regions of dorsal cilia in the supposedly neural crest origin area, particularly in the attic. The ciliated cells in the attic are believed to have a dual role in balancing air pressure and conducting sound signals across the ME, as well as in fluid and pathogen clearance (Figure 10). [24]

It is noteworthy that ciliated airway epithelial cells, such as those residing within the lungs and the tongue, play a role in chemosensory-based defence mechanisms, which is potentially shared by their ME ciliated epithelial counterparts. [35]

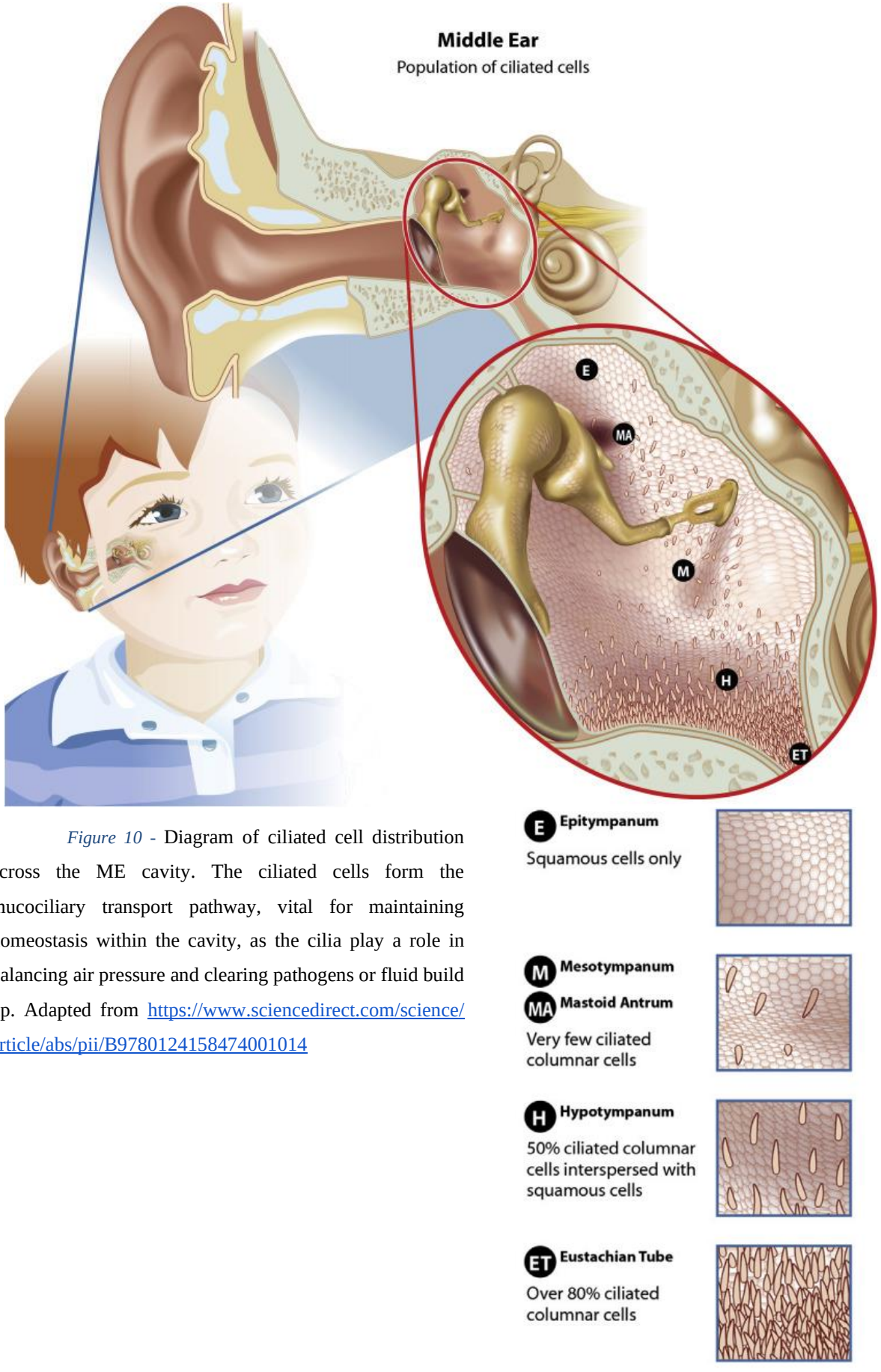


Figure 10 - Diagram of ciliated cell distribution across the ME cavity. The ciliated cells form the mucociliary transport pathway, vital for maintaining homeostasis within the cavity, as the cilia play a role in balancing air pressure and clearing pathogens or fluid build up. Adapted from https://www.sciencedirect.com/science/article/abs/pii/B9780124158474001014

1.4. Otitis media

Dysfunction of the ET is potentially linked to ear pathologies. This dysfunction typically occurs when the ME or nasopharynx are infected (e.g., viral upper respiratory tract infection (URTI)), leading to inflammation and/or swelling of the ET. However, ET dysfunction can also occur due to adenoid hypertrophy (swelling of the adenoids and tonsils) or failure of the muscles that connect, wrap around and pass through the ET. These factors prompt blockage of the ET, impairing drainage of ME contents and disrupting pressure equalisation. A typical result of ET dysfunction is otitis media (OM) (Figure 11). [36]

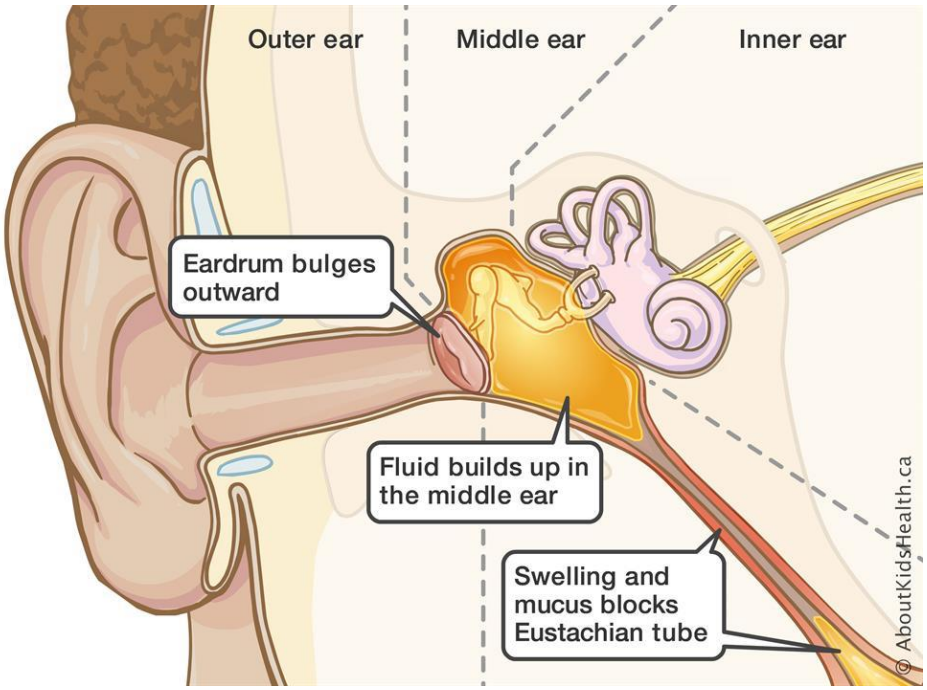
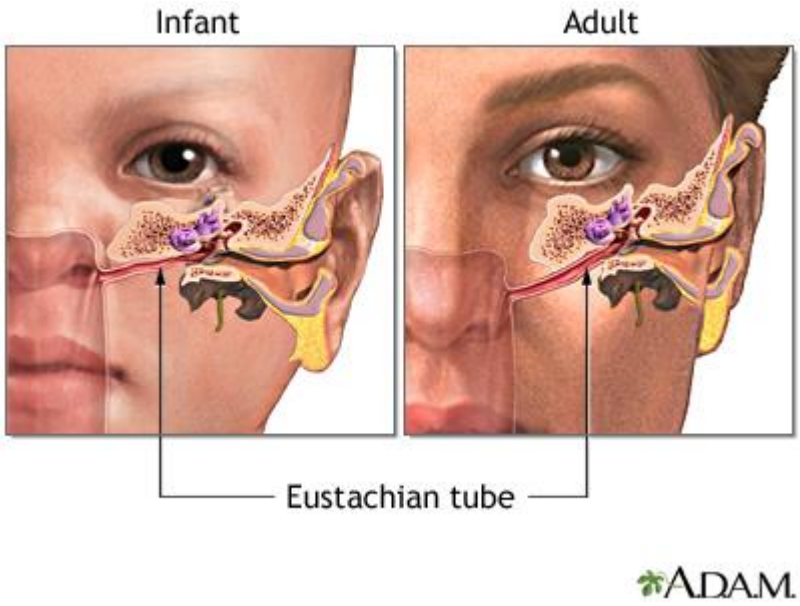


Figure 11 - Diagram of ear affected by OM. In cases of otitis media, a trigger initiates inflammation within the Eustachian tube or ME, leading to partial or complete occlusion, due to either swelling or mucous blockage, of the Eustachian tube. The inflammation is commonly triggered by pathogens and is followed by an increase in mucus production, and consequently, fluid build-up within the ME cavity. The tympanic membrane also becomes inflamed, and the increased pressure within the cavity leads to the tympanic membrane bulging outwards. This process can cause immense pain in affected individuals, as well as diminishing the individual's ability to hear. Adapted from https://www.aboutkidshealth.ca/article?contentid=8&language=english.

Although more evidence is required to support this fact, it has been hypothesised that the anatomic differences between adult and children's ETs may be one of the main determinants underpinning the increased susceptibility of children to OM. For example, a

child's ET is shorter, more horizontally aligned, softer, and of smaller calibre than an adult's ET. When this is considered alongside a child's immature immune system, it leads to the hypothesis that pathogens travel more easily from a child's nasopharynx into the ME, consequently leading to the onset of OM (Figure 12). Other OM-susceptibility factors include



socioeconomic status, environmental factors and genetic background. [37]

Figure 12 - Diagram of anatomical differences between adult and children's Eustachian tube. A child's Eustachian tube is shorter, more horizontally aligned, softer and of smaller calibre, facilitating pathogenic migration from the nasopharynx into the middle ear cavity. Adapted from: https://medlineplus.gov/ency/article/000638.htm

1.4.1. Acute otitis media

OM is typically an infection of the ME that leads to redness and swelling of the tympanic membrane, followed by fluid build-up inside the ME cavity. It commonly affects new-borns, toddlers and young children. During this period, ME ossicle movement is drastically reduced, leading to conductive hearing loss. [38]

OM initiates as a result of a host-pathogen interaction, either in the ET or ME, which prompts congestion and swelling of the nasal mucosa, nasopharynx and ET, leading to occlusion of the tube and poor drainage of ME contents. In turn, this prompts accumulation of secretions in the ME cavity, which promotes the onset of secondary infections by commensal

or newly introduced bacteria, leading to inflammation of the area, and the development of acute OM (AOM). [39,40] Although ET and nasopharynx dysfunctions are primarily caused by pathogenic triggers, either viral or bacterial, leading to OM onset, as recently shown by Aslier *et al* (2021), adenoid hypertrophy is closely linked to OM with effusion, resulting in a decline in hearing abilities in children aged 3-11. Thus, sterile inflammation leading to disease should not be an overlooked occurrence. [41]

In response to the pathogenic trigger and unregulated inflammation taking place during AOM, the ME epithelium undergoes extensive hyperplasia, meaning an increase in cell proliferation and its numbers, including in the epithelium overlying the cochlea and the attic areas, which normally present low levels of proliferation. The simple squamous epithelium transitions to a pseudostratified respiratory-like epithelium, increasing the number of goblet and secretory cells. [9] Hyperplasia is a hallmark feature of OM. Palacios *et al* (2014) have found that p38 MAPK plays an important role in promoting a mucosal hyperplastic response and recovery to a baseline physiology during bacterial OM. The p38 pathway is involved in a broad range of cellular functions, including cell proliferation, differentiation and apoptosis. p38 is highly expressed upon bacterial stimuli and, in rats, its early inhibition reduced bacterial-induced mucosal proliferation and led to fewer OM episodes, lessening the severity of OM sequelae in these animals' ME. However, when p38 was inhibited at later stages of disease, due to its role in apoptosis signalling, it affected cell death, potentially increasing duration of hyperplasia and the mucosal return to basal levels. [42]

1.4.2. Chronic otitis media

If the ME inflammation does not resolve, either spontaneously or through administration of treatment, leading to pathogen clearance, persistent inflammation develops with a permanent remodelling of the ME epithelium. Remodelling induces overproduction of mucin and antimicrobial peptides, prompting fluid build-up, which can cause pressure to significantly increase in the ME and lead to spontaneous perforation of the tympanic membrane. Subsequently, this allows for frequent ear discharges into the outer ear, a condition known as suppurative OM (SOM). SOM can have two distinct outcomes: chronic SOM (CSOM), characterised by a chronic infection that hampers healing of the perforated tympanic membrane, or resolved OM, where the perforated membrane heals and returns to a new

baseline. If the tympanic membrane is not perforated, the pathology is characterised by proliferation of mucus-secreting goblet cells in the ME and inflammation subsides but leaves residual fluid in the ME cavity. This condition is referred to as OM with effusion (OME), which is usually asymptomatic, resolving within 3 months. However, during this resolution period, the ME environment is prone to reinfection and if these symptoms do not clear and the effusion persists, the condition is then referred to as chronic OME (COME) (Figure 13 and 14). [43]

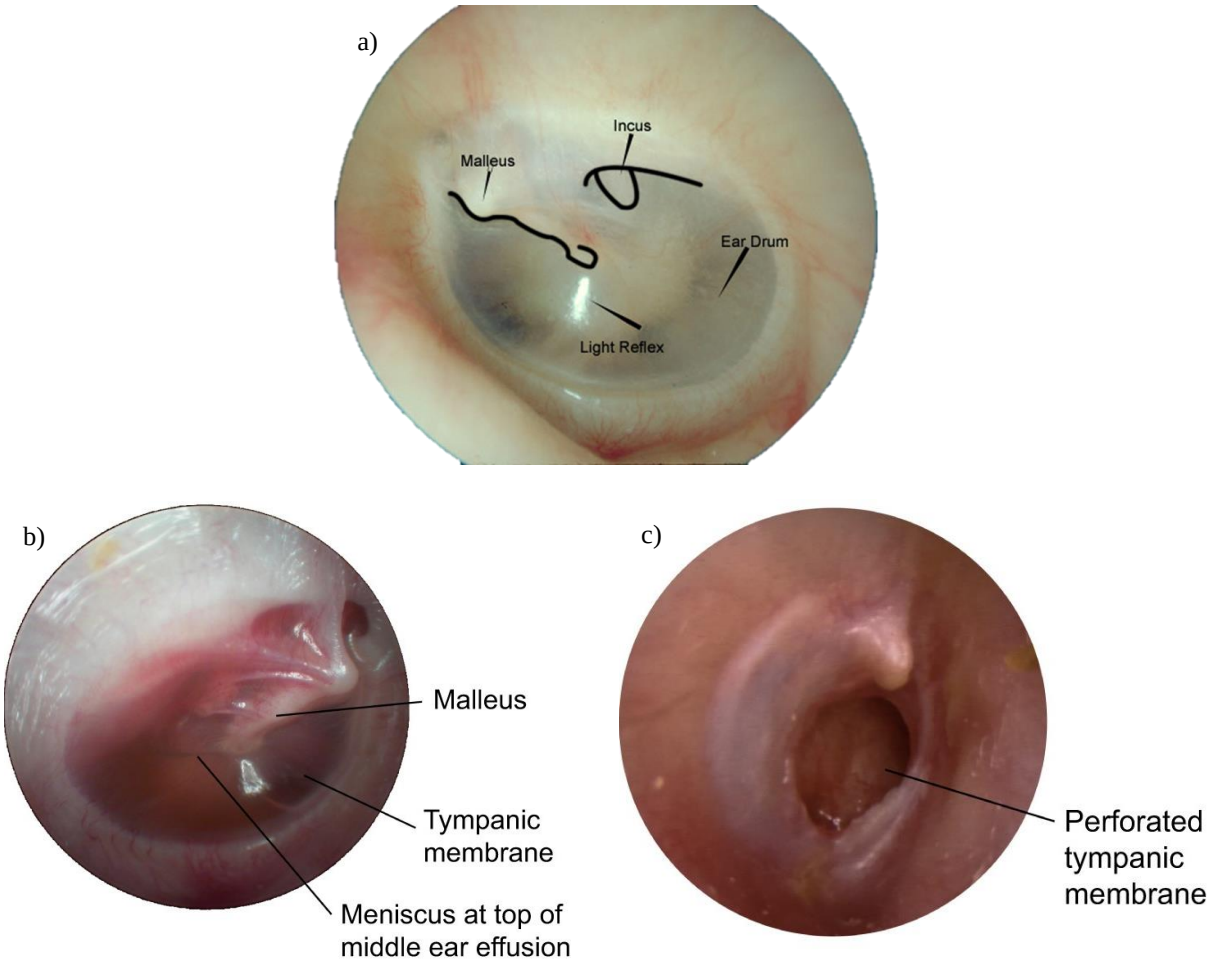


Figure 13 - Otoscopic visualisations of the tympanic membrane and its different appearances in COM affected ears. (a) Healthy ME; with no inflammation, redness or swelling, tympanic membrane is clear and reflective, and it is possible to visualise the outline of auditory ossicles. (b) COME affected ear; most of the inflammation, redness and swelling has subsided and the tympanic membrane remains intact, but the fluid build-up within the ME cavity is still visible. (c) CSOM affected ear; tympanic membrane has been perforated, leaving an open gap between the outer and ME, redness and signs of inflammation are still visible due to the ongoing infection hindering the membrane healing. Adapted from: https://med.uth.edu/orl/online-ear-disease-photo-book/chapter-3-ear-anatomy/ear-anatomy-images/ and https://journals.biologists.com/dmm/article/10/11/1289/53024/Understanding-the-aetiology-and-resolution-of

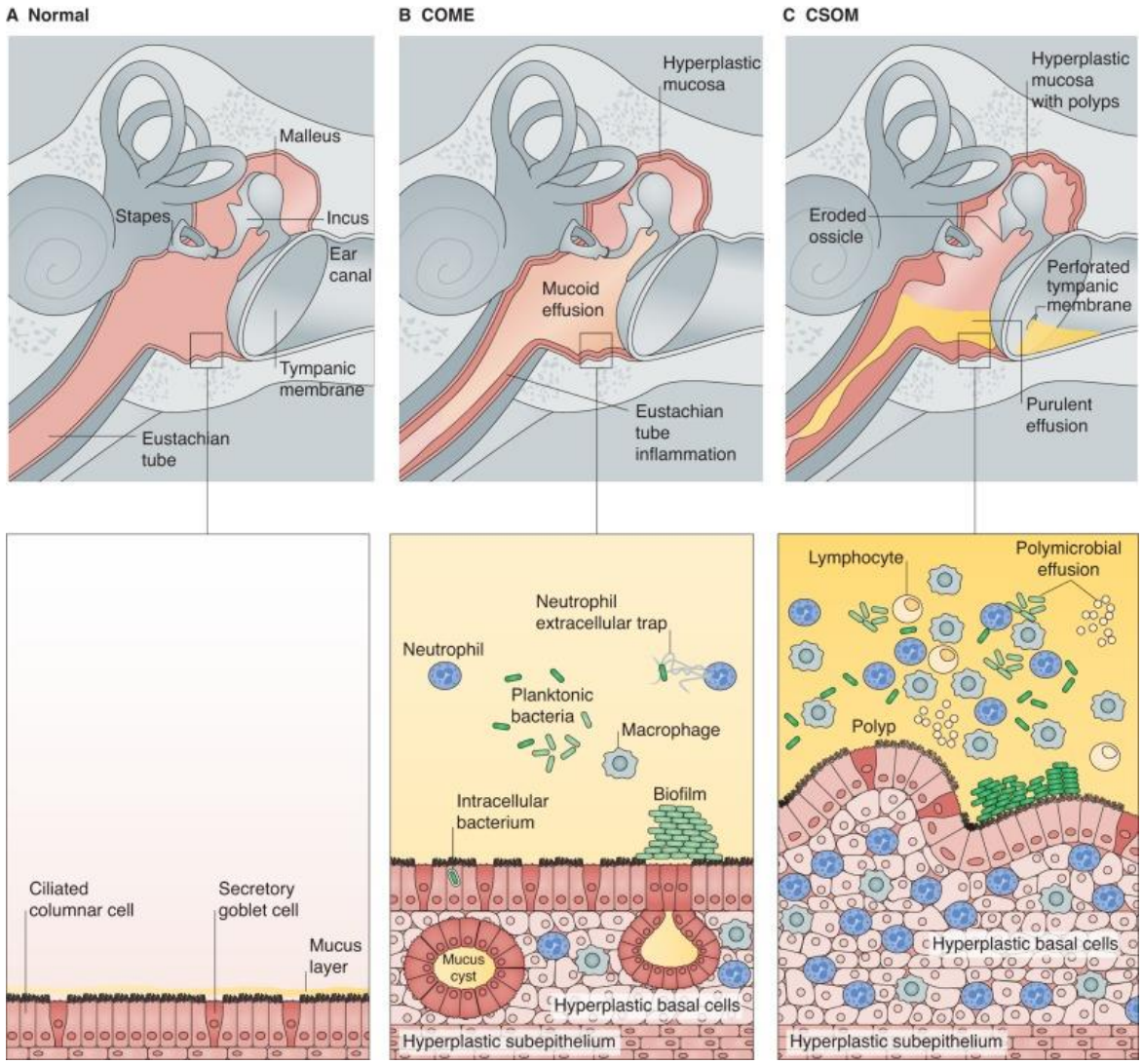


Figure 14 - Diagram of the ME cavity and how different subtypes of COM affect the structure and function of the ME epithelium. (a) Healthy ME; normal ciliated and non-ciliated epithelial lining, overlaid by a thin layer of mucus. (b) COME affected ear; cavity is filled with a mucoid effusion The epithelium lining is inflamed and features mucosal hyperplasia. Bacteria can be found in the cavity in a planktonic state, as a biofilm or intracellularly. Recruitment and activation of macrophages and neutrophils is a common response to infection. (c) CSOM affected ear; cavity is filled with a purulent effusion that spills onto the outer ear through the perforated tympanic membrane. The epithelium lining is inflamed and features mucosal hyperplasia, which can be excessive and form polyps. Bacteria can be found in the cavity in a planktonic state, as a biofilm or intracellularly, often as polymicrobial communities. Recruitment and activation of macrophages and neutrophils, as well as lymphocytes is a common response to this condition. [39] Adapted from: https://journals.biologists.com/dmm/article/10/11/1289/53024/Understanding-the-aetiology-and-resolution-of

1.4.3. Incidence and prevalence of otitis media

Liese *et al* (2014) conducted a study in European countries (Germany, Italy, Spain, Sweden and the UK) involving 5,776 children. They reported that 1 in 4 children were affected by AOM before the age of 6 and that spontaneous perforation of the tympanic membrane occurred in 7% of the cases. [44] In the USA, Kaur *et al* (2017) performed a similar study involving 615 children and reported that by the time a child reaches 1 year of age, 23% will have suffered at least one episode of AOM. This percentage drastically increases by the age of 3, where 60-80% of children will have experienced at least one episode, and 24% will have suffered three or more episodes. Nonetheless, these figures represent a downward trend when compared to a similar study conducted two decades earlier, by Teele *et al* (1989), before the introduction of the pneumococcal conjugate vaccine, where over 80% of children in high-income countries suffered at least one AOM episode by the age of 3 and approximately 40% had had more than three episodes. [45,46] Tonnaer *et al* (2009) have highlighted the long-term impacts of multiple OM episodes, by reporting that 15-20% of teenagers in the Netherlands suffer from tympanic membrane fibrosis and scarring, a consequence of persistent OM. [47]

Leach *et al* (2020) focused on hearing loss amongst poorer populations, in low to medium income countries, combining data from 18 studies, involving a total of 35,173 participants (17,521 from Africa, 16,182 from Asia and 1,470 from Oceania). The prevalence rates for any type (acute or chronic) of OM were 8% in Africa, 14% in Asia, and 50% in Oceania; the respective overall hearing loss (conductive and/or neurosensorial) prevalence rates were 5%, 8% and 1%, but region-specific rates for hearing loss presented higher values of 12%, 12% and 26%, respectively. This illustrates issues arising from the use of pooled data whereby the severity of the disease, especially in regions with niche and highly diverse socio-economic backgrounds, is underestimated. Interestingly, Bhutta *et al* (2015) noted that in indigenous groups, such as the Inuit (Arctic regions), Native American (USA), Maori (New Zealand) and Australian Aborigine (Australia), the rates of AOM incidence are almost always 1.5 to 3 times higher than non-indigenous populations in those regions. [48] Key reasons for increased OM rates in indigenous populations may include a range of socioeconomic factors, such as overcrowded and poorer housing conditions, high exposure to tobacco smoke, malnutrition, restricted access to healthcare facilities, and poorer economic backgrounds. Interestingly, reports have also found high carriage of common otopathogens in the nasopharynx of indigenous children. Mackenzie *et al* (2010) reported that Australian Aborigine populations possess a higher nasopharyngeal load of *Streptococcus pneumonia, Haemophillus*

influenzae, and *Moraxella catarrhalis* when compared to individuals in high-income countries, namely Belgium and Denmark, and even low-income countries, Gambia and Kenya. The authors were able to assert that frequent exposure to smoke plays a central role in maintaining high bacterial load rates, particularly pneumococcal carriage. [49–52]

To conclude, a recent report from the World Health Organisation (WHO) estimates that by 2050, 1 in 4 people worldwide will be affected by hearing problems due to poor infrastructures on hearing health. The report also singles out COM as one of the leading causes for hearing loss in childhood and adolescence, further encouraging an effort to develop better support and hearing health structures, as OM can be prevented by early diagnosis and management. [53,54]

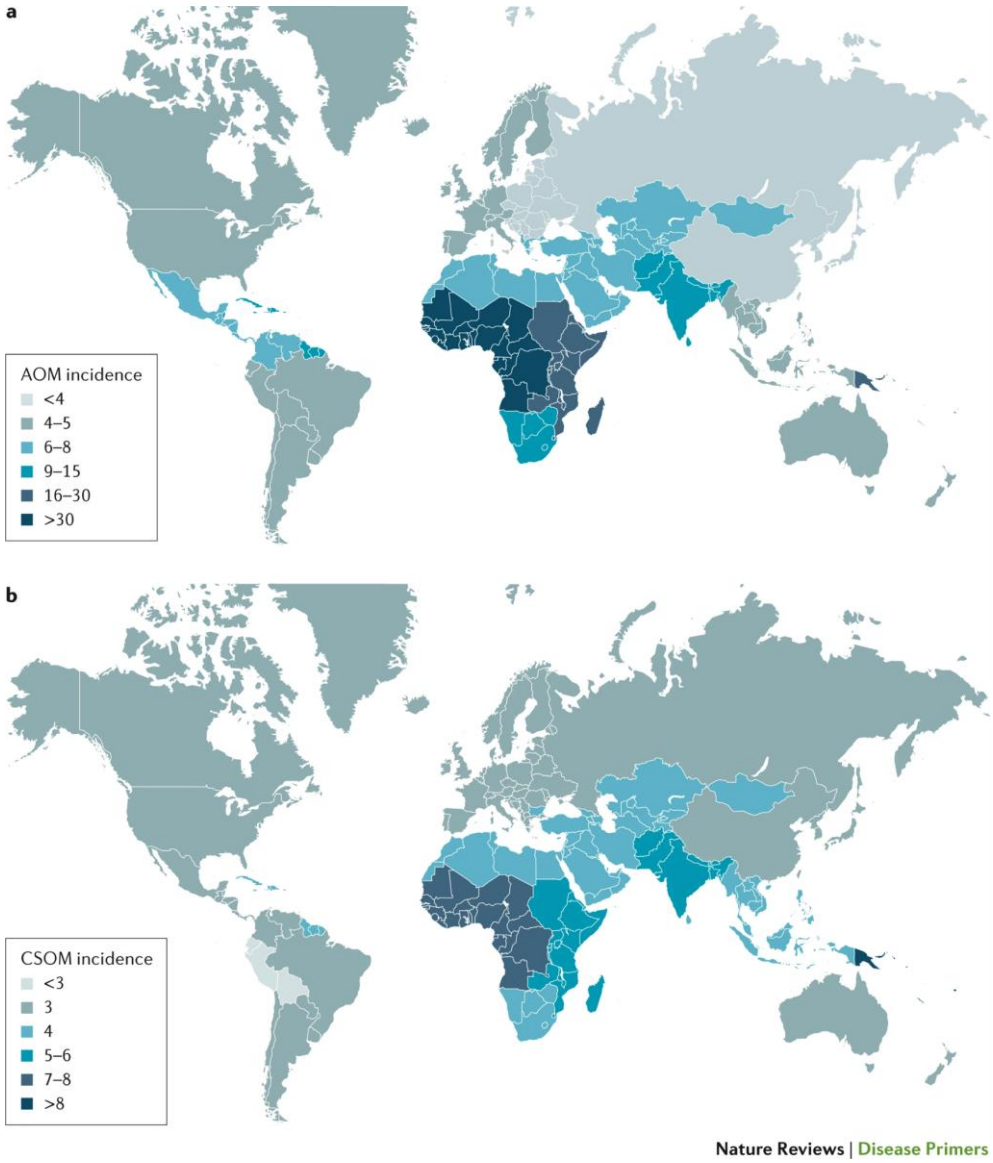


Figure 15 - Geographical distribution and worldwide incidence rates, in 2005, of (a) AOM (per 100 people) and (b) CSOM (per 1000 people). Adapted from https://www.nature.com/articles/nrdp201663.

1.4.4. Further complications associated with otitis media

Serious clinical complications of OM include a variety of intracranial aggravations, such as: the extension of infection to the mastoid air cells, known as mastoiditis; the formation of intracranial, subarachnoid and subperiosteal abscesses; facial nerve palsy; labyrinthitis or inflammation of the inner ear; febrile seizures; cholesteatoma; and meningitis. Mastoiditis is a serious infection that typically occurs as a result of OM. As the mastoids contribute to the maintenance of air pressure, and protection of ear structures and the temporal bone, infections in this region can contribute to hearing loss and spread of infection to adjacent structures. Further, although a cholesteatoma is a rare condition, it is one of the most common consequences of COM as it consists of an accumulation of simple squamous epithelial cells within the ME cavity that can lead to damage and degradation of the surrounding structures, such as the ME ossicles, and irreversible hearing degradation. Meningitis is an infection of the protective membranes that surround the brain. The disease carries a high risk of morbidity and mortality. Though this condition is not exclusively linked with OM, due to its proximity of the brain, ME infections are a weighty threat for the extension of the infection to the area. [55–59]

Additionally, COM is linked to high morbidity rates, particularly in young children, as hearing loss leads to speech development problems and/or language acquisition delays. Treatment of COM and its sequelae often require expensive or complex solutions, such as invasive ear and upper respiratory tract surgeries, as well as a continuous use of antibiotics, contributing to their overprescription and the antimicrobial resistance crisis.

1.4.5. Societal impact of otitis media

As mentioned previously, OM commonly affects neonates, toddlers and young children. In high-income countries, OM presents a massive burden to healthcare systems, as children require multiple doctor visits and follow-up appointments, the administration of different forms of treatments, including antibiotics and decongestants, and, potentially, invasive surgeries. In fact, OM-associated surgery, such as tympanostomy, is one of the most common reasons for paediatric surgery requiring anaesthesia, and OM is the leading cause for antibiotic prescription in paediatric settings. In low-income countries, however, the disease presents as a burden to society. Individuals without access to treatment face the complications

associated with unhampered progression of disease, which hinders their growth and development. [38]

Prevalence of COM has been shown to be associated with social-economic status, as in high-income countries 5-6% of children suffer from COME and less than 1% suffer from CSOM, but in low-income countries COME affects 10% of children while CSOM affects more than 4-6% of children, with much higher incidences in indigenous populations, 8%-50%. [48,60] Furthermore, Monasta *et al* (2012) estimate that annually 21,000 people die due to OM-related complications. [61]

1.4.6. Current and new treatments for otitis media

1.4.6.1. Treatment of acute otitis media

The latest European treatment guidelines suggest that once AOM is diagnosed, in individuals without underlying conditions, an observation period of up to 48 hours should be followed, whereas in at risk groups, such as immunocompromised individuals or babies under 6 months of age or with underlying birth defects (e.g.: cleft palate), treatment should start immediately. During the observation period, pain management and analgesia can be provided to avoid patient discomfort (e.g., paracetamol and ibuprofen). If no improvement is seen after the observation period, a 5-to-10-day course of systemic antibiotics course, commonly amoxicillin, a broad-spectrum antibiotic, should be started. (See table 3). [62]

The frequent hesitancy in immediate antibiotic administration post OM diagnosis aims to prevent antibiotic overuse in infections that can self-resolve. Unrestricted antibiotic intake, particularly broad-spectrum systemic antibiotics, may lead to further adverse effects, such as gastrointestinal symptoms, allergic reactions and *Clostridium difficile*–associated colitis. [43,63] However, due to concerns regarding poor membrane diffusion and the ototoxicity of ototopical (local) antibiotics have driven the medical community, in the vast majority of AOM cases, to opt for systemic antibiotics instead of the local-specific medication, or, in cases of COM, to use multiple otoantibiotics combined with corticosteroids. [64–67]

Despite a global update in treatment guidelines in 2013, moving OM treatment away from immediate antibiotic administration to careful watching, Marom *et al* (2021) have noted

that there has been a moderate adherence to the new approach, with only 46-52% of the cases evaluated (out of a total of 491,106 cases) following the updated recommendations, while the remainder started immediate antibiotic administration on OM diagnosis. It is important to note that following updated clinical treatment guidelines is essential to administer the best possible care, as the updates are based on evidence and studies to guide clinical decision-making. [68]

Table 3 - According to USA treatment guidelines, early antibiotic prescription in AOM affected patients is dependent on the patient's age, previous history and risk of subsequent complications, as well as severity of symptoms presented. Limited evidence suggests amoxicillin is more effective than other commonly administered antibiotics. Pink bottle represents immediate antibiotic prescription. Pneumatic otoscope represents up to 48 hours observation period. Adapted from https://pubmed.ncbi.nlm.nih.gov/23439909/

Symptoms severity Patients' age	Severe symptoms (unilateral or bilateral)	Mild symptoms (unilateral)	Mild symptoms (bilateral)
< 6 months			
6-23 months		 or 	
> 24 months		 or 	 or 

For OM treatment, oral steroids and decongestants have commonly been prescribed alongside antibiotics. Francis *et al* (2018) studied the effects of oral steroids and found no effect on the resolution of OM-associated hearing loss in children aged 2 to 8 years old. Similarly, Ranakusuma *et al* (2018) investigated the effects of corticosteroids for AOM treatment and found that the duration of symptoms was not affected by its administration. [69,70] Shaikh *et al*

(2017) found that, for children under the age of two, immediate administration of amoxicillin was the most cost-effective treatment for AOM, while Spurling *et al* (2017) noted that immediate amoxicillin was more effective in treating OM when compared to delayed antibiotic administration. Hoberman *et al* (2016) concluded that a reduced antibiotic administration was not as effective as a full regimen for OM resolution in children between the ages of 6 to 23 months. [71–73] However, these findings must be taken on a case-by-case consideration, depending on disease severity upon diagnosis, as immediate antibiotic administration is not supported by the new treatment guidelines, paediatricians' decisions should balance the costs and benefits of immediate administration and its consequences to the current antimicrobial resistance crisis. Santos-Cortez *et al* (2019) alerted the medical community to the overprescription of medicines such as steroids, corticosteroids and decongestants, that do not halt disease progression. Furthermore, they also reminded the medical community that antibiotics (particularly amoxicillin) are still the golden standard for OM treatment, warning that in an era of insurgent antibiotic-resistant bacteria, alternative treatments to fight disease progression are of utmost importance. [74]

Regarding drug delivery to the ME, alternative methods of administration have been investigated. For example, Yang *et al* (2016) found that therapeutic levels of ciprofloxacin could be delivered to the chinchilla ME by applying drug-containing hydrogels to the external surface of the intact tympanic membrane. Kurabi *et al* (2017) identified peptides that promote the active transport of large particles, such as bacteriophage, across the intact *ex vivo* human and *in vivo* rat tympanic membrane. Both studies facilitate routes that target the specific site without a need for systemic administration. Furthermore, Kurabi *et al* (2018) showed that the tympanic membranes of healthy rat MEs allows for trans-tympanic membrane transport at the same rate as tympanic membranes from infected MEs, that the rate for transportation of different sized particles is similar and that healthy rat and human tympanic membranes present similar transportation rates. These findings are of great importance in propelling alternative delivery mechanisms for OM treatment. [75–77] Non-ototoxic local antimicrobial administration (for example: eardrops) could replace systemic antibiotic administration, which carries significant side effects, including potential allergic reactions, disruption of commensal microbial flora, particularly in the gastrointestinal tract, and increased bacterial resistance. Alternatives to systemic antibiotic delivery are essential to ensure better health outcomes, on an individual and community level.

1.4.6.2. Treatment of chronic otitis media

As previously mentioned, once AOM has been resolved without tympanic membrane perforation, if fluid has built up in the ME, it progresses to OME. At this stage, direct visualisation shows no soreness or redness of the tympanic membrane, however, due to high fluid-air levels in the ME cavity, there is reduced mobility of the tympanic membrane. OME can resolve within a few weeks without surgical intervention, but recurrent AOM (rAOM) patients or patients whose OME is not resolved after 3 months may be considered for surgical intervention for the insertion of tympanostomy tubes. The tubes, also known as ventilation tubes or grommets, are inserted into the tympanic membrane to promote aeration of ME, preventing fluid accumulation and allowing for pressure normalisation (Figure 16). [55] COME can also be resolved through the insertion of ventilation tubes, though the underlying mechanisms for disease clearance are unknown. The leading hypothesis suggests that, in humans, oxygen tension is an important factor in maintaining ME homeostasis, thus it can be theorised that, as seen in COME-affected mouse models with surgical tympanic membrane incisions mimicking ventilation tubes, the tubes reduce the hypoxic environment and inflammatory effusion, promoting resolution of inflammation. [78]

Though tympanostomy largely improves a child's quality of life in the short-term, it is important to reiterate that it constitutes an invasive surgical procedure. Further, tympanostomy may be combined with adenoidectomy, another invasive surgery, generally requiring anaesthesia, inpatient care and follow-up procedures, such as antibiotics and analgesic administration. The consequences of this form of treatment can range from postoperative nausea and vomiting to airway obstruction or cardiovascular instability. [79–81] Furthermore, tympanostomies are not always successful as up to 50% of children with ventilation tubes get discharge from the tube causing bad odour, pain and fever; 5-10% of the tubes become blocked due to ME effusions or bacterial biofilms and/or fall out too early and 1% of children may suffer from tympanostomy-derived cholesteatoma or sensorineural hearing loss. In order to prevent postoperative ear discharge and facilitate ME healing, guidelines advise frequent saline washouts or regulated antibiotic administration (possibly combined with corticosteroid ear drops to reduce inflammation). [55,64]

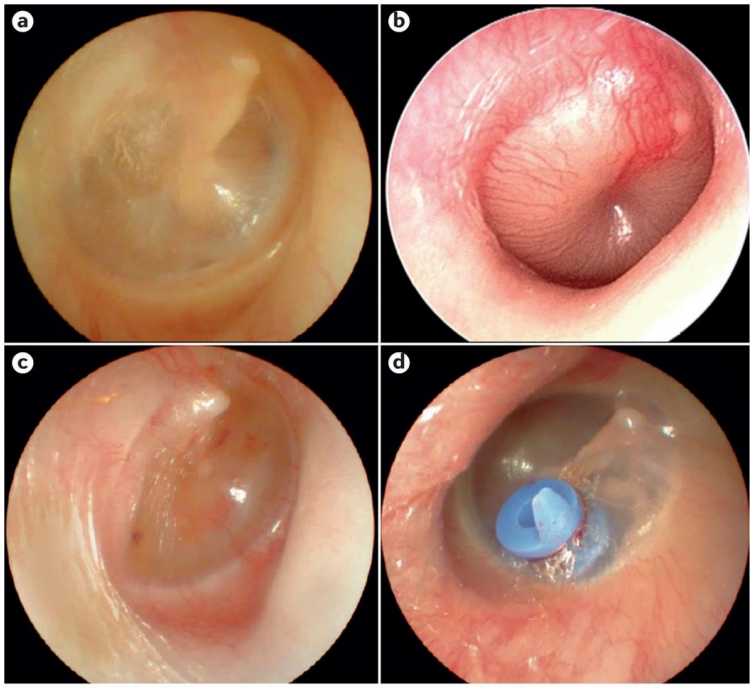


Figure 16 - Otoscopic visualisations of the tympanic membrane and its different appearances in OM-affected ears. (a) Healthy tympanic membrane. (b) Swollen, red and bulging tympanic membrane, typically seen in cases of AOM. (c) OME-affected ear. (d) Tympanic membrane with ventilation tube, a common form of treatment for COME-affected ears, as it improves oxygen circulation, aiding in the resolution of inflammation. Adapted from: https://www.nature.com/articles/nrdp201663

In CSOM, resolution can be achieved by administration of topical non-ototoxic antibiotics, such as quinolone. The infection clearance is more easily achieved in CSOM than COME, as in CSOM the tympanic membrane is naturally perforated allowing for better diffusion of the antibiotic into the ME. However, it has not yet been reported how often antibiotic treatment enables long-term resolution of the disease and, as CSOM is characterised by ongoing intermittent infections that impair tympanic membrane recovery, full membrane healing frequently fails. Thus, surgical repair, through tympanic membrane replacement using autologous cartilage or fascia grafts, is the most successful long-term option despite 1 in 6 attempts failing. [43]

Even though the number of OM cases has decreased over the past decade, due to better diagnostic tools, more accurate treatment administration and improved public health, OM is still among the most common diseases of childhood and a leading cause of paediatric surgery and antibiotic prescription. Furthermore, the rising number of antibiotic-resistant pathogens,

alongside the increased vaccine hesitancy movement, will affect treatment of future cases of OM.

1.4.7. Causes, risk factors and prevention of otitis media

OM is a multifactorial disease, influenced by pathogen virulence, host immunity status, environmental factors, previous OM infections and genetic predisposition. Around 60-80% of children under the age of 3 will suffer from at least one AOM episode; only a small subset will develop recurrent OM (rOM) or COM. Guidelines for prevention of OM include basic ear hygiene practices, such as avoiding dirty hand-nose contact and keeping sensible distances from URTI patients. Reports have shown that inhalation of air pollutants, such as tobacco and indoor smoke, both in utero or in childhood, are linked to increased cases of ear diseases in young individuals, underlying the environmental component of an individual's susceptibility to OM. [51,82]

1.4.7.1. The ear microbiome and immunisation efforts

The microbiome composition of the ME also plays a role in susceptibility to OM, as disturbances to the normal microbial flora, due to migration of commensal bacteria, opportunistic pathogens or immunological clearance of bacteria residing in the ME or ET, may trigger disease. To support this fact, it is of relevance to look into immunisation data against otopathogens. [83] Vaccination efforts against *H. influenzae*, *Pneumococcal spp.*, and seasonal influenza have led to a reduction of OM cases in immunised individuals when compared to non-immunised individuals. Block *et al* (2004) and Casey *et al* (2004) have previously shown, in studies involving 419 and 551 AOM-diagnosed children, in USA, that introduction of heptavalent pneumococcal conjugate vaccine (PCV7) significantly decreased cases of OM, from 48 to 31%, in both rural and suburban settings. [84,85] More recently, Soysal *et al* (2020) reported that, in Turkey, introduction of 13-valent pneumococcal conjugate vaccine (PCV13) in 2011/2012 led to an AOM decrease of 54% in 2016/2017, in children younger than 5 years old, in a study involving 23,005 AOM-diagnosed children. Further, their data also shows a 65% decrease in tympanostomies performed, compared to pre-PCV13 period. [86] However, introduction of PCVs has led to an increase in the colonisation of the ME by other bacterial species, most notably, *H. influenzae*. Introduction of the *H. influenzae* serotype b (Hib) vaccine

in the 1990s has greatly reduced the incidence of Hib-related diseases, including meningitis and OM, but has also forced a shift in microbial populations from encapsulated Hib to non-typeable *H. influenzae* (NTHi). [87] To further reduce the microbial shift post immunisation, NTHi's Protein D was also conjugated into some PCV vaccines, such as the 10-valent pneumococcal NTHi protein D conjugate vaccine (PHiD-CV10). Sigurdsson *et al* (2018) show a 22% decrease in OM incidence rates, in Iceland, post introduction of PHiD-CV10 in 2010. The vaccine significantly reduced the first and second episodes of AOM and their data included evidence of herd immunity effect and AOM protection in children too young to receive the vaccine. [88] Finally, Wu *et al* (2018), performed a retrospective cohort analysis of the impact of the introduction of the trivalent influenza vaccine (TIV) on AOM cases, in Taiwan. Data from 803,592 children less than 10 years old in influenza seasons was evaluated. TIV became available for children less than 2 years old, in 2004, and its introduction led to a significant reduction, 32.8%, in AOM cases, and consequently a reduction in the number of outpatient visits for AOM in the influenza season in the following years, 2005 and 2006. However, these reductions were only observed in vaccine-eligible children, as older groups, who were not enrolled in the vaccine programme, did not experience the same effect. [89] Unsurprisingly, reports have also shown that passive immunisation (antibody transference through maternal milk and breastfeeding of new-borns and toddlers) also leads to reduced numbers of OM cases, when compared to non-breastfed children. [90] These findings highlight the importance of active and passive immunisation, whenever possible, as an important tool in the prevention and management of OM.

Further work into more cost-effective vaccines and its global availability should be carried out, as these are clear contributors to the reduction of disease cases. A more thorough analysis on the effects of vaccination and subsequent emergence of non-capsulated pneumococcal variants and NTHi cases should also be conducted, as the long-term effects of this populational shift are still unknown.

1.4.7.2. Genetic predisposition and susceptibility to otitis media

Genetics of OM have been thoroughly studied as, within patients of similar regions and economic status, this is often the differentiating factor to disease susceptibility. A plethora of genes with different biological functions have been associated with increased susceptibility to

OM, but genes with protective functions against OM onset have also been identified. These range from genes encoding proteins that participate in both inflammatory and anti-inflammatory responses, to immune receptors and to genes that possess roles in cellular structure and differentiation. [91,92]

Fucosyltransferase 2 (FUT2) is an enzyme responsible for regulating the secretor status and surface expression of both Lewis and ABO(H) antigens on the mucosal epithelium. Different bacteria utilise these antigens as an anchor to epithelial cells, and thus specific variants of FUT2 have been associated with different OM susceptibilities and bacterial infections. For example, Santos-Cortez *et al* (2018) reported that the FUT2 rs601338 p.Trp154* variant is linked to an increased abundance of *Lactobacilli* and Gammaproteobacteria in the ME, and greater susceptibility to OM. FUT2 p.Trp154* regulates expression of the A-antigen, used by bacteria to adhere to cells. However, evidence also shows that, in non-secretor ABO(H) carriers of FUT2 p.Trp154*, where the variant co-regulates expression of the Lewis antigen, the polymorphism may confer protection against infection of other bacteria, but augmented predisposition to *S. pneumoniae* infection. Furthermore, FUT2 populational variants have also been linked to different OM developments, where carriers of reduced levels of FUT2 are more vulnerable to cholesteatoma. Thus, combinations of various common and rare variants of *Fut2*, associated with environmental factors, increase the complexity of OM. [93]

Polymorphisms in genes with functions in the cell or molecular biology of the ME have also been proven to have an impact on the development of OM. Alpha-2-Macroglobulin Like 1(A2ML1) is a member of the A2M family and a ME-specific protease inhibitor that acts as a marker for vascular permeability of ME mucosa during infection. A2ML1 is responsible for protease ensnaring. A study by Santos-Cortez *et al* (2015) on the effects of rare variants of A2ML1 concluded that duplication of the gene leading to protein truncation affected protease trapping and lysosomal clearance. Furthermore, it was shown that serous ME infections present decreased levels of A2M, but acute purulent OM exhibit increased levels of the antiprotease. Children affected by rAOM also present low levels of A2ML1, which can lead to severe ME mucosa damage due to unchecked levels of proteases on site. Likewise, Santos-Cortez *et al* (2015) found that certain *A2ml1* variant products affect thiol-protease trapping as the conformation of A2ML1 and formation of A2ML1-protease complexes affects normal functions of the protease inhibitor. Interestingly, bacitracin, a common component of antibiotic ear drops, has been shown to competitively inhibit binding of A2M-complexes to macrophages

and fibroblasts, thus dampening A2M responses. This property could be exploited for dysfunctional A2ML1 variants. [94]

A recent study by Van Ingen *et al* (2016) has also associated gene polymorphisms of Fibronectin Type III Domain Containing 1 (FNDC1), an extracellular matrix protein involved in multiple cellular processes, such as cell adhesion, migration, proliferation and differentiation, with a higher risk of AOM, namely *Fndc1* SNP rs2392989, that decreases levels of FNDC1 methylation. As unfolded FNDC1 activates the NFκB pathway, constant upregulation of the protein is linked to increased inflammation, due to secretion of IL-8 and TNF-α. Increased levels of inflammation within the ME are linked to AOM. [95]

Additionally, inflammation is associated with decreased on-site levels of glucose and oxygen leading to cell hypoxia. As upregulation of hypoxia pathways, such as the vascular endothelial growth factor (VEGF) pathway, in white blood cells from COME patients is seen across both mucoid and serous samples and the ME mucosa, it has been concluded that hypoxia plays a role in COME. It has also been hypothesised that oxygenation of the ME can lead to significant improvements of chronic inflammatory conditions. [78]

In OM, deficiencies in cellular structures, as with the previously mentioned defects in molecular pathways, can have consequences in the susceptibility to disease. This has been demonstrated in mice with partial knockout (hemizygotic) of *Fl1* and *Ets1*. These ETS transcription factors play a role in development of the nose, ME cavity and auditory ossicles, and are linked to accentuated hearing impairments associated with COM, inflamed ME and abnormally small ME cavities. [96]

According to a study by Crompton *et al* (2017), young adult mice (3-20 weeks) who possess point mutations in the *Enpp1* gene (*asj* mice) manifest OM-related symptoms, with non-infectious origin, such as effusions in the ME, thickened ME epithelium, ET tube dysfunction due to augmented epithelial proliferation, fusion of the auditory ossicles and calcification of several ME structures, hence suffering from conductive hearing loss. [97] On the other hand, homozygous young adult mutant mice (20 weeks) with point mutations in the *Nisch* gene (*Edison* mice) spontaneously develop COM, presenting with mild craniofacial defects, fluid-filled ME cavities lined with thick mucus and, in more severe cases, inflamed tympanic membranes. Additionally, Crompton *et al* (2017) reported that the effect of the point mutation in the *Nisch* gene causes disturbances in the LMK1 and NFκB pathways, leading to the development of COM. [98]

As with any other infection, pathogenic bacteria in the ME will elicit an immune response, with the innate immune system becoming active soon after entrance of the pathogen. Studies using mice with reduced expression of proinflammatory cytokines, namely IL-1β, TNF-α and CCL3, have shown diminished but prolonged leukocyte recruitment, defective macrophage function and failure to clear common otopathogens, such as NTHi. [99,100] Moreover, mouse mutants deficient in PRRs, such as Toll-like receptor (TLR) 2, 4 and 9, NOD-like receptors (NLRs), and adaptor proteins (MyD88 and TRIF), lack significant production and maturation of the proinflammatory cytokines IL-1 and TNF-α, leading to poor leukocyte recruitment but prolonged inflammatory signals without clearance of bacteria. These findings suggest that, in mice, a poor innate immune response leads to prolonged periods of disease. [92]

The ME epithelium is composed of a layer of ciliated cells interspersed by goblet cells. Goblet cells are responsible for secreting proteins that contribute to the maintenance of homeostasis in the ME. However, alterations in the expression and secretion of proteins occurs in response to external triggers, such as infection. Dysregulation of protein expression associated with host-defence factors has been shown to positively affect development of OM. For example, MUC5B is produced and secreted by goblet cells. This mucin plays an important role in host innate immunity and is commonly found in COME-affected patients. Roy *et al* (2014) have shown that mice with knocked-out *Muc5b* expression in their ME developed infectious OM. Conversely, mice that transgenically overexpressed *Muc5b* presented improved macrophage functions. This study determined MUC5B to play an essential role in the interplay of airway defence. [101,102]

Recently, the role of BPIFA1 in OM has also been closely investigated, as the protein is reported to be one of the most abundant secretory proteins in the upper respiratory tract, with a multifunctional role in host defence and antimicrobial activity. A study by Mulay *et al* (2018) concluded that although deletion of *Bpifa1* did not lead to spontaneous development of OM nor impairment of a proinflammatory response, in established COM mouse models (*Junbo* mice), it led to severe exacerbation of the phenotype, demonstrating a fundamental protective role of BPIFA1 in the mucosal regions. [103]

In conclusion, OM is a heritable disease as genetic makeup confers increased susceptibility or protection, with studies suggesting a strong correlation between genetics and length, severity and number of episodes. Due to small human cohorts, the complexity of the human genome and the uncertainty when translating results obtained in animal models to

humans, it is difficult to define the exact conditions that render an individual more susceptible to OM. Moreover, as an individual's response involves complex interactions of factors from ME epithelium, sub-epithelium, inflammatory cells and ME effusions, it is difficult to pinpoint cell-specific functions. These issues, however, could be tackled using *in vitro* ME epithelium cultures replicating the phenotype of human genetically modified OM models, which are easy to manipulate and allow cell differentiation and cell infection with multiple pathogens.

1.4.8. Models for the study of otitis media

The anatomical location of the ME presents problems in terms of accessibility to the cavity for treatment and research purposes. Studies utilising human ME epithelium often rely on recovery of epithelium from the ME of cadavers or foetuses or from ME swabs performed during invasive surgeries that access the ME through the tympanic membrane. As such, due to their wide availability, most of our current knowledge on the ME epithelium and OM has been derived from experimental animal studies and relies on recovery of ME effusions or the ME bulla post animal culling.

1.4.8.1. *In vivo* models

The use of animal models is a longstanding practice in research and medicine, particularly mammals, due to their physiological similarities to humans. Due to the complexity of humans, animal models provide the closest reliable and equally complex model to many diseases and mechanisms. Animals offer the advantage of not being an isolated environment and relying on the physiological interactions occurring in the system as a whole, a particularly useful tool in studies focusing on the dissemination of microorganisms during infection and subsequent immunity responses. [104]

Most OM studies involving animal models are carried out in mice or rat, due to the availability of reagents, low cost of animal maintenance, ease in tracing genetic backgrounds, well-characterised immune responses and well-defined microbiological status. Thus, mutant murines presenting OM-like characteristics are frequently utilised in research. Different phenotypes such as variable proportions of polymorphonuclear cells (including foamy large

macrophages), lymphocytes, plasma cells and apoptotic or necrotic cells, thickened inflamed bulla mucosa, oedematous and with polyps, capillary and lymphatic proliferation, and a loss of ciliated cells and increased goblet cell numbers have been described and utilised as OM models. Their anatomical and physiological similarities to the human ME, the natural occurrence and high heritability of OM, the ease with which the genome can be altered creating different transgenic models make them ideal OM research tools. It is relevant, however, to note that surgical procedures are challenging, and their immune systems and responses are not always comparable to humans, e.g.: induction of OM in mice using human pathogens often requires experimentally induced conditions. [105–110]

Davidoss *et al* (2018) reviewed the use of animal models in the study of AOM and found that the rat and chinchilla were the most commonly used models, but mice, gerbils, and guinea pigs have also been used. The rat was a favoured AOM model due to its significant similarity in ME anatomy and histology when compared to humans. They have similar cell types and distribution, with a well-defined area of simple, squamous epithelium, that can present small clusters of secretory cells, and a complex epithelium with ciliated cells, interspersed with secretory cells, continuous to the ciliated epithelium of the Eustachian tube, suggesting the existence of a mucociliary clearance system, and similar AOM pathology. [111–117] Chinchillas also provide a useful OM model due to their large bullae, which facilitate inoculation and recovery of effusions. They rarely develop natural OM but share the same viral and bacterial pathogens as humans and have a similar disease progression. However, their multi-loculated bulla is prone to the development of fibrosis, limiting the spread of infection within the ME. In addition, they are not as hardy as rats, in that they easily shed when stressed and develop sepsis more frequently. [118–120]

Several models for the study of COM have also been developed, particularly genetically modified mice where the models chronically portray COM phenotypes. More recently, a significant number of studies have used, for example, *Jeff* mice carrying a missense mutation in the *Fbxo11* gene, responsible for a number of pathways associated with cell proliferation, differentiation and apoptosis, that have been linked to COME, and *Junbo* mice carrying a mutation in *Evi1* (also known as *Mecom*), which plays a role in host innate immune regulation. [103,121–125]

1.4.8.2. *In vitro* models

Cell cultures provide a versatile tool, often associated with homogeneity and data reproducibility, particularly with the use of cell lines. Cell culture usage is not as restricted as *in vivo* models, as it does not necessarily come at the expense of an animal, and it allows for frequent biochemical, cellular and molecular studies. Nevertheless, *in vitro* models fail to fully resemble the complexity of *in vivo* organism models, by lacking the ability to replicate the cellular and biochemical interactions between the various organ systems. [126]

1.4.8.2.1. Development of cell lines

When compared to primary cells, immortalised cell lines present an advantage in terms of propagation, proliferation, and reproducibility of results. Cell lines also overcome the senescence issue primary cells face, as the latter possess a limited number of mitoses. However, the immortalisation of cells requires changes to the genome, the effects of which may lead to differences in the data collected between cell lines and primary cells.

Ueyama *et al* (2001) immortalised a rat ME cell line utilising an adeno 12-SV40 hybrid virus, rMEE-1, which appeared to maintain primary epithelial cultured cell characteristics, such as "dome" formation, hexagonal appearance and tight junctions. These cells, however, displayed a slightly smaller size and a shorter doubling time compared to the primary cells. [127]

Moon *et al* (2002) successfully immortalised a human ME epithelial cell line, HEI-HMEEC-1 (hMEEC-1), using a replication-defective retrovirus construct, pLXSN 16E6-E7, coding for the HPV type 16 transforming oncoproteins E6 and E7. hMEEC-1 cells are phenotypically similar to primary human MEEC, forming monolayers with a hexagonal shape. However, as hMEEC-1 is derived from male adult cells and OM is primarily a paediatric disease, this may represent a limitation to the usage of the cell line. Moon *et al* (2002) also noted that hMEEC-1 cells maintain the capacity to produce secretory gene products, such as lysozyme and mucin, although recent studies show that this expression seems to be very limited when compared to primary cells. [128]

Tsuchiya *et al* (2005) established a temperature-sensitive mouse ME epithelial cell line utilising a simian virus 40 A-gene. At permissive temperatures, 33ºC, the cell line proliferates, but at non-permissive temperatures, 39ºC, the cells undergo differentiation. Although epithelial

markers are expressed by the cell line, mesenchymal markers are expressed too. The authors highlight that an epithelial-mesenchymal transformation can be common when epithelial cell lines are genetically modified, leading to expression of both epithelial and mesenchymal markers. [129]

The ongoing need for a ME epithelial cell line for studies of bacterial interaction with host cell receptors, particularly in OM cases, led to the development of the above-mentioned cell lines. However, human cell lines, particularly of paediatric origin, that retain the ability to differentiate into a pseudostratified epithelium that closely resembles the ME are of the utmost importance to further the research of ME disease.

1.4.8.2.2. *In vitro* studies in animal cells

Schousboe *et al* (1996) evaluated the growth and synthesis of rabbit ME epithelial cells from explants. Their model, comprising a single cell type, provided valuable information under controlled conditions, but lacked an approximation to physiological conditions. This suggests the need for a model that truly mimics the middle ear, requiring exposure of the cells to air and delivering nutrients by diffusion. [130]

Nakamura *et al* (1991a) cultured chinchilla ME epithelium from explants and characterised the cells, confirming the different area-specific epithelial phenotypes seen throughout the ME, simple and complex. Their research highlighted the cobblestone appearance of ME epithelial cells, resulting from the presence of tight junctions, and the "dome" formation, an elevated monolayer of epithelial cells that indicates active ion and fluid transportation. [131] Further, Nakamura *et al* (1991b) applied a serial culture technique to the cells, observing that early passage epithelial cells conserved the cobblestone appearance, but in later passages, the cell morphology changed to more closely resembled a fibroblast-like morphology, losing most of its cobblestone appearance and dome structures. Nakamura *et al* (1991b) hypothesised that reduction of contact inhibition might have led to these changes. [132]

Amesaral *et al* (1992) used a dissociated cell culture system to establish a chinchilla ME epithelium, highlighting for the first time the importance of establishing 2D models that undergo fibroblast differential separation. Additionally, the authors reported that despite observing ciliary movement in the initial explant, following 7 days in culture, the cellular outgrowth presented no ciliary movement. This suggests that explanted differentiated ME

epithelial cells undergo dedifferentiation when the culture conditions do not resemble the ME. [133]

Herman *et al* (1992) investigated the transport properties of the Mongolian gerbil ME epithelium in culture. They confirmed the pluripotency of ME epithelium, through the presence of secretory, ciliated, and simple squamous epithelium in the ME. Their cultured cells presented polarised monolayers and exhibited dome structures on non-porous layers. Within physiological scenarios, the authors hypothesised that modulation of the water transport system could hinder the homeostasis of mucociliary clearance mechanisms, leading to COM. [134]

Although 2D models are useful tools for research, there are clear limitations in its use, leading to a strong demand for efficient 3D models that more closely resemble *in vivo* conditions, taking into account factors such as layered and diverse differentiated cell populations or organoid arrangements. [126]

Mulay *et al* (2016) developed a model for the culture and differentiation of primary mouse MEEC at air-liquid interface (ALI) using a transwell system and defined the transcriptome of these cells. This model mimicked the *in vivo* upper airway epithelium, by exposing the apical chamber, where the cells are seeded, to air and feeding them through the basal chamber. The cells recovered in this study exhibited characteristic epithelial features such as cobblestoned morphology, formation of tight junctions, apical-basal polarisation, presence of desmosomes and apical microvilli, however, non-ciliated flat cells were also noted, as described previously by Moon *et al* (2002) and Thompson and Tucker (2013). [101] Mulay *et al* (2021) also demonstrated that the differentiated cultures presented a similar morphologic and genetic profile to that seen *in situ*, and regions of distinct mucociliary epithelium were positive for ciliated cell markers, mucus-producing cell markers and antimicrobials. [135]

1.4.8.2.3. *In vitro* studies in human cells

Information gathered from animal studies cannot always be extrapolated and translated into human physiology. Although 95% of the human and mouse genome are homologous, the differences in anatomy and physiology are significant. Furthermore, results from carefully selected and inbred models do not give accurate representations of whole populations, as a single animal model cannot mimic human disease, taking into consideration all polymorphisms

shared by the population. Development of multiple characterised human cell lines and isolation of primary cells is needed to permit research relevant to humans. [104]

Nell *et al* (1999) investigated the effects of *Salmonella typhimurium*'s endotoxin, similar to the endotoxin derived from NTHi, on air-exposed cultured primary normal human ME epithelium, from patients of undisclosed age undergoing ear surgery without ME pathology, to illustrate that disturbances in the mucociliary system may prompt ME pathologies. [136] Choi *et al* (2002) established a normal primary human ME epithelium cell culture system using the air-liquid interface (ALI) technique to induce ciliary differentiation, utilising cells from patients of undisclosed age (Figure 17). Their data suggested that secretory functions develop before ciliogenesis, and transcription of lysozyme and mucin mRNA increases due to the maturation of cells. [137] Choi *et al* (2003) also showed an association between metaplasia of the simple squamous ME epithelium and ME pathogenesis. This is of relevance as ME squamous metaplasia is a hallmark feature of COM and cholesteatoma. [138]

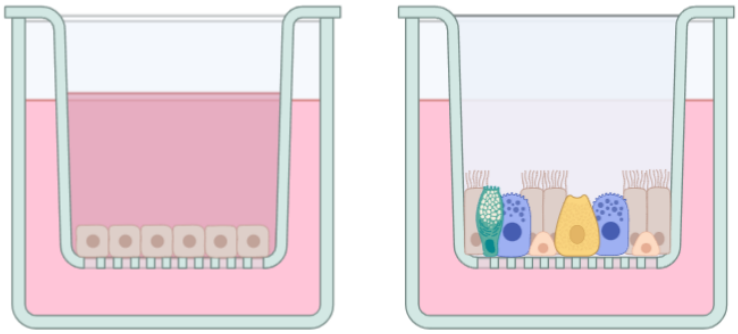


Figure 17 - Diagram of an air-liquid interface cell model. (a) Expansion phase, where the seeded epithelial cells in the apical chamber proliferate submerged in culture media, up to 7 days. (b) Maintenance phase, where the media in the apical chamber is removed and the cells are exposed to air and begin to differentiate into a pseudostratified epithelium; cells can be maintained for a limited period of time. In an air-liquid interface, the cells are seeded on top of a porous-membrane and fed through the basal chamber. Adapted from https://app.biorender.com/.

Buchman *et al* (2009) found that normal human ME mucosal cells, from patients of undisclosed age, support influenza A virus infection. Viral infection is a common OM trigger that is thought to lead to congestion of the ET and subsequent fluid, debris and bacterial accumulation in the ME. However, the possibility of direct infection of ME mucosal cells, and not ET, and the initiation of inflammatory cascades on-site in connection to ME cell infection

needs to be considered. Since the ME mucosa is a modified respiratory epithelium, devoid of MALT, it could possess similar response mechanisms to that of the airways. [139]

During the immortalisation process of hMEEC-1, Moon *et al* (2002) successfully serially cultured primary human MEE, again from patients of undisclosed age. The cells did not proliferate further than passage 5 (P5), and alterations to their morphology were observed, i.e., increase in number of intracellular vacuoles. Moreover, they hypothesised that utilisation of ALI systems could present a more adequate method for studies involving ME epithelium cells, as the cells they isolated lost differentiation characteristics (cilia), after 5 days in culture. [128]

To overcome the need for cell lines and due to previous reports of ME epithelium cell challenges at later passage, Chen *et al* (2019) developed a promising method for sustained culture of primary human cells, from ME cavities of children aged 1-10. Adapting a reprogramming protocol that utilises irradiated fibroblast feeder medium and Rho kinase inhibitor, Chen *et al* (2019) developed a culture medium that promoted proliferation of primary cells, without the need for viral transformation or activation of oncogenes. When placed in a specialised differentiation media, the cells were still able to differentiate into their original phenotype. Wide usage of this method could be very effective in utilising paediatric primary cells as a tool to study OM. [140]

Espahbodi *et al* (2021) compared the responsiveness of paediatric primary cells, from patients with noninflamed ME, rAOM and OME, and hMEEC-1 to stimuli in 2D culture settings. Their data showed that, overall, paediatric ME cells provide an appropriate research tool for the study of OM. Expression of targeted genes, *Tnf-α*, *Il-1β*, *Il-6*, *Il-8*, *Il-10* and *Muc5b*, and protein, IL-8, were significantly higher in paediatric cells than in hMEEC-1, both at basal levels and in response to TNF-α and NTHi lysate stimuli, suggesting that hMEEC-1 is less responsive to stimuli than paediatric cells. Further, Espahbodi *et al* (2021) also highlighted their ongoing work in the development of paediatric cell lines from healthy, rAOM and OME for comparative studies *in vitro*. [141]

Recently, Mather *et al* (2021) developed a 3D model of the ME, using fetal human ME epithelial cells. Cells were harvested from the ME of a 14-week-old fetus, expanded for 7 to 10 days, transferred onto transwell membranes, where they were exposed to air after a 4 day expansion period, and, after 4 weeks, differentiation levels were validated. Differentiated fetal ME cell models were up to 3 cell layers tall and expressed markers for epithelial (cytokeratin

14/16), basal (p63), ciliated (FOXJ1), and secretory (MUC5B/AC) cell subtypes, suggesting the presence of complex cell populations in these models, closely resembling the *in vivo* environment. However, this study only presented a single biological repeat, raising concerns regarding the reproducibility and feasibility of such a system, potentially hindering its use in further investigations of ME characterisation or response to infection. [142]

1.4.8.2.3.1. Cell co-culture

As 3D models and ALI provide an approximation of the *in vivo* physiology *in vitro*, the use of cell co-cultures might also be of value. The ME possesses a subepithelial layer under the epithelium, populated with fibroblasts. ME cells have not yet been successfully co-cultured with fibroblasts in the same way that lung epithelial cells and cholesteatoma cells have been. [143–146] Thus its effects and interactions with the ME epithelium, in presenting a more representative human model, are of great interest to the study of OM.

It's noteworthy that utilisation of both 2D and 3D models may be required to further the understanding of the ME. Although 3D models offer a closer mimic to the ME's epithelium, the timeframe of obtaining a differentiated functional model can be quite lengthy, and 2D models offer a convenient rapid tool for preliminary results.

1.5. The middle ear microbiome

The ME microbiome is variable in terms of bacterial communities, ranging from barely detectable bacterial load to significant levels of many different populations. In a normal ME, when a microbiome is present, the most abundant bacterial phyla are Proteobacteria, followed by Actinobacteria, Firmicutes and Bacteroidetes. Interestingly, these microorganisms are also found in COM without active inflammation, but in COME ears the bacteria populations are significantly different, with a lower prevalence of Proteobacteria and a higher prevalence of Firmicutes. Neeff *et al* (2016) did not culture any bacteria from surgically collected swabs of healthy MEs, and 68% of samples were negative for bacteria using molecular methods. As not all bacteria are easily cultured in a laboratory setting, molecular methods are essential for

identification of unculturable bacteria; further, molecular methods provide quicker and more accurate identification of species than bacterial cultures. [147] In their evaluation of the ME microbiome, Minami *et al* (2017) found that the microbiome of children with normal ME of differs significantly from that of adults, but in both the bacterial load is low. Alterations in microbiomes, as seen in COM with active inflammation when compared to normal ME, may contribute to the aggravation of inflammation. Minami *et al* (2017) speculated that the bacteria found in dry COM have the ability to cause active inflammation and stress the importance of treating chronic perforations and removing cholesteatoma to prevent the development of inflammation. [83]

Importantly, the studies above demonstrate low bacteria prevalence in the healthy ME, and how changes in the microbiome can contribute to disease development.

1.5.1. Middle ear pathogens

As previously mentioned, onset of bacterial OM requires an initial stimulus, typically a viral URTI, to trigger the host's inflammatory response and induce the conditions necessary for increased bacterial pathogenesis. Recent studies have positively correlated viral epidemics to spikes in the number of AOM cases. [148,149] Interestingly, reports show a decline in OM appointments, up to 63% compared to previous years, during the COVID-19 pandemic, but that can probably be attributed to the infection control measures introduced during 2020 and 2021. [150–154]

A study carried out by Yatsyshina *et al* (2016) in AOM-affected children showed that 14.5% of the children diagnosed with bacterial AOM still carried a significant viral load in their ME. In AOM-affected patients, the most commonly detected viral pathogens were respiratory syncytial virus, rhinoviruses, influenza and adenoviruses, whereas the most commonly detected bacterial pathogens were *S. pneumoniae*, *H. influenzae, Staphylococcus aureus*, *Streptococcus pyogenes* and *M. catarrhalis*. Bacterial presence was detected by both bacterial cultures and PCR analysis, and the study highlights the use of multiplex qPCR as potential diagnosis tool, as the molecular technique allows for detection of a wide variety of pathogens in shorter time periods than bacterial cultures. Furthermore, Yatsyshina *et al* (2016) attempted to correlate detection of pathogens in the ME fluid and their presence in the nasopharynx. Their data shows that bacterial detection through nasopharynx PCRs had variable

positive predictive values and high negative predictive values when compared to ME fluids PCRs. Once again, although their evidence may not significantly correlate the presence of one pathogen in the nasopharynx to its presence in the ME, it allows to accurately exclude which pathogens are not present in the ME. [155]

Ngo *et al* (2016) determined in a systematic review, which included reports from January 1970 to August 2014, that the most commonly detected bacteria in cases of AOM without discharge were *S. pneumonia*, NTHi and *M. catarrhalis*. On the contrary, in a systematic review compiling studies from July 2009 to June 2018, Hullegie *et al* (2021) showed that, in the PCV era, in children affected by AOM with discharge, the most commonly detected bacteria included *S. pneumonia*, NTHi, *S. aureus* and *S. pyogenes*. It is important to note that samples from AOM-patients with discharge are typically cultured from the discharge present in the outer ear, where *S. aureus*, as a skin flora commensal, is highly abundant, and thus its detection could be biased. Conversely, in AOM without discharge cultures, the samples are typically obtained from tympanocentesis, thus the probability for cross contamination is lower. [43,156,157]

Although bacterial species found in AOM cases can also be found in cases of COM, in these instances, they are usually detected alongside several other bacterial species. Kolbe *et al* (2019) have shown that pathogens commonly associated with AOM were only present in ~50% of the samples from COME patients. Interestingly, presence of *Haemophilus*, *Staphylococcus*, and *Moraxella* in the ME cavity was significantly higher in children with COME and asthma or bronchiolitis than those without lower respiratory tract disease. *Turicella* and *Alloiococcus* loads were significantly lower in COME-patients with lower respiratory tract disease. These data provide the basis for a link between the microbiome of COME-affected children and those with lower airway disease, and the bacterial diversity of COME-affected ME compared to normal ME or AOM-affected ME. [158] Furthermore, studies focusing on CSOM in low-income countries have reported that bacterial cultures from effusions of affected children contain significant levels of *Staphylococcus spp., Pseudomonas spp., Proteus spp.* and NTHi, which can be found as both plankton-form or biofilms. [60] Polymicrobial biofilms are structures that allow persistence in host environments, due to the possibility of interspecies signalling and the combined abilities to protect the bacterial community against antimicrobial factors. They are often recovered from rAOM or COM-affected patients, suggesting a role for biofilm formation in the development of COM. [159,160] In COME, the role of bacteria in the inflammatory process is still unclear; live bacteria can be found as both biofilms, either on mucosal surfaces or in

effusions, or planktonically within the effusion. In CSOM the role of bacteria in disease development and progression is clearer; as CSOM is associated with lower socioeconomic status, there is an inherent increased risk to pathogen exposure, increased bacterial loads and their presence hampers healing of the tympanic membrane. [39]

Bair and Campagnari (2019) investigated the biofilm viability of the three major otopathogens, *M. catarrhalis*, *S. pneumonia* and NTHi. Their study found that NTHi viability decreased when in a dual species biofilm with *S. pneumonia*, but not with *M. catarrhalis*. Furthermore, *M. catarrhalis* protected NTHi from *S. pneumonia* bactericidal activity in a polymicrobial biofilm, suggesting that *M. catarrhalis* promotes stable environments for polymicrobial formation with other species. Although this study was conducted in an *in vitro* nasopharyngeal model rather than a ME model, the results are likely still applicable in the ME, as the ME epithelium is in functional continuity with the respiratory system and, as previously mentioned, both the nasopharynx and ME epithelia are similar. [161]

Previous studies have shown that *Pseudomonas aeruginosa*, *S. aureus*, *Proteus spp.*, *Klebsiella pneumoniae*, and *Corynebacterium spp.*, which are all facultative anaerobic bacteria, are commonly found in chronic draining ears. [162–169] These bacteria species are able to form biofilms, promoting their own survival within the ME and prolonging the period of infection. Biofilm formation may explain the nature of recurrent and COM, as it is frequently seen in OM with effusion, as the lack of bacterial clearance leads to constant mucin production. In CSOM, biofilm presence also hampers healing of the tympanic membrane, as it does not allow the membrane tissue to heal and seal again. [170] Yadav *et al* (2017), investigated multispecies biofilm formation of *P. aeruginosa* and *S. aureus in vitro* and assessed the *in vivo* response to colonisation in the rat ME. Their data found that polymicrobial colonisation induced differential expression of genes involved in immune response and inflammation that were not expressed during single organism infection, suggesting that polymicrobial infections may be more virulent and should be addressed accordingly when selecting treatment. [171]

The frequency with which bacteria are found, and association between otopathogens and other bacteria, varies depending on socio-economic conditions. In their systematic review including reports from June 1996 to September 2015, Coleman *et al* (2018) highlight the differences in OM microbiomes from indigenous populations. AOM and AOM with discharge affected individuals presented similar bacterial profiles to the previously mentioned, however, in cases of AOM with discharge, *A. otitidis* was also present. As previously mentioned, the

rates of OME and CSOM are elevated in these populations. *Corynebacterium spp*., *Alloiococcus* and *Pseudomonas aeruginosa* were also frequently detected in cases of OME in Australian Indigenous children, as well as, perhaps more concerningly, multiple fungi species. These included *Candida, Aspergillus, Fusarium, Alternaria, Rhodotorula, Aurobasidium* or *Acrinomium* species. Fungi present a more complex challenge to treat than bacteria due to their evolutionary proximity to eukaryotic cells, as many of the same basic cell structures and machinery are shared between the two. Thus, some antifungal drugs that target fungal components can also target human cellular components, potentially leading to serious side effects in patients, including gastrointestinal disturbances, cardiac effects, nephrotoxicity, hypokalemia, and hepatotoxicity. Finally, in their review, Coleman *et al* (2018) came across few reports regarding biofilms in relation to OM in indigenous children. Given the high rates of COM, particularly CSOM, in these populations, the lack of data on the subject represents a significant overlook in the research of OM. [50,172]

The role of anaerobic and facultative-anaerobic species in OM requires further investigation. As anaerobic bacteria can be harder to culture than aerobes, their presence can often be missed. The use of molecular techniques for diagnosis would contribute to a better detection of anaerobic species. Furthermore, biofilm formation and secretion of ME effusions contribute to a hypoxic environment during inflammation, which allows anaerobic and facultative anaerobic bacteria to grow, and there are currently not enough reports on the consequences of the presence of these bacteria in COM.

1.5.1.1. *Streptococcus* / Pneumococcal species

Most pathogenic bacteria require virulence and persistence factors, which allow for enhanced bacterial invasion of the host cells and immune system evasion, both events being key to bacterial survival within a host. Keller *et al* (2016) have reported that in *S. pneumonia,* the non-encapsulated Pneumococcal variants express a specific surface protein, the pneumococcal surface protein K (PspK), which is fundamental in mediating adhesion to host epithelia and biofilm formation, promoting persistence within the host. [173] Furthermore, Pneumococcal species (spp) have also been shown to utilise the LuxS quorum-sensing systems in order to construct single species and polymicrobial biofilms that are mutually beneficial to bacteria. Quorum-sensing is a mechanism of bacterial cell-cell communication that allows for

inter and intraspecies communication to coordinate population efforts, from proliferation to virulence to biofilm formation. This mechanism has been proven to be vital for biofilm formation, as Yadav *et al* (2018) reported that inactivation of *luxS* led to bacteria's inability to produce biofilms and reduced ability to colonise the rat ME, suggesting that loss of *luxS* renders the bacteria unfit for successful disease onset. [174]

1.5.1.2. Moraxella catarrhalis

In *M. catarrhalis*, the role of Ubiquitous surface protein (Usp) family members, as mediators of bacterial attachment to the extracellular matrix and host epithelial cells, has been studied in detail. Singh (2015) demonstrated that the bacterial Usp proteins bind to plasminogen, which upon activation degrades proteins C3b and C5, key mediators of the complement cascade that promotes bacteria killing. [175] A study by Tan *et al* (2019) evaluating the role of NucM, a nuclease that degrades extracellular DNA and RNA, showed that mutants lacking this nuclease produced biofilms of larger mass. *In vitro* testing demonstrated that decreased expression of NucM by *M. catarrhalis* led to early infection followed quickly by biofilm formation while a later increase in expression promoted bacterial dispersal to other sites, spreading the infection. [176] Finally, Armbruster *et al* (2019) also evaluated quorum sensing effects on *M. catarrhalis*. Although the bacteria do not produce AI-2, the product of *luxS* expression, the presence of this interspecies signalling mechanism promotes increased *M. catarrhalis* biofilm formation and resistance to antibiotics. Furthermore, AI-2's secretion by NTHi was essential to the establishment of polymicrobial biofilms with *M. catarrhalis*, as NTHi mutants that did not express *luxS* were not able to form polymicrobial biofilms, and increase *M. catarrhalis* persistence in the chinchilla ME. [177]

1.5.1.3. Non-typeable *Haemophilus influenzae*

NTHi is a small gram-negative coccobacillus that, unlike *H. influenzae*, does not have the ability to produce a polysaccharide capsule. This bacterium is an opportunistic pathogen in the respiratory tract and has been linked to multiple diseases but is most frequently associated with diseases of the human mucosa and airway infections, in both children and adults (Figure 18). These diseases typically have high mortality and morbidity rates. NTHi's successful

colonisation of the respiratory tract is due to its ability to exploit multiple mechanisms of attachment to cells, its multiple virulence factors and rapid response to host defences through antigenic variation of its proteins, the phasevarion. [178,179]

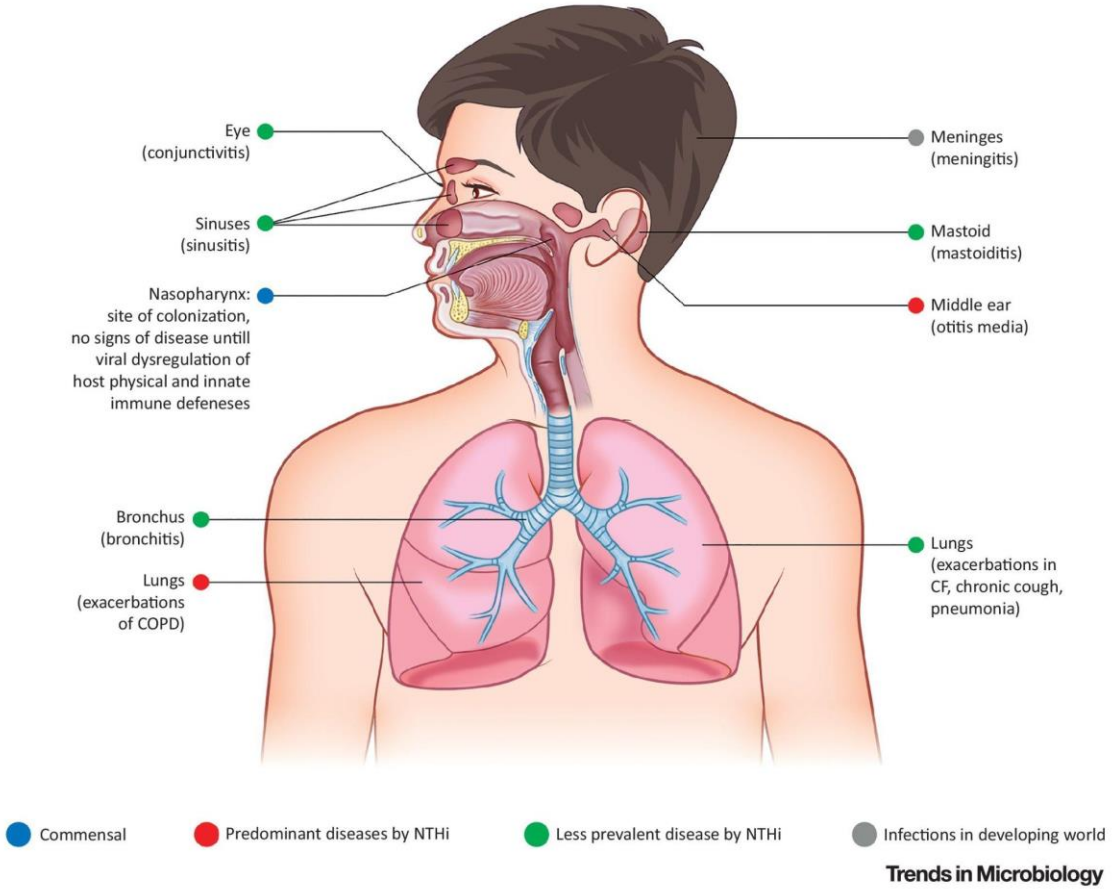


Figure 18 - Diagram highlighting the association between non-typeable *Haemophilus influenzae* and its role in several diseases. Adapted from https://www.sciencedirect.com/science/article/pii/S0966842X18301033.

NTHi is capable of forming biofilms that confer bacterial protection against antimicrobial substances and immune effectors. NTHi biofilms promote bacterial persistence in the respiratory tract and ME and thus play an important role in respiratory diseases and OM. Biofilm formation is also responsible for disease exacerbation and modulation of the immune response. [180,181] NTHi release DNA and DNA-binding proteins, also known as nucleoid-associated proteins (NPAs), accessory to the formation of biofilms and they are thought to confer structure to the biofilm. Devara *et al* (2015) and Das *et al* (2017) reported that NPAs,

alongside the type IV secretion system, are fundamental for biofilm integrity and disruption of these two components compromises the whole biofilm. [182,183] However, biofilm formation is not seen in all clinical strains, as a study carried out by Kress-Bennett *et al* (2016), in chinchilla, investigated the role of virulence factors in the progression of infection and demonstrated only certain NTHi strains contributed to biofilm formation and persistence within macrophages, due to the upregulation and expression of a novel virulence factor: Msf protein. Furthermore, in the ME, biofilm formation is highly dependent on the conditions of the surrounding environment, as the optimal biofilm growth conditions include a low oxygen environment, microaerophilic and anaerobic, such as that found in rAOM and OME, but not always in AOM. [184,185] Additionally, NTHi is able to exploit its collection of regulons, the phasevarion, whose expression is adaptable to conditions faced by the bacteria. Through methyltransferases associated with type III restriction-modification, the phasevarion allows NTHi to quickly and reversibly adapt to the challenges posed by the surrounding environment, from immune system effectors to antimicrobial administration. To date, twenty-one phasevarion types, regulating NTHi's different phenotypes, have been identified. [186,187]

Kaur *et al* (2013) reported that NTHi is the main cause of OM relapse after antibiotic treatment and the main pathogen found in rAOM. [188] In a study by Krueger *et al* (2017), the relationship between the COM ME effusion microbiomes and mucin secretion profiles was evaluated. Using 16s RNA sequencing, Krueger *et al* (2017) analysed the ME effusions of 55 children with COM, between the ages of 3 months and 14 years, in USA, undergoing myringotomy with tympanostomy tube replacement. Their analysis most commonly identified *Haemophilus*, *Moraxella* and *Streptococcus* as the most abundant genera, but *Turicella* and *Alloiococcus* abundances were also significant. Interestingly, *Stenotrophomonas* was also detected, although the pathogen had not been previously identified in association with OM. Identification of novel pathogens highlights the powerful tool molecular techniques provide in the betterment of diagnosis and treatment. Furthermore, in this study it is shown that *Haemophilus* species were found to be more abundant in patients with conductive hearing loss, and in those with higher proportions of mucin glycoproteins in their ME effusions, namely MUC5AC and MUC5B. *Haemophilus* presence was greater in patients expressing both MUC5AC and MUC5B than those expressing only one or neither mucin. Additionally, almost all samples that contained MUC5AC, also contained MUC5B, but the opposite was not seen. When MUC5B was present alone in the effusions, *Haemophilus* was not the predominant species in the sample, suggesting that expression of MUC5AC, but not MUC5B, occurs

simultaneously to *Haemophilus* infection. This evidence formed a previously unrecognised linked between *Haemophilus* presence and overproduction of mucins. It also suggests that *H. influenzae* infection contributes to genetic expression of MUC5AC, potentially promoting progression of disease from AOM to COM, as a previous report by Kerschner *et al* (2010) established that MUC5AC expression was highly associated with recurrent and chronic OM. [189,190]

1.5.1.3.1. NTHi's phosphorylcholine

Like many bacteria that inhabit the human mucosa, NTHi possesses lipooligosaccharides (LOS) which, unlike the lipopolysaccharides (LPS), lack the repeating O-side chains as a bacterial toxin and cell wall component (Figure 19). However, due to its phasevarion, NTHi is able to regulate the expression of genes involved in the assembly of LOS, altering its makeup in response to environmental pressures. For example, LOS epitope analogues can be found in host cells, and thus NTHi's mimicry of host proteins enables the bacteria to evade detection and clearance.

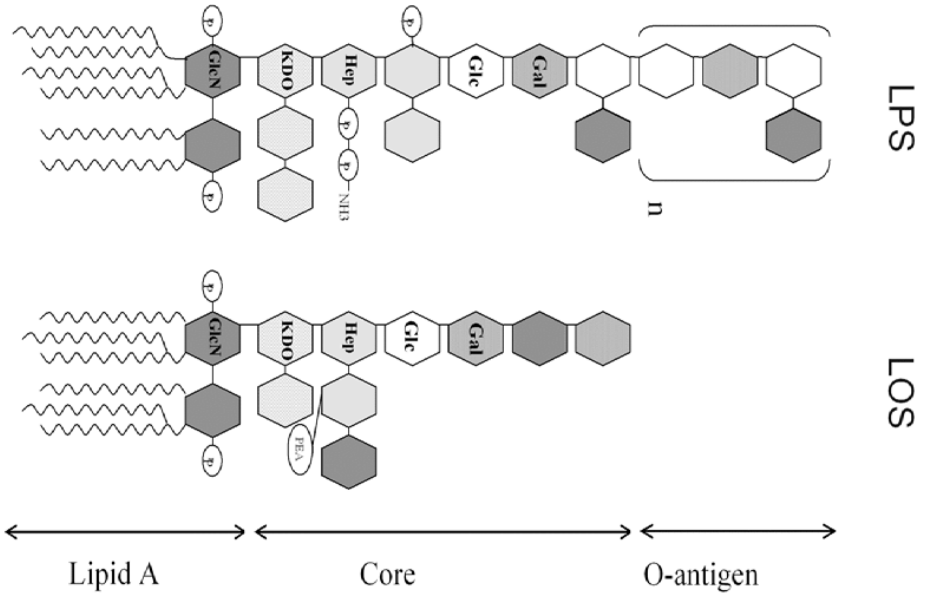


Figure 19 - Diagram highlighting the differences between LPS and LOS structures. LPS structures are organised in 3 portions: Lipid A, Core and O-antigen, whereas LOS lacks the O-antigen. The lipid A is a phospholipid with a high number of long-chain fatty acids. The core domain constitutes 9 or 10 sugar branched oligosaccharides. The O-antigen is a repetitive sequence of carbohydrate polymers, linear or branched, with a

variable number of oligosaccharide residues. Adapted from https://www.researchgate.net/figure/Fig-1-Schematic-structure-of-LPS-and-LOS-In-the-scheme-LPS-Escherichia-coli-and_fig1_221883547.

One of the structures shared by NTHi and their host is phosphorylcholine (PCho), which on host membrane lipids aids bacterial persistence as the protein has been linked to host cell adherence and invasion (Figure 20). PCho is also expressed by *S. pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, which are also known to cause respiratory tract infections. [191] PCho expression is upregulated in NTHi populations growing in biofilms where it leads to weaker innate immune responses. However, a higher incorporation of PCho into LOS, or the presence of PCho expressing bacteria in a given environment, does not equate to higher biofilm formation. [192,193]

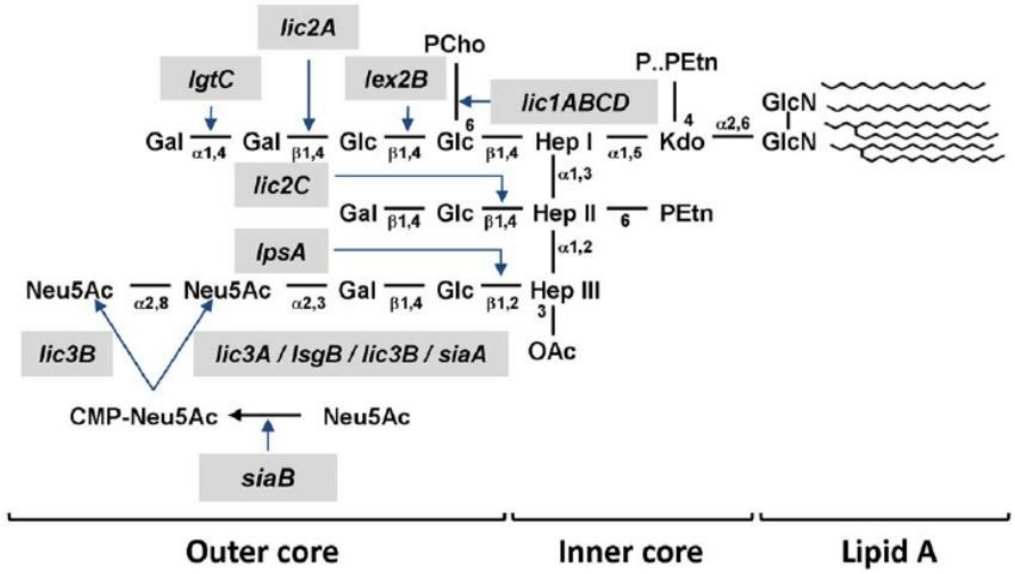


Figure 20 - Representation of NTHi's lipooligosaccharide structure. Each LOS structure possesses a heptone backbone comprising of HepI, HepII and HepIII. The relevant sugars are linked by the products of the relevant genes (lgtF, lic2C, lpsA, lic3A, lic3B, siaA, lsgB, lic1ABCD, lic2A, lgtC and lex2B. Lipooligosaccharide is an endotoxin that lacks the O-side chain and is expressed on bacterial surfaces by members of the genera *Neisseria*, *Haemophilus*, *Bordetella*, *Campylobacter* and *Branhamella*. Due to the phasevarion, NTHi is able to exchange the decorations of the structure to mimic different human proteins. Adapted from https://www.researchgate.net/figure/Schematic-representation-of-NTHi-LOS-structure-Relevant-sugars-linked-by-the-products_fig1_51243146.

NTHi in the nasopharynx benefits from expressing PCho, as it increases bacterial adherence to epithelial cells subsequently leading to higher survivability rate, however, this benefit is not seen across all other body sites. Langereis *et al* (2019) found, that in the blood, where there is a high concentration of PCho-specific antibodies and C-reactive protein, NTHi with low PCho expression possess higher survival rates. In this scenario, low PCho expression strains are not recognised by PCho specific IgM, nor bind to C-reactive protein and trigger complement C3, thus increasing their survival rates. [194]

1.5.1.3.2. Interactions between NTHi and airway epithelial cells

The airway epithelium is inhabited by a variety of bacteria that compete for space and resources. Not only have the interactions between airway epithelial cells and NTHi been well documented, so have the interactions between NTHi and its bacterial competitors. *Haemophilus haemolyticus* is a respiratory tract commensal that can be misidentified as NTHi due to its high levels of genetic relatedness. It was observed that *in vitro* pre-treatment of nasopharyngeal and bronchoalveolar epithelial cells, namely D562 and A549, with *H. haemolyticus* significantly reduces NTHi attachment, suggesting an interference or competition between the two species and highlighting the importance of studies that take into account both poly-infections and the diversity of the ME microbiome, as it may affect interactions between the epithelial cells and the pathogen of study. [195]

NTHi possesses well-known proteins that function as adhesins including the Hia adhesins and High Molecular Weight (HMW) adhesins, which are essential for colonisation and pathogenesis but are not expressed simultaneously within the same strain. [196] Different NTHi strains can express unique HMW adhesins with affinities to different lectins, allowing the bacteria to bind to different sites in the respiratory tract. [197]

Su *et al* (2016) investigated the role of P4, a protein expressed by NTHi that binds to extracellular cellular matrix proteins of human epithelial cells such as laminins, fibronectins and vitronectins. *In vitro* experiments showed that bacteria lacking P4 struggled to adhere to cells and did not persist in murine ME. [198] *Haemophilus* adhesion protein (HAP), is an NTHi adhesin that is ubiquitously expressed and also binds to basement membrane components such as fibronectin, laminin and collagen IV. [199] Ronander *et al* (2008) found that NTHi binds to the host's vitronectin through protein-E (PE), a bacterial receptor that has been shown to play a

role in the attachment to, and subsequent invasion of, bronchial and alveolar epithelial cells. [200] It was shown that, when compared to wild-type counterparts, PE-deficient mutants lose part of their adhesive capacities and that survival of bacteria is dependent on the presence of host vitronectin. [201,202]

NTHi's *H. influenzae* fimbriae (HiF) binds to mucins. In mucus and mucin-rich environments, such as COPD-related mucus hypersecretion in the lungs and rAOM in the ME, the ability to bind to mucin is advantageous to the bacteria as it allows progression to other disease stages, such as tissue damage and invasion. In cases of cystic fibrosis and chronic bronchitis in the lungs and COME in the ME, where the clearance of mucus is impaired, mucin adherence allows for chronic colonisation of the epithelium. [203]

Furthermore, Janson *et al* (1999) found that NTHi's Protein D negatively affects ciliated cells by impairing their ciliary functions. It was shown that, unlike Protein D deficient strains, NTHi expressing Protein D significantly reduced the ciliary beat frequency of nasopharyngeal epithelial cells. *In vivo* these results suggest that protein D may impact bacteria clearance in the airways and promote bacterial adhesion to the cells, or that the protein is expressed in response to the increase in ciliary beat frequency prompted by the immune response upon detection of a pathogen (Figure 21). [204] Utilising the host's β-Glucan receptor, NTHi is also able to adhere to and invade monocytic and epithelial cells, via receptor mediated endocytosis, meaning that NTHi can enter human monocytic cells through a phagocytosis-like process, whereas it enters non-ciliated epithelial cells by macropinocytosis. [205,206]

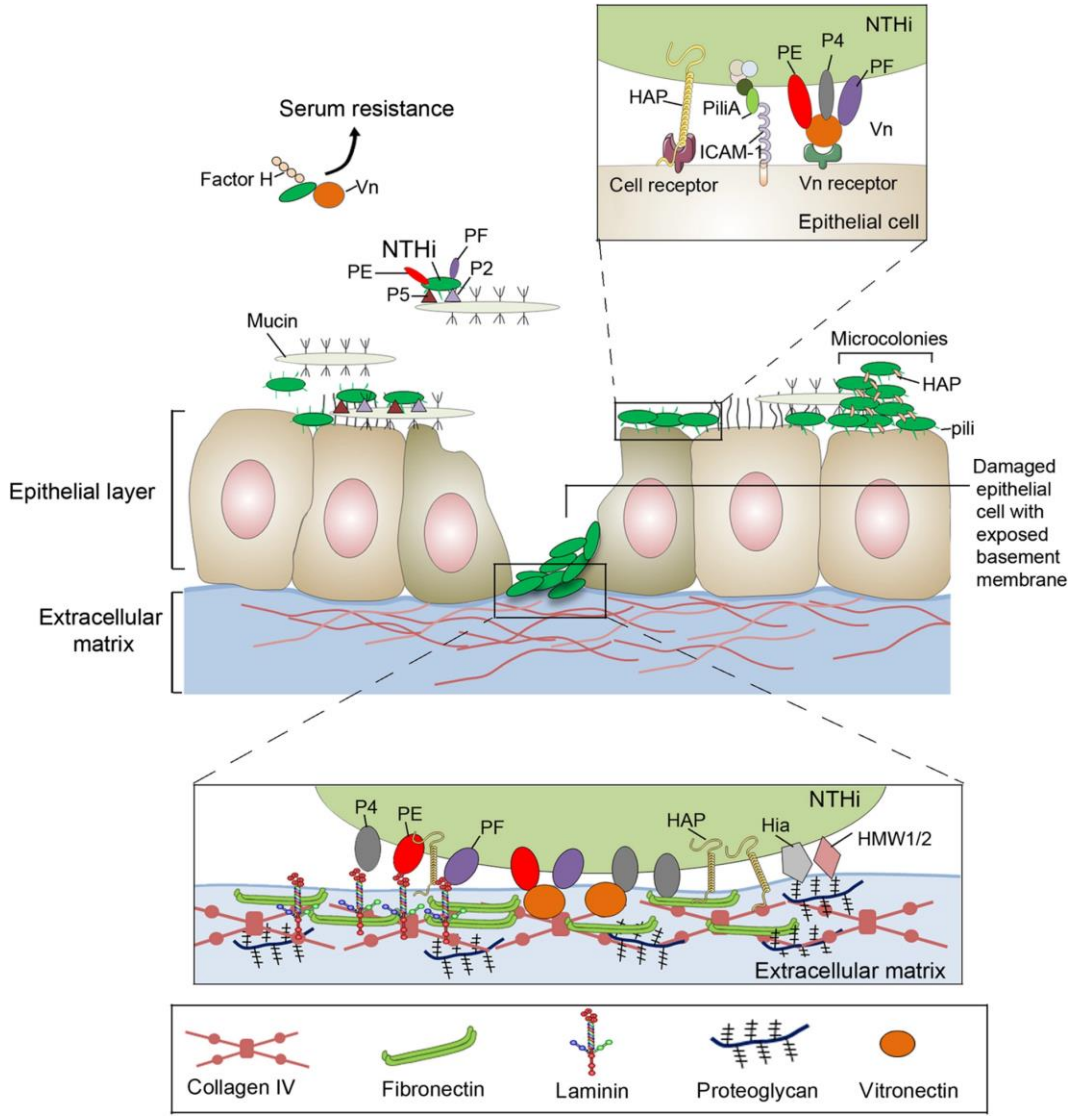


Figure 21 - Diagram of NTHi's mechanisms of adhesion to host proteins. NTHi is able to bind to a plethora of host proteins, from both epithelial cells and the underlying extracellular matrix. Adhesion to host proteins facilitates colonisation and invasion of cells, but also provides defensive mechanisms as it assists in the evasion of the immune response and biofilm formation. Adapted from https://pubmed.ncbi.nlm.nih.gov/27508518/.

A well-studied host receptor that mediates NTHi invasion is the platelet activating factor receptor (PAFr). PAF is a phospholipid activator and regulator of platelet aggregation and degranulation, inflammation, chemotaxis and anaphylaxis. PAF binds to PAFr, a G-protein coupled receptor that has been associated with a variety of pathological processes such as respiratory diseases - allergy, asthma, and chronic obstructive pulmonary disorder (COPD) -,

septic shock, arterial thrombosis, and kidney failure. However, it has also been shown to have protective effects in other diseases, such as colitis [207–212] Blockage of bacterial linkage and adhesion to antagonists of PAFr have been shown to prevent adhesion of NTHi and *S. pneumoniae* and thus, decrease disease exacerbations. [213] The ability of *S. pneumoniae* to invade respiratory epithelium through PAFr has been evaluated and the interaction between the two has been associated with severe pneumonia. [214,215] Viruses also exploit PAFr negatively impacting the host. For example, Garcia *et al* (2010) showed that influenza A binding to PAFr is followed by an intense inflammatory response, leading to lung injury and even death. [216]

PAF is a chemokine released, upon stimulation, by neutrophils, eosinophils, macrophages, mast cells, and epithelial and vascular endothelial cells. PAF stimulates the release and metabolism of arachidonic acid (AA), which increases vascular permeability, and activates neutrophils, monocytes, and macrophages, as well as regulating neutrophil adhesion to endothelial cells. AA metabolism produces a variety of metabolites, such as leukotrienes (LTs) and prostaglandins (PGs). PAF, IL-1, and TNF can induce each other's release, self-generating positive feedback cycles, but synthesis of AA is also promoted by LTB4, LTC4 and LTD4. [217] PAF and AA metabolites have also been linked to the production of ME effusions by stimulation of mucous glycoprotein release, increase of vascular permeability, stimulation of epithelial secretory activity and hindrance of mucociliary activity, which can potentially lead to chronic ME effusions. [218–220]

NTHi's PCho is a molecular mimic of PAF's PCho segment. NTHi's LOS interacts with PAFr through its PCho, enabling bacterial adherence and invasion. Swords *et al* (2000) showed that in bacterial strains that failed to express PCho adherence to bronchial epithelial cells was significantly hindered, and pretreatment with PAFr antagonist significantly reduced NTHi invasion. [221] As shown by Clementi *et al* (2014), the internalisation of NTHi through PCho-PAFr seems to be independent of lipid rafts and, rather, indirectly clathrin-mediated. PCho-PAFr interactions promote NTHi intracellular trafficking through early endosomes, however, NTHi inhibits acidification and maturation of the endosomes into lysosome, thus avoiding killing (Figure 22). [222]

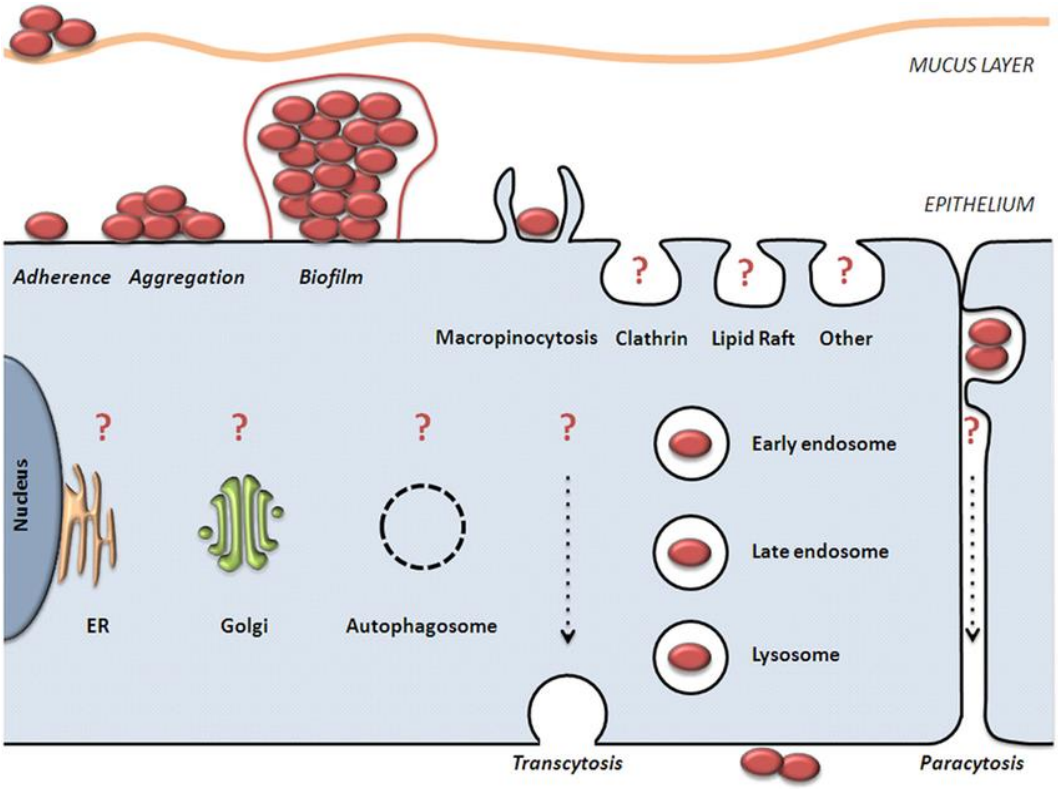


Figure 22 - Diagram of alternative invasion strategies for NTHi. NTHi is able to adhere to mucous layers and non-ciliated epithelial cells, then it can aggregate and mature into biofilms. It is well-established that NTHi can be internalised by macropinocytosis and trafficked within vesicles that are involved in endolysosomal processes, which can often mature into lysosomes, allowing NTHi bacterium to avoid, escape or neutralise the fatal process, however, NTHi can also utilise other internalisation pathways, namely receptor-mediated pathways, which include β-glucan receptor-mediated endocytosis in macrophages, and PAFr-mediated endocytosis in epithelial cells. The latter has since been found to be independent of lipid rafts, but indirectly clathrin-mediated. Adapted from https://pubmed.ncbi.nlm.nih.gov/22919570/.

Despite the wide utilisation of host receptors for cellular invasions, in heme-iron restricted environments NTHi utilises macropinocytosis as an invasion method, where the bacterial cells escape or evade the early endosome to form intracellular bacterial communities. Swords *et al* (2001) showed that inhibition of macropinocytosis significantly reduced the invasion of certain NTHi strains, but not others, suggesting that some bacteria utilise macropinocytosis as their main route of invasion, whereas others favour receptor-mediated invasion mechanisms, or have the ability to combine both (Figure 23). Moreover, the study compared the invasion efficiency between macropinocytosis and PAFr mediated-invasion, and the data suggest that invasion mediated by PAFr activation is more efficient than macropinocytosis. [223]

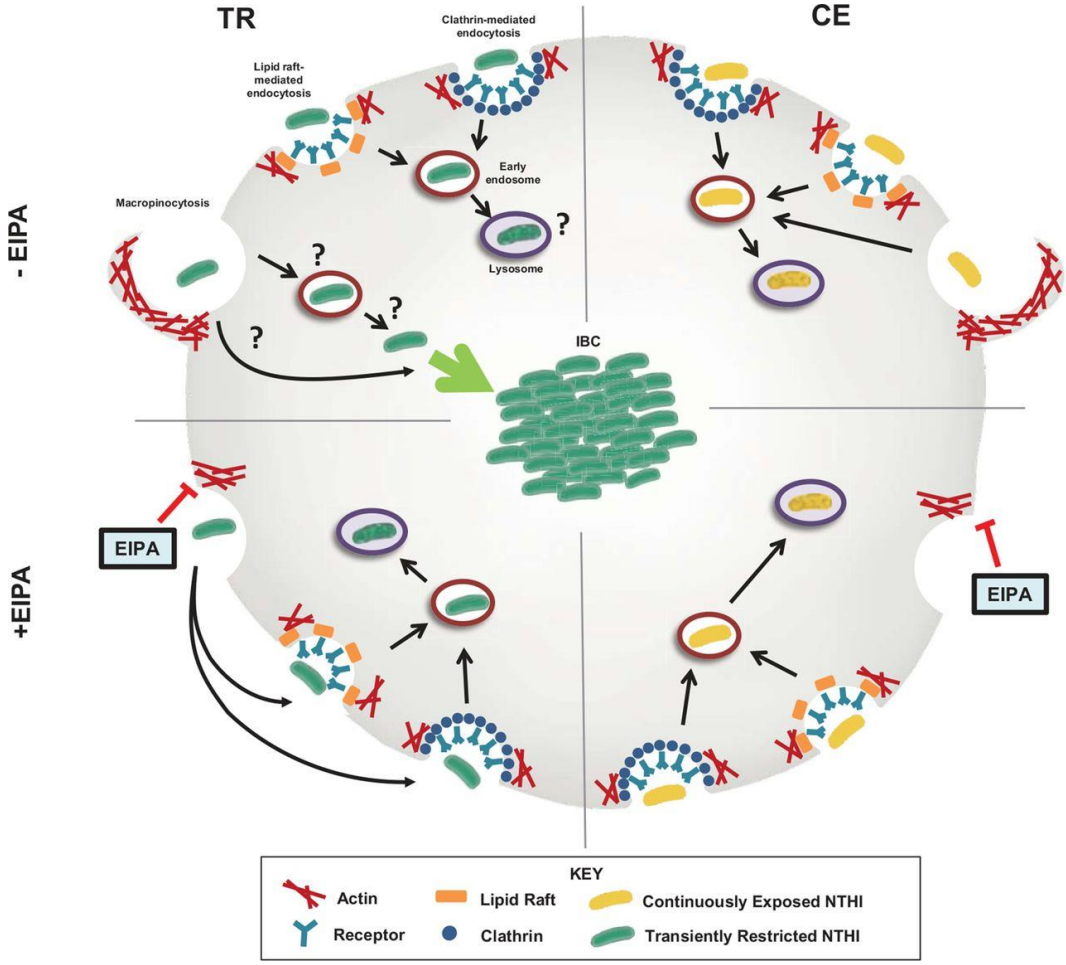


Figure 23 - Diagram of differential trafficking of NTHi under exposed and restricted heme-iron conditions, and its effect on the formation of intracellular bacterial communities in bronchial epithelial cells. EIPA is a compound used to inhibit macropinocytosis; TR -transiently restricted of heme-iron; CE - exposed to heme-iron. In the absence of EIPA, NTHi in both the presence and absence of iron enters the cells through endolysosomal pathways, that can be clathrin mediated (blue circles), lipid raft-mediated (orange circles) or through macropinocytosis (membrane ruffling by actin (red lines) polymerisation), thus the ability to invade the cell through these pathways is independent of heme-iron exposure. Once inside the cell, continuously exposed NTHi (yellow bacteria) are trafficked to the early endosomes (red circles), which mature into lysosomes (purple circles) where the bacteria are degraded; transient-restricted NTHi (green bacteria) also enter the cells through endolysosomal pathways and are trafficked into early endosomes and lysosomes, however, the subpopulations that enter through macropinocytosis are able to either completely evade or escape this pathway to form intracellular bacterial communities. When exposed to EIPA, trafficking of NTHi continuously exposed and transiently restricted to heme-iron remains unchanged within the endolysosomal pathways, significantly decreasing survival rate and reducing the number of intracellular bacterial communities. Adapted from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6135960/.

1.5.1.3.3. Interactions between PCho and airway epithelial cells

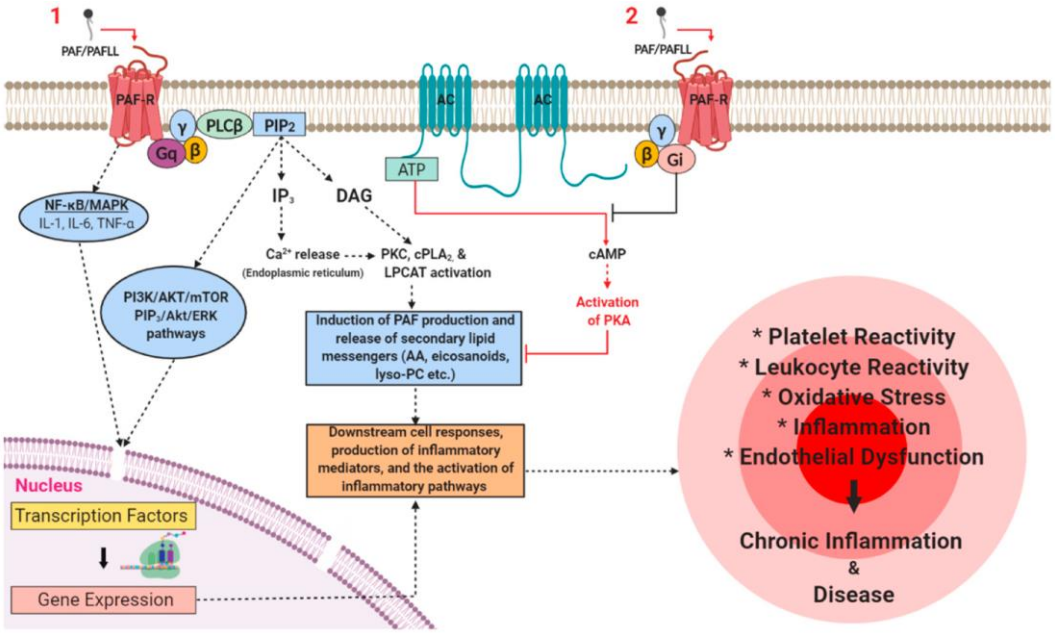
Interactions between PCho and the airway epithelium have been extensively studied. For example, mouse nasopharyngeal bacteria with PCho-decorated LOSs are able to avoid antibody binding more easily than non-expressing strains, as the PCho's positive molecular charge affects the physical properties of the bacterial membrane, negatively impacting bacterial recognition and antibody-dependent clearance. [224]

Further, lipids are important components of host immunity as they organise membrane signalling complexes and release lipid-derived mediators. Changes in the airway's epithelium lipid composition have been linked to cystic fibrosis, COPD and asthma. Fernández-Calvet *et al* (2018) showed that a *vacJ* gene deficiency in NTHi increases the bacterial global fatty acid and phospholipid composition, increasing also the expression of PCho expression in LOS, contributing to an increased surface hydrophobicity, which can reduce the interaction of NTHi with hydrophobic and lipophilic molecules, such as hydrophobic antibiotics and synthetic antimicrobial peptides, but also free fatty acids in the host's lungs. Though this could be advantageous to pathogenic processes, the continuous excessive hydrophobicity can also destabilise the bacteria's surface, potentially affecting its growth and survivability. Thus, PCho expression brings advantages to the bacteria under situational circumstances, and bacteria that can regulate their phasevarions present better survival rates. [225] Furthermore, PCho protects NTHi against other antimicrobials, such as the bactericidal peptide LL-37/hCAP18 which is transcribed on the epithelial surface of the human nasopharynx *in situ* and in epithelial cells derived from the upper airway. *Lysenko et al* (2000) found that in the presence of LL-37/hCAP18 the NTHi population increased its selectiveness for the $PCho^+$ phase. Additionally, they showed that bacteria which constitutively express PCho have a significantly higher survival rate than those in which PCho expression has been knocked out, likely due to their altered surface charge, which prevents effective binding of the LL-37/hCAP18 to LOS and thus alters the susceptibility of the bacteria to the antimicrobial. [226]

As well as offering protection against antimicrobials, PCho also acts as an anchor for adhesion of bacteria to host cells. Binding of PCho to PAFr initiates a receptor coupling to a PTX-sensitive heterotrimeric G protein complex, resulting in a multifactorial host cell signal cascade and, ultimately, bacterial invasion. This signalling cascade involves phosphoinositide and arachidonic acid metabolism, intracellular calcium changes and protein phosphorylation. The release of arachidonic acid is phospholipase A_2-regulated and initiates responses in

platelets, neutrophils, eosinophils, macrophages, smooth muscle cells, epithelial cells (Figure 24). The activation of phospholipase A_2 is regulated by intracellular levels of Ca^{2+}, which may explain the association between PAFr signalling and the mobilisation of intracellular calcium. Furthermore, Ca^{2+} regulates epithelial-specific functions, such as the transepithelial solute transport, cell growth and differentiation. [227,228]

Figure 24 - Diagram highlighting the main pro-inflammatory signalling pathways induced by binding of



PAF to its receptor. Adapted from https://www.mdpi.com/1420-3049/24/23/4414/htm/.

1.5.1.3.4. Interactions between PCho and middle ear epithelial cells

As described above, the potential for interactions between PAFr and PCho is high, particularly in the airway epithelium. The ME epithelium is a modified respiratory epithelium, and given the number of pathophysiological outcomes related to PAFr in the airways, it is of interest to investigate the interactions between the PAFr of ME epithelial cells and the PCho of NTHi and subsequent effects in OM. [224]

Furukawa *et al* (1995) has previously reported that ME effusions have high PAF activity and hypothesised that the lipid mediator could play a role in the pathogenesis of OM, particularly OM with effusion. [229] Diven *et al* (1998) described PAF as an active mediator of inflammation in AOM, as they showed that chinchilla MEs pretreated with PAFr antagonists

presented significantly fewer histopathological signs of inflammation, such as mucosal thickness, infiltration of innate immune cells, and reduced levels of effusions, in response to bacterial infection. [230] Ogura *et al* (2008) observed the effects of PAF on the induction of IL-8 production in the rat ME. Their research showed that IL-8 production was induced by PAF in a dose-dependent manner, but incubation with a PAFr antagonist (WEB2170) eradicated this effect. [219] A report by Karasen *et al* (2000) also showed that blockage of PAFr utilising antagonists significantly reduced the levels of IL-8 present in the ME of guinea pigs. [231] Ogura *et al* (2008) showed that although PAF alone could not induce chronic OM, when administered directly into the rat ME through the tympanic membrane, it led to a cytokine storm and production of effusion, causing OME that lasted up to 7 days before clearance.

Yokota *et al* (2010) previously showed that during respiratory syncytial virus infections PAFr expression was upregulated. Their research further showed that fosfomycin, an antimicrobial agent, significantly suppressed RSV-associated PAFr upregulation and expression, consequently leading to reduced adhesion of *S. pneumoniae* and *H. influenzae* to PAFr. This effect prevented secondary-bacterial infection post viral infection, the most common OM trigger. Thus, the link between activation of PAF/PAFr and disease is strong. [232] The potential for hindering the role in bacterial adherence, reducing inflammation and halting disease progression that PAFr antagonists offer in OM-associated scenarios, makes them appealing targets for future research as potential therapeutic agents.

1.6. The middle ear response to infection

The mechanisms leading to the resolution of AOM and OME are still unclear, however, due to reports in similar tissues such as the nasopharynx or lungs, it is possible to establish comparisons and formulate hypotheses as to how resolution in the ME is achieved. Moreover, these early resolution mechanisms could also potentially have therapeutic applications to forms of COM.

As previously mentioned, inflammation within the ME leads to mucosal changes, hyperplasia of the epithelium, with increased levels of goblet and basal cells, and oedema of the subepithelial space, resulting in increased vascular dilation and permeability and infiltration of phagocytic cells that can lead to tissue damage. However, after inflammation has subsided, the epithelial profile of ME cells is very distinct, with submucosal fibrosis, calcification and

squamous epithelium hypertrophy taking place. As OM is hallmarked by its exacerbated inflammatory profile, regulating inflammation levels is essential in clearance of disease, which alongside tissue healing, is usually achieved by a complex interplay of immune factors, such as depletion of chemokines, downregulation of proinflammatory cytokines, upregulation of pro-resolution mediators, leukocyte apoptosis and alternative activation of macrophages. However, it is noteworthy that the ME is an immune privileged site, due to its high tolerance to microorganisms invading from the upper respiratory airways, self-regulated inhibition to inflammatory stimuli and remote location that delays recruitment and migration of immune cells. Thus, it is dependent on the epithelial antimicrobial response and immune effectors of surrounding tissues, namely the mucosa-associated lymphoid tissues (MALT) of adjacent organs and appendixes, such as the tonsils or nasopharynx. [233,234]

1.6.1. Mechanical and chemical barriers of the middle ear mucosa

The human body's first line of defence is its mechanical and chemical barriers, as these are the first blockades pathogens encounter when infecting an individual.

Pathogens that cause OM are either inhaled or are commensal to the nasopharynx and will encounter the ET before reaching the ME. The combined action of tight junctions, secretions and cilia of epithelial cells in the ET promotes both the mechanical mucociliary flush of pathogens and the formation of a chemical barrier. Certain secretory epithelial cells express antimicrobial peptides, such as lactotransferrin, lysozymes, defensins, surfactants and mucins, which allow maintenance of a pathogen-free environment. Lysozymes catalyse breakdown of the bacterial cell wall, cleaving its peptidoglycans and forming pores on their surface. [235] Some epithelial cells are responsible for producing lysozymes that combine with mucoid and serous mucous from nearby goblet cells; the combined synergistic action of these molecules increases the bacteria-killing ability. [236] Defensins can inhibit the action of bacterial toxins, flag the bacteria for opsonisation and create pores in the membrane of the pathogen leading to destabilisation and cell lysis. [237] Three groups of defensins have been identified, however, humans only express two, namely α-defensins, expressed in granulocytes, and β-defensins, expressed in epithelial cells. Human β-defensins (HBD) are secreted by ME epithelium and HBD1 and 2, upregulated by IL-1α, TNF-α and lipopolysaccharide, have been shown to kill

NTHi, *S. pneumoniae* and *M. catarrhalis*. [238] The human β-defensin 3 (HBD-3) plays a vital role in planktonic NTHi eradication. Jones *et al* (2013) reported that HBD-3 activity is dampened by the formation of biofilms, showcasing the transition from planktonic to biofilm states can impact the protective action of a molecule. Jones *et al* (2013) also investigated the combined activity of HBD-3 and DNAse I. As DNA structures constitute a significant component of the biofilm matrix scaffolding, the addition of DNAseI, an enzyme that degrades DNA, is expected to disrupt the biofilm. Results on the combined action of DNAseI and HBD-3 proved that, *in vitro*, biofilm matrix disruption occurs, and the biofilm presents diminished biological activity. This combined action could have a therapeutic use, as if this activity is maintained *in vivo* it would assist in clearance of the bacteria from the ME. [239] Surfactant proteins can also contribute to clearance of bacteria through opsonisation. SPLUNC1 (also known as BPIFA1) is a multifunctional host defence peptide critical for homeostasis of the mammalian upper airway. It is found in the mucous of the nasopharynx of mice and chinchilla and is reported to have surfactant-like properties that has been shown to disrupt biofilm formation by *P. aeruginosa*. [240,241] Mucins promote mucous secretion and mucous cell expansion which, as mucus traps pathogens, creates a protective barrier, hindering bacterial binding to cells. While ME homeostasis is important, overproduction of mucin may halt clearance of OM pathogens, by preventing normal function of immune effectors due to excess mucus, providing bacteria with a proliferation favourable environment. [242]

1.6.2. Innate immune response to otitis media

Jecker *et al* (1995) surveyed the numbers of immunocompetent cells in the ME of rats aged between 3 to 6 weeks old, before and after OM-induction. Their study reported that macrophages and dendritic cells populated the healthy ME in the highest numbers, whereas NK cells and T and B-lymphocytes were rarely present, and granulocytes, such as mast cells and neutrophils, were generally absent. This absence supports the hypothesis that granulocytes are recruited to the site upon trigger of inflammation. Jecker *et al* (1995) also highlighted that the distribution and localisation of immunocompetent cells in the ME resembles that of the mucosa of the respiratory tract, suggesting functional continuation between the two. Upon induction of OM, it was reported that the ME mucosa underwent morphological and populational changes. The highest populational increases happened in granulocytes, followed

by a moderate increase in NK, T and B-cells, and macrophages and dendritic cells. Interestingly, it was reported that while granulocytes migrated from the inflamed mucosa to the effusion fluid, other immunocompetent cells did so in lower numbers, indicating that the composition of the ME fluid is not representative of the cellular population of the ME. [243]

Neutrophils and macrophages are typically the first responders to an infection, playing an essential role in clearing otopathogens. These cells are known for their ability to actively kill bacteria by releasing antimicrobial peptides stored in granules, and through phagocytosis. Neutrophil extracellular traps (NETs), extracellular fibres, composed mostly of neutrophil DNA, immobilise and trap pathogens, facilitating neutrophil killer actions towards eliminating infectious agents, however, their persistence hinders resolution of inflammation. ME effusions of children with either multiple AOM episodes, rAOM, or COM demonstrated the presence of NETs in the ME fluid. [244] Although NK and mast cells have also been reported in association with OM, particularly in CSOM, as they both present increased activity and numbers, their role in the disease requires further investigation. As the healthy ME has been widely regarded as devoid of immunocompetent cells, due to their low numbers in the absence of disease, it is unsurprising that there is a limited number of reports on the role of NK and mast cells, which are typically only present during disease. [236] Interestingly, Seppanen *et al* (2018) have found that otitis-prone children have elevated numbers of NK circulating cells, in comparison to healthy controls, potentially due to frequent exposure to respiratory pathogens, or even persistence of the cells post-infection (NK cell memory). However, the authors were unable to determine a role for the presence of these cells. NK cells are highly efficient at directing cellular cytotoxicity and thus beneficial to the elimination of pathogens, however dysregulation and overactivation of NK cells can have detrimental consequences for the host, as the increased activity may lead to damaging levels of inflammation, increased bacterial counts and poor outcomes. Therefore, NK cells activity and impact on OM outcomes is situational dependent. [245] Similarly, the role of mast cells has not yet been fully understood, but Ebmeyer *et al* (2005) suggest that these work as "sentinels" of innate immunity in the ME mucosa. Ebmeyer *et al* (2005) investigated the consequences of the presence or absence of mast cells in 6 week old OM-challenged mice. Mice lacking mast cells presented delayed early responses to bacterial infection compared to wild-type or mast-cell replenished mutant mice, suggesting that although mast cells are not required to mount an innate immune response, its presence and subsequent release of immunoregulatory mediators and cytokines, greatly improves the promptness of the early innate immune response. [246]

Essential components in the initiation of the innate immune response are the PRRs, such as TLRs, responsible for identifying pathogen-associated molecular patterns (PAMPs). [40] TLRs are expressed on both immune and epithelial cells, and can trigger a molecular cascade, activating the NFκB, MAPK or IRF pathways, leading to changes in expression of genes involved in innate and adaptive immune cell maturation and proliferation. Activating an inflammatory response can, however, result in two different outcomes: clearance of infection or progression to chronic infection. Intracellular bacteria, like NTHi, are able to interact with surface PRRs, such as TLR-1,2,4-10 and 3,4, as well cytoplasmic PRRs, such as NOD1,2 and TLR9 (Figure 25). [247]

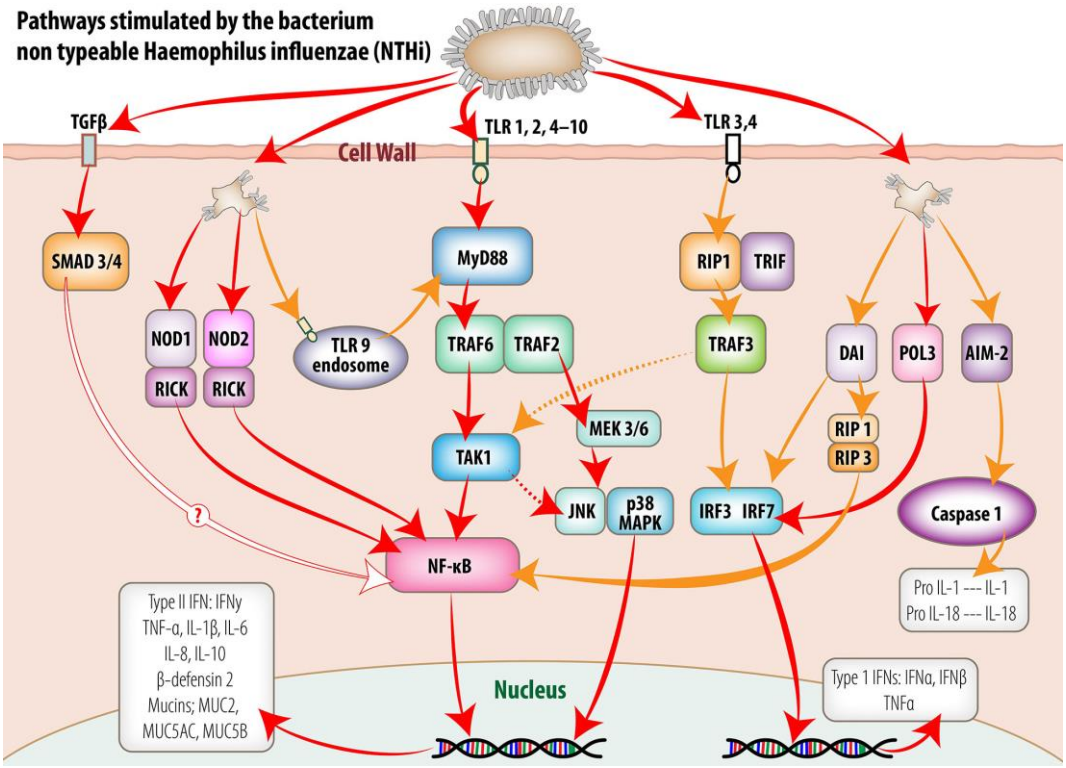


Figure 25 - Diagram of intracellular cascade signalling pathways triggered in ME mucosa in response to infection by NTHi. Both surface or intracellular pattern recognition receptors detect conserved motifs of pathogens and promote synthesis of downstream cytokine cascades. Activation of the surface pattern recognition receptors TLR 1, 2, and 4-10 results in a MyD88-dependent translocation of NFκB to the nucleus, and upregulation of type II interferons and other proinflammatory and antimicrobial effectors. TLR3 initiates a MyD88-independent pathway that utilises TRIF and IRF3 to upregulate expression of type I interferons. Importantly, TLR3 and 4 activate a delayed response through TAK1 and the JNK p38 MAPK pathway to stimulate type II and proinflammatory effector production. In respiratory epithelia TGF-β activates SMAD3 and SMAD4 upregulating NFκB, but the mechanism has not yet been confirmed for ME mucosa. Cytoplasmic pattern recognition receptors NOD-1, NOD-2 and TLR9 activation can also upregulate NFκB stimulation of type II interferons and proinflammatory and antimicrobial effectors. DAI induces production of both type II and type I interferons, POL3

via IRF7 utilises the same path as TLR3, while AIM2, through caspase 1, promotes synthesis of IL1 and IL18. Adapted from https://www.frontiersin.org/articles/10.3389/fcimb.2021.764772/full.

Activation of PRRs often leads to production of cytokines, small proteins that act as mediators of interactions and communications between cells. Chemokines are a subset of cytokines, small peptides that act as chemoattractants and potent activators of leukocyte subpopulations. Interaction between chemokines and their receptors triggers a flux of intracellular calcium leading to chemotaxis, recruitment of cells to the site where the stimuli are. Interleukins are another subset of cytokines, low molecular weight proteins with pleiotropic effects on cells as they can affect tissue growth and repair, homeostasis and are important modulating factors of the immune system. A number of these inflammatory effectors play a central role in OM (See table 4).

Table 4 - Source and role of inflammatory effectors in OM.

Inflammatory effector	***Source***	***Role***
Cytokines		
Tumor necrosis factor - α (TNF-α)	Macrophages	Release of prostaglandins; activation of neutrophils, eosinophils and macrophages; release of cytokines
	Lymphocytes	
	Epithelial cells	
	Endothelial cells	
Interleukin-1 (IL-1)	Macrophages	Activation of B- and T-cell; promoting the proliferation of epithelial cells and
	Neutrophils	

	Fibroblasts	fibroblasts; synthesis of cytokines; release of histamine; fever induction and bone resorption
	Epithelial cells	
	Endothelial cells	
Interleukin-2 (IL-2)	T-cells	Activation of T-cells
Interleukin-4 (IL-4)	T-cells	Differentiation and proliferation of T-2 cells; promoting the anti-inflammatory action of T- and B-cells, and monocytes
	Mast cells	
	Basophils	
Interleukin-5 (IL-5)	T-cells	Production of IgA by B-cells; eosinophil chemotaxis and promotion of eosinophil production in bone marrow
	Mast cells	
	Basophils	
Interleukin-6 (IL-6)	Macrophages	Activation of B- and T-cells; stimulating antibody production; fever induction and bone resorption
	Lymphocytes	
	Epithelial cells	
Interleukin-10 (IL-10)	Macrophages	Promoting down regulation of IL-1, IL-6, TNF-α
Interleukin-12 (IL-12)	Macrophages	Promoting interferon release; activation of macrophages; proliferation of T-cells;
	Neutrophils	

	Langerhans cells	production of cytokines; stimulating cytotoxicity of lymphocytes and T_H1 development.
Interleukin-13 (IL-13)	T-cells	Similar to IL-4; links allergic inflammation cells to non-immune cells in contact with them
Transforming growth factor - β (TGF-β)	Neutrophils	Initiation and maturation of inflammatory processes; recruitment, activation and proliferation of inflammatory tissues and cells
	Macrophages	
Granulocyte-macrophage colony stimulating factor (G-CSF)	Macrophages	Production of granulocytes
Chemokines		
Interleukin-8 (IL-8)	Macrophages	Neutrophil chemotaxis and activation; angiogenesis
	Fibroblasts	
	Epithelial cells	
	Endothelial cells	
RANTES (CCL5)	Epithelial cells	Monocyte and T-cell chemotaxis

MCP-1	Epithelial cells	Monocyte and T-cell chemotaxis
Histamine	Mast cells	Increasing vascular permeability, vasodilation, neutrophil and eosinophil chemotaxis
	Basophils	
Vascular endothelial growth factor (VEGF)	Thrombocytes	Increasing vascular permeability; vasodilation; promoting infiltration of inflammatory cells
Platelet activating factor (PAF)	Monocyte	Chemotaxis and degranulation of neutrophils; increasing vascular permeability
	Neutrophils	
	Lymphocytes	

Certain inflammatory cytokines, including IL-8, TNF-α, IL-1β, IL-6, and IFN-γ, have been closely associated with COM. Expression and activation of different ME cell surface TLRs leads to production of the aforementioned plethora of inflammatory cytokines, which provides a link between the variability of expressed mucosal TLRs and disease (as low levels of TLR expression will lead to a weak response to infection, persistent inflammation and enhanced susceptibility to COM). Cytokines play a role in inter-cell communication and coordination of inflammatory cells, such as neutrophils, macrophages and lymphocytes. Different cytokines are involved in the early and late stages of inflammation with IL-1β and TNF-α being early response cytokines, inducing expression of glycoprotein adhesion molecules on the surface of vascular endothelial cells, that promote recruitment of circulating leukocytes to the site of infection. Conversely, IL-10 and IL-2 are late-stage cytokines, that contribute to resolution of inflammation, providing negative feedback of TNF-α, and induction

of proliferation and differentiation of T-cells, B-cells and NK cells, as well as, monocytes and macrophages, respectively. [248]

Another aspect of the innate immune system is the complement system, a cascade of small proteins that circulate in the blood as inactive precursors. The complement system is activated early in response to inflammation and once activated it triggers a series of proteases that culminates in amplification of inflammatory responses, such as release of cytokines, recruitment of leukocytes, and cytotoxic responses to infection (Figure 26). Tong *et al* (2014) reported that complement deficient mice (C1qa-/- and factorB-/-) exhibited delayed viral and bacterial clearance in the ME and experienced significant mucosal damage to the ME and ET, when compared to wild-type mice, challenged by influenza A virus and *S. pneumoniae*. This showcases that both the classical and alternative complement pathways play important roles in the susceptibility against otopathogens. Further, the role of C5a and the interactions with its receptor, C5aR, were also evaluated. Interestingly, C5ar1−/− mice present lower levels of IL-6 and MCP-1, as well as reduced inflammatory cell recruitment and inflammation levels, but improved bacterial clearance. The deletion of C5aR may lead to a more efficient clearance of *S. pneumonia*, by lowering exacerbated inflammation levels allowing for a transition into the resolution phase. [249] He *et al* (2013) showed that children between 5 and 38 months old with rAOM present with significantly higher levels of complement fragments, namely C3 and C5a, in their ME effusions than in the serum. *In vitro* data, utilising primary human middle epithelial cells from a 19 year old patient, showed that cells infected with *S. pneumoniae* secreted higher levels of C3 and factor B when compared to uninfected cells. Although complement activation may be beneficial for clearance of pathogens, unregulated complement processes lead to excessive inflammation which can be harmful to ME mucosa. [250]

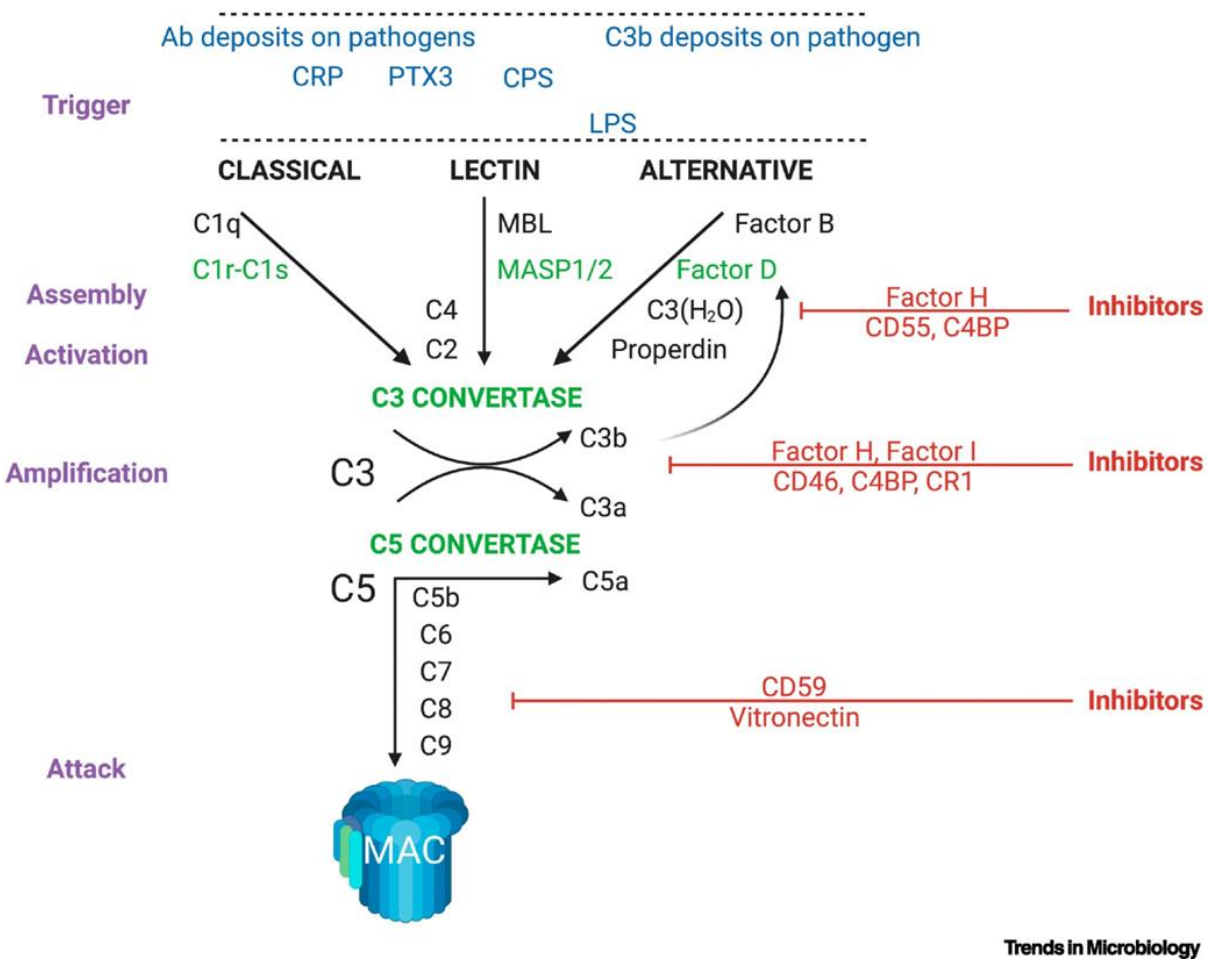


Figure 26 - Diagram of the complement cascade system. The complement system can be activated via 3 pathways - the classical pathway, the lectin pathway and alternative pathway - through assembly of different proteins. These pathways are triggered by various bacterial surface components, which induce a cascade of host molecules subsequently cleaved by proteases. These three pathways converge into similar outcomes at the formation of the C3 convertase. The cascade descends until the formation of the membrane attack complex, which destabilises bacterial cell membranes, promoting cell lysis. Adapted from: https://www.cell.com/trends/microbiology/fulltext/S0966-842X%2821%2900213-4.

Through its phasevarion, NTHi has the ability to change its surface makeup, and consequently the immunological response of the host. As previously shown by Brockman *et al* (2016) ModA2 phasevarion affects the progression and severity of OM, and a phasevarion shift within the ME leads to greater disease severity when compared to environments where the shift does not occur. [187] In a study utilising chinchilla, Robledo-Avila *et al* (2020) found that the animals infected with an NTHi 723 strain, a paediatric OM clinical isolate constitutively expressing *modA2* (locked ON), had significantly higher levels of mucosal biomass, fluid within the ME, antigen presenting cells (APCs) and neutrophils, 14 days post infection, than

those infected with the same strain whose modA2 was locked OFF. Furthermore, APCs and macrophages isolated from the MEs of chinchillas challenged with modA2 locked OFF, expressed and produced more pro-inflammatory cytokines than those infected with bacteria modA2 locked ON, suggesting that the virulence factors involved in infection by modA2-lacking bacteria induced greater activation or recognition by the immune cells. The modA2-lacking bacteria were also more sensitive to killing by macrophages, but this may be due to a greater activation of the macrophages in comparison to those reacting to modA2-expressing bacteria. [251] Thus the ability of bacteria to adapt to the environment, by avoiding recognition and suppressing macrophage activation, also contributes to increased survival rates. Brockman *et al* showed that in chinchilla ME, inoculated with NTHi 723 modA2 OFF, a consistent shift in the NTHi population takes place from OFF to ON, highlighting the importance of phasevarion in bacterial survivability and the pathogenesis of OM. [187]

When regulated, the inflammatory response is beneficial to the host. Innate immune cells and APCs, such as dendritic cells, play a vital role in the transition to the adaptive response, where memory is created, and further defence mechanisms engage in pathogen clearing. When persistent or unregulated, however, exacerbated inflammatory responses can ultimately lead to cell death, tissue damage and hampering of tissue healing. To better comprehend OM, a full understanding of the immune response to various ME infections is required.

Hypothesis

This project hypothesises that fetal ME epithelial cells can be utilised to create *in vitro* human ME 3D cell models, providing reliable tools for the study of the pathogenesis of OM. Furthermore, as NTHi causes intracellular infections in the respiratory epithelium, it is expected a similar pattern should be seen in ME epithelium infections.

Aims and Objectives

This project aspires to address the current, concerning, lack of suitable *in vitro* models for the study of OM, namely, the lack of a paediatric epithelial cell line and *in vitro* ME epithelial model. Developing a reproducible human ME *in vitro* model, that accurately represents the ME epithelium, will provide a reliable tool which will allow for a representative study of OM, from its onset to later stages of disease. This will be achieved by collecting paediatric/fetal ME cells and exposing them to an air-liquid interface to promote differentiation. If possible, paediatric/fetal ME cells will be transgenically modified to expand the lifespan of the cells.

This project also aims to evaluate the interactions between NTHi and the ME epithelium, and to investigate the mechanisms of infection and, potentially, invasion by the bacterium. This will be achieved by analysis of intracellular invasion of NTHi through antibiotic protection assays and assessment of cytokine expression by ME epithelial cells in response to NTHi infection.

Finally, the project will attempt to address the current uncertainty regarding the development and origin of the ME. This will be achieved through immunohistochemical analysis of tissues recovered from terminations of pregnancies ranging from 23CS to 17 weeks.

2. Chapter 2 - Materials and Methods

2.1. Tissue culture

2.1.1. Dissection of fetal human skulls from early termination of pregnancies for recovery and isolation of middle ear cells

The dissection of human fetal skulls from early terminations of pregnancy (TOP) was used for the recovery of primary ME epithelial cells as, due to the COVID-19 pandemic, previously planned access to ME tissue from otorhinolaryngology surgeries was not possible. Human fetal skulls were obtained from the MRC/Wellcome trust-funded Human Developmental Biology Resource (HDBR), in London and Newcastle. Samples sent in the scope of this project were either fresh samples, used for cell harvesting and isolation, or samples fixed with formalin, that were used for immunohistochemical analysis.

Although the specimens obtained varied in age, between Carnegie stage (CS) 23 to 20 post conception weeks (PCW), recovery of ME epithelial cells was only feasible in TOPs from 14 PCW onwards. Harvesting of cells was significantly easier to achieve in "older samples", from 18 to 20 PCW, due to a more visible definition of the ear in these later stages.

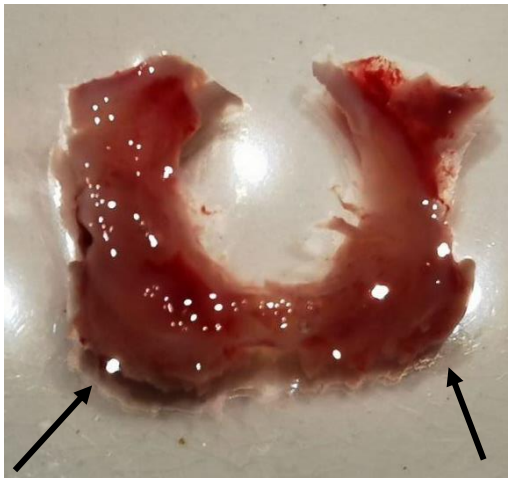
Under direct visual observation, and utilising fine dissecting forceps and scissors, the fetal skulls were dissected into smaller sections nearing the inner and outer ear, in 90 mm sterile Petri dishes. After discarding excess tissue and cartilage, the ear pinna and the cochlea served as a guide to narrow the position of the ME. A previous study from our group, by Apoorva *et al* (2016), demonstrated that in young adult mice the ME is easily found as an encapsulated bone structure, however, no distinct cartilaginous or round bone structures were found in the human fetal samples. [101] The ME cavity can be found by locating the developing auditory bones within the growing mesenchyme of the cavity and the tympanic membrane and ring, which are easier to identify in fetal samples older than 16 PCW (See table 5).

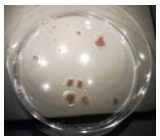
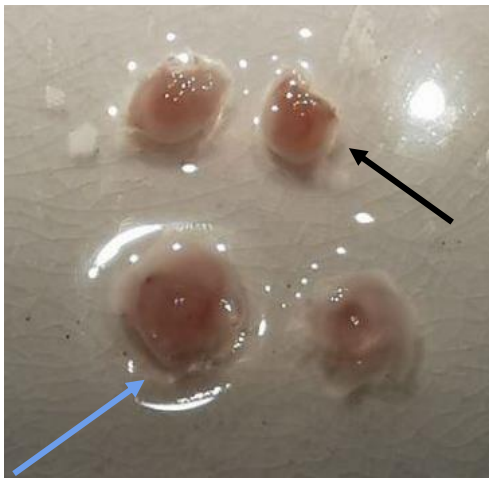
In most dissections performed, in order to reach the ME cavity, the inner ear would sustain heavy damage and thus be discarded. The areas corresponding to the ME cavity, namely the tissue walls lining the outside of the inner ear, the tympanic membrane and the tympanic

ring, and the lining surrounding the auditory ossicles were recovered for treatment overnight with dispase (1U/ml) in PneumaCult™ ExPlus medium (STEMCELL™ Technologies), supplemented according to the manufacturer's instructions, at 4°C. The following day, the solid tissue components were recovered and placed in a T-25 TC-treated flask with supplemented ExPlus medium. The supernatants were centrifuged at 500xg for 5 minutes, resuspended in ExPlus medium and transferred into a separate T-25 TC-treated flask.

When confluent monolayers had formed, the flasks presented a heterogeneous population of epithelial-like and mesenchymal-like cells, which were separated by two rounds of differentiational trypsinisation. The mesenchymal cells detached from the surface within the first minute of exposure to trypsin-EDTA, whereas the epithelial cells required incubations of over five minutes.

Table 5 - Chronological progression of dissected tissue, depending on fetal developmental stage.

<table>
<tr><th colspan="2">8 PCW TOP</th></tr>
<tr>
<td> 
 
Dissected skull

Ears (black arrows)</td>
<td> 
 
Further dissection

Cochleas (black arrows, top row), ME mesenchyme (bottom row)</td>
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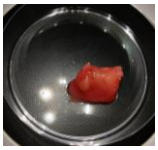
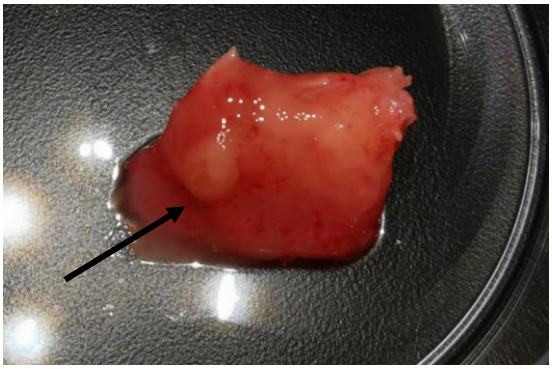
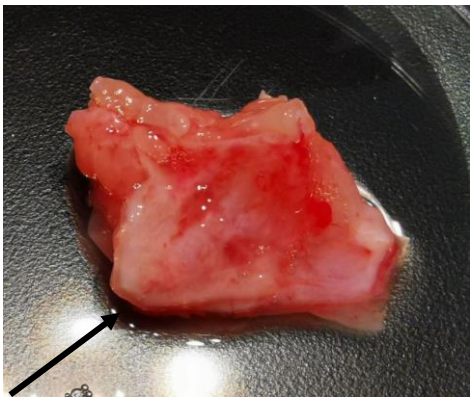
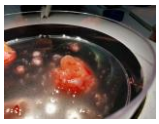
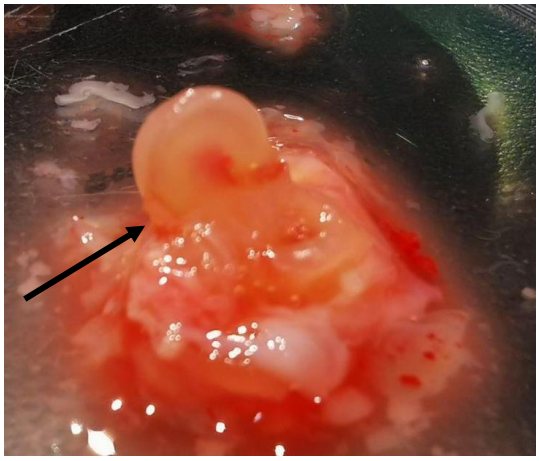
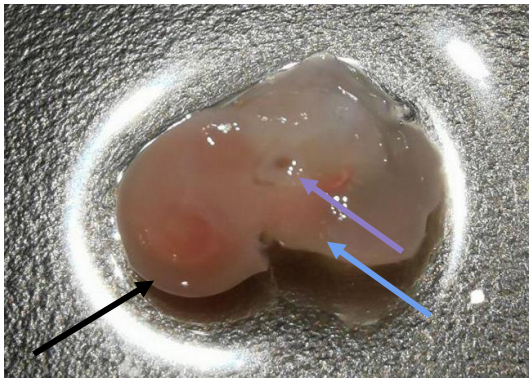
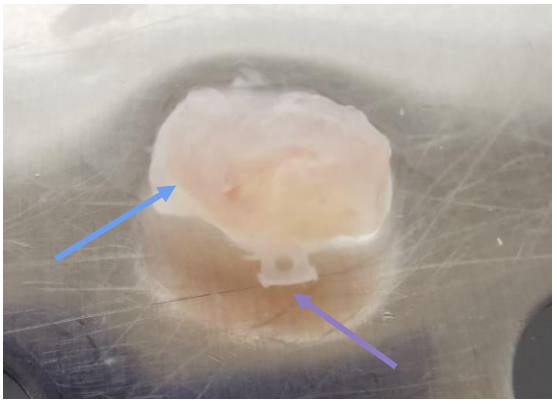
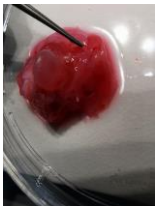
12 PCW TOP	
  Dissected skull Pinna (black arrow)	  Dissected skull Inner ear (black arrow)
  Further dissection Cochlea (black arrow)	  Further dissection Cochlea (black arrow), stapes (purple arrow), ME mesenchyme (blue arrow)

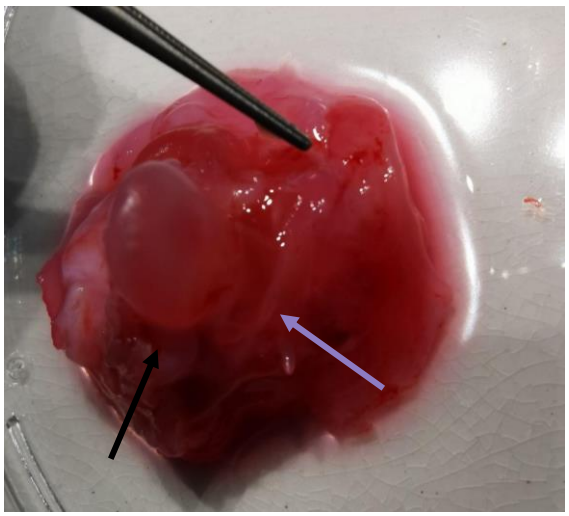


Further dissection

ME mesenchyme (blue arrow) and stapes (purple arrow)

16 PCW TOP





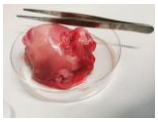
Dissected skull

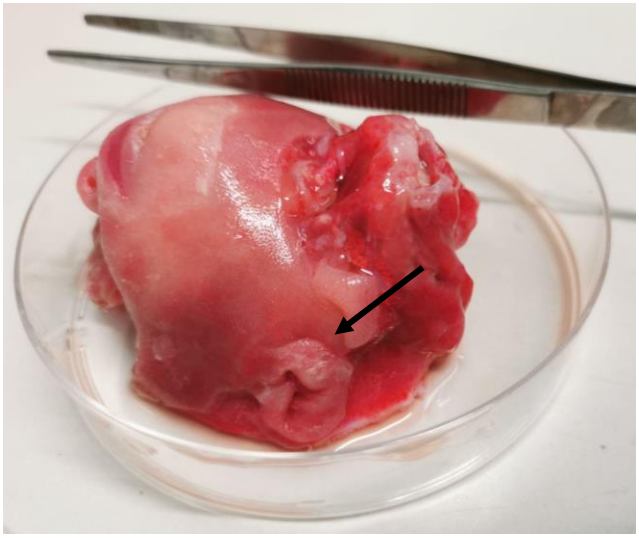
Inner ear (black arrow) and ME cavity (purple arow)

Dissected skull

Dislodged tympanic ring (black arrow)

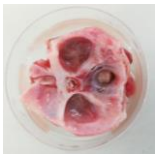
19 PCW TOP

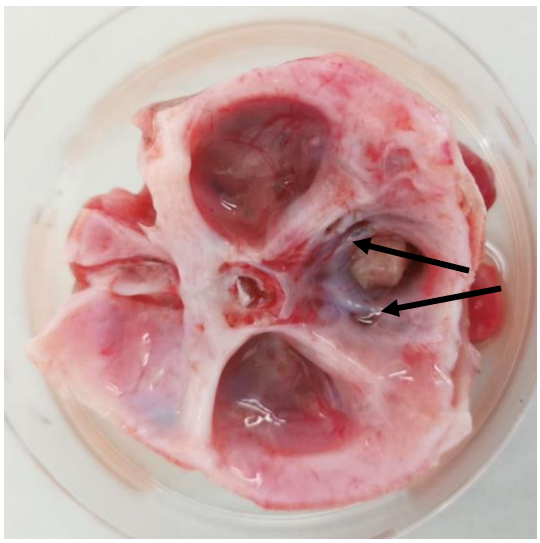




Lower half of fetal skull (lateral view)

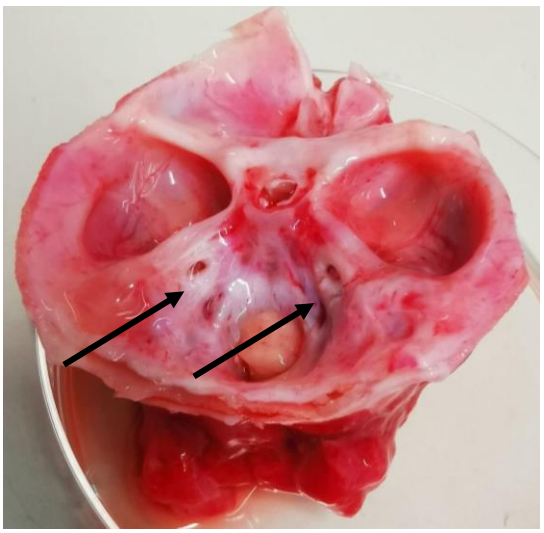
Pinna (black arrow)





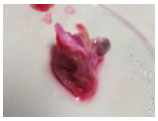
Lower half of fetal skull (top view)

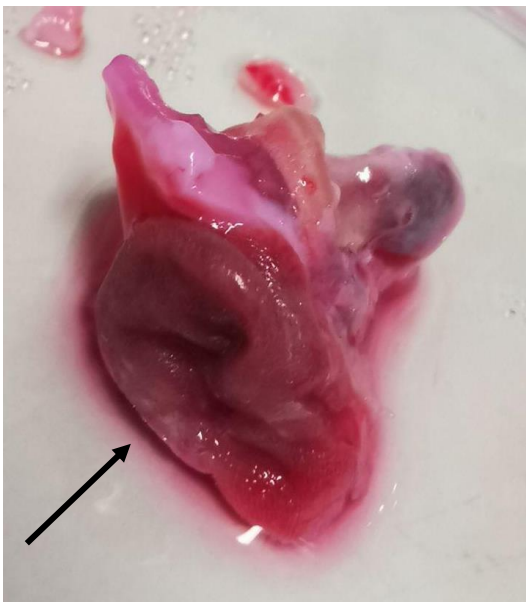
Ears (black arrows)



Lower half of fetal skull (top lowered view)

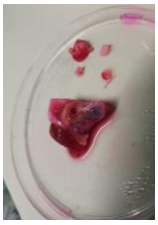
Ears (black arrows)

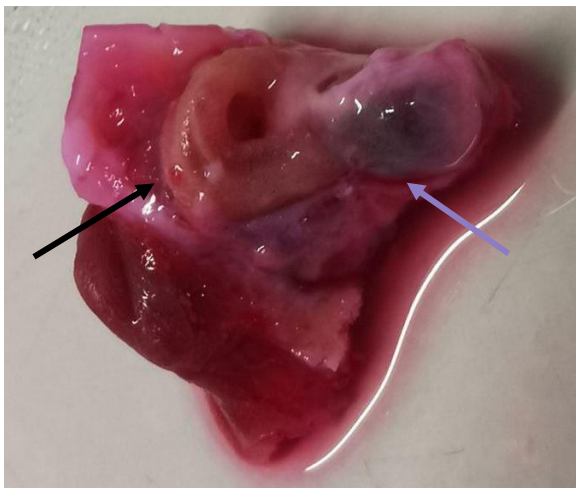




Further dissection

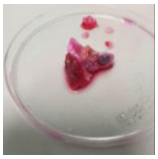
Pinna (black arrow)

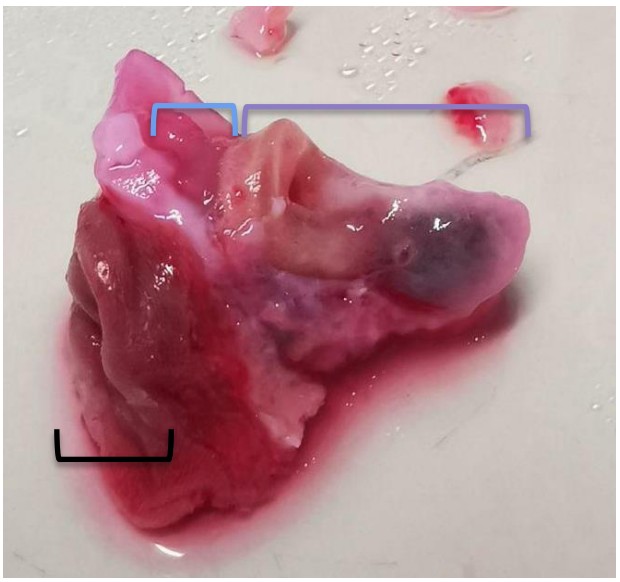




Further dissection

Semicircular canal (Black arrow) and cochlea (purple arrow)

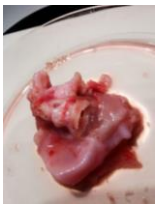


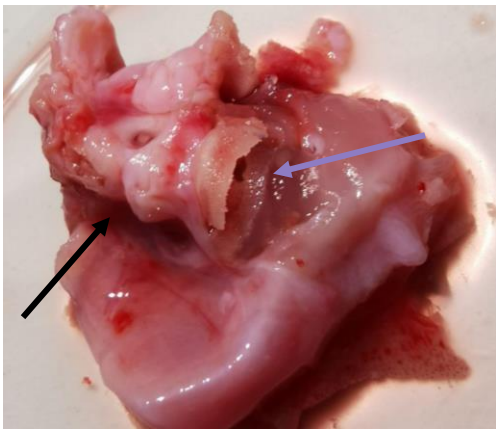


Further dissection

Outer ear (Black bracket), middle ear (blue bracket), inner ear (purple bracket)

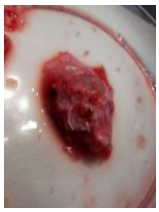
19.5 PCW TOP

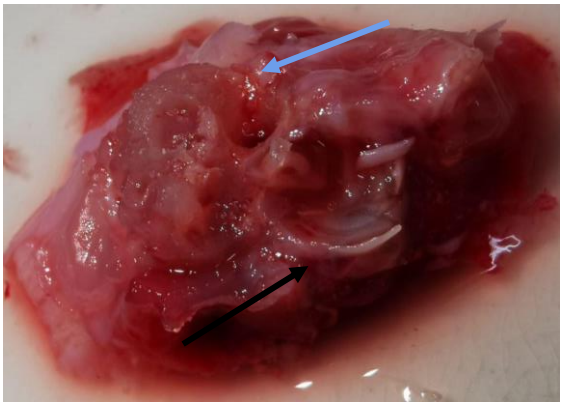




`Dissected ear / Middle ear towards inner ear

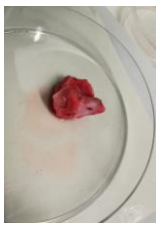
Cochlea (black arrow) and semicircular canal (purple arrow)

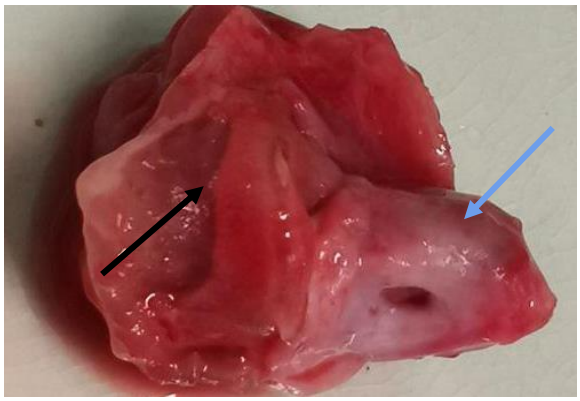




Dissected ear / Middle ear towards outer ear

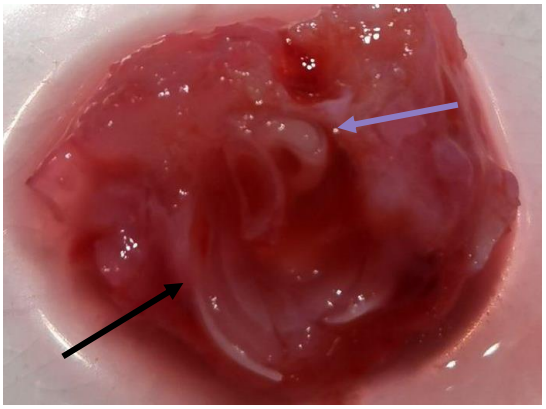
Tympanic ring and membrane (black arrow) and ME mesenchyme (blue arrow)





Further dissection towards inner ear

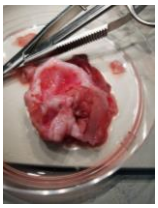
Inner ear (blue arrow) and semicircular canal (black arrow)

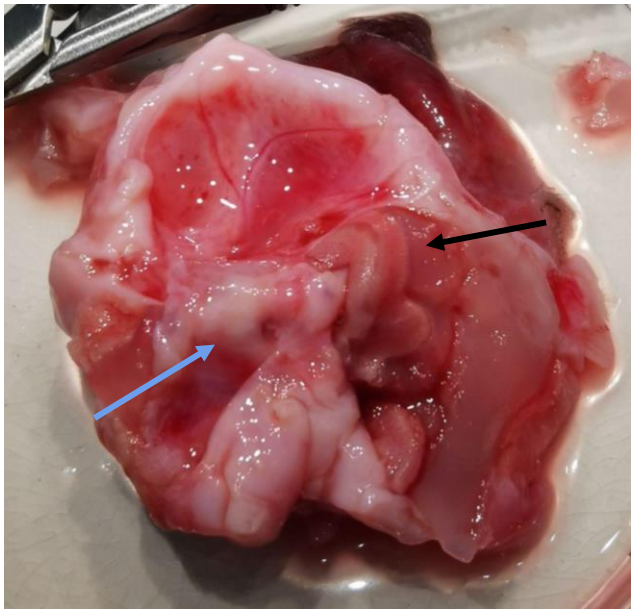


Further dissection towards outer ear

Tympanic ring and membrane (black arrow) and stapes (purple arrow)

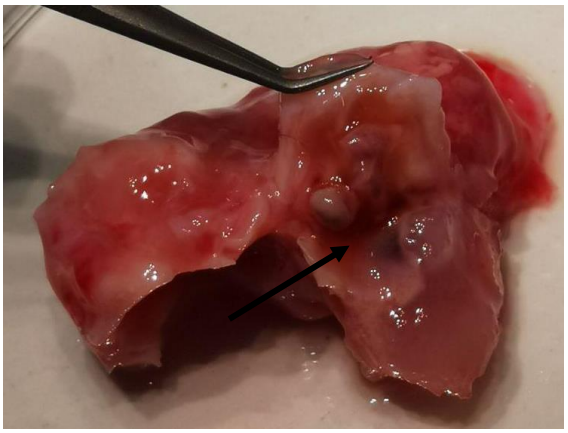
20 PCW TOP





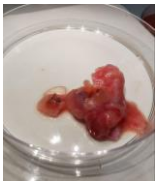
Dissected fetal skull

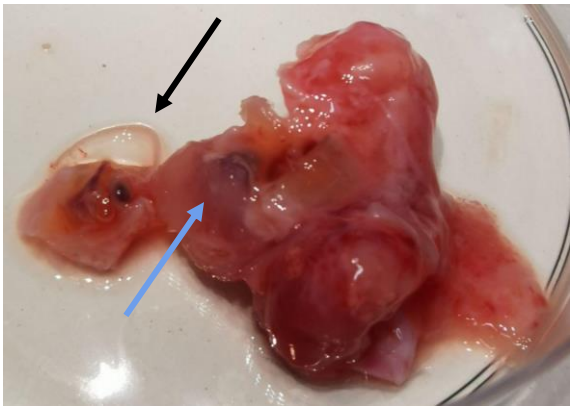
Cochlea (blue arrow) and semicircular canal (black arrow)



Further dissection

Middle ear cavity with auditory ossicles (Black arrow)





Further dissection

Exposed middle ear with tympanic membrane ring (black arrow) and auditory ossicles (blue arrow)

2.1.2. Maintaining cell cultures

hMEEC-1 is an adult male ME cell line, established utilising a retrovirus containing human papillomavirus type 16 E6/E7 genes. For determining optimal culture conditions for the growth of ME epithelial cells, hMEEC-1 was seeded and grown in four different media: PneumaCult™ ExPlus Medium, supplemented according to manufacturer's instructions (ExPlus) (STEMCELL™ Technologies, 05040); low glucose Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum (DMEM); Keratinocyte Growth Medium, supplemented as follows: 335ml low glucose DMEM, 115ml F12, 50ml fetal bovine serum, 5ml L-Glutamine, 5ml Adenine, 2ml Hydrocortisone, 250µl Insulin, 500µl EGF (KGM); and Keratinocyte Serum Free Medium, supplemented according to manufacturer's instructions (KSFM) (Gibco™, 17005042). Cell viability and proliferation were evaluated using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, G3580). Cells were seeded at a density of 5,000 cells per 100µl. After analysis of results, all ME epithelial cells described in this work were grown in ExPlus, at 37°C, 5% CO_2 (See table 6).

Table 6 - Cell types and media utilised to grow cells throughout the research project.

Cell type	Origin	Growth medium
Primary ME epithelial cells recovered during dissection (MEEC)	TOP dissection	ExPlus
hMEEC-1	Adult male ME, immortalised utilising human papillomavirus type 16, genes E6/E7	ExPlus
Primary ME mesenchymal stromal cells (MSCs)	TOP dissection	ExPlus, supplemented with 10% Rocki (1µM)
HBEC3-KT	Adult female bronchial, immortalised utilising hTERT	KSFM
Normal oral fibroblasts 817	Adult oral primary fibroblasts	DMEM

2.1.3. Air liquid interface cultures of human middle ear cells

MEEC and hMEEC-1 were seeded, in duplicate, into Falcon® 0.4µm pore-sized transparent Polyethylene Terephthalate (PET) cell culture inserts (Corning, 353180) in Falcon® 12 well cell culture insert companion plates (Corning, 353503), at a density of 90,000 cells per transwell. Cells were initially cultured submerged in ExPlus media, with 300µl of media in the apical chamber and 1.5ml of media in the basal chamber. After 1 to 3 days, when the cells were confluent, media from the apical chamber was removed and media from the basal chamber was replaced with 1ml of PneumaCult™ ALI Medium (STEMCELL™ Technologies, 05001), supplemented according to the manufacturer's instructions.

In an ALI system, exposure to air and humidity is known to promote differentiation of cells by mimicking *in vivo* physiological conditions, however, this was not observed in my study. To promote differentiation, following the protocol mentioned above, cells were further exposed to three growth conditions when transferred to ALI, by (1) seeding 15,000 cells/ml of NOF-817 in the basal chamber, (2) adding 1µM retinoic acid and 20ng/ml TNF-∝ to ALI media, further supplementing it with 20ng/ml TGF-∝ on day 3 at ALI, and (3) adding 2mM $CaCl_2$ to ALI media. Later, a fourth growth condition was introduced, where transwells were coated with a 10% mixture of rat-tail collagen type I (4.3mg/ml) (Sigma-Aldrich, C3867) in ExPlus and incubated for 30 minutes at 37°C, 5% CO_2. MSCs and MEEC cell density was adjusted to 450,000 cells/ml, and the two populations were mixed together as a 50/50 cell suspension. 200µl of this cell suspension was seeded onto the collagen coated transwells.

Cells were grown at ALI for 14 or 21 days (Figure 27). Apical surfaces were gently washed with sterile PBS and the media was changed every 48-72 hours to clear cellular debris and secretions. Cells were lysed in 200µl of TRI Reagent® for RNA extraction.

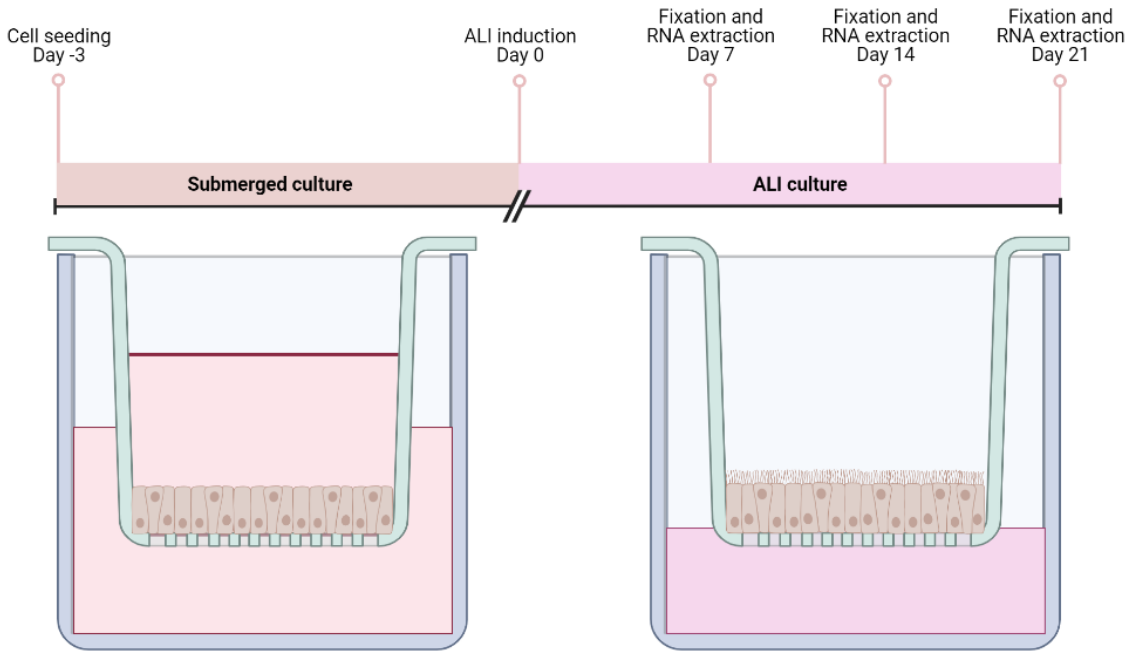


Figure 27 - Timeline and methods for ALI culture of hMEEC-1 and MEEC. Post dissection of ME and pronase dissociation, cells were expanded, and the mixed population was separated into mesenchymal-looking and epithelial-looking cells through trypsinisation at different time points. After seeding, cells were allowed to expand for 3 days and then exposed to air, with basal chamber media changes every 2 days. ALI promotes differentiation into cilliated and secretory cells. Adapted from https://www.biorender.com/.

2.1.4. Apical-out airway organoids

Apical-out airway organoids are generated in an ECM-free environment from 2D-expanded ME epithelial cells and differentiated in suspension to form organoids with cilia (Figure 28). Utilising the PneumaCult™ Apical-Out Airway Organoid system (STEMCELL™ Technologies, 100-0620), MEEC and hMEEC-1 were seeded at a density of 120,000 cells/ml per well into a pre-treated AggreWell™400 and incubated at 37°C, 5% CO_2 in supplemented PneumaCult™ Apical-Out Airway Organoid basal medium, all according to the manufacturer's instructions. Post cell aggregation (24-72 hours), the spheroids were transferred onto a pre-treated 24- or 96-well flat bottom plate. Medium was partially changed every 48-72 hours, up to 14 days.

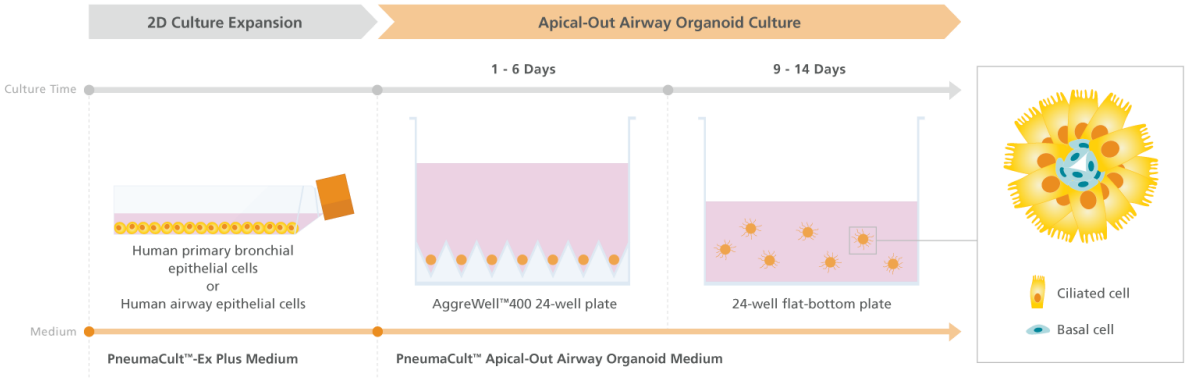


Figure 28 - Timeline and methods for ApAO culture of hMEEC-1 and MEEC. Adapted from https://www.stemcell.com/products/pneumacult-apical-out-airway-organoid-medium.html

2.1.5. Life span extension of primary middle ear epithelial cells

The work in this section (2.1.5.) was carried out by Dr Ruhina Maeshima, in Prof Stephen Hart's laboratory, at the Genetics & Genomic Medicine Department, University College London.

To expand their lifespan, MEEC were transfected with a lentivirus carrying *Bmi-1* inserted into a pLVX-Puro vector (Clontech, 632164) digested with *Xho*I and *Bam*HI (LV-BMI-Puro), as previously described (Figure 29). [252,253] To achieve this, MEEC at passage 4 (P4) were seeded onto 6-well plates at a density of 100,000 cells/well and incubated overnight at 37°C, 5% CO_2. The following day, 1 ml of LV-BMI-Puro solution (stock: $2x10^8$ TU/ml) was added to the wells at different multiplicities of infection (MOI): 1 (0.5µl LV-BMI-Puro in 200µl OPTIMEM + 800µl ExPlus), 4 (2µl LV-BMI-Puro in 200µl OPTIMEM + 800µl ExPlus) and 16 (8µl LV-BMI-Puro in 200µl OPTIMEM + 800µl ExPlus). Plates were incubated overnight at 37°C, 5% CO_2. On the following day, 1ml of ExPlus media was added to each well, for a total of 2ml per well. The cells were moved to T-75 flasks when confluency was achieved in the 6-well plates, approximately 3 days later.

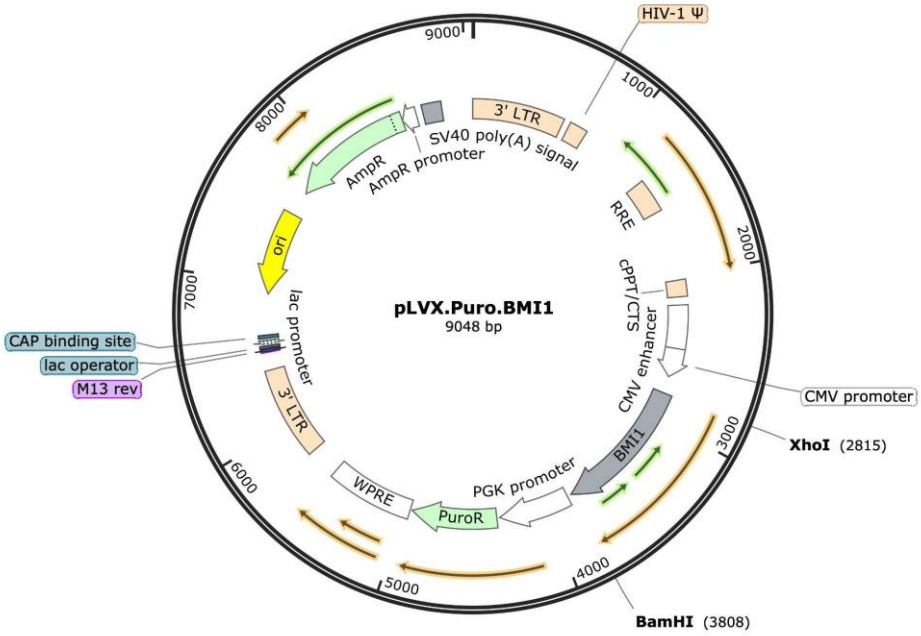


Figure 29 - Diagram of plasmid carried by lentivurs with inserted Bmi-1.

2.1.5.1. Assessment of BMI-1 transfected cells

The success of the transfection was assessed by the presence of the puromycin-resistant cassette in cells. A puromycin gradient was set up to estimate optimal puromycin concentration for selection of transfected cells: 0, 0.5, 1, 2.5, 5, 7.5, 10µg/ml. Non-transfected MEEC and the different MOI BMI-1-transfected cells were seeded in 96-well plates at a density of 20,000 cells per 100µl and incubated overnight at 37°C, 5% CO_2. Non-transfected MEEC acted as a control. The following day the appropriate volumes of 1mg/ml puromycin (Gibco™, A1113803) were added to achieve the concentrations of the gradient stated above. Plates were incubated overnight at 37°C, 5% CO_2. The following day cells were washed, the medium was changed, with puromycin added again. Cells were incubated for another 72 hours.

To select transfected cells growing in T-75 flasks a concentration of 2.5 and 5µg/ml of puromycin was added. The cells were washed 24 hours later, and fresh medium with puromycin was added. Cells were exposed to puromycin for approximately 2 weeks.

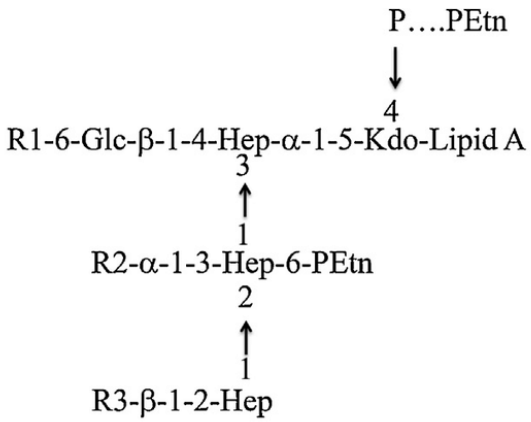
2.2. Microbiology

2.2.1. NTHi strains

For *in vitro* infections of hMEEC-1 and MEEC, laboratory strains Rd, H446, H457, H491 and GFP-tagged streptomycin-resistant clinical isolate NTHi-375 were used (See table 7) (Figure 30 and 31). All laboratory strains were kindly provided by Dr Jeroen Langereis, at the Radbound Institute for Molecular Sciences, Netherlands.

Table 7 - List of bacterial strains utilised in my research project. H446 is a *lic1D* mutant, unable to incorporate PCho, H457 is a *lic1D* mutant complemented with *lic1* from Eagan, a cerebrospinal fluid clinical isolate NTHi strain, with a PCho lock-on on HepIII, and H491 is a mutant strain with a PCho lock on HepI. The *lic1* operon (*lic1A* to *lic1D*) encodes for a transferase responsible for incorporating PCho fragments into LOS.

Strain	Description	Reference
Rd	Type d, unencapsulated isolate	Fleischmann *et al* (1995) [254]
H446 (Contains kentamicin resistance cassette)	Rd with *lic1D*::Km, constitutive PCho-	Lysenko *et al* (2000) [255]
H457 (PCho attached to hexose extension from $Heptose_{III}$ - H3)	Rd with *lic1D* (Eagan), constitutive PCho	Lysenko *et al* (2000) [255]
H491 (PCho in natural position for Rd, attached to hexose extension from $Heptose_{I}$ - H1)	Rd with *lic1A*Δ$(CAAT)_n$, constitutive PCho+	Lysenko *et al* (2000) [255]
NTHi-375	Paediatric otitis media clinical isolate	Hood *et al* (1999) [256]



For Eagan: R1=H, R2=Gal-α-1-4-Gal-β-1-4-Glc-β-1-4-Glc & R3=Gal
For Rd: R1=PCho, R2=H & R3=GalNAc-β-1-3-Gal-α-1-4-Gal-β-1-4-Glc
For 375: R1=Pcho, R2=H & R3=Gal-α-1-4-Gal-β-1-4-Glc (PEtn can be non-stoichiometrically attached at the 4-position of Hep III).

Figure 30 - Schematic representation of LOS from NTHi strains Eagan, Rd and NTHi 375. Represented in the LOS structure: (Kdo) 2-keto3-deoxyoctulosonic acid; (Hep) heptose; (Glc) glucose; (Gal) galactose; (GalNAc) N-acetylgalactosamine; (P) phosphate; (PCho) phosphorylcholine; (PEtn) phosphoethanolamine. Adapted from https://www.sciencedirect.com/science/article/pii/S0264410X15002492

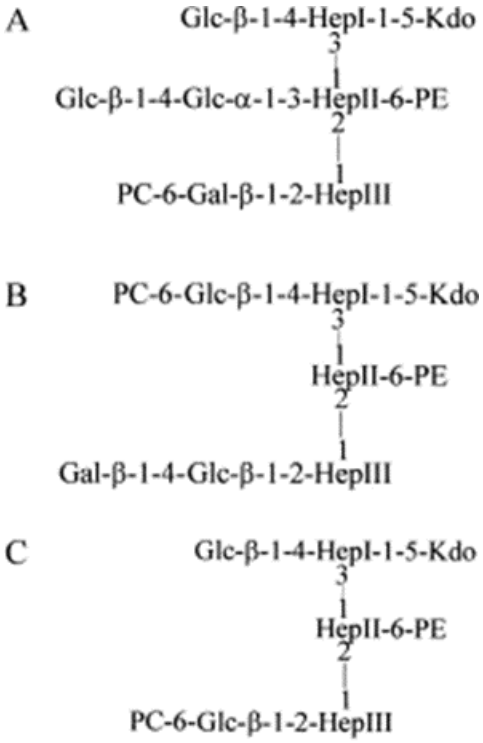


Figure 31 - Schematic representation of LOS from NTHi strains (A) Eagan, (B) Rd and (C) H457. Strains Eagan and Rd elaborate mixtures of glycoforms in which further chain extension can occur from HepII and HepIII respectively (Masoud et al., 1997; Risberg et al., 1999). Strain H457 (C) is an allelic variant of Rd containing the lic1 locus of Eagan. Strain H473 Adapted from https://onlinelibrary.wiley.com/doi/full/10.1046/j.1365-2958.2000.01707.x

2.2.2. Maintaining bacteria

Bacteria were streaked from 25% glycerol stocks on chocolate agar round Petri dishes (plate). Chocolate agar plates were prepared by melting blood agar base no.2 (Thermo Scientific Oxoid, CM0271B), cooling for 15 minutes or until the temperature reached approximately 60°C, adding 7% oxalated horse blood (Thermo Scientific Oxoid, SR0049C), and mixing the solution in a 60°C water bath until a homogeneous chocolate brown colour formed. For plates that would be streaked with NTHi-375, 200µg/ml streptomycin sulphate solution was added to the agar. The agar was poured onto round or square Petri dishes and left to cool for up to 3 hours. Plates were stored at 4°C, up to 2 months. Bacteria were streaked onto new chocolate agar plates every week.

When necessary, overnight inocula were picked from streaked chocolate agar plates into supplemented BHI (sBHI) broth. sBHI broth was prepared by adding 10µg/ml Nicotinamide adenine dinucleotide hydrate (NADH) (Cayman Chemical, 16078), 2µg/ml hemin (Merck, H9039) and, if the bacteria inoculated were NTHi-375, 200µg/ml streptomycin sulphate to BHI broth (Thermo Scientific Oxoid, CM1136B).

2.2.3. NTHi susceptibility to antibiotics

Bacterial susceptibility to four antibiotics (Metronidazole, Azithromycin, Amoxicillin, and Gentamicin) was evaluated through disk diffusion tests. Blank disks (Oxoid™, CT0998B) were carefully placed onto a NTHi-375 streaked chocolate agar plate, and 10µl of each antibiotic solution (100µg/ml) was loaded onto the disks (Figure 32).

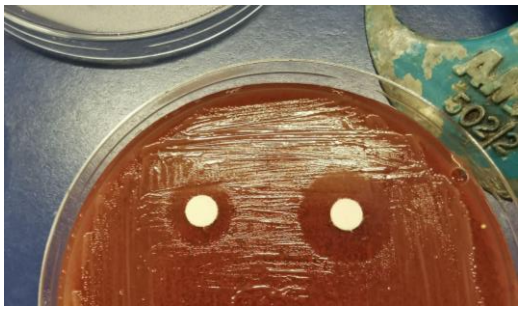


Figure 32 - NTHi-375 streaked on chocolate agar with disks loaded with 10µl of azythromicin (left) and amoxicillin (right) at a concentration of 100µg/ml.

Amoxicillin was submitted to a minimum inhibitory concentration (MIC) test to determine the lowest concentration of antimicrobial agent required to prevent visible growth of NTHi. The MIC was performed by carrying out a serial dilution of the antibiotic, from 0.25µg/ml to 256µg/ml, in sBHI, in a 96 well plate. 10µl of bacterial inocula growing in sBHI, OD_{600}=1, was added to wells with the antibiotic and to a well without antibiotics, which acted as a positive control (0µg/ml of amoxicillin). A well with sBHI that was not inoculated acted as a negative control. Cells were incubated at 37°C for 5 days. OD_{600} was measured using a plate reader (Infinite M200, TECAN) (Figure 33).

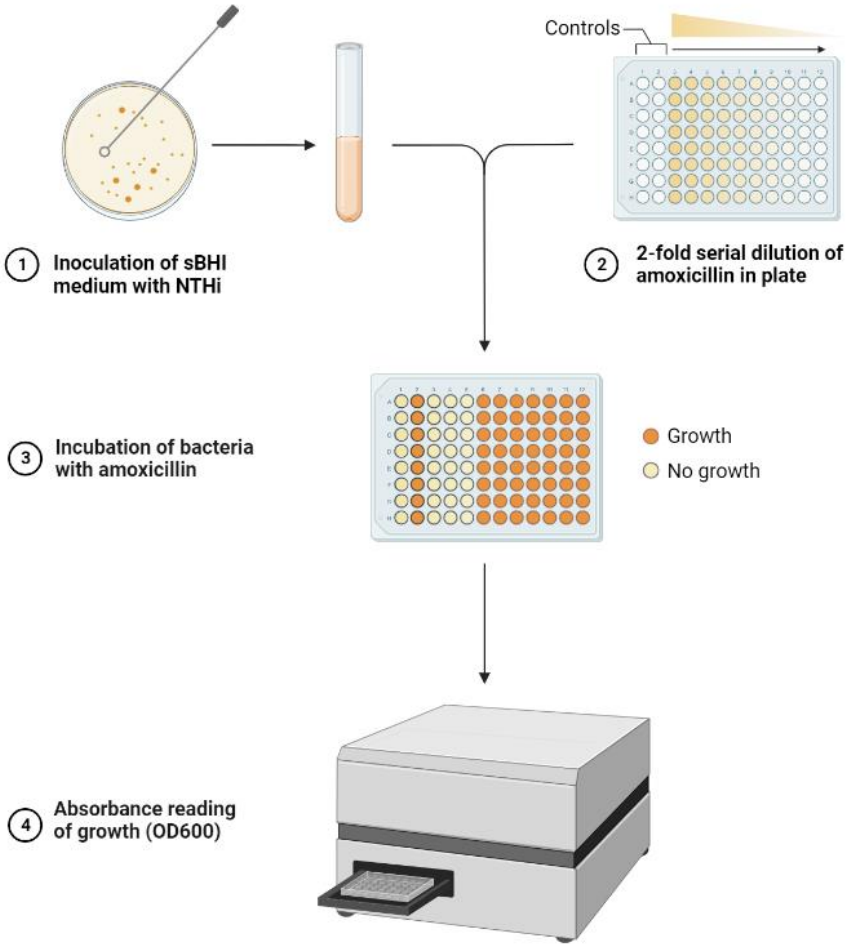


Figure 33 - Diagram of MIC protocol.

2.2.4. hMEEC-1 sensitivity to antibiotics

Cell sensitivity to antibiotics was evaluated to determine any effects on cell proliferation or viability. hMEEC-1 were seeded at a density of 20,000 cells per 100µl, in triplicate, in a 96-well plate, with 200µg/ml of each antibiotic, and their proliferation was assessed utilising a CellTiter 96® AQueous One Solution Cell Proliferation Assay, as described above.

2.2.5. Antibiotic protection assay

Antibiotic protection assays assess the intracellular invasiveness of bacteria, by determining the levels of bacterial adhesion to the cell surface and of surviving intracellular bacteria. Both hMEEC-1 and MEEC were infected with NTHi-375, Rd, H457, H491, and H446 at a MOI of 100, and treated with or without 100µM of ABT-491 (Santa Cruz Biotechnology, sc-214459), a PAFr antagonist.

500µl of cell suspension was seeded, in triplicate, at a density of 500,000 cells/ml, with an additional sacrificial well for counting purposes, in two 24-well plates. One plate was used for assessing total association (TA), the number of bacteria attached to the cell surface and present intracellularly, and one for assessing invasion (INV), the number of bacteria present intracellularly. Both were incubated overnight at 37°C, 5% CO_2. The following day, cells were gently washed twice with 500µl of PBS and incubated with 2% BSA in unsupplemented ExPlus, for 1 hour, at 37°C, 5% CO_2, with or without 100µM of ABT-491. Bacterial colonies streaked on the previous day were suspended in unsupplemented ExPlus media, and a 1:100 dilution was carried out for counting purposes. The number of bacteria/ml was assessed utilising a Hawksley-Heber counting chamber. Cells were washed twice with 500µl of PBS, the bacterial suspension was diluted to an MOI of 200 per ml (MOI defined based on the number of cells counted from the sacrificed well), and 500µl of the suspension was added to the wells with cells, as well as three empty wells, which were used to calculate the bacterial viability (BV). The plates were incubated for 90 minutes, at 37°C, 5% CO_2 and washed afterwards. In the invasion plate, the cells were washed twice with 500µl of PBS and 500µl of amoxicillin (200µg/ml) was added and plates incubated for an additional hour, at 37°C, 5% CO_2. Cells were lysed by adding 200µl of sterile deionised water to each well and scraping the

well surface with a micropipette tip. Finally, a Miles-Misra was carried out and dilutions were loaded, in triplicate, onto a square chocolate agar plate (Figure 34).

% Association = CFU/ml of NTHi associated after 1 hour / CFU/ml of NTHi in the inoculum

% Invasion = CFU/ml of NTHi invaded after 1 hour / CFU/ml of NTHi in the inoculum

CFU/ml of NTHi associated after 1 hour = # of CFU in TA * Dilution factor * 200

CFU/ml of NTHi invaded after 1 hour = # of CFU in INV * Dilution factor * 200

CFU/ml of NTHi in the inoculum = # of CFU in BV * Dilution factor * 200 * 2.5

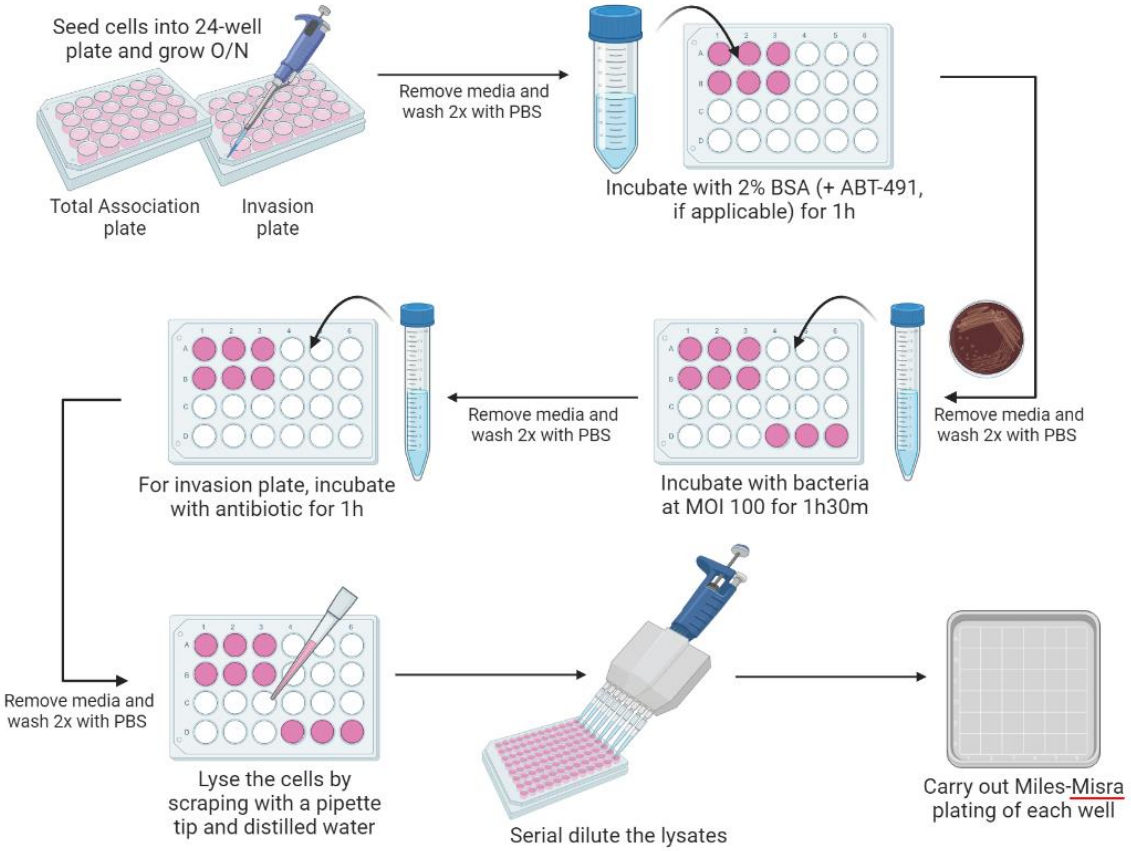


Figure 34 - Diagram of antibiotic protection assay protocol.

2.2.6. Infection of models

In order to assess the cytokine response of hMEEC-1 and MEEC to infection by NTHi at an MOI of 100, 500µl of cell suspension was seeded into 24 well plates, at a density of 400,000 cells/ml and incubated overnight, at 37°C, 5% CO_2. A sacrificial well was used to

determine the number of cells per well, in order to calculate the appropriate bacteria density for an MOI of 100, as described above. hMEEC-1 and MEEC were incubated with NTHi-375, H457, H491 and H446, in unsupplemented ExPlus for 1 hour at 37°C, 5% CO_2; whereas negative controls were incubated with unsupplemented medium only. Wells were washed twice with PBS to remove non-adherent bacteria, and fresh supplemented medium was added to all wells. The plates were incubated at 37°C, 5% CO_2 for 4, 24, 48 and 72 hours. At these time points, medium was collected and centrifuged for 5 minutes, at 12,000xg, and the supernatant was stored at -80°C for further analysis. Cells in the wells were lysed in 300µl of Tri-Reagent for RNA extraction.

2.3. Molecular biology

2.3.1. RNA extraction and RT-PCR

Cells were lysed using Tri-reagent (Merck, T9424) and mRNA purified using a Direct-zol RNA miniprep kit (ZymoResearch, R2052), as per the manufacturer's instructions. mRNA was eluted in 8µl of RNAse and DNAse-free distilled water and the RNA quantified using a Nanodrop™2000. Samples were considered of good quality when the absorbance ratios 260/280 and 260/230 were between 1.8 and 2.2. Reverse transcription polymerase chain reaction (RT-PCR) was performed to convert mRNA templates into complementary DNA (cDNA), utilising High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, 4368814). The 2x RT master mix, RT reactions and thermal cycler were set up according to the manufacturer's instructions. The final RT reaction consisted of 10µl of mRNA at an adjusted final concentration of 50ng/µl and 10µl of 2x RT master mix (See table 8 and 9), making up a final volume of 20µL. cDNA was stored at -20°C or used immediately.

Table 8 - RT-PCR reagents list.

2X RT master mix	Volume
10X RT Buffer	2.0 µl

25X dNTP Mix (100 mM)	0.8 µl
10X RT Random Primers	2.0 µl
MultiScribe™ Reverse Transcriptase	1.0 µl
Nuclease-free H_2O	4.2 µl

Table 9 - RT-PCR machine cycle.

Temperature	Cycle duration	Number of cycles
25°C	10 minutes	1 cycle
37°C	60 minutes	2 cycles
85°C	5 minutes	1 cycle
4°C	Hold	Hold

2.3.2. End-point PCR and electrophoresis

Specific segments of cDNA were amplified through PCR, utilising DreamTaq Green PCR Master Mix (2X) (ThermoFisher, K1081), according to the manufacturer's instructions. The final PCR reaction consisted of 1µl of cDNA (~50ng/µl) and 19µl of 2x PCR master mix, making up a final volume of 20µL (see table 10, 11 and 12).

Table 10 - PCR reagents list.

2X PCR Master Mix	Volume

Dreamtaq Green PCR Master mix	10µl
Primer Forward	1µl
Primer Reverse	1µl
Nuclease free water	7µl

Table 11 - PCR machine cycle.

Temperature	Cycle duration	Number of cycles
94°C	5 minutes	1 cycle
94°C	1 minute	35 cycles
60-64°C	1 minute	
72°C	1 minute	
72°C	5 minutes	1 cycle
4°C	Hold	Hold

Table 12 - PCR primer designs.

Gene	Primer type	Sequence (5'-3')
GAPDH	Forward Reverse	TGATGACATCAAGAAGGTGGT TCCTTGAGGCCATGTGGGCC
E-cadherin	Forward Reverse	GAAGGAGGCGGAGAAGAGGA GGGTCAGTATCAGCCGCTTT
Cytokeratin 5	Forward Reverse	CCAGGAGCTCATGAACACCA GCTTCCACTGCTACCTCCG
Vimentin	Forward Reverse	CTCTGGCACGTCTTGACCTT CAGCTCCTGGATTTCCTGCA
N-cadherin	Forward Reverse	CCTTTCAAACACAGCCACGG TGTTTGGGTCGGTCTGGATG
ICAM-1	Forward	TCTTCCTCGGCCTTCCCATA

	Reverse	CAGGTACCATGGCCCCAAAT
cKIT	Forward Reverse	GAAGGCTTCCGGATGCTCAG GTCTACCACGGGCTTCTGTC
ZO-1	Forward Reverse	TCACGCAGTTACGAGCAAGT TGAAGGTATCAGCGGAGGGA
a-Tubulin	Forward Reverse	CTATCCCCGCATCCACTTCC TTTACCATGGCGAGGGTCAC
Sox2	Forward Reverse	TTACGCGCACATGAACGGCT TGCGAGTAGGACATGCTGTA
PAFr	Forward Reverse	AAATTCCTGTGCAACGTGGC CAGTCTTGATGGGCCGAGTT
MUC5AC	Forward Reverse	TCGGCTCCTACAACCAACAC GGCAGAAGTTGTGCTGGTTG
MUC5B	Forward Reverse	CAACAGCCATGTGGACAACT CTCGCAGAAGGTGATGTTGA
TEKT-1	Forward Reverse	CAGTGCGAAGTGGTAGACG TTCACCTGGATTTCCTCCTG
BPIFA1	Forward Reverse	ACAGAGGAGCCGACGTCTAA CCAAGAAAGCTGAAGGTTC

A 2% agarose gel was made using 2g of agarose powder (Sigma-Aldrich, A9539) in 100ml of 1X Tris-acetate-EDTA (TAE) buffer and 1µl of Ethidium Bromide (1mg/ml). 8µl of amplicon solution and 3µl of GeneRuler 1kb DNA ladder (ThermoScientific™, SM0311) were loaded onto the gel. The gel was run at 120V for 45 minutes in Bio-Rad Sub-Cell GT Electrophoresis Cell and visualised using Syngene InGenius 3.

2.3.3. Quantitative Real-time PCR

Specific mRNA segments were amplified through real-time quantitative-PCR, utilising the qPCRBIO Probe Blue Mix Lo-ROX (qPCRBIO, PB20.25-05). The final qPCR reaction consisted of 1µl of the cDNA solution (~25ng/µl) and 9µl of qPCR master mix, making up a final volume of 10µL (See table 13 and 14).

Table 13 - qPCR reagents list.

qPCR Master Mix	Volume
qPCR Probe Blue Mix Lo-ROX	5µl
Taqman primer	0.5µl
B2M Endogenous Control (ThermoFisherScientific, 4326319E)	0.5µl
Nuclease free water	3µl

Table 14 - qPCR Taqman primers.

Primer	Assay ID, catalog number
IL-6	Hs00985639_m1, 4331182
IL-8 (CXCL8)	Hs00174103_m1, 4331182

2.3.4. Cell Viability assays

CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) (Promega, G3580) is a colourimetric assay that relies on the production of formazan, a product obtained due to tetrazolium reduction, and was utilised to assess cell proliferation and viability. Cells that underwent the assay had been seeded in 100µL of appropriate media at desired cell density, in a TC-treated 96 well plate, and incubated overnight, at 37°C, 5% CO_2. The following day, the assay was performed by adding 20µL of CellTiter solution, in a dark laminated flow hood,

wrapping the plate in aluminium foil and incubating the cells for 1 hour at 37°C, 5% CO_2. Colourimetric changes were recorded using a plate reader to measure absorbance at 490nm. In all readings, a blank well was created by adding CellTiter solution to 100µL of appropriate media only.

2.3.5. Evaluation of senescence of human middle ear cells

In order to evaluate senescence, cells at late passage were compared to earlier passage and immortalised cells, by analysing β-Galactidose activity. Cells were seeded in a Nunc™ Lab-Tek™ II 8-well Chamber Slide™ System (Thermo Scientific™, 154534) at a density of 20,000 cells per well, in 500µl, and incubated O/N at 37°C, 5% CO_2. The following day, media were harvested, cells were fixed, and the procedure carried out using a Senescence Detection Kit (abcam, ab65351), according to manufacturer's instructions. Images were acquired from three regions within the well, and blue stained cells and total cell numbers were counted using ImageJ's Cell Counter plugin.

2.3.6. Lactate dehydrogenase assay

CytoTox 96® Non-radioactive Cytotoxicity Assay (Promega, G1780) is lactate dehydrogenase (LDH) assay, which indicates cytotoxicity, through colourimetric change due to conversion of a tetrazolium salt into a red formazan product. 100µl of conditioned media collected in 2.3.5. was transfered to a 96-well plate and incubated with 50µl of substrate mix, according to manufacturer's instructions. After incubation, the reaction was stopped with 50µl of Stop Solution. Colourimetric changes were recorded using a plate reader at 490nm.

2.3.7. Protein isolation, quantification and western blot

For analysis of protein expression, cells growing in TC-treated 24 wells plates were washed with PBS twice and lysed with RIPA lysis buffer (ChemCruz, Santa Cruz Biotechnology, sc-24948A) supplemented with protease and phosphatase inhibitors (Roche, 04693159001). Using micropipette tips, cells were scraped from the well, and further lysed

through mechanical action. After 10 minutes of incubation on ice, samples were centrifuged at maximum speed for 15 minutes at 4°C. The supernatants were collected and stored at -80°C.

Protein concentration was assessed using a Pierce™ BCA Protein Assay kit (Thermo Scientific, 23225). In a 96 well plate, 10µl of the cell lysates were added to 190µl of the BCA reagent solution, as per manufacturer's instructions. The standard control was prepared utilising 10µl of bovine serum albumin (BSA) and 190µl of the BCA reagent solution, followed by a serial dilution according to the manufacturer's instructions, ranging from 0µg/ml to 2000µg/ml. The plate was incubated at 37°C for 10-15 minutes, and the colourimetric change was measured using a plate reader at 562nm. Absorbance values were analysed through Excel by interpolation, by comparing absorbance of each sample to the known BSA concentrations of the standard curve.

Protein samples were prepared for western blot analysis by mixing an appropriate volume of lysate at the desired concentration, 10µg, with water and 5X SDS loading dye buffer, to make up a final total volume of 30µl. The mix was vortexed and spun for 10 seconds and incubated at 95°C for 10 minutes in a thermo-block (Jencons-PLUS). The samples were centrifuged again and stored at 4°C, for short-term storage, or -20°C, for long-term storage.

Samples were loaded onto a 10% SDS-polyacrylamide gel alongside 5 µl of protein ladder (BioLegend, 773302). The electrophoresis ran in a SDS-Tris-glycine buffer, at 80-100V. Proteins were then transferred onto a nitrocellulose blotting membrane (GE Healthcare Life Science, 10600003), by semi-dry transfer using the Trans-Blot® Turbo™ Transfer system (Bio-Rad,1704150EDU), at 25V for 30 minutes. Once the transfer was complete, efficiency was assessed by incubation of the membrane with Ponceau S solution (Sigma-Aldrich, 27195) for 30 seconds to 1 minute, and then washed with distilled water. To block nonspecific antibody binding, the membrane was incubated with 5% skimmed milk (VWR Prolabo Chemicals, 84615.0500) in PBS, for 1 hour at RT, on a shaker at 80rpm. After removal of the blocking buffer, the membrane was incubated with primary antibody diluted in fresh blocking solution (See table 15), at appropriate dilution as per the manufacturer's instructions, overnight at 4°C.

The membrane was then washed 3 times for 5 minutes with TBS-0.1%Tween, and subsequently incubated with a secondary antibody conjugated with horseradish peroxidase (HRP), diluted in fresh blocking solution for 1 hour at RT.

Table 15 - Western blot primary and secondary antibodies.

Primary antibody	Dilution	Size
Rabbit Anti-Human Vinculin (Cell Signaling Technology, 13901S)	1:1000	123,8 kDa
Rabbit Anti-Human BMI-1 (Atlas Antibodies, HPA030472)	1:1000	36,95 kDa
Secondary antibody		
Goat Anti-Rabbit HRP-linked (Cell Signaling Technology, 7074S)	1:3000	–

The membrane was washed again 3 times with TBS-T and incubated with Clarity Western ECL substrates (BioRad, 1705060) for 1-2 minutes at RT. Finally, the membrane was digitally developed using the C-digit Blot Scanner (LI-COR).

2.3.8. Immunofluorescence for detection of cell surface receptors

For detection of cell surface receptors, hMEEC-1 and MEEC were seeded at a density of 400,000 cells/ml in 1ml of medium, in TC-treated 24 well plates, and incubated O/N at 37°C, 5% CO_2. The following day, cells were washed with PBS twice and fixed using 4% paraformaldehyde for 20 minutes at RT. Afterwards, wells were incubated with blocking solution, 10% goat serum with 0.5% Triton X in PBS, for 1 hour at RT on a shaker at 80 rpm. Blocking buffer was then removed and wells washed twice with PBS. Primary antibody was added at the appropriate dilution, as per the manufacturer's instructions, and the plate incubated at 4°C, overnight on a shaker at 80 rpm. Subsequently, wells were washed with PBS twice, and, in a dark room, fluorescent secondary antibody solution in PBS was added at an appropriate dilution (See table 16).

Table 16 - Immunofluorescence primary and secondary antibodies.

Primary antibody	Dilution
Rabbit Anti-Human PAFR Polyclonal Antibody (Bioss Antibodies, BS-1478R)	1:150
Secondary antibody	
Goat Anti-Rabbit Alexa Fluor™ 488 (Thermo Fisher™, A-11008)	1:500

The plate was then wrapped in foil to avoid contact with light and photobleaching. Wells were washed again, twice with PBS, and diluted DAPI solution (1:1000) was added to the wells for 1 minute. After the DAPI was removed, wells were washed with PBS once, and 1 ml of fresh PBS was added. Plates were visualised utilising a ZOE™ Fluorescent Cell Imager (Bio-Rad, 1450031).

2.3.9. ELISA

bio-techne® Human IL-6 and IL-8 DuoSet ELISAs (R&D Systems, DY206 and DY208) are antibody sandwich arrays for quantitative assessment of the specific cytokines. These arrays were used to assess cytokine concentrations in 100µl of supernatant of NTHi-375 infected hMEEC-1 and MEEC. The assay was performed following the manufacturer's instructions. Colourimetric change was detected at 450nm using the plate reader.

2.3.10. Cytokine arrays

bio-techne® Proteome Profiler Human Cytokine Array Kit (R&D Systems, ARY005B) is a membrane-based antibody sandwich array for the quantitative assessment of relative levels of human cytokines and chemokines (See table 17). 700µl of the supernatants recovered from uninfected (control) and infected hMEEC-1 and MEEC with NTHi-375, Rd, H457, H446 and H491 were used for this assay. The assay was performed following the manufacturer's

instructions. Membranes were exposed to x-ray film for approximately 5 minutes. Membrane analysis was performed utilising ImageJ's Protein Array Analyzer extension.

Table 17 - Cytokines, chemokines and acute phase proteins that can potentially be detected in bio-techne® Proteome Profiler Human Cytokine Array Kit.

C5a	IL-4	IL-27
CD40 Ligand	IL-5	IL-32 alpha
G-CSF	IL-6	CXCL10/IP-10
GM-CSF	IL-8	CXCL11/I-TAC
CXCL1/GRO alpha	IL-10	CCL2/MCP-1
CCL1/I-309	IL-12 p70	MIF
ICAM-1	IL-13	MIP-1 alpha/MIP-1 beta
IFN-gamma	IL-16	CCL5/RANTES
IL-1 alpha	IL-17	CXCL12/SDF-1
IL-1 beta	IL-17E	Serpin E1/PAI-1
IL-1ra	IL-18	TNF-alpha
IL-2	IL-21	TREM-1

2.4. Histology

2.4.1. Fixation of specimens, tissue processing, embedding and microtome cutting

TOP dissected tissue was fixed in 10% neutral buffered formalin and fixed, depending on size, for 24 to 72 hours. The samples were placed in the appropriate size embedding cassettes with removable cover, and tissue embedding sponges when necessary, and placed in the Citadel™ 2000 Wax Bath (Thermo Scientific).

The tissue processing programme was as follows:

1. 70% Alcohol - 1 hour
2. 80% Alcohol - 1 hour
3. 90% Alcohol - 1 hour and 30 minutes
4. Absolute alcohol I - 1 hour and 30 minutes
5. Absolute alcohol II - 1 hour and 30 minutes
6. Absolute alcohol III - 2 hours
7. Toluene I - 1 hour and 30 minutes
8. Toluene II - 2 hours
9. Wax I - 2 hours
10. Wax II - 2 hours

Samples were embedded into paraffin blocks using embedding base moulds and the Leica EG1150 Modular Tissue Embedding Center. Once solidified, 5µm-thick sections were cut and placed onto SuperFrost Plus™ Adhesion slides (Epredia™, J1800AMNZ).

2.4.2. Immunohistochemistry

Immunohistochemistry allows for identification of antigens in tissue samples by utilising antibodies and enzymatic dyes. Slides with paraffinised tissue were "baked" at 60°C for at least an hour to ensure softening of the paraffin. Afterwards, slides underwent rehydration and removal of endogenous peroxidase, which can lead to background staining, as follows:

1. Xylene I - 5 minutes
2. Xylene II - 5 minutes
3. 100% Ethanol I - 5 minutes
4. 100% Ethanol II - 5 minutes
5. 3% H2O2 in MetOH - 20 minutes
6. Rinse in PBS

When necessary, slides underwent antigen retrieval by placing in 0.01M sodium citrate buffer (pH=6) and heating in a microwave for 8 minutes. Slides were rapidly cooled to room temperature and nonspecific binding blocked with 100% serum at RT for 30 minutes. The serum used was dependent on the antibody utilised to reduce cross-reactivity, as it is dependent on the animal used to raise the secondary antibody. Primary antibody was diluted to appropriate concentration, according to manufacturer's instructions, and sufficient solution added to cover the tissue on the slides (See table 18). The slides were incubated at 4°C O/N.

Table 18 - IHC antibodies.

Primary antibody	Dilution
Mouse Anti-Cytokeratin 5/6 (Life Technologies™, 180267)	1:100
Rabbit Anti-E-cadherin (Abcam, ab40772)	1:300
Rabbit Anti-Sox2 (Proteintech®, 11064-1-AP)	1:100
Rabbit Mucin5B (Santa Cruz Biotechnology, H-300: sc-20119)	1:200

Rabbit Anti-FOXJ1 (Sigma-Aldrich / Atlas Antibodies, HPA005714)	1:200

On the second day of staining, slides were washed twice in PBS for 5 minutes to ensure removal of unbound antibodies. Primary antibody detection was performed utilising the appropriate (anti-rabbit or anti-mouse) Elite ABC-HRP Kit (Peroxidase, Universal) (VECTASTAIN®), according to manufacturer's instructions. Slides were then washed in distilled water, counterstained with haematoxylin and dehydrated in linear stainer as follows:

1. Harris' haematoxylin I - 40 seconds
2. Harris' haematoxylin II - 40 seconds
3. Harris' haematoxylin III - 40 seconds
4. Harris' haematoxylin IV - 40 seconds
5. Running tap water - 40 seconds
6. 0.1% acid alcohol - 40 seconds
7. Running tap water - 40 seconds
8. Scott's Tap Water Substitute
9. 99% IMS I - 40 seconds
10. 99% IMS II - 40 seconds
11. 99% IMS III - 40 seconds
12. 99% IMS IV - 40 seconds
13. Xylene I - 40 seconds
14. Xylene II - 40 seconds
15. Xylene III - 40 seconds
16. Xylene IV - 40 seconds

Finally, slides were mounted with DPX (Sigma-Aldrich, 06522) and coverslipped, ensuring removal of air bubbles and excess of mounting medium.

3. Chapter 3 - Developmental biology of the fetal middle ear

This chapter will focus on this project's research on the embryonic development of the human middle ear, from ages 23CS to 17 PCW.

Materials and Methods: The methods utilised in this chapter can be referred back to in Materials and Methods, section 2.1.1., 2.4.1., 2.4.2..

3.1. Results

In association with HDBR, throughout my research project, it was possible to obtain a generous number of fetal skulls from early TOPs that often, due to their young age (less than 16 PCW old) and reduced size, made epithelial cell extraction extremely difficult. To prevent tissue wastage, these samples were utilised to contribute to the understanding of the development of the origins of the middle ear. As previously mentioned, two conflicting hypotheses are currently being investigated to better understand the potentially single endodermal or dual neural crest-endodermal origin of the middle ear. To evaluate the veracity of either hypothesis, it was of interest to assess the presence of certain neural crest and endodermal markers, such as Sox2 and E-cadherin and cytokeratin-5, respectively, as well as differentiation markers, such as MUC5B and FOXJ1. As early TOPs sample arrived, they were fixed in formalin, for the appropriate amount of time depending on size, and prepared for tissue processing. Preparation typically involved reducing the size of the sample in order for it to fit in a tissue processing cassette and sectioning it to the desired anatomical plane. Three anatomical planes were tested: sagittal, coronal/frontal and axial/transverse, which will be colloquially referred to as towards inner and outer ear, towards front and back (of the head) and towards top and bottom (of the head), respectively (Figure 35).

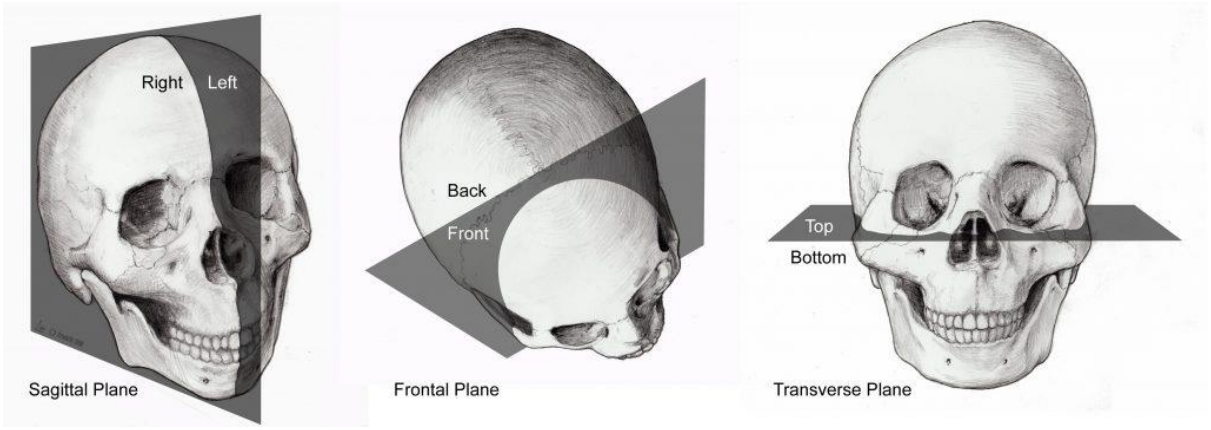
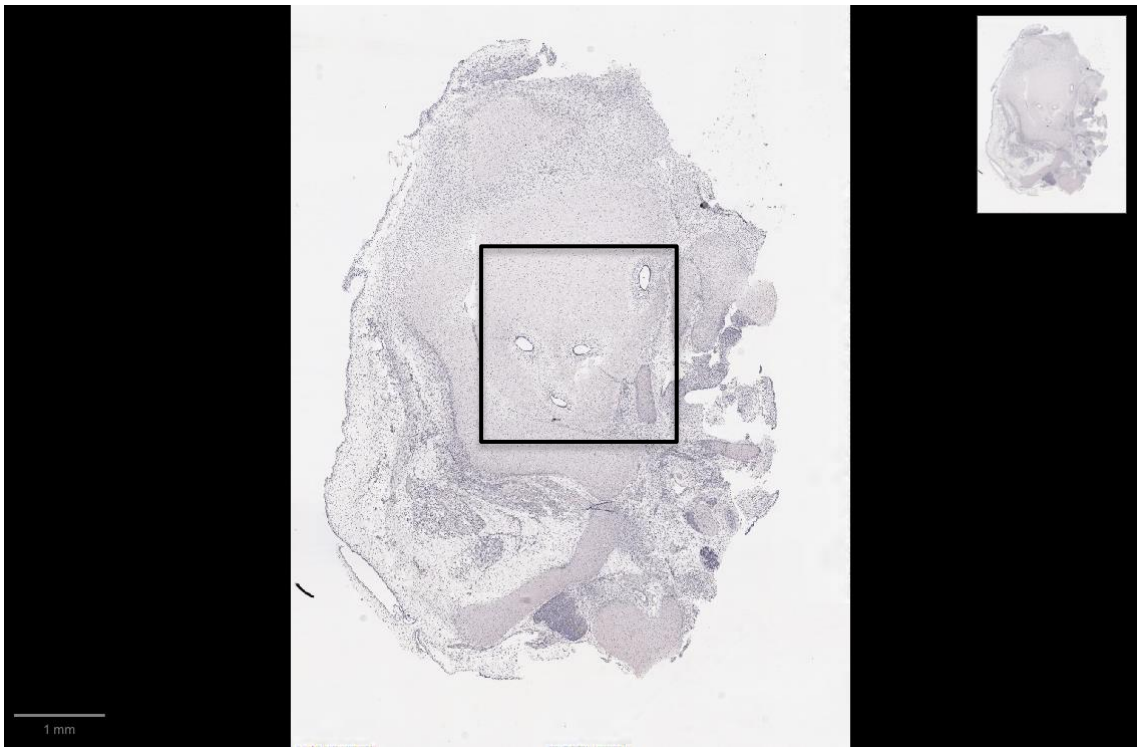
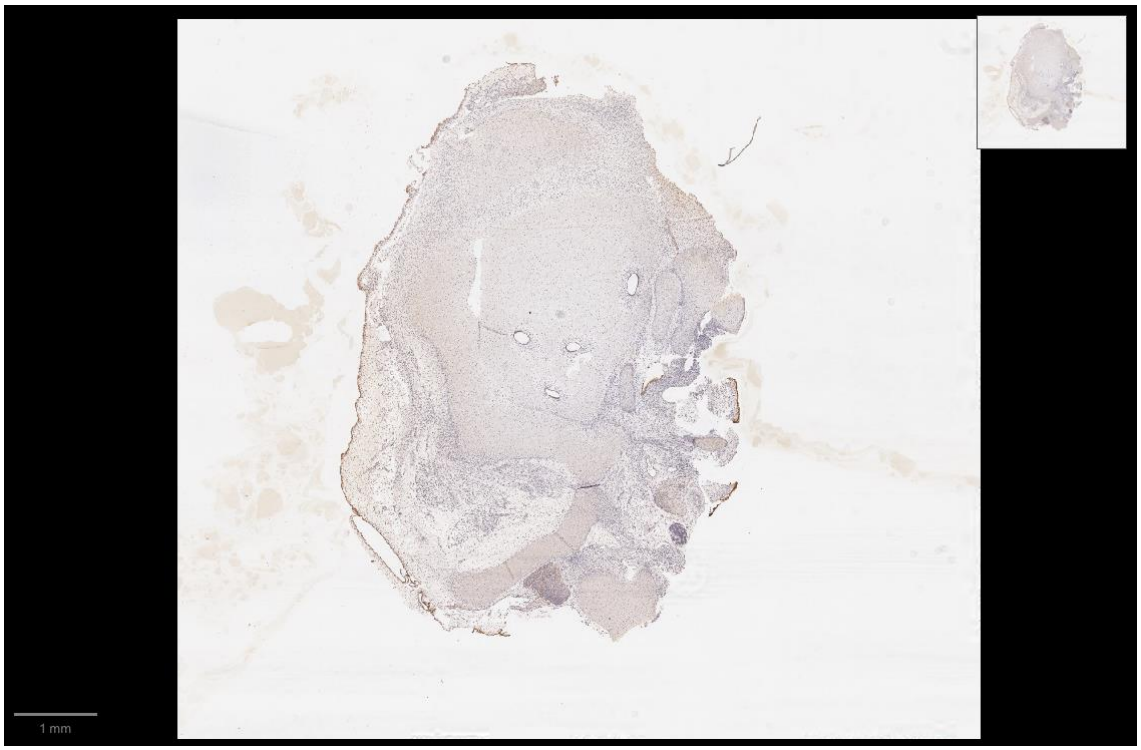


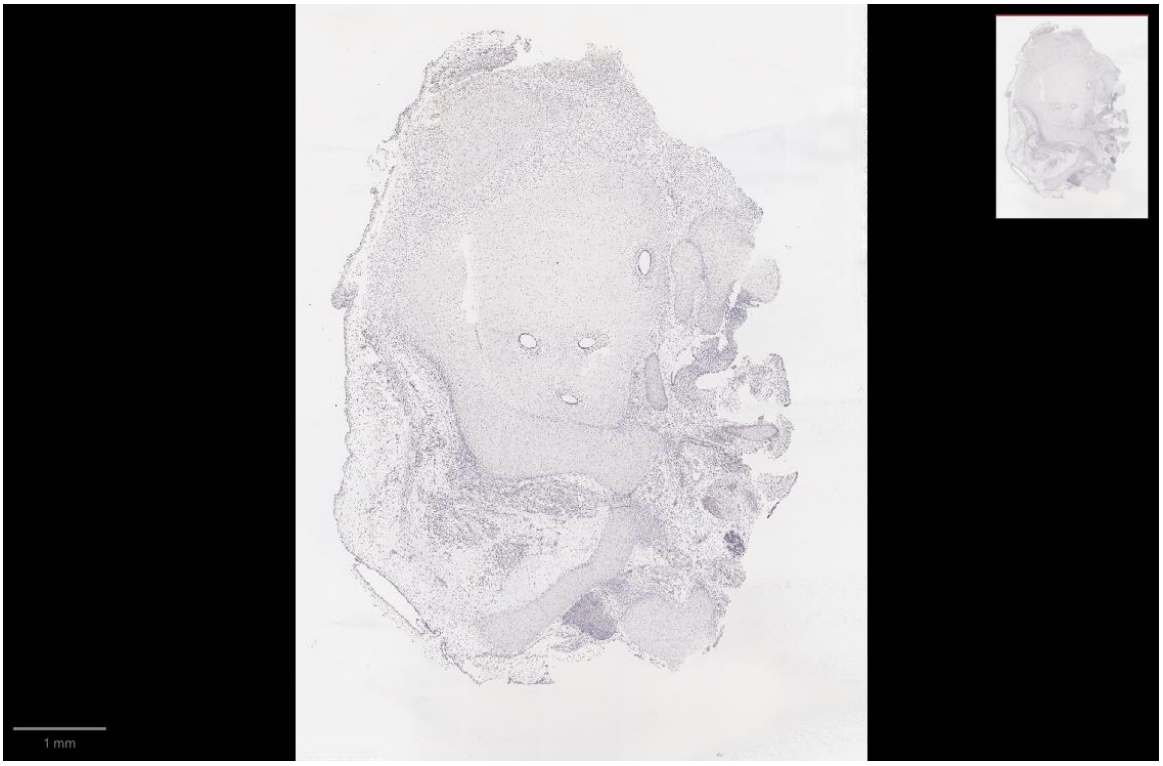
Figure 35 - Common anatomical planes for dissection and sectioning of head samples. Adapted from https://www.crossfit.com/essentials/anatomical-planes-axes.

Table 19 - Scanned images of sagittal sections of IHC samples of early TOPs. Samples ranged from 23CS to 16 PCW and were stained for CK5/6, E-cadherin, Sox2, MUC5B and FOXJ1. Potential location of the middle ear (black gate).

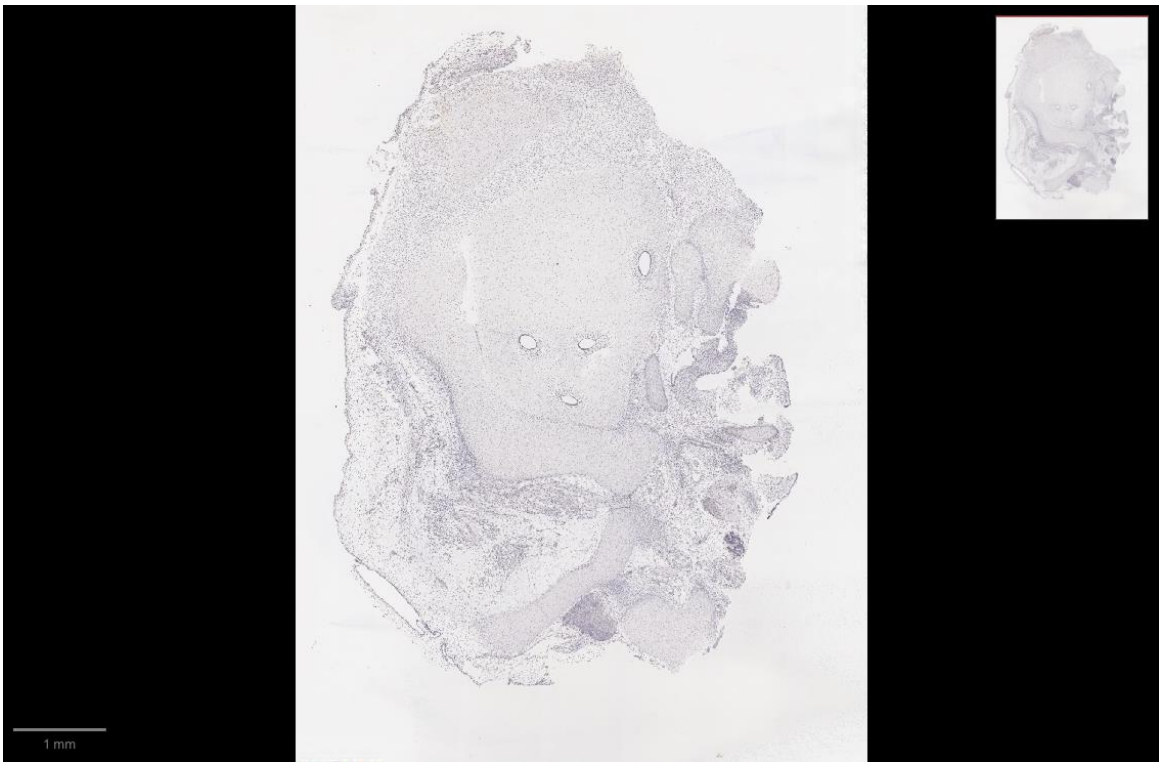
23 CS - Towards Inner Ear
CK5/6 
E-cadherin 

Sox2

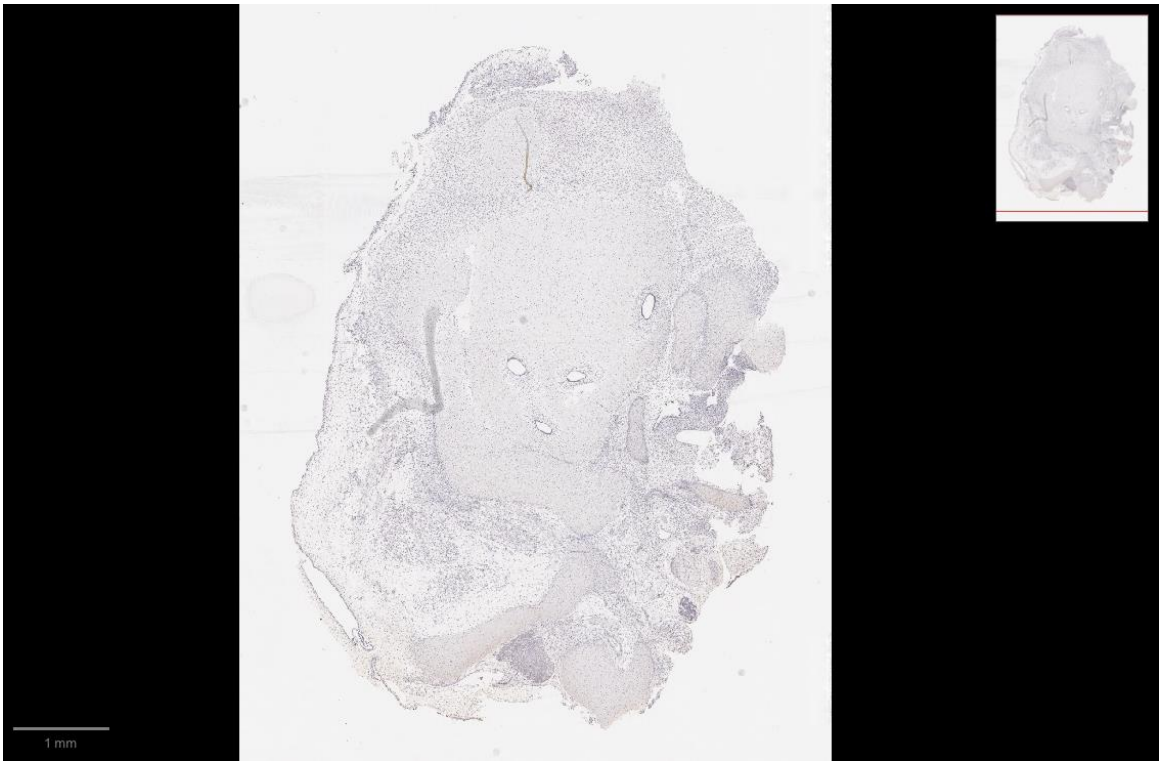
FOXJ1



MUC5B

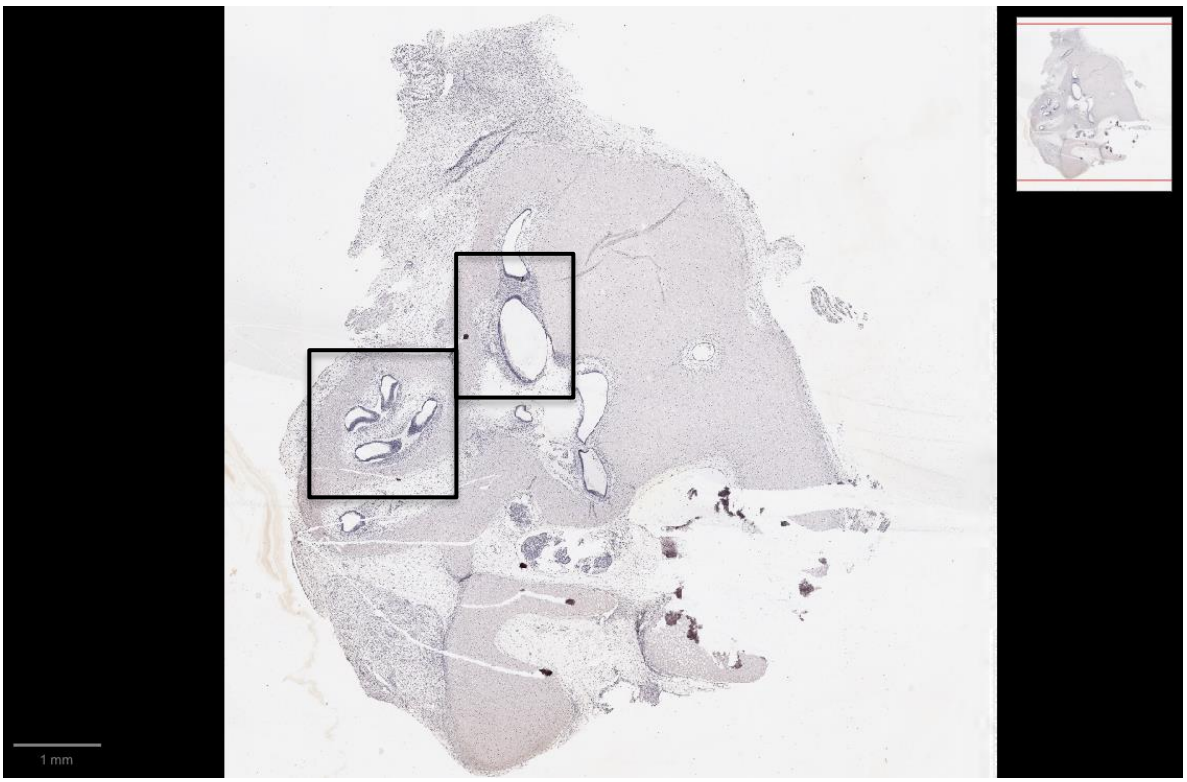


Negative

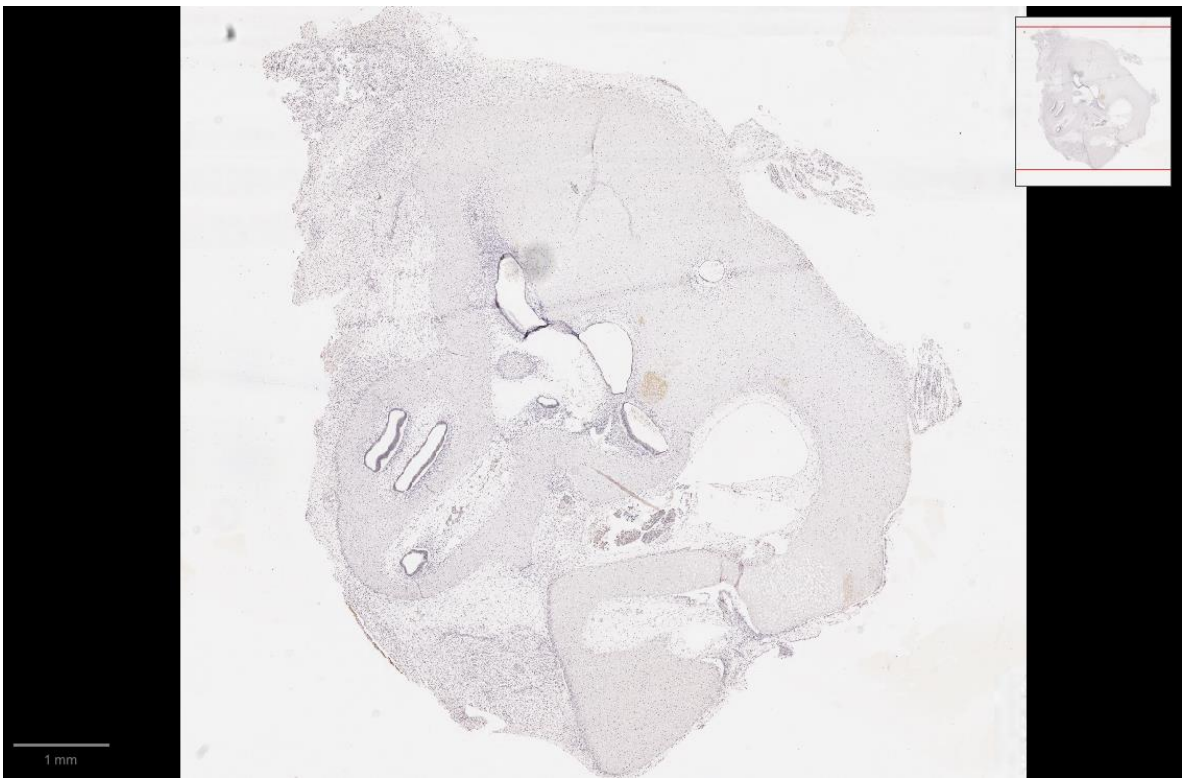


9 PCW - Towards Inner Ear

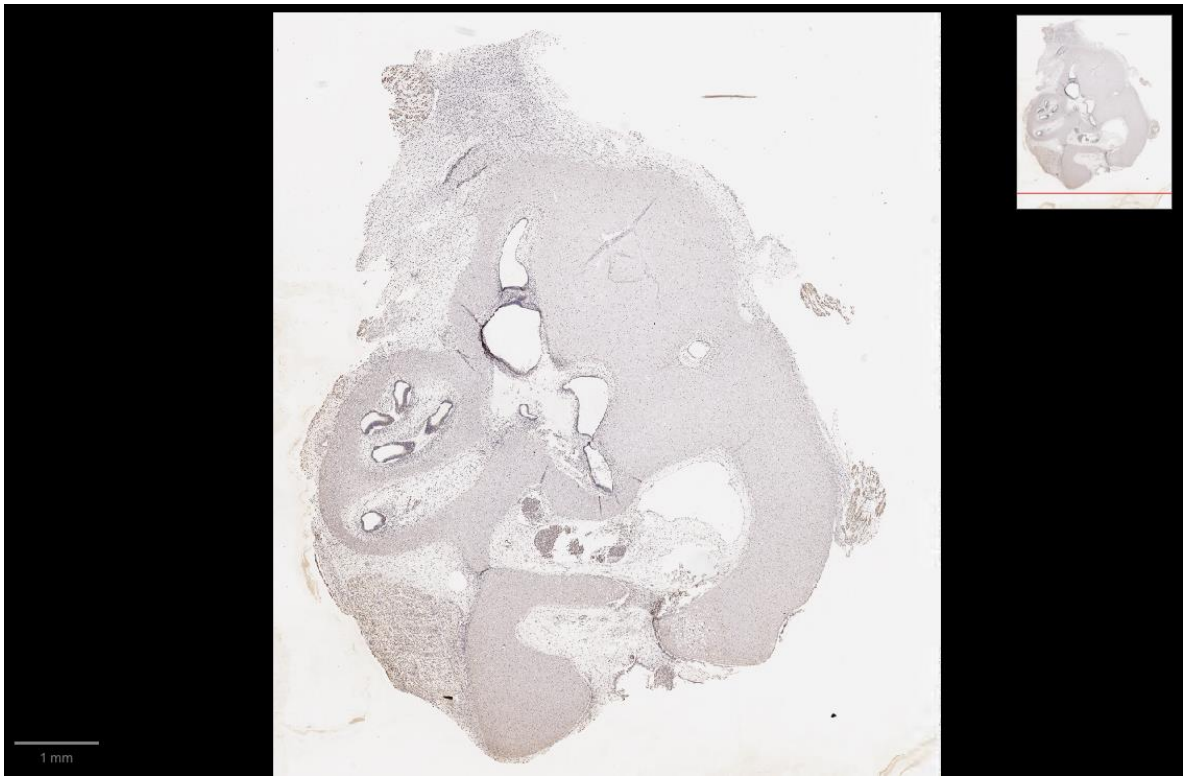
CK5/6



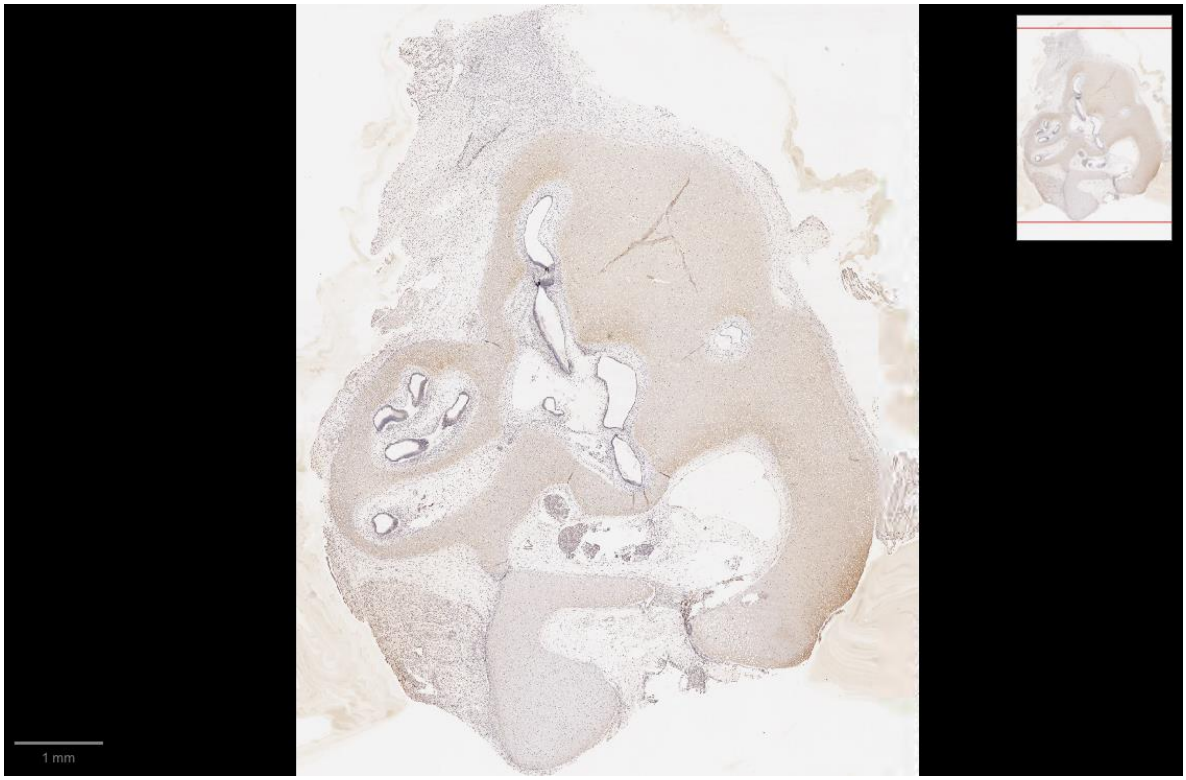
E-cadherin



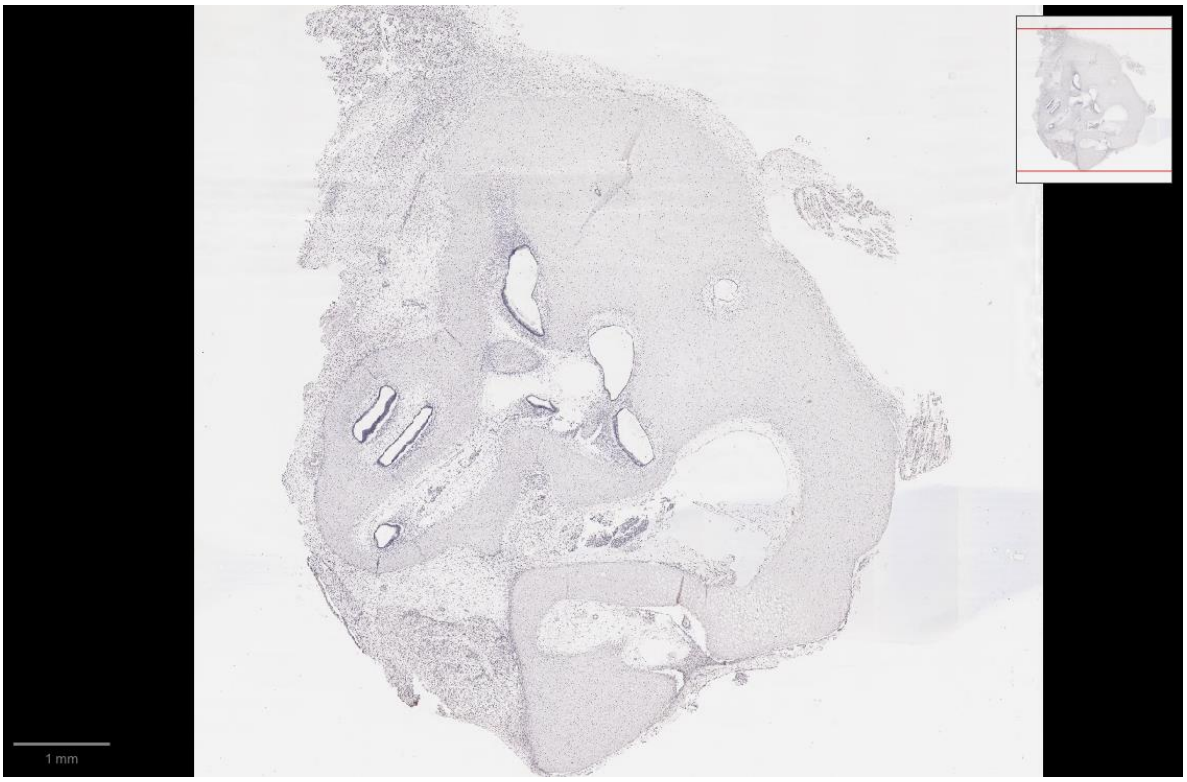
Sox2



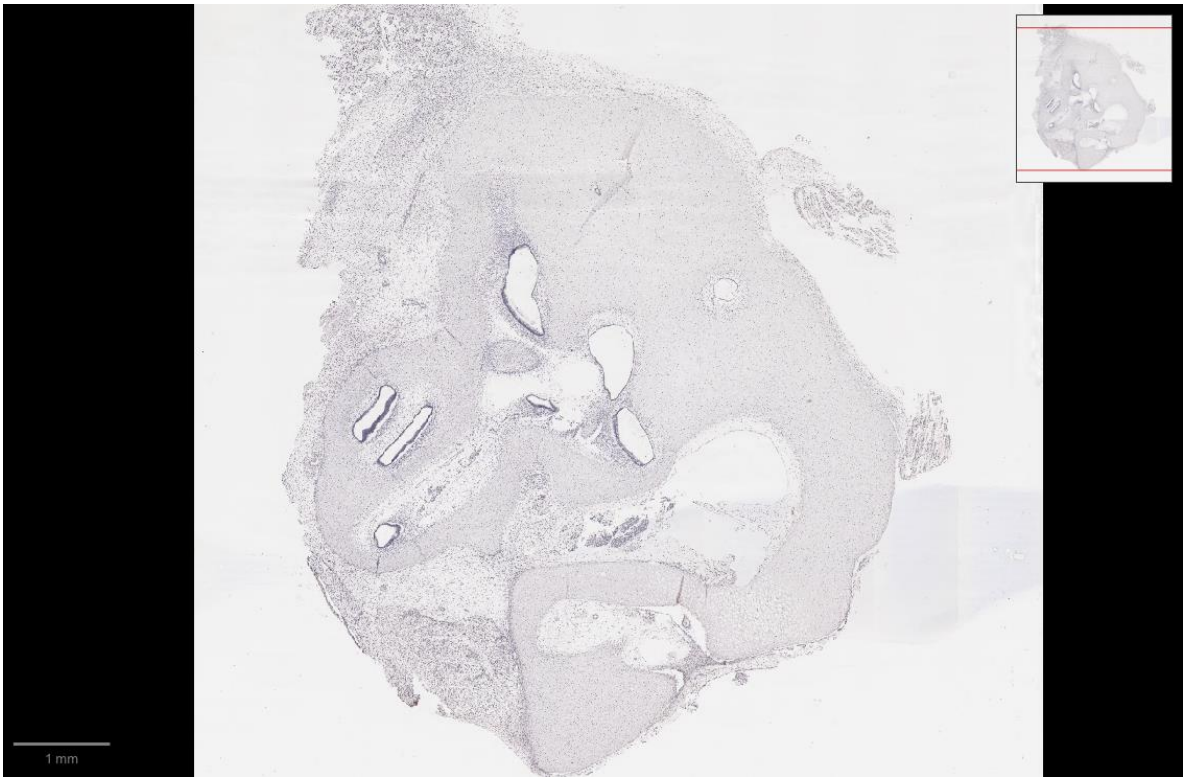
FOXJ1



MUC5B

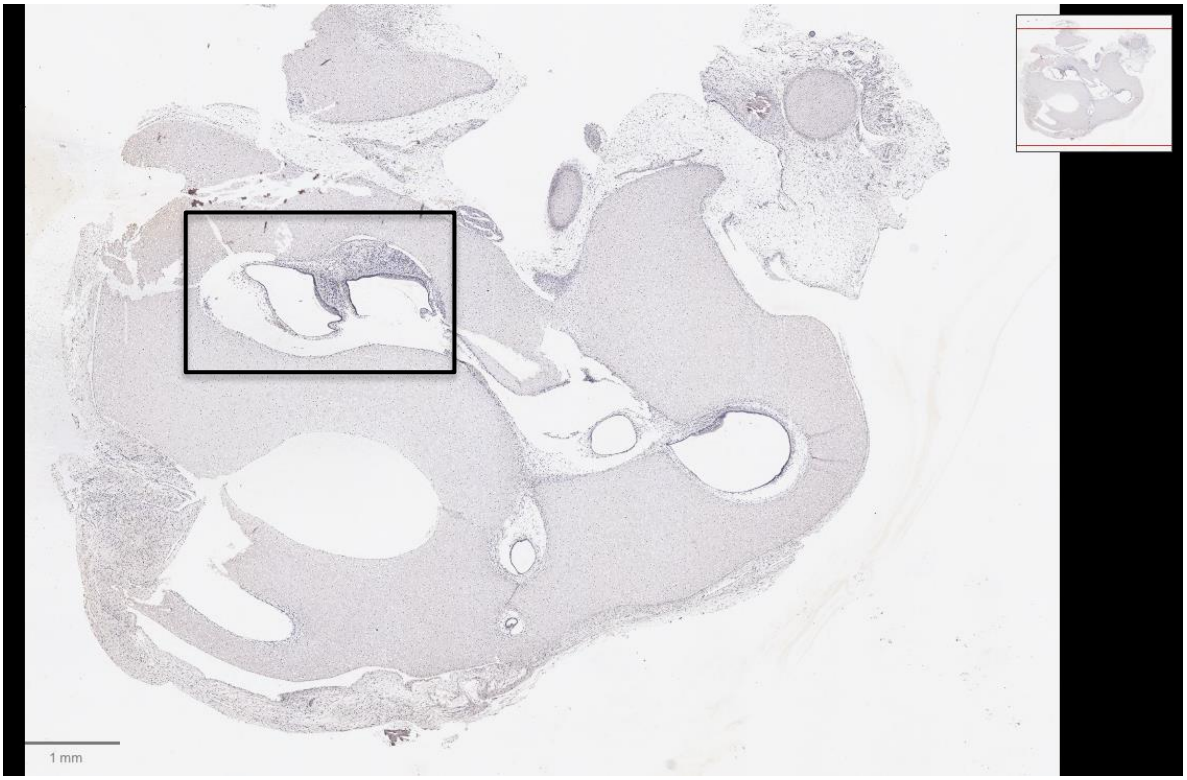


Negative

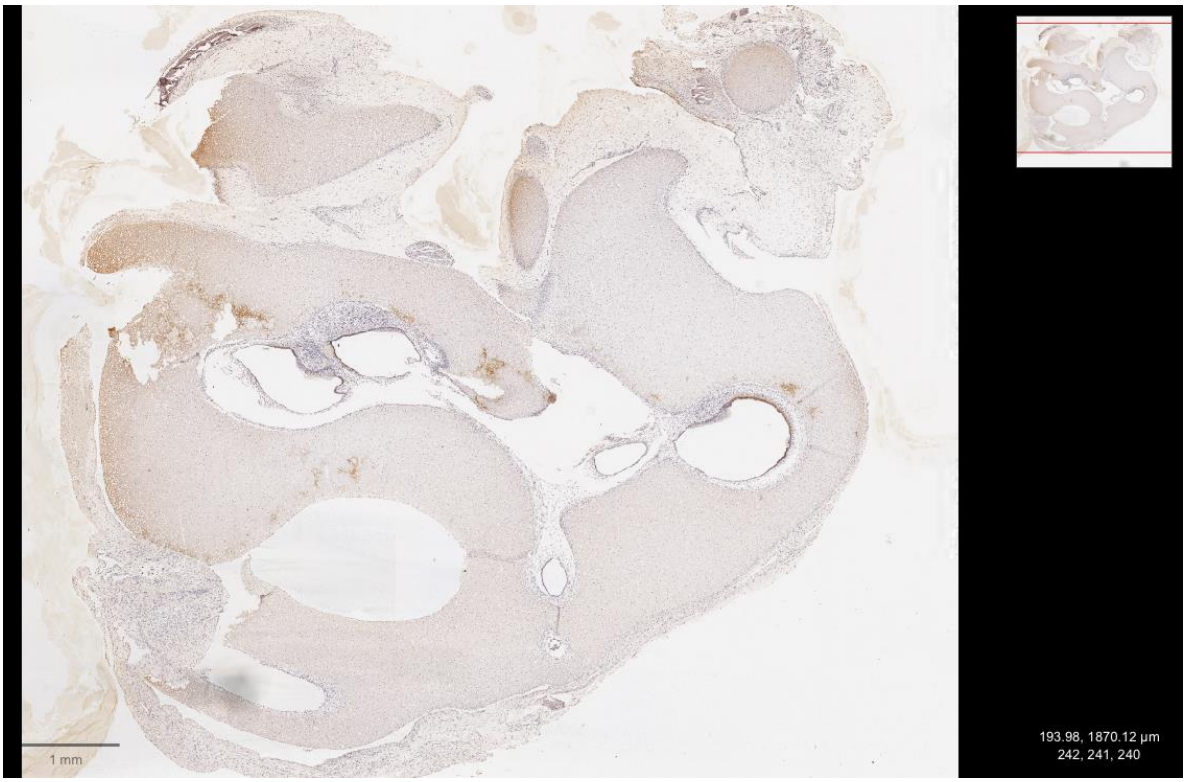


12 PCW - Towards Inner Ear

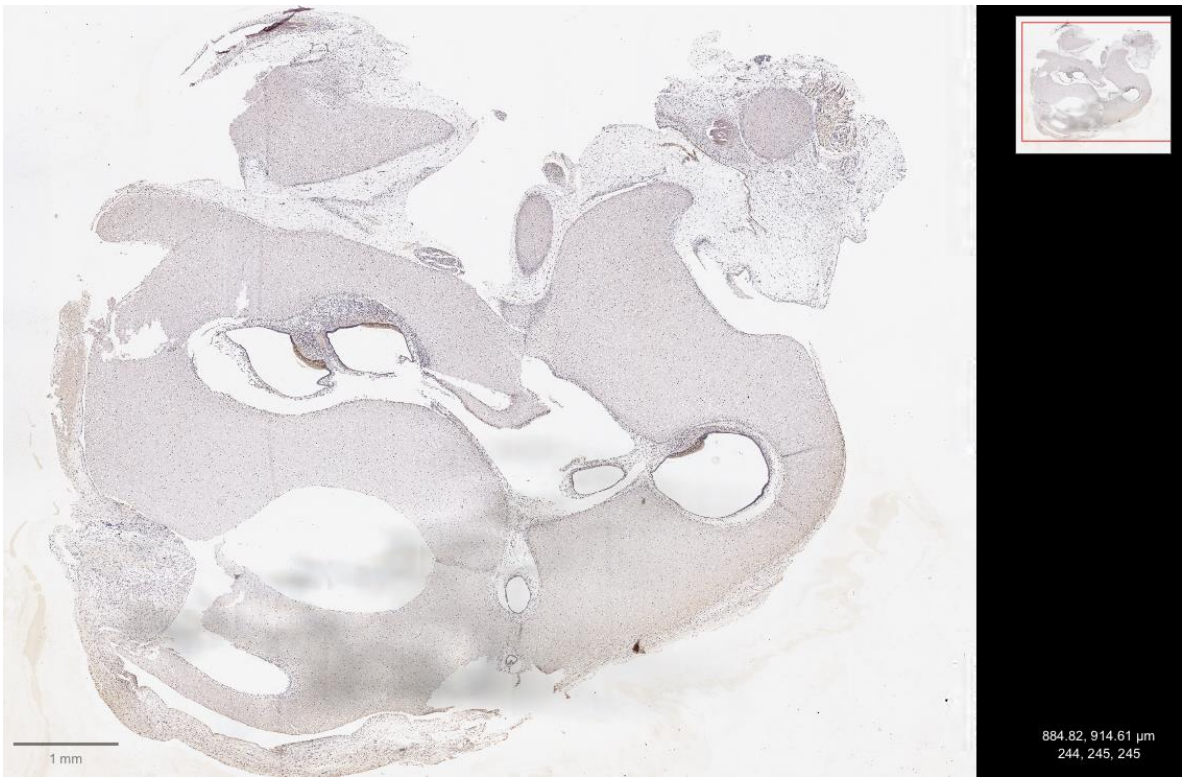
CK5/6



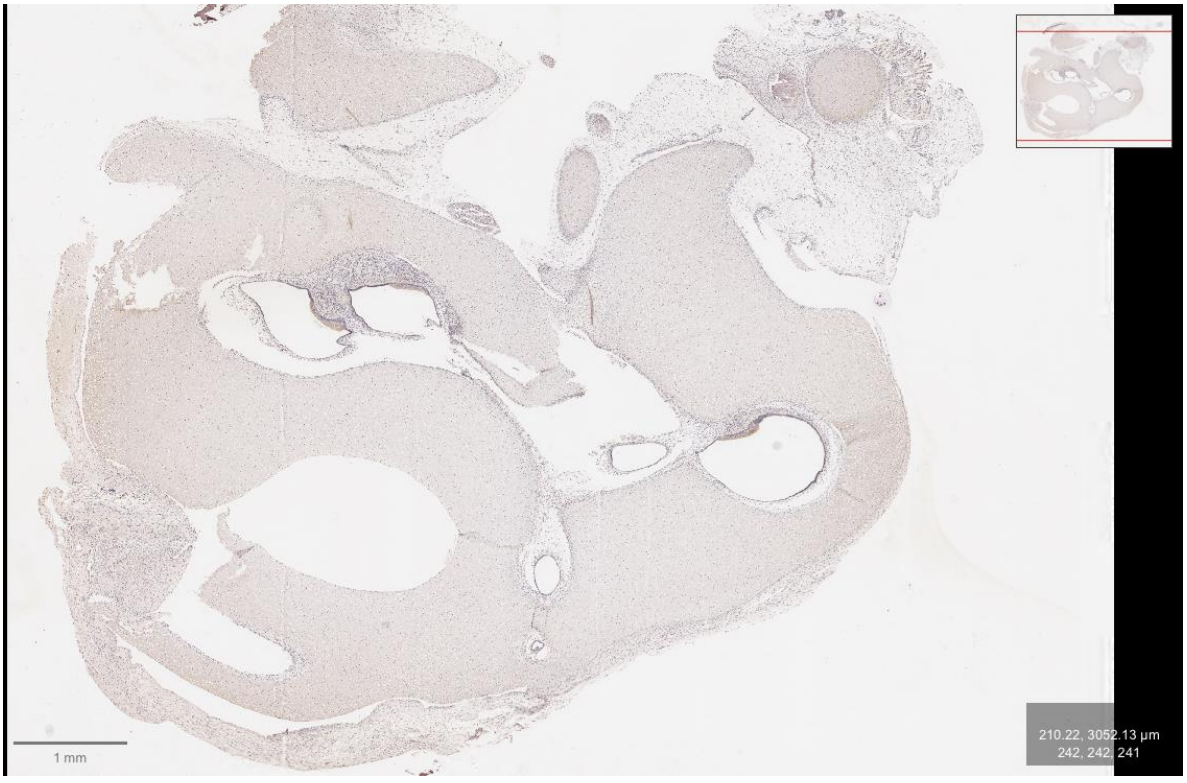
E-cadherin



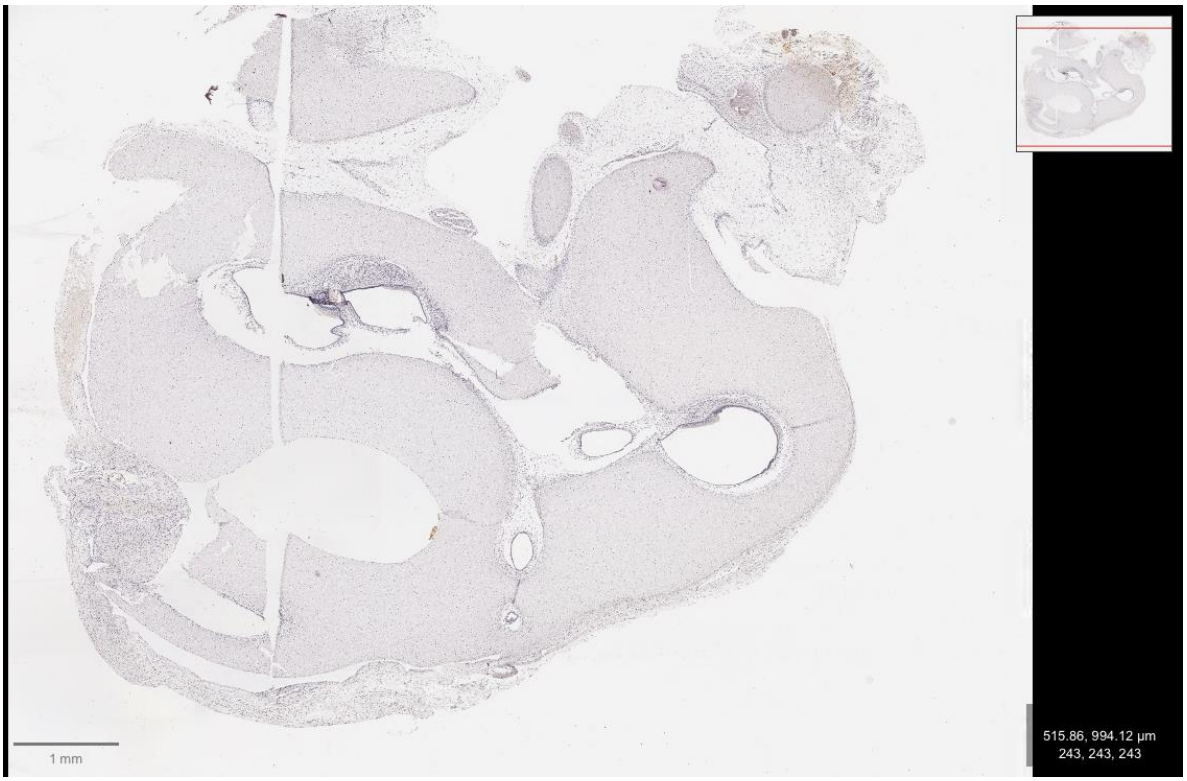
Sox2



FOXJ1



MUC5B



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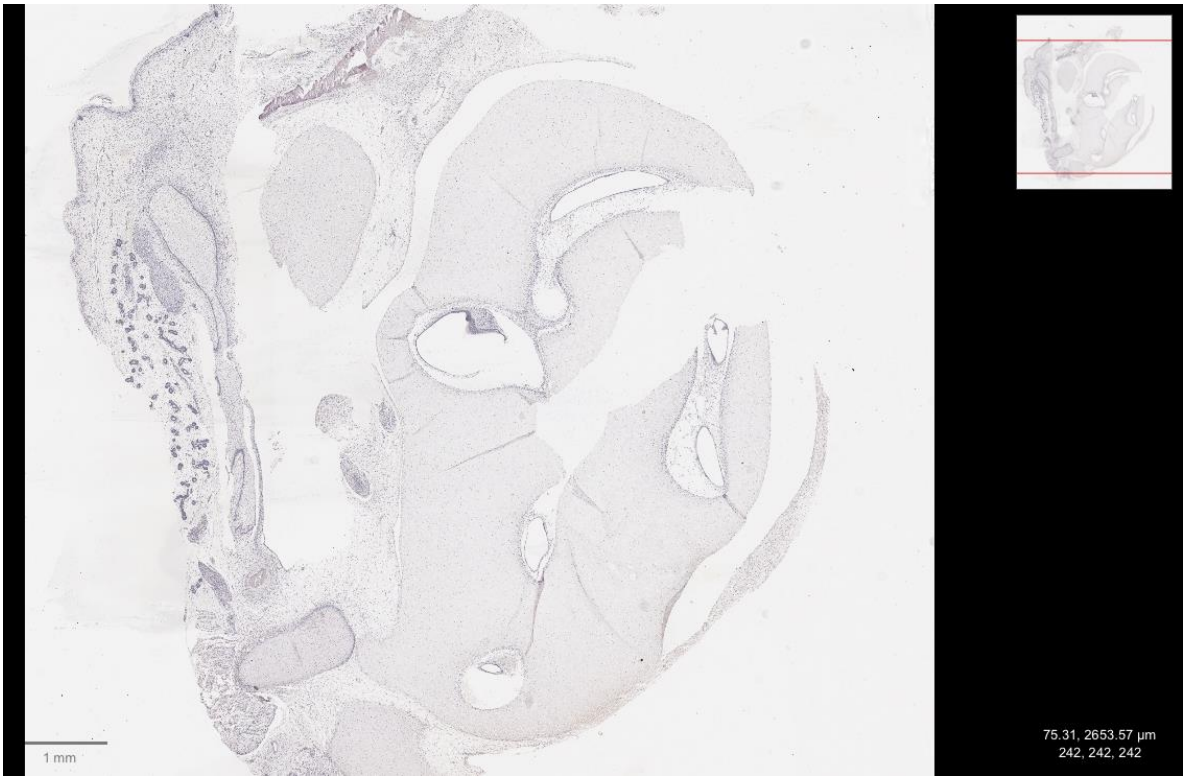
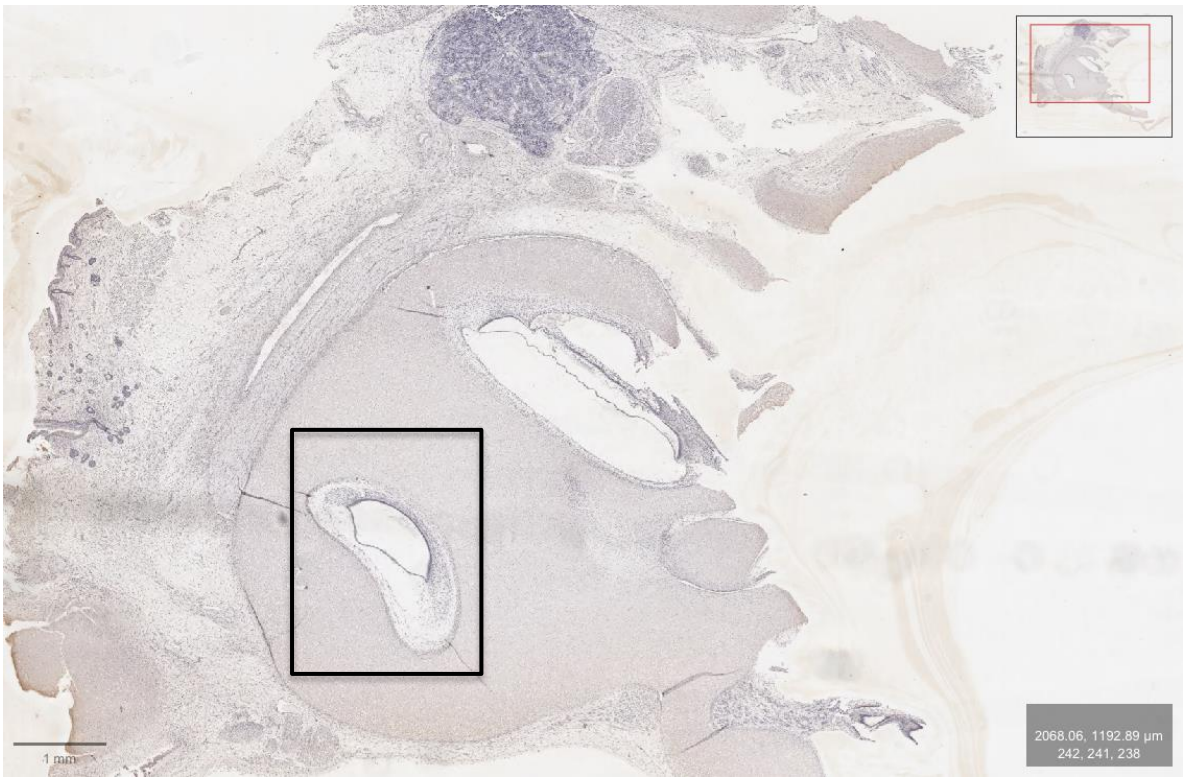
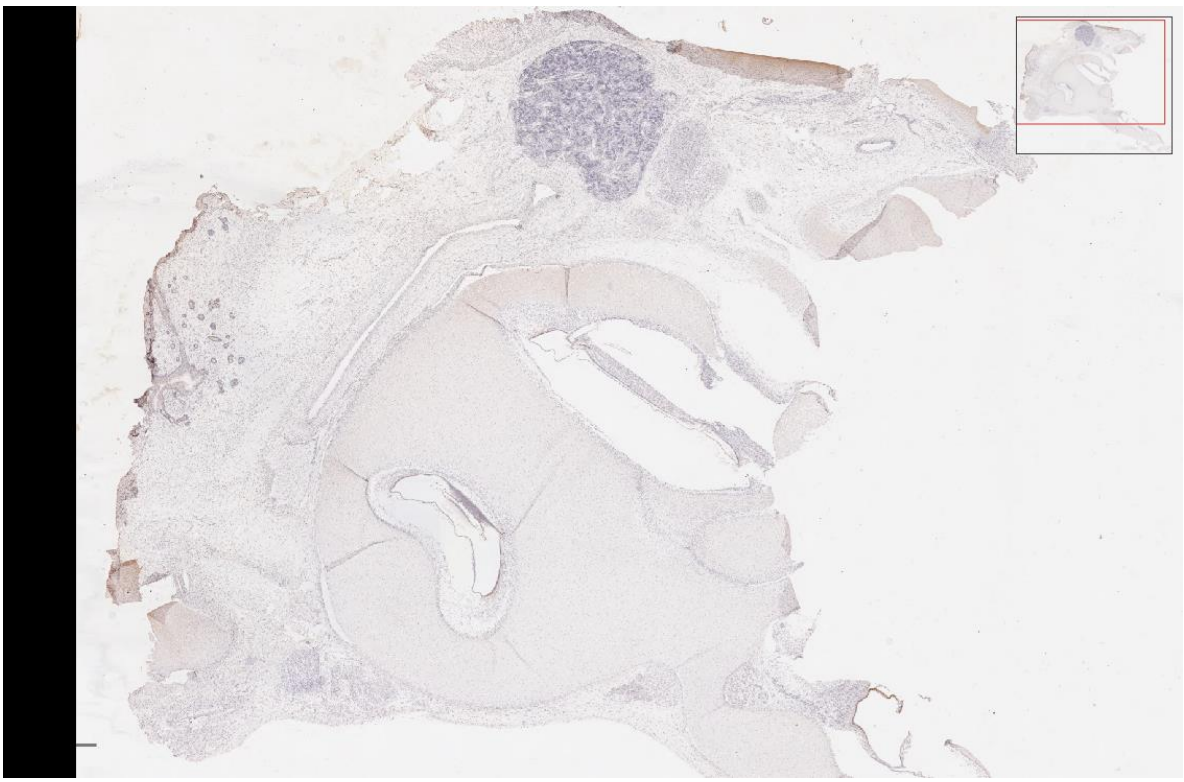


14 PCW - Towards Inner Ear

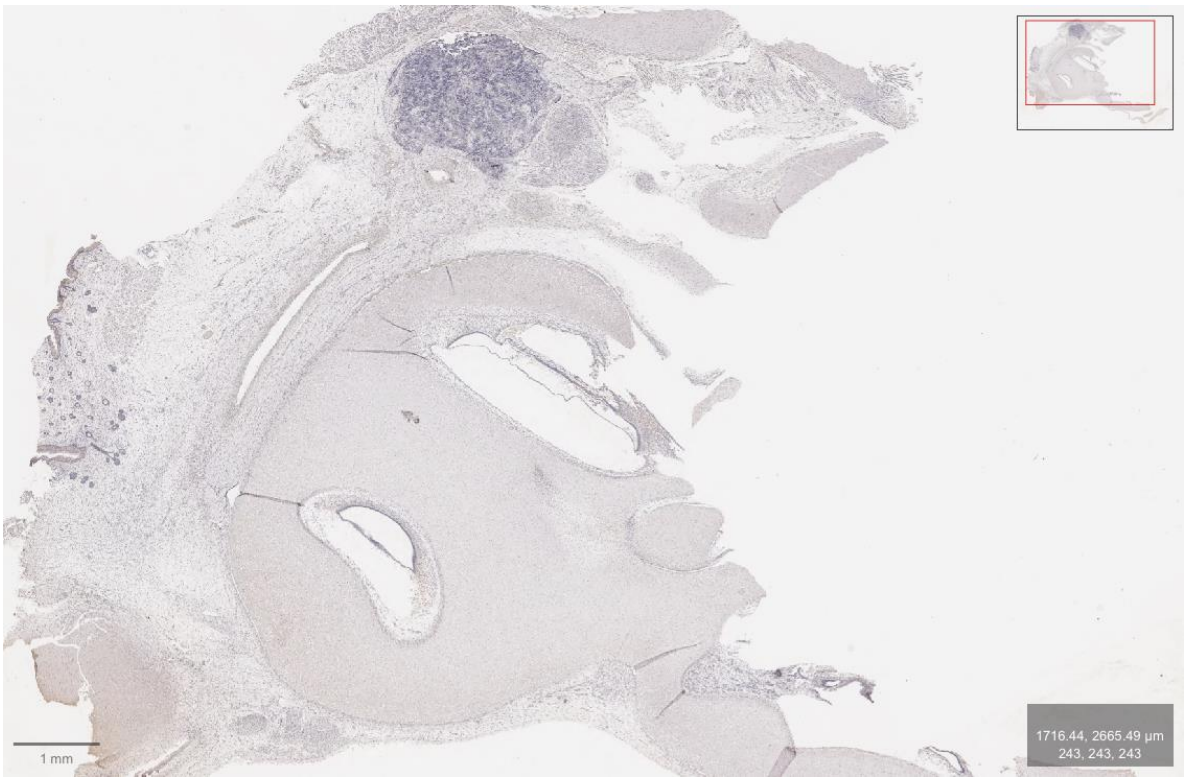
CK5/6



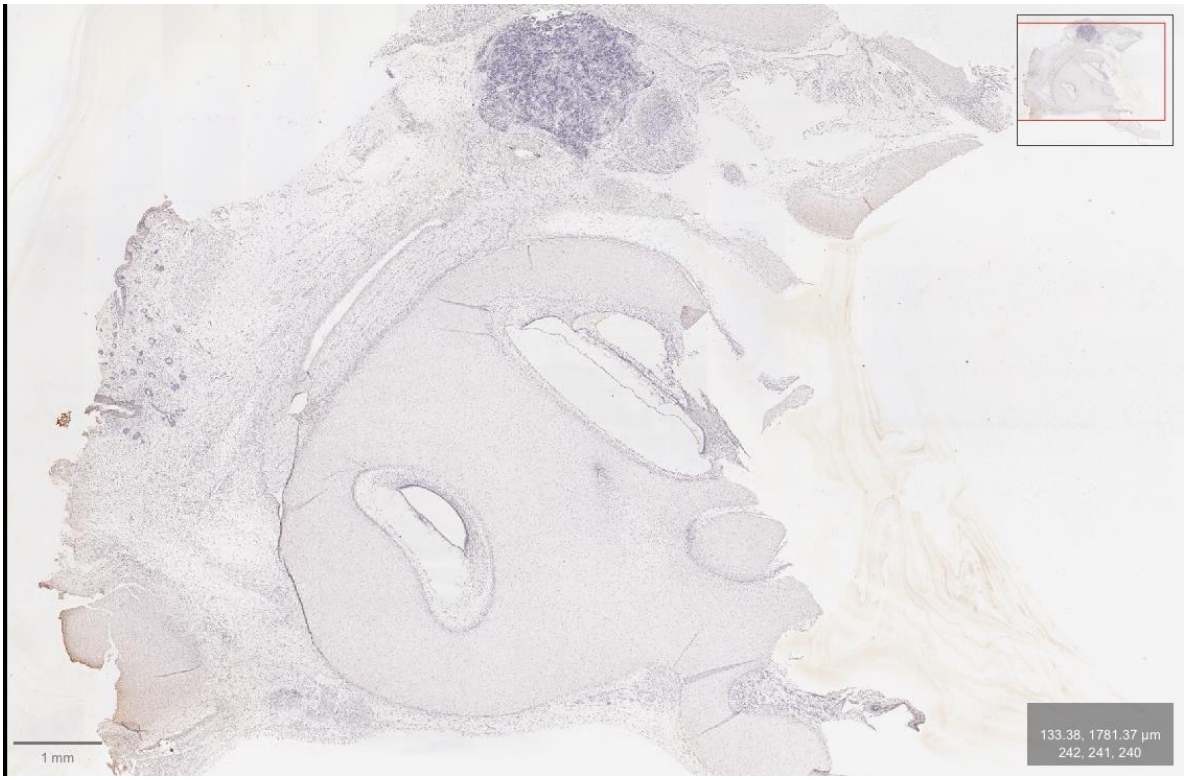
E-cadherin



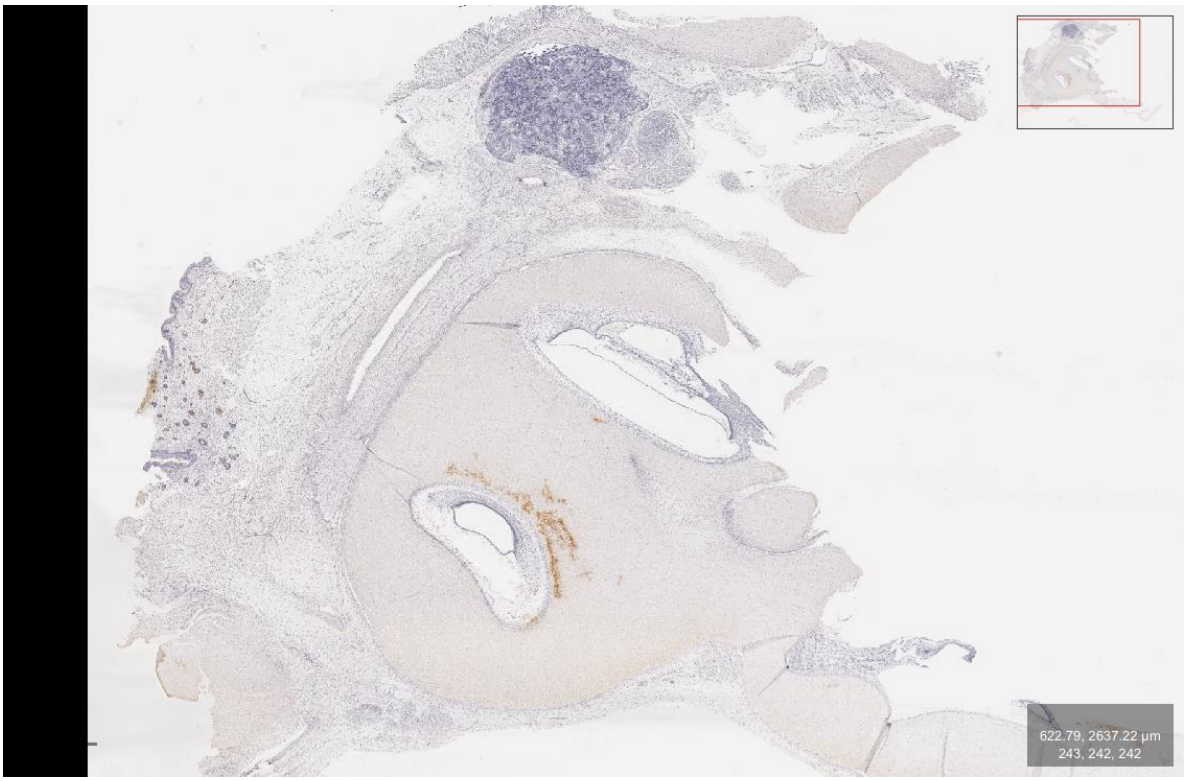
Sox2


1 mm
1716.44, 2665.49 µm
243, 243, 243

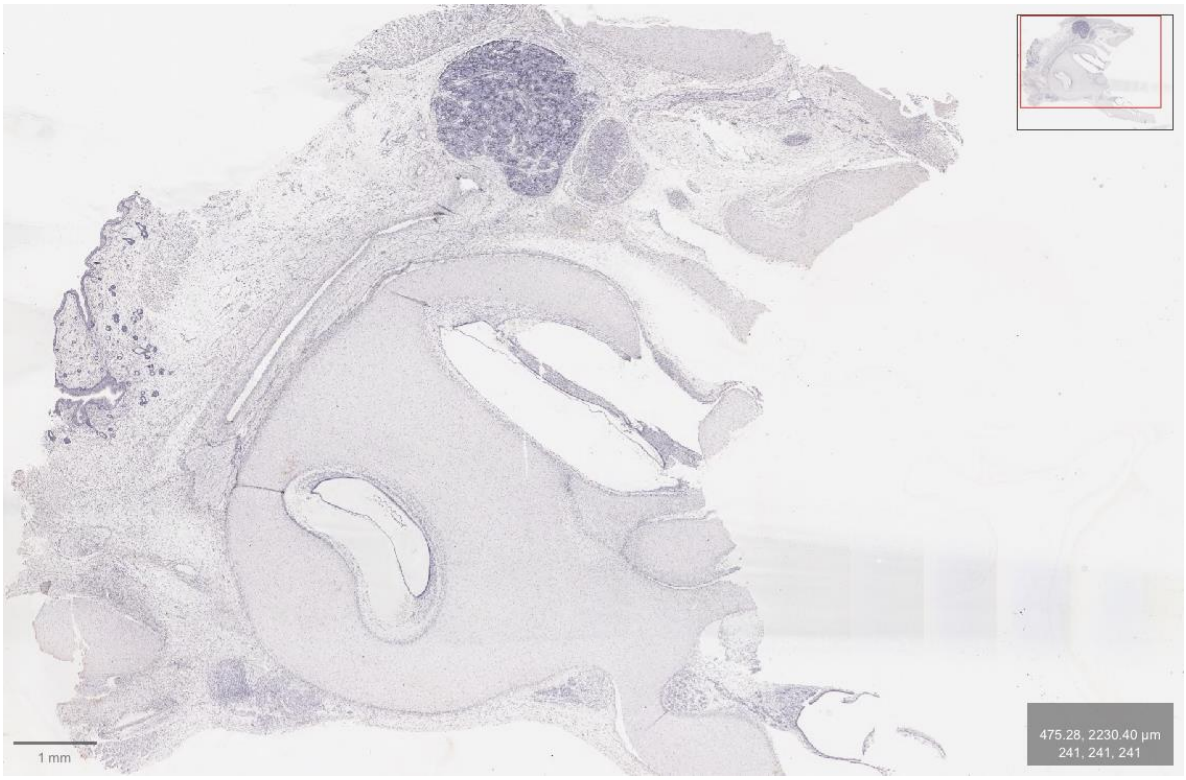
FOXJ1


1 mm
133.38, 1781.37 µm
242, 241, 240

MUC5B

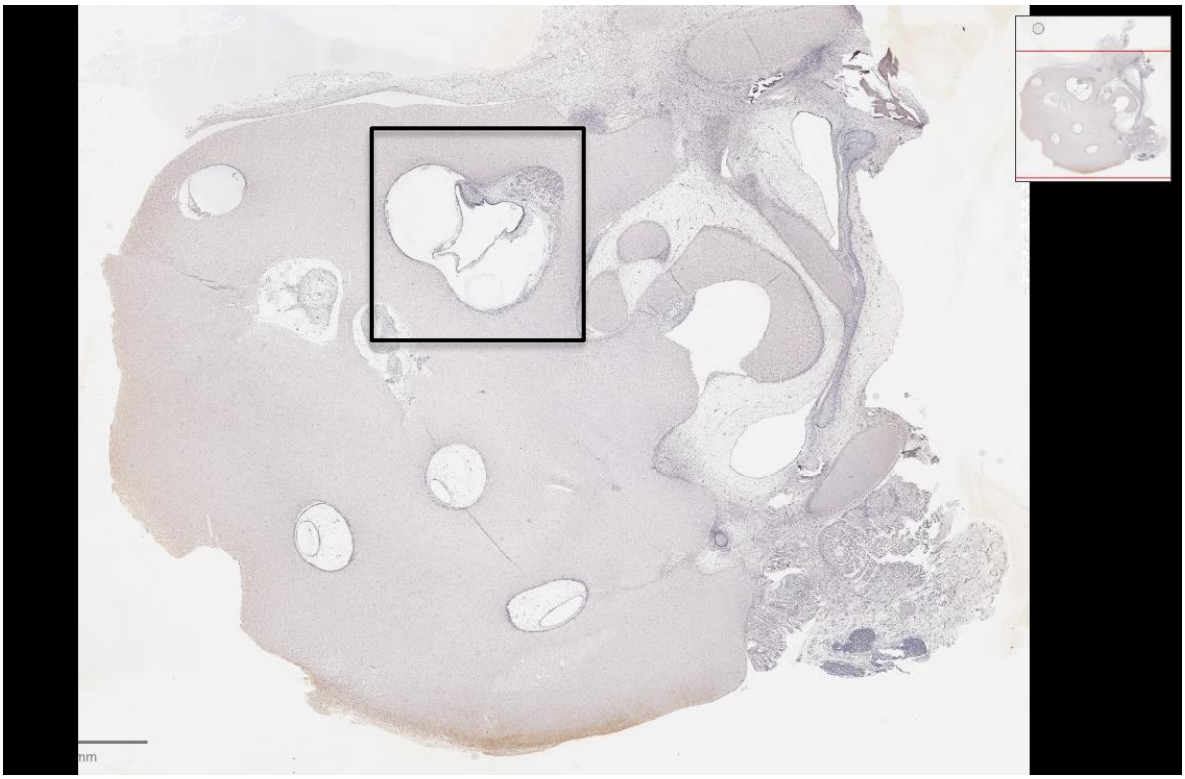


Negative



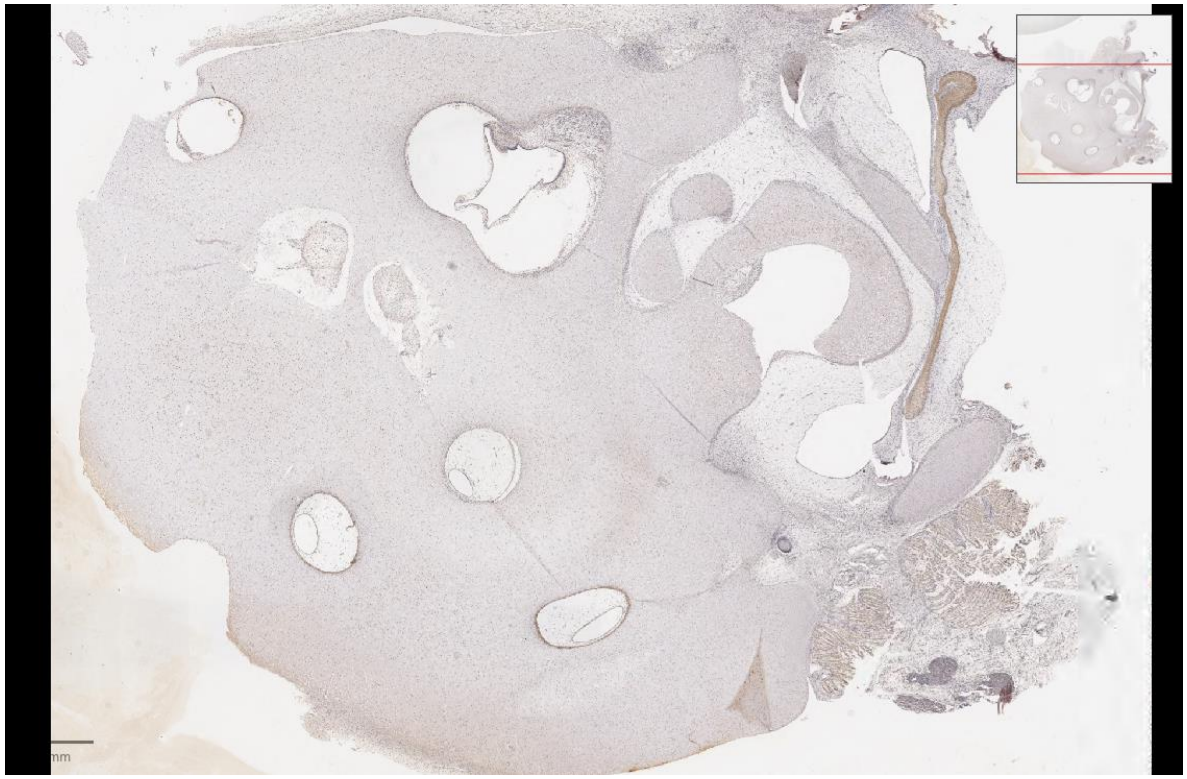
16 PCW - Towards Outer Ear

CK5/6

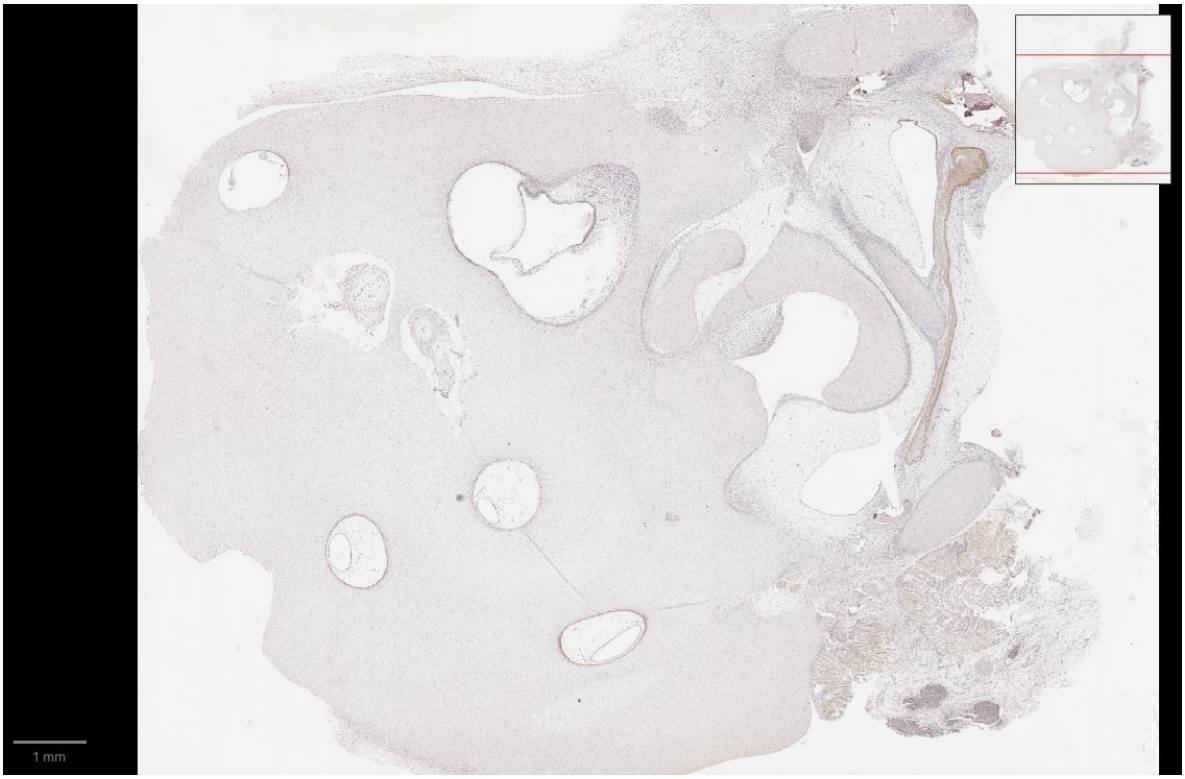


E-cadherin

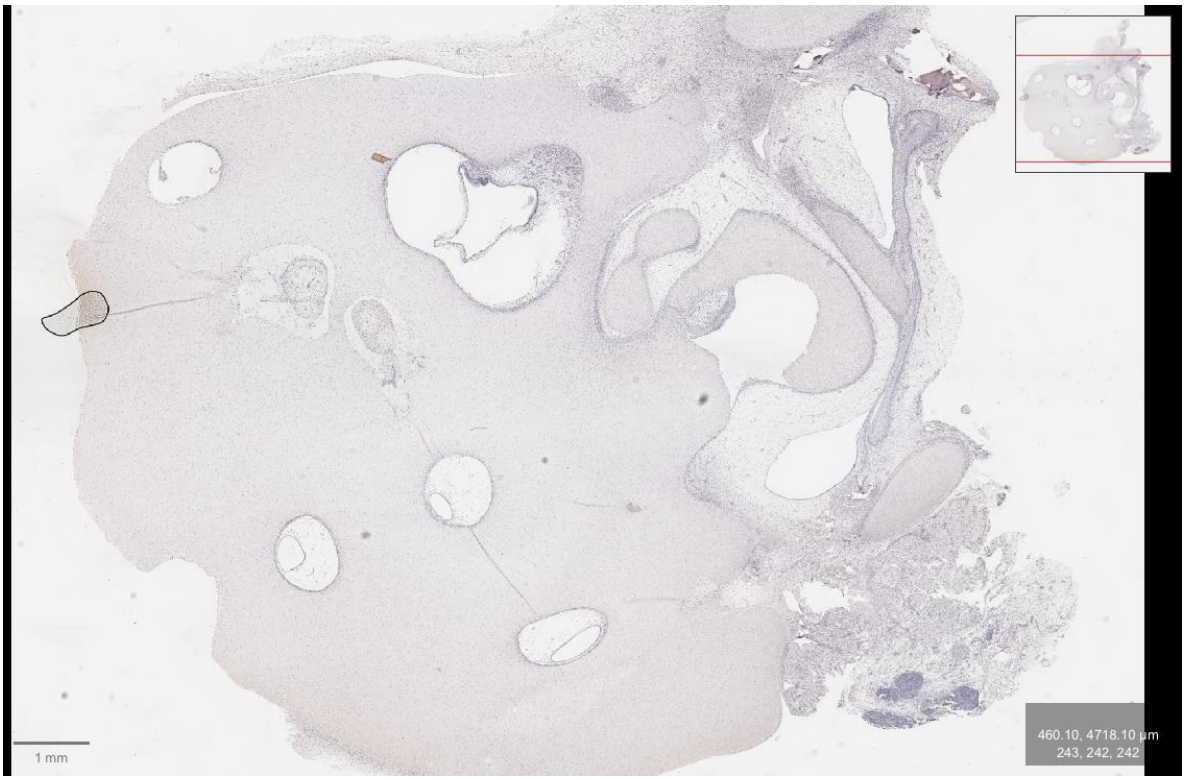
Sox2



FOXJ1



MUC5B



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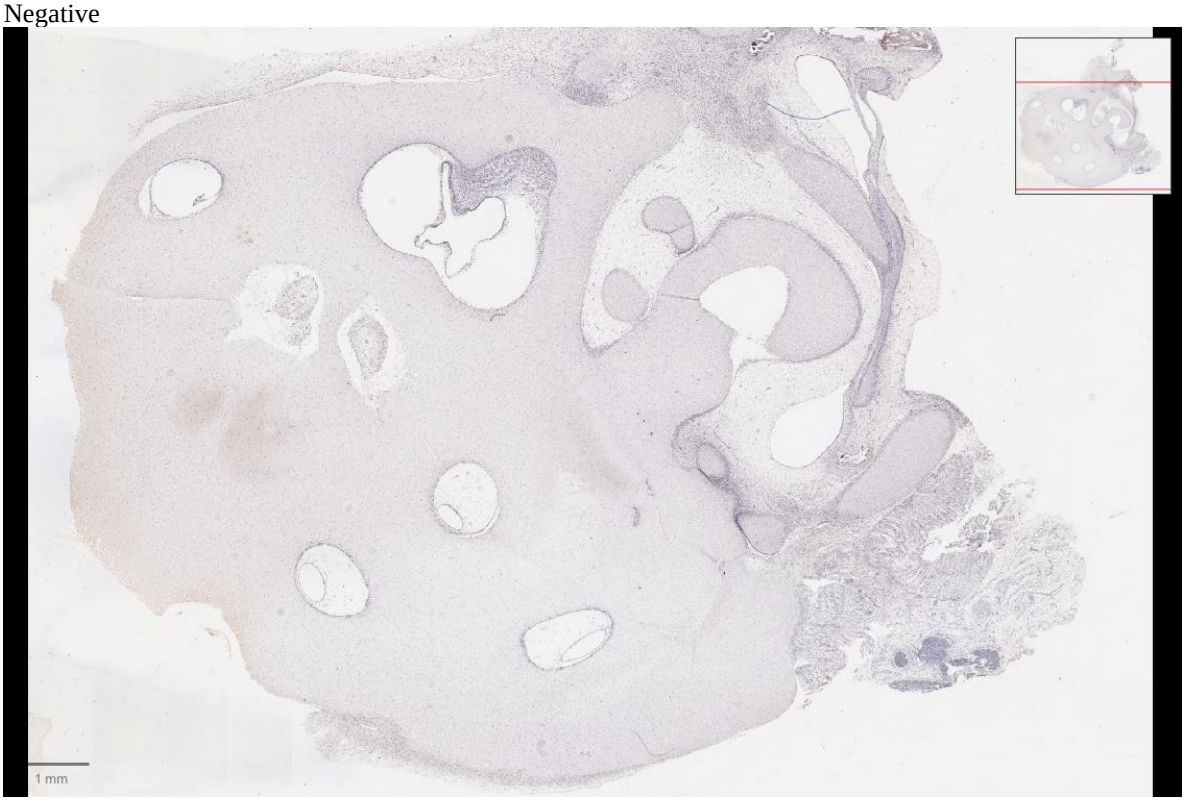
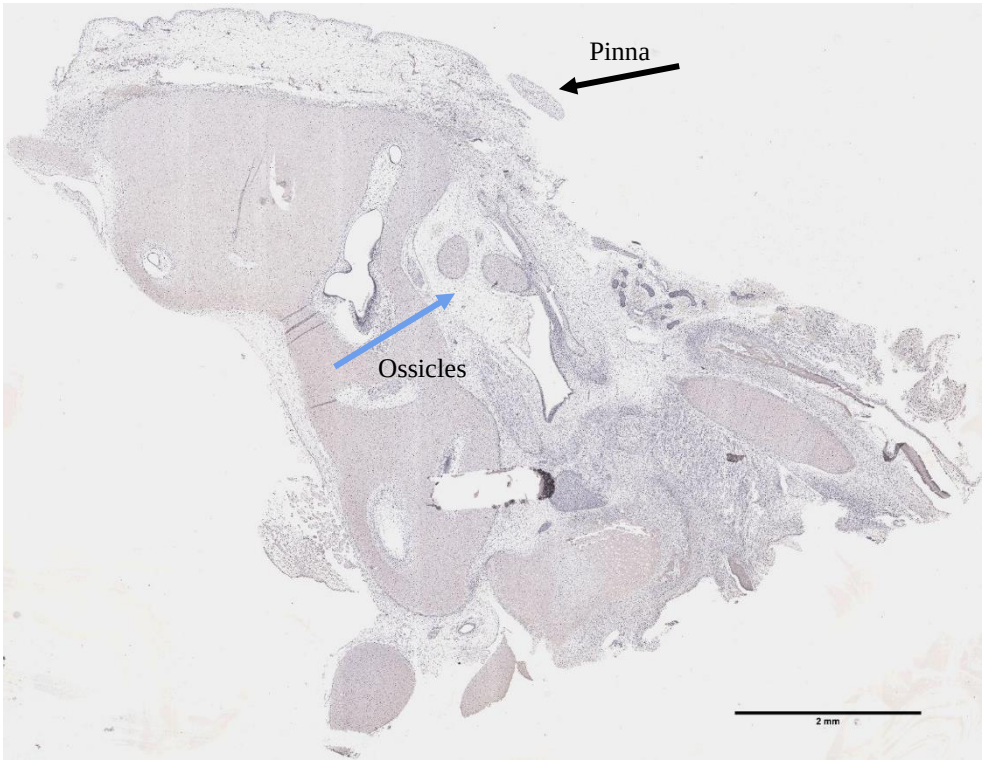
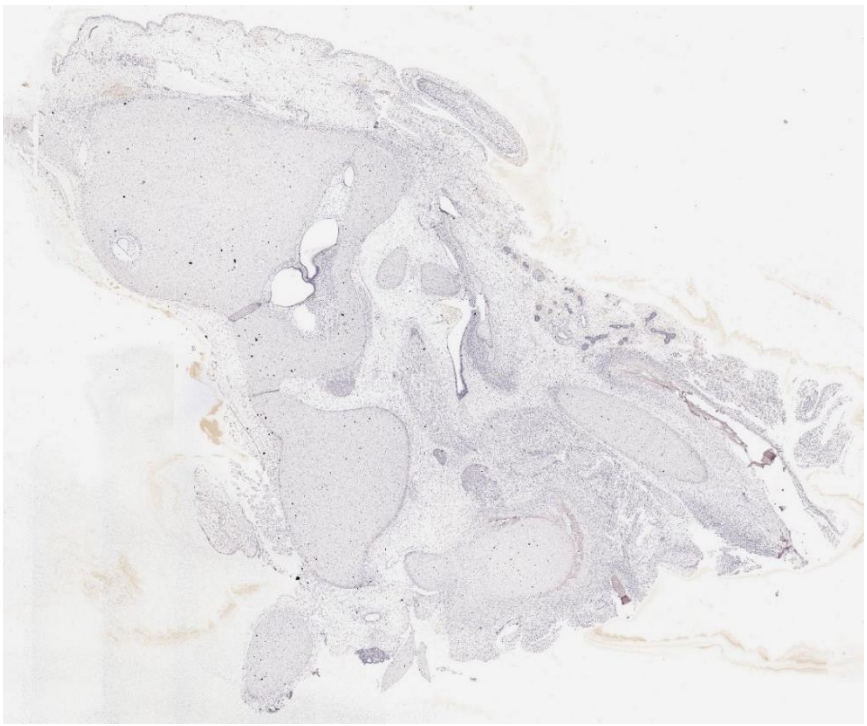
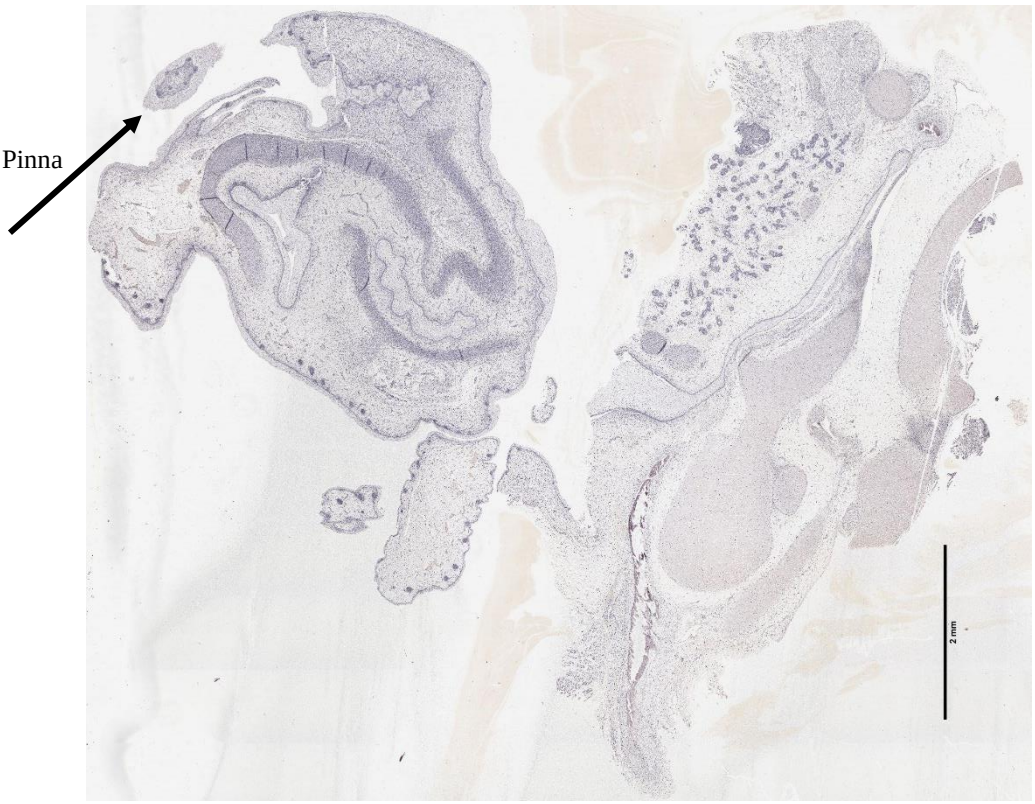


Table 20 - Scanned images of coronal and axial sections of IHC samples of early TOPs. Samples ranged from 10 to 17 PCW and were stained with CK5/6. Pinna (Black arrow), middle ear ossicles (blue arrow).

10 PCW - Towards Bottom
CK5/6 
Negative 

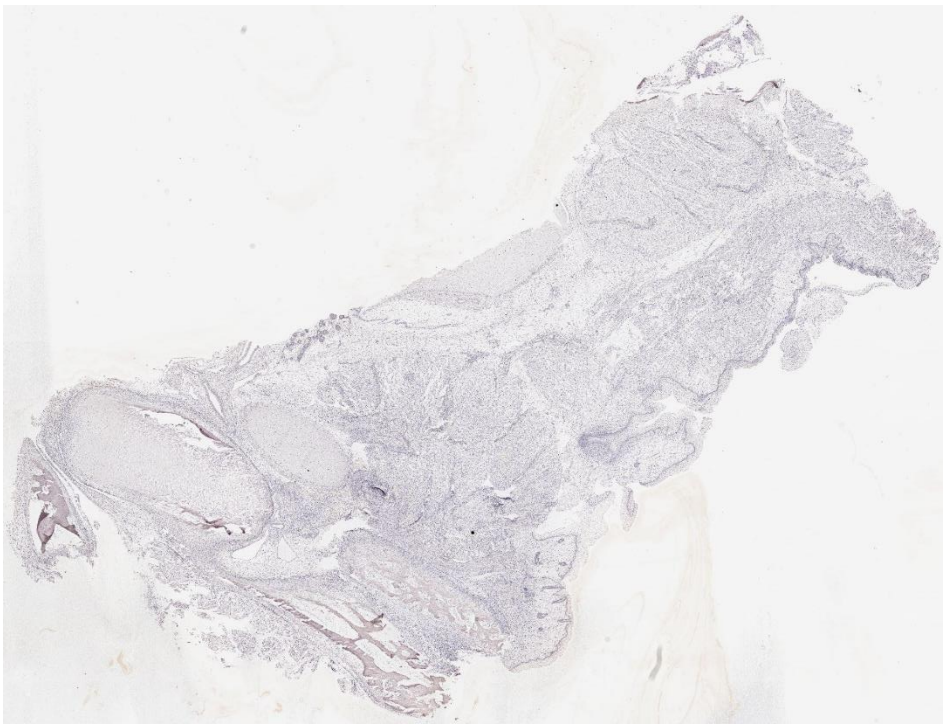
12 PCW - Towards Top

CK5/6



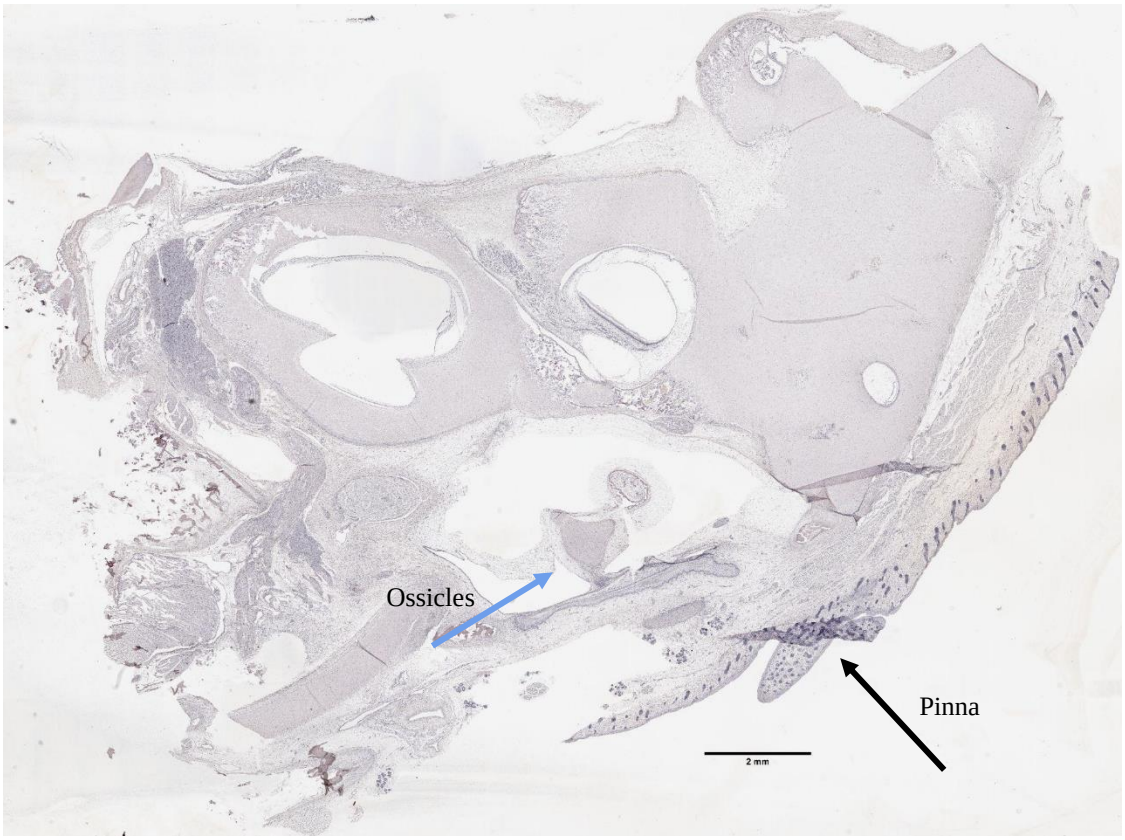
12 PCW - towards bottom

Negative



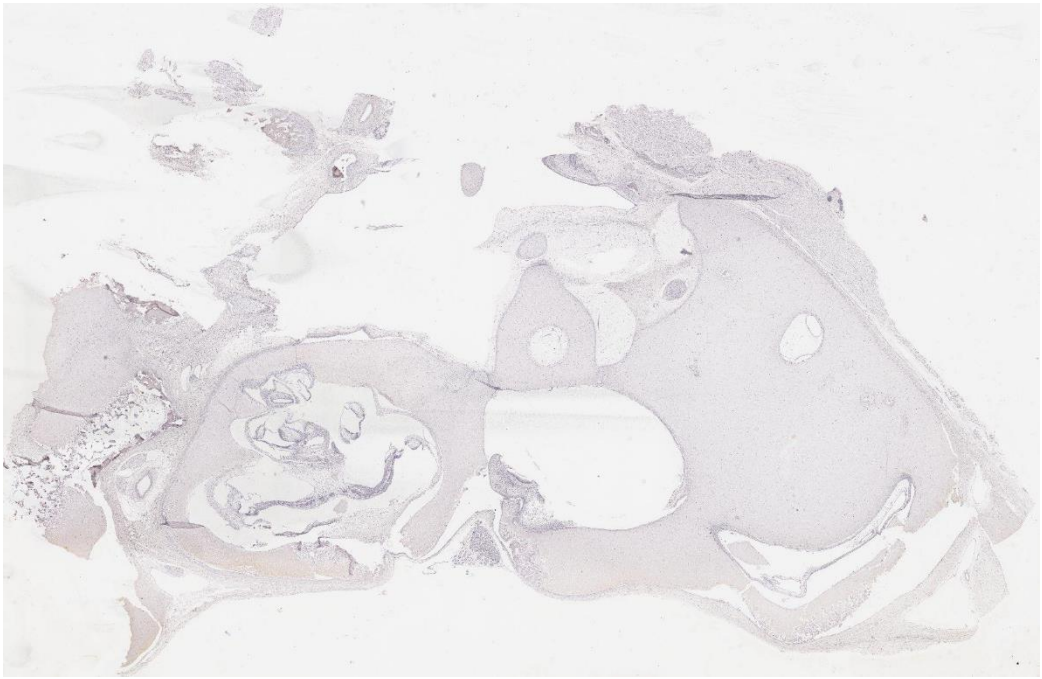
17 PCW - Towards Top

CK5/6



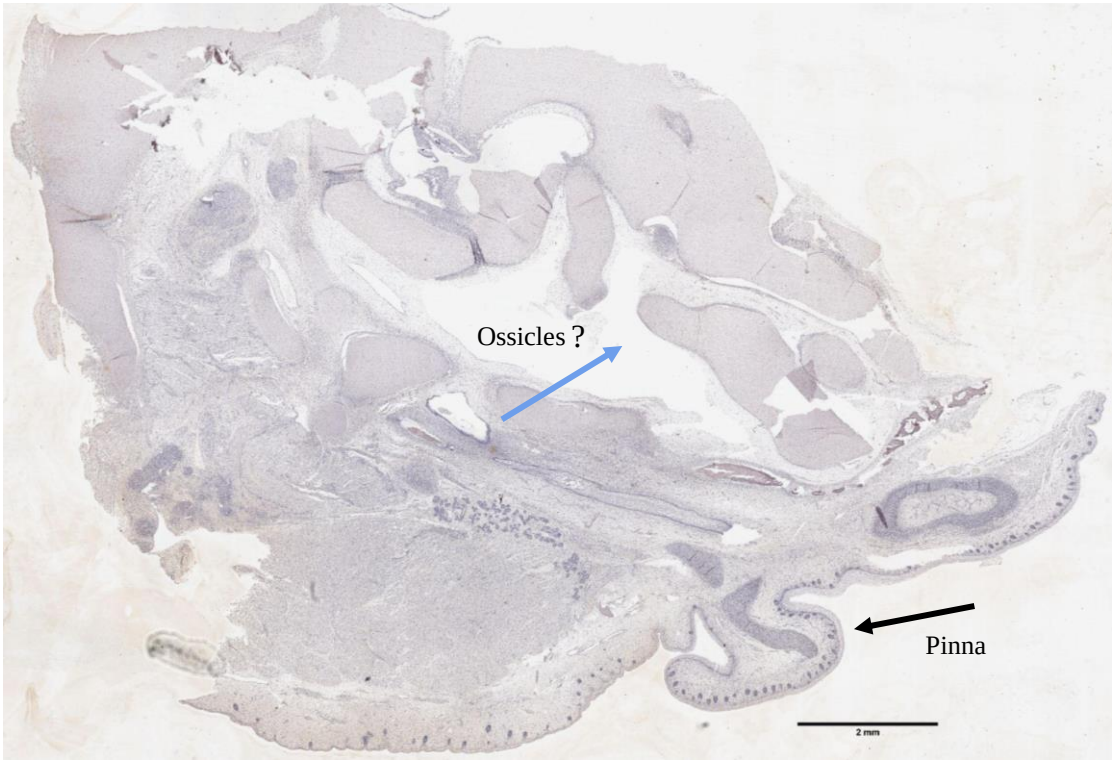
17 PCW - towards bottom

Negative

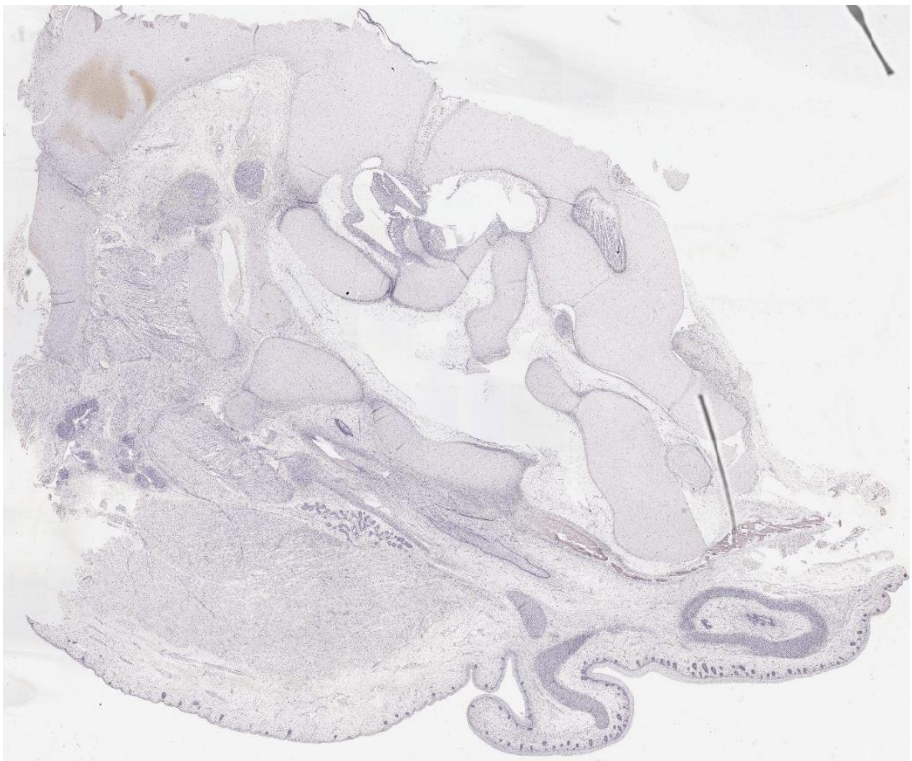


14 PCW - Towards Back

CK5/6

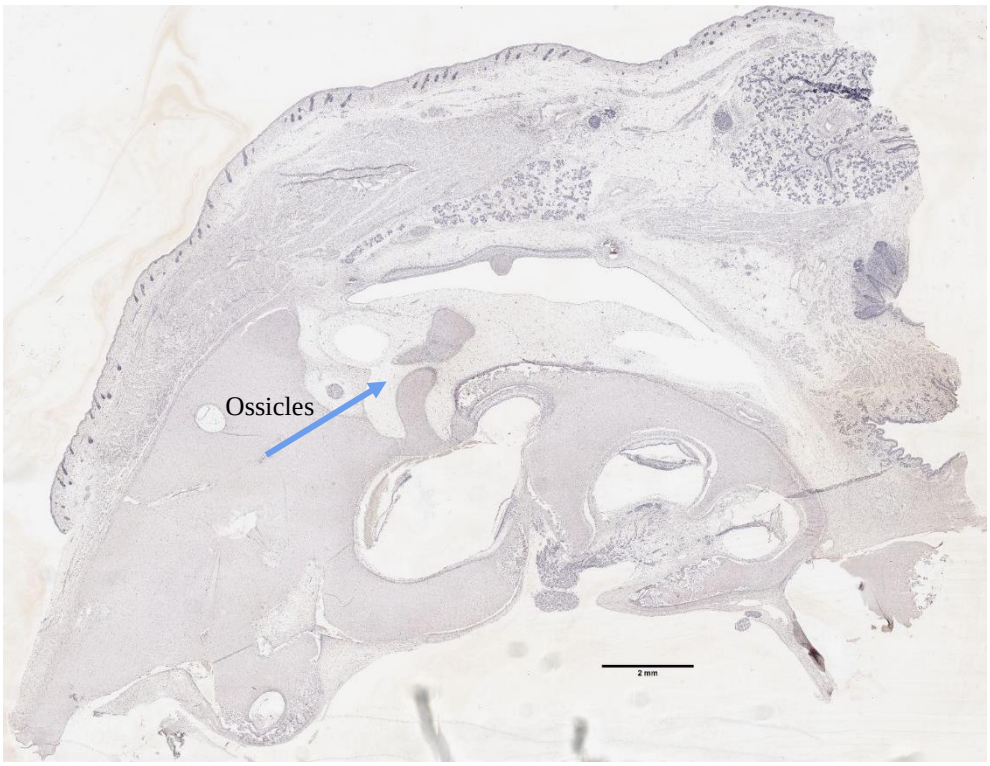


Negative



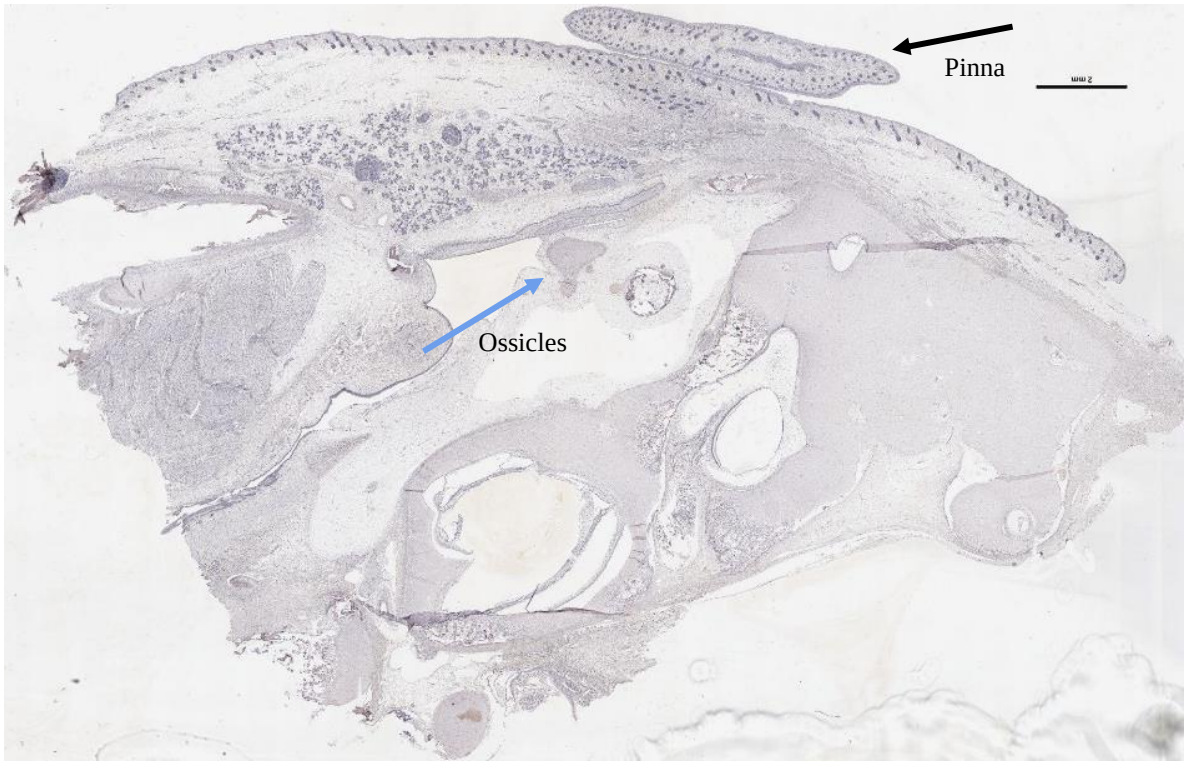
17 PCW - Towards Back

CK5/6

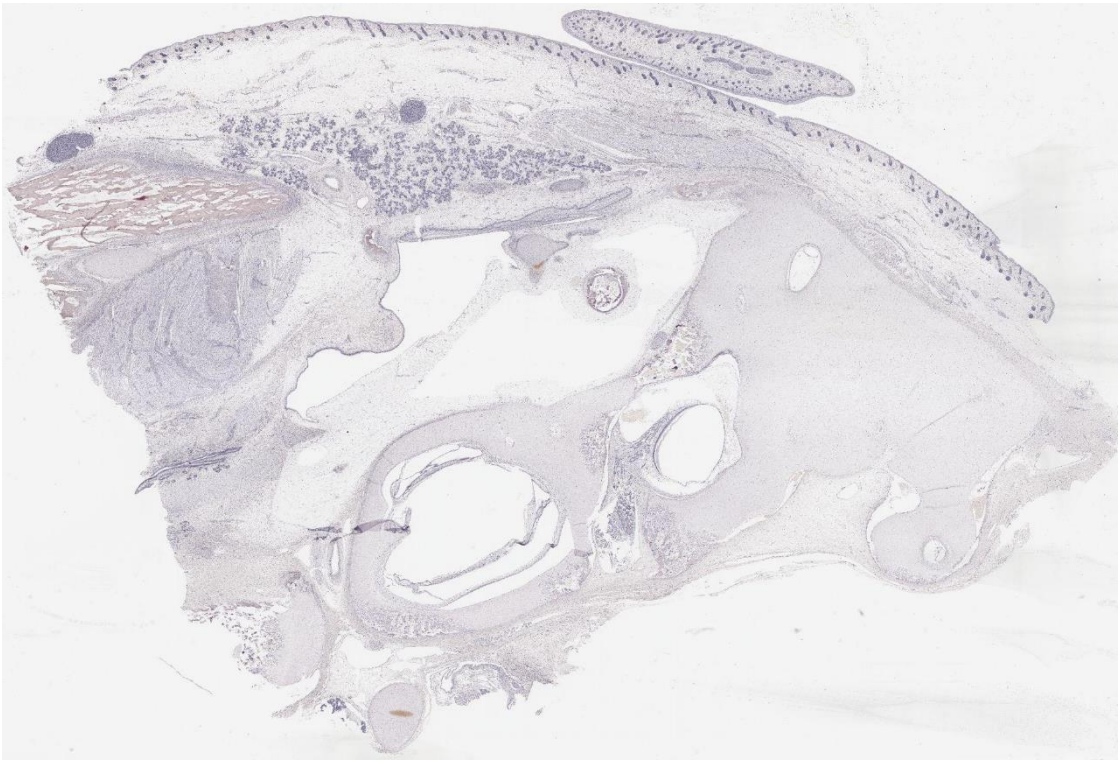


17 PCW - Towards Face

CK5/6



Negative



3.2. Discussion

I attempted to build a timeline of the development of the fetal ME, by examining the progression of certain cavities throughout the different PCW. However, this proved to be a significant challenge due to technical issues that arose during the study. I initially hypothesised that sagittal plane sectioning would provide the best information regarding ME development, as it would allow for a full view of the epithelium in the ME cavity, from attic to base, however, the lack of visibility of identifiable ear structures, such as the pinna or ME ossicles, hindered the localisation of the ME cavity, and therefore of its epithelium (Table 19). Thus, identification of the ME proved to be difficult in samples dissected at a sagittal cut. Upon analysis of the different planes, it became evident that more coronal and axial sectioning of samples would have provided a clearer understanding of the ME formation, as these section planes provide a more obvious view of the ear structures mentioned above (Table 20).

In all 17 PCW sections, it is possible to identify the pinna, as well as the auditory ossicles. Closer examination of the shape suggests that the ossicles may be the incus (17 PCW towards back) and malleus (17 PCW towards face). However, although it is possible to identify the pinna, in the 14 PCW sectioning towards back, the location of the ME cavity is far less

clear due to a lack of visibility of the auditory ossicles. This may be due to the fact that at this stage the auditory ossicles are not fully separated from each other, but rather form a more condensed pre-structure, although it could potentially be simply due to sectioning in an area where the ossicles are not visible, or that they have been lost during sectioning. At 17 PCW towards top, it is also possible to distinguish the pinna, as well as the ME cavity due to the presence of a triangular structure, likely the incus, that locates where the auditory ossicles should be, enveloped in a cell formation, possibly of loose mesenchyme. Interestingly, the ME cavity seems to also be visible in the 10 PCW towards bottom and 12 PCW towards top sectioning, identified by the presence of the pinna and amorphous structures that are likely to be the auditory ossicles. At both these stages however, the loose mesenchyme seems to be more condensed than at 17 PCW, and the cavity naturally presents smaller dimensions (Table 20).

As previously mentioned, IHC was performed to understand the mapping of neural crest (Sox2), endodermal (E-cadherin and cytokeratin-5), and differentiation markers (MUC5B for secretory cells and FOXJ1 for ciliated cells). However, staining of the slides did not align with the intended plan. Although I believed that staining of sagittal sectioned slides would assist with the identification of the ME, through identification of epithelial cells stained positively for E-cadherin, that did not prove to be the case and the ME is still difficult to identify in these slides. Interestingly, in the slides where the ME is identified through localisation of the pinna and/or ME ossicles, staining of markers is mostly imperceptible throughout the walls of what was expected to be the ME lining. As all primary antibodies presented positive staining during the optimisation of dilution, lack of staining is not likely due to defective reagents.

As Tucker *et al* (2017) reported that, in mice, cytokeratin-5 and Sox2 expression were associated with distinct populations of ME cells, staining for these two markers was expected to be visible within the lining of the ME cavity. In 3 week old mice ME, Tucker *et al* (2017) described expression of both cytokeratin 5 and Sox2 restricted to the hypotympanum close to the ET and the area surrounding the eardrum, but barely any expression of the markers in the non-ciliated epithelium lining of the attic and cochlea. Furthermore, they noted that cytokeratin 5 expression in ME basal cells had little overlap with Sox-2 positive cells, thus hypothesising that, during homeostasis, cytokeratin 5-positive cells differentiated into ciliated Sox-2 positive cells. [9] Furthermore, Luo *et al* (2017) reported, in the ME of 5 week old mice, cytokeratin 5 expression in both basal and goblet cells and in the non-ciliated squamous epithelial cells within the hypotympanum region, as well as sporadic positive labelling of cytokeratin 5 in the attic region. Thus, although cytokeratin 5 expression might not have been exclusively limited to

basal cells, Luo *et al*'s (2017) work supported the hypothesis that cytokeratin 5 positive basal cells give rise to ciliated cells. [24]

It is of the utmost importance to understand that the findings reported by Tucker *et al* (2017) and Luo *et al* (2017) are seen in animal models post-birth, where the ME has cavitated and the cells are expected to have undergone differentiation, giving rise to an aerated and functional cavity, which is not the case in the human fetal ME observations. However, lack of staining for E-cadherin, CK5/6, and Sox2 in the lining of the ME cavity is surprising. Lack of differentiation markers, namely MUC5B and FOXJ1, is not unexpected, as at this stage the ME lining is still expanding.

4. Chapter 4 - Middle ear epithelial cells

This chapter will focus on this project's research on the usage of human middle ear epithelial cells, both cell lines and primary cells, whether its employment has been appropriate to investigate the pathogenesis of OM, the need for a reliable model that accurately mimics the ME, the collection of ME primary cells from fetal tissue and the lifespan extension of primary ME epithelial cells.

The methods utilised in this chapter can be referred back to in Materials and Methods, section 2.1.2., 2.1.3., 2.1.4., 2.1.5., 2.3.1., 2.3.2., 2.3.4., 2.3.5., 2.3.6., 2.3.7., 2.3.8..

4.1. Results

4.1.1. Characterisation of hMEEC-1 and primary middle ear epithelial cells

The first goal of my study was to thoroughly characterise hMEEC-1, its growing conditions and cell markers, and to compare them to the ME primary cells recovered from the fetal human skull dissections. When originally immortalised, hMEEC-1 were cultured in supplemented bronchial/tracheal epithelial cell growth medium (BEGM, Clonetics) containing penicillin (100µg/mL) and streptomycin (100µg/ml). For my study, hMEEC-1 were cultured in four different media to assess optimal proliferation and cell viability conditions: Pneumacult ExPlus (ExPlus), Dulbecco's Modified Eagle Medium (DMEM), Keratinocyte Growth Medium (KGM) and Keratinocyte Serum-Free Medium (KSFM), supplemented as described in the Materials and Methods chapter (Figure 36).

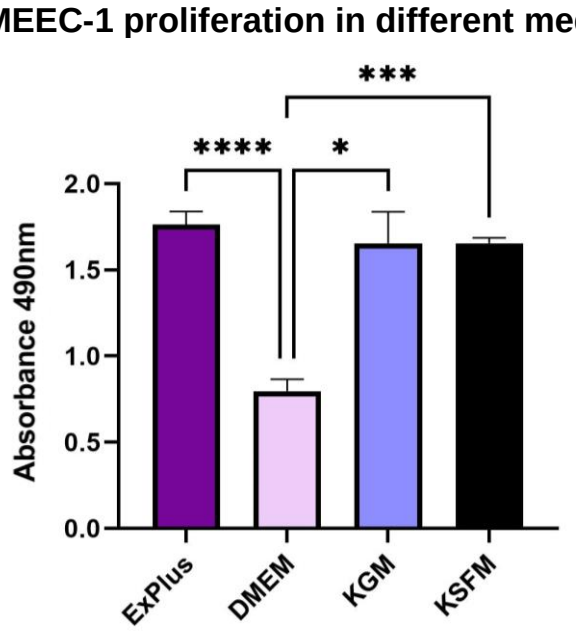


Figure 36 - Assessment of hMEEC-1 cell proliferation rates in different media by CellTiter 96® AQueous One Solution Cell Proliferation Assay. hMEEC-1 were able to proliferate in ExPlus, KGM and KSFM, but not DMEM. (n=3, RM One-way ANOVA)

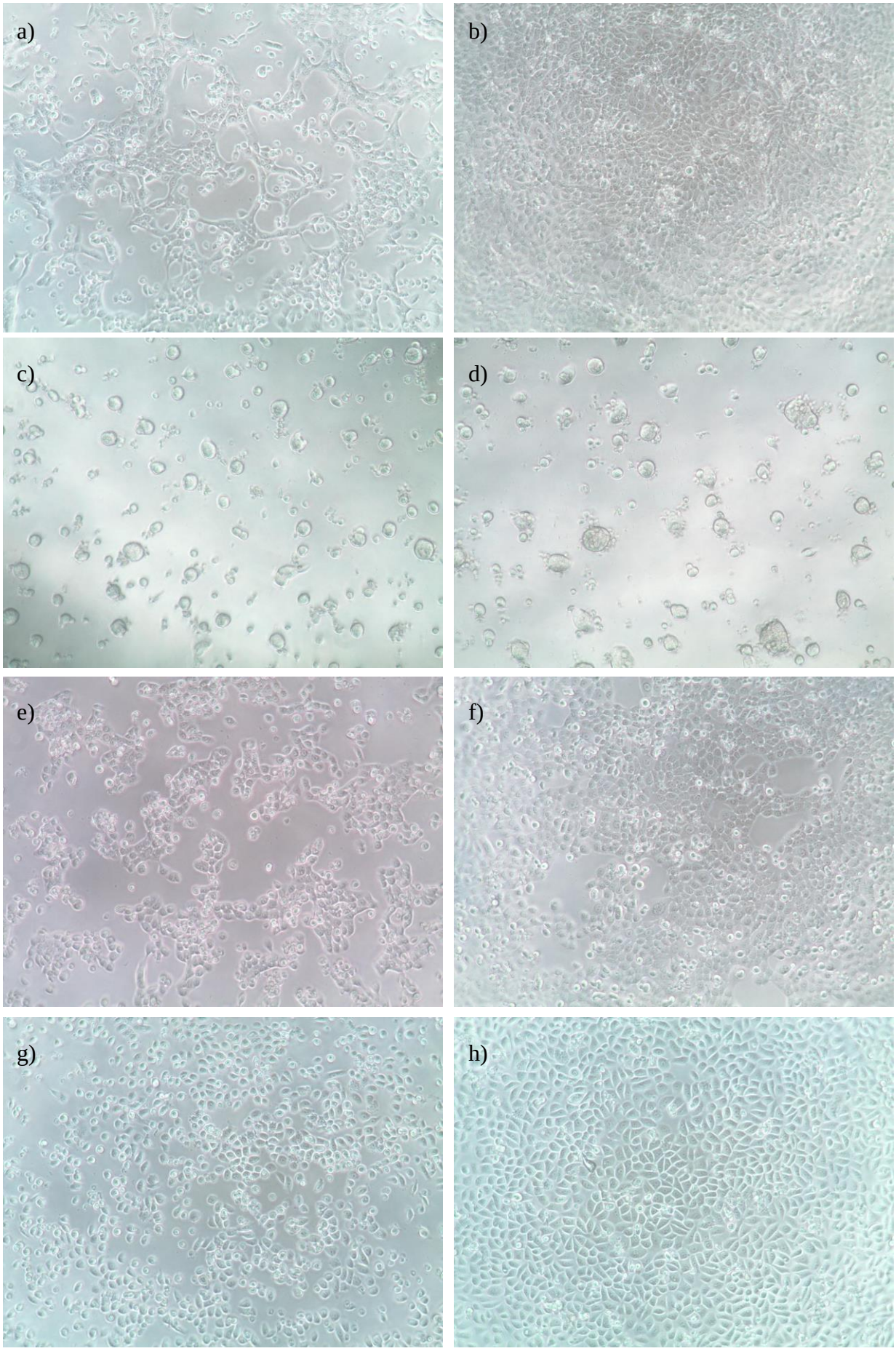


Figure 37 - Observation of hMEEC-1 phenotypic profile in different media. a) ExPlus 24 hours post

seeding, b) ExPlus 48 hours post seeding, c) DMEM 24 hours post seeding, d) DMEM 48 hours post seeding, e) KGM 24 hours post seeding, f) KGM 48 hours post seeding, g) KSFM 24 hours post seeding, h) KSFM 48 hours post seeding. Unlike cells growing in KSFM, cells growing in ExPlus and KGM media preserve the hexagonal shape, phenotype described by Moon *et al* (2002). [128]

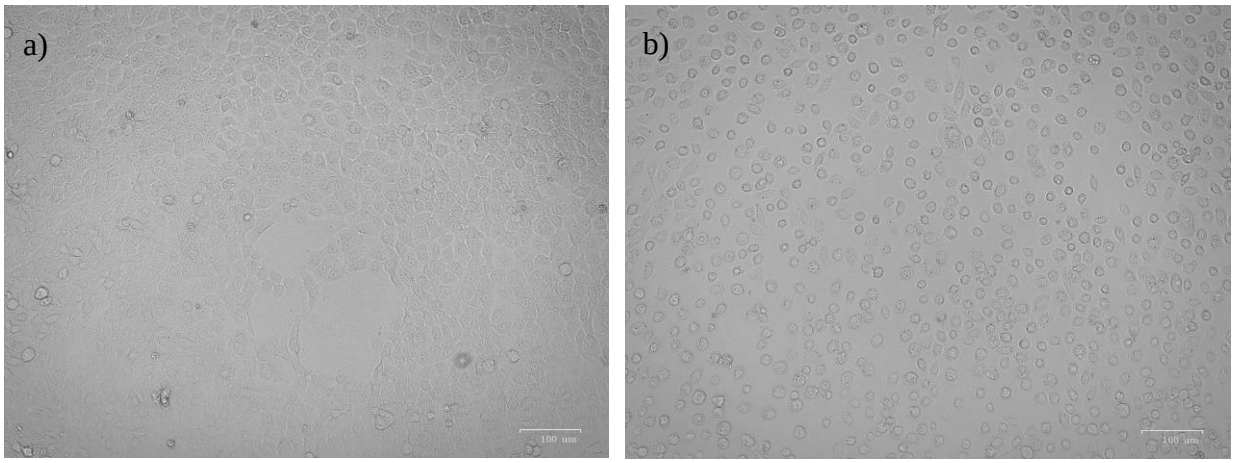


Figure 38 - Observation of hMEEC-1 phenotypic profile changes after four passages in a) ExPlus and b) KSFM medium.

As seen, cells grown in DMEM formed clusters of various sizes that did not attach to the surface of the tissue culture flask. After seeding, cells grown in KSFM were able to proliferate, however when passaged for prolonged periods of time (from passage 4 (P4) onwards) in this medium, replication was slower, and the cells did not adhere to the flask. Further, hMEEC-1 in KSFM did not present as the same phenotype initially described by Moon *et al* (2002). [128] hMEEC1 cultured in either KGM or ExPlus appeared with the same, expected cell phenotypic profiles and proliferation rates, and these were not affected over time with increasing passage number. Thus, I concluded that DMEM and KSFM were not suitable media for the cell utilised in my study (Figure 37 and 38). Although both KGM and ExPlus supported hMEEC-1 2D cell cultures in a similar manner the compatibility of ExPlus with Pneumacult ALI media made it more appropriate for the 3D model work this project would undertake.

After selecting an appropriate growth medium for ME epithelial cells, it was necessary to source primary human middle ear cells. The cells were recovered through dissection of fetal skulls, ranging from 14 to 20 PCW.

The population of ME cells that initially grew from the sectioned tissue presented at least two cell populations, distinguishable due to their different phenotypes. These two seemingly distinct populations presented an epithelial-like appearance and a mesenchymal stromal cell (MSC)-like appearance. Occasionally fibroblast-like cells would also be seen in the mixture of populations. The cell populations were easily separated through differential trypsinisation, a short incubation (less than a minute) that lifted MSC-like cells and, when present, fibroblasts followed by a longer trypsinisation (up to 8 minutes) that lifted the epithelial cells (Figure 39).

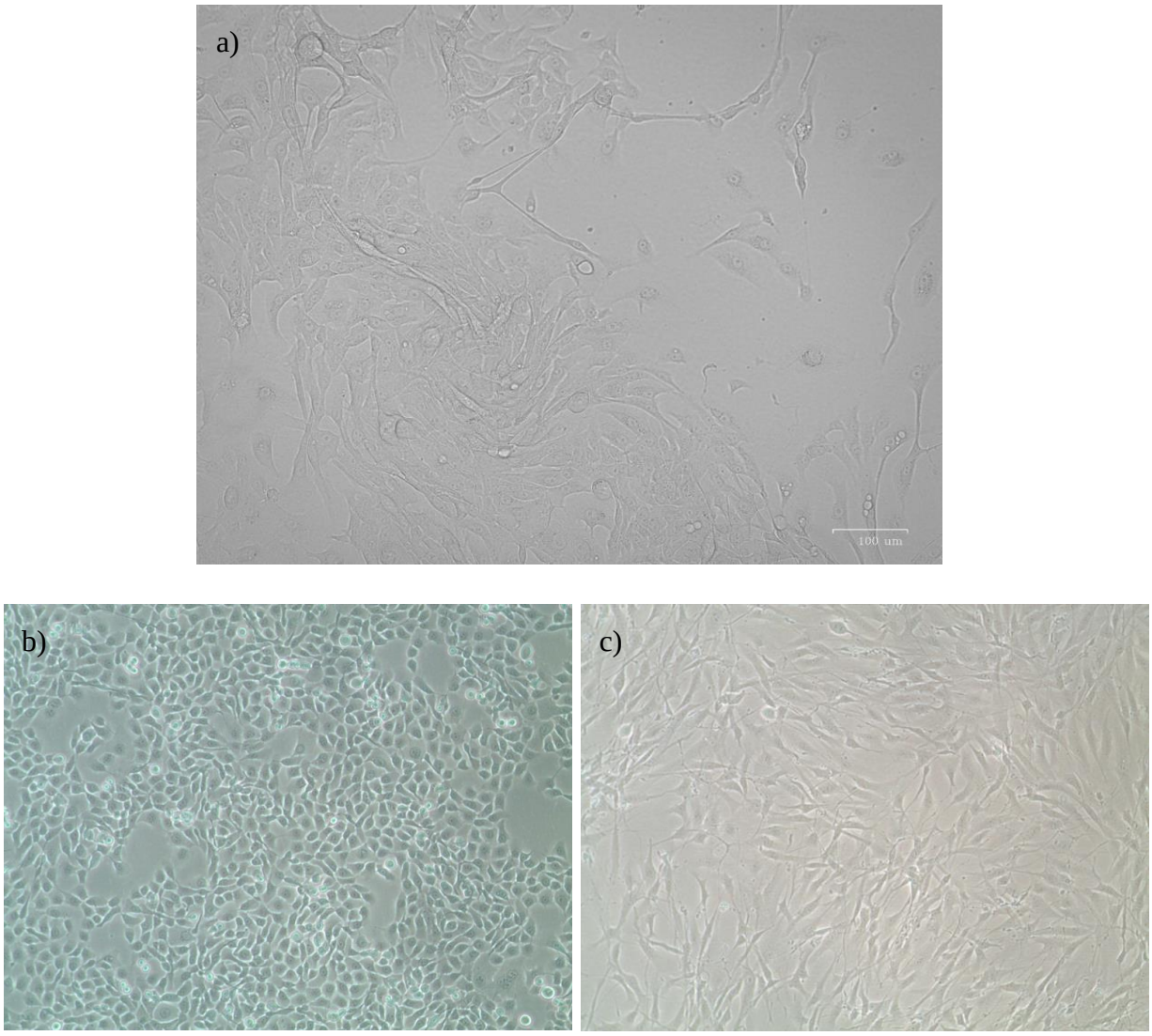


Figure 39 – Observation of cell populations post expansion of material retrieved from TOP dissections. a) Mixed population. b) Epithelial-like population. c) MSC-like population

Two cell populations were selected, one of epithelial-like cells only referred to as middle ear epithelial cells (MEEC) and one with both epithelial and MSC-like cells, virtually the whole middle ear population recovered, referred to as mixed population (MixPop). Later, a third cell population was selected by isolating the cells that rapidly lifted when incubated with trypsin (MSC-like cells). End-point PCRs and agarose gel electrophoresis were used to detect mRNA expression of specific cell markers to characterise these populations and to compare them to hMEEC–1 (Figure 40 and 41).

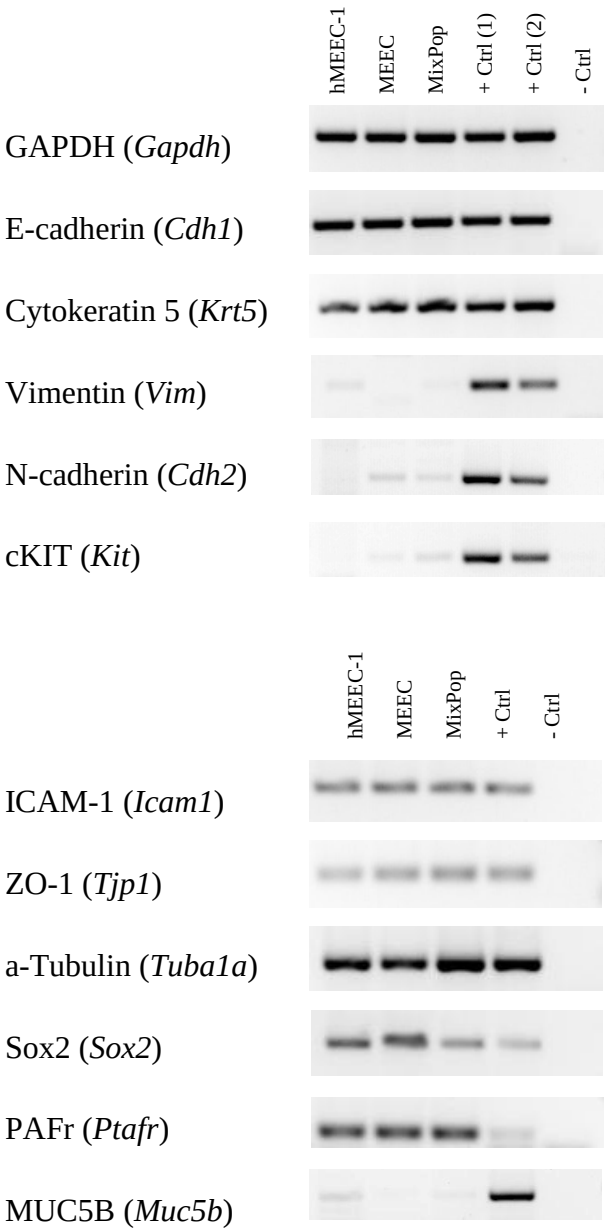


Figure 40 - hMEEC-1, MEEC and MixPop characterisation through End-point RT-PCR. + Ctrl (1) and + Ctrl is mRNA extracted from dissected fetal labial gland primary epithelial cells, + Ctrl (2) is mRNA extracted

from dissected fetal sublingual primary epithelial cells. Two positive controls were utilised in the first PCR run to attest the presence of markers on both tissues.

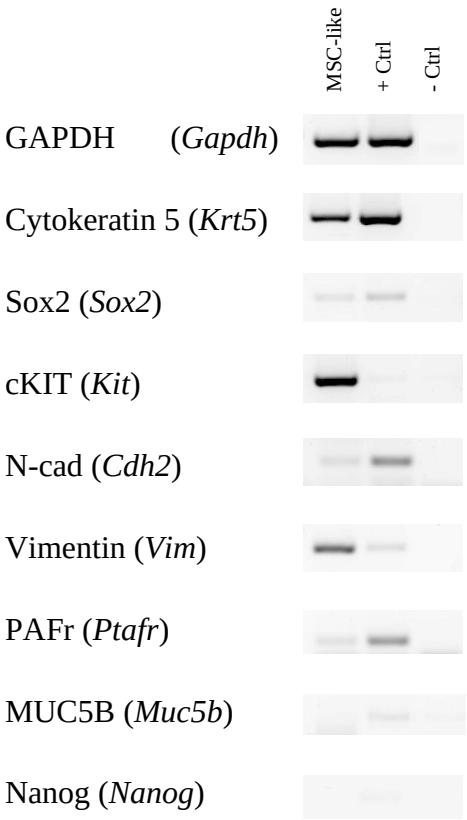


Figure 41 - MSC-like characterisation through End-point RT-PCR. + Ctrl is mRNA extracted from dissected fetal labial gland primary epithelial cells, except for Nanog, where + Ctrl is mRNA extracted from mshef11, embryonic stem cells, where differentiation had been initiated.

hMEEC-1, MEEC (P6) and MixPop (P5) presented significant levels of transcription for: *E-cad*, a surface epithelial cell marker that plays a vital role in cell adhesion, contact inhibition and the maintenance of epithelial phenotypes; *Krt5*, a cytoplasmic marker expressed by epithelial cells. Cytokeratin 5 dimerises with cytokeratin 14 to form intermediate filaments that maintain the cytoskeleton of basal epithelial cells; *Tuba1a*, expresses a highly conserved heterodimer responsible for the assembly of dynamic microtubules that offer structural support, pathways for transport and force generation in cell division; *Ptafr*, encodes a surface receptor that binds to PAF, a cell-to-cell communication mediator; and *Sox2*, a transcription factor that plays an important role in the formation of different tissues during embryonic development. All cells expressed moderate levels of transcription for: *Icam-1*, which encodes a cell surface glycoprotein expressed at low basal levels in immune, endothelial and epithelial cells, with

central roles in cell-to-cell adhesion and epithelial injury-resolution responses; and *Tjp1*, which coordinates epithelial cells' organisation by regulating actinomyosin contraction, membrane traffic, and generation of unified apical surfaces. MEEC and MixPop presented mild to weak expression of *Kit* which, although regarded as an oncogene, in the context of embryonic and fetal development, is vital in maintaining stem and hematopoietic progenitor cells. Weak expression by hMEEC-1 of *Vim*, a marker for epithelial-mesenchymal transitions (EMT) may be due in part to the insertion of the HPV 16 E6/E7 genes, used to immortalise the cells. This change in vimentin expression in epithelial cells undergoing HPV16 E7 transformation has previously been reported by Hellner *et al* (2009). [257] MEEC and MixPop weak expression of *Kit* and *Cdh2* is indicative of the presence of mesenchymal cells in the culture, likely MSCs, as the genes are unlikely to be expressed by epithelial cells but are a cell marker for the mesenchymal cell population. MSC-like cells demonstrated significant levels of transcription of *Krt5* and *Kit*, and mild to weak levels of *Sox2*, *Cdh2*, *Vim* and *Patfr*, however no levels of *Nanog*, an embryonic stem cell marker, were detected. Interestingly, only hMEEC-1 expressed moderate levels of *Muc5b*. This finding does not invalidate the potential for MEEC and MixPop to present more prominent expressions of the gene in response to stimuli, but rather suggests that insertion of HPV 16 E6/E7 might have also affected expression of genes other than vimentin.

PAFr is a cell surface receptor present in epithelial cells, that has previously been reported to be utilised by respiratory tract pathogens, some of which are also OM pathogens, such as NTHi, to invade epithelial cells. For the purposes of my study, additional to the confirmation of its mRNA transcription, protein expression on the surface of middle ear epithelial cells was also assessed through immunofluorescence (Figure 42 and 43).

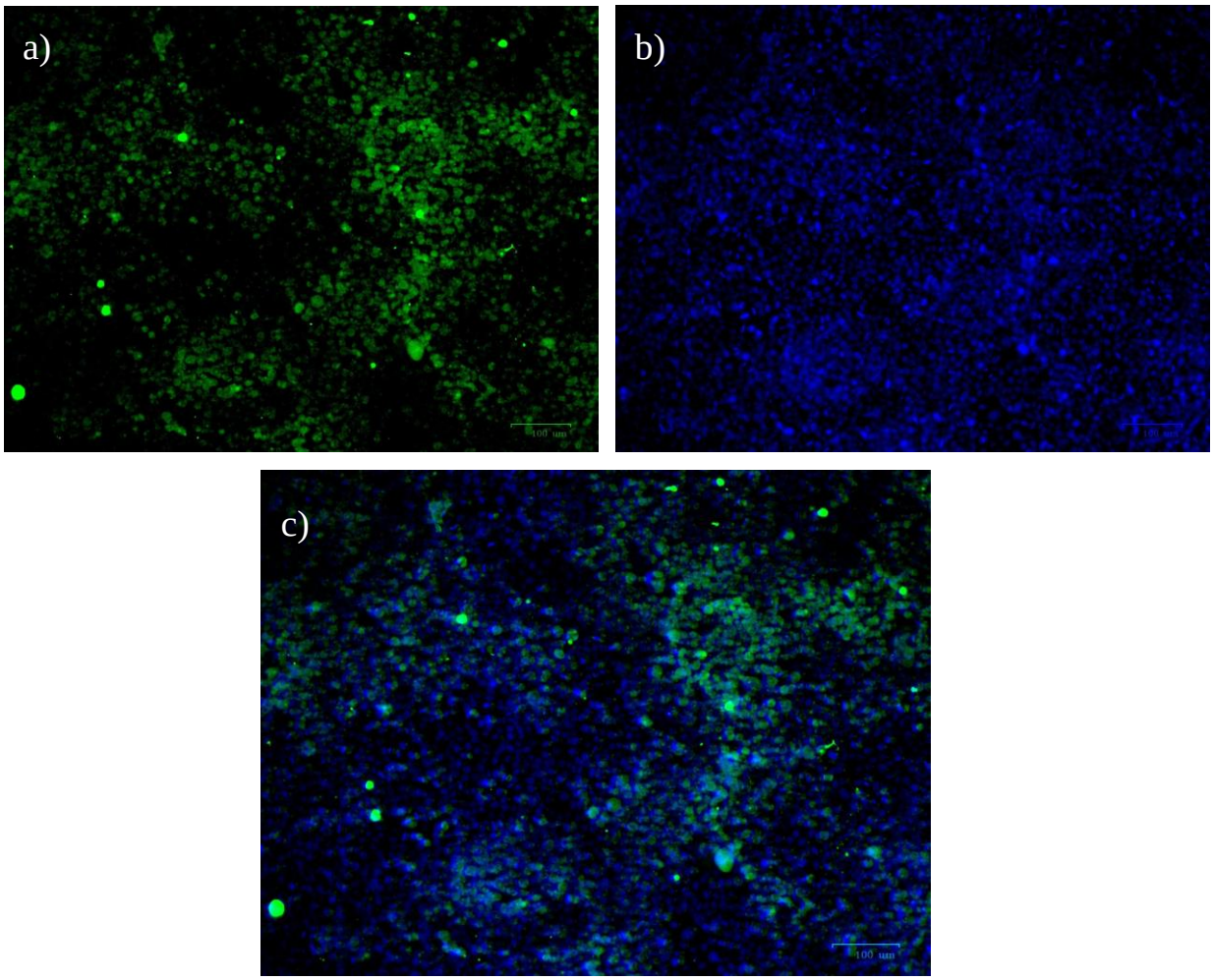


Figure 42 - Assessment of expression of PAFr on the surface of hMEEC-1 through immunofluorescence. Green labelling indicates the presence of PAFr through antibody binding, blue labelling indicates nuclei through DAPI. a) hMEEC-1 monolayer tagged with PAFr antibody conjugated with GFP-tagged secondary antibody, b) hMEEC-1 monolayer with DAPI, c) Merged construct of a) and b).

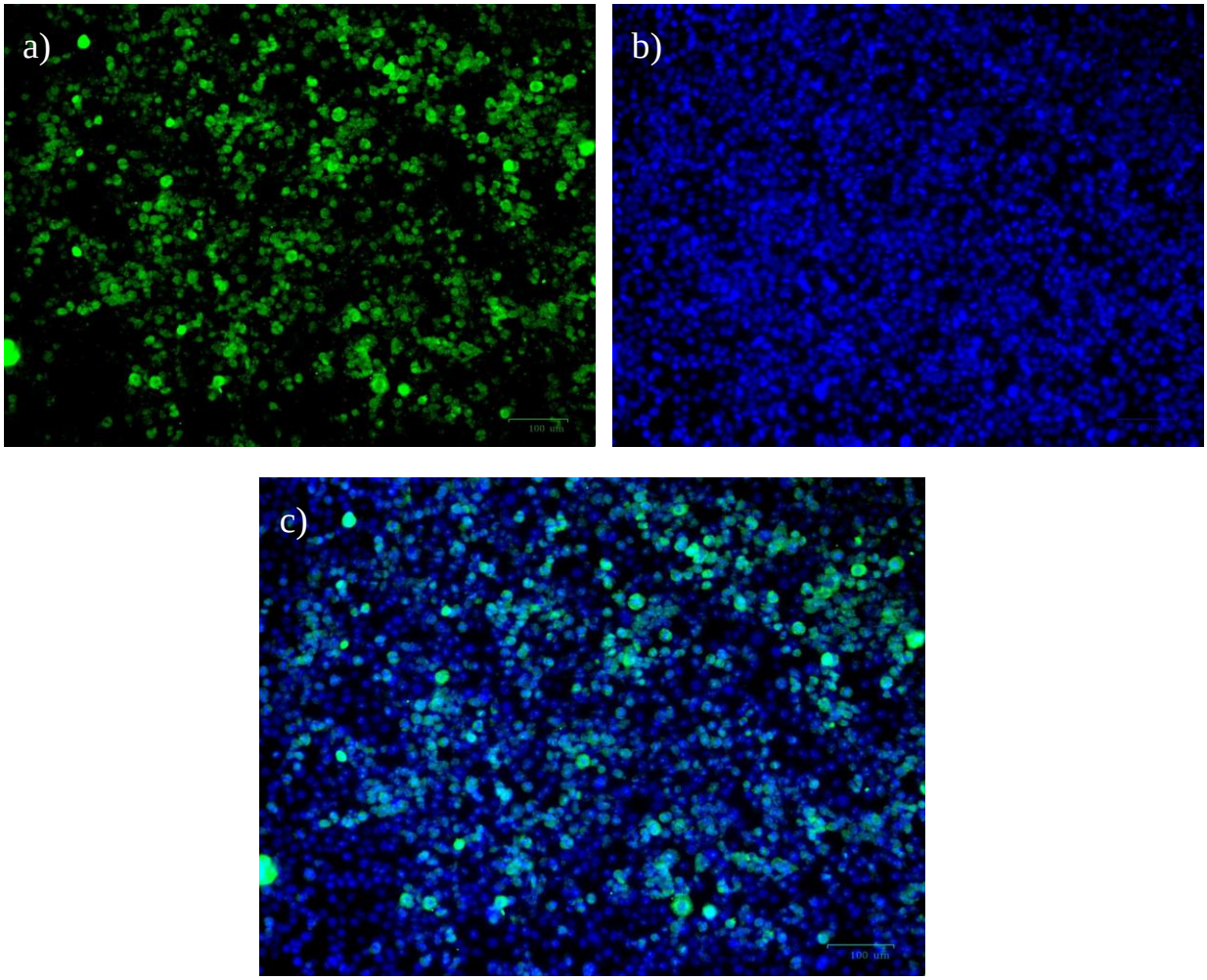
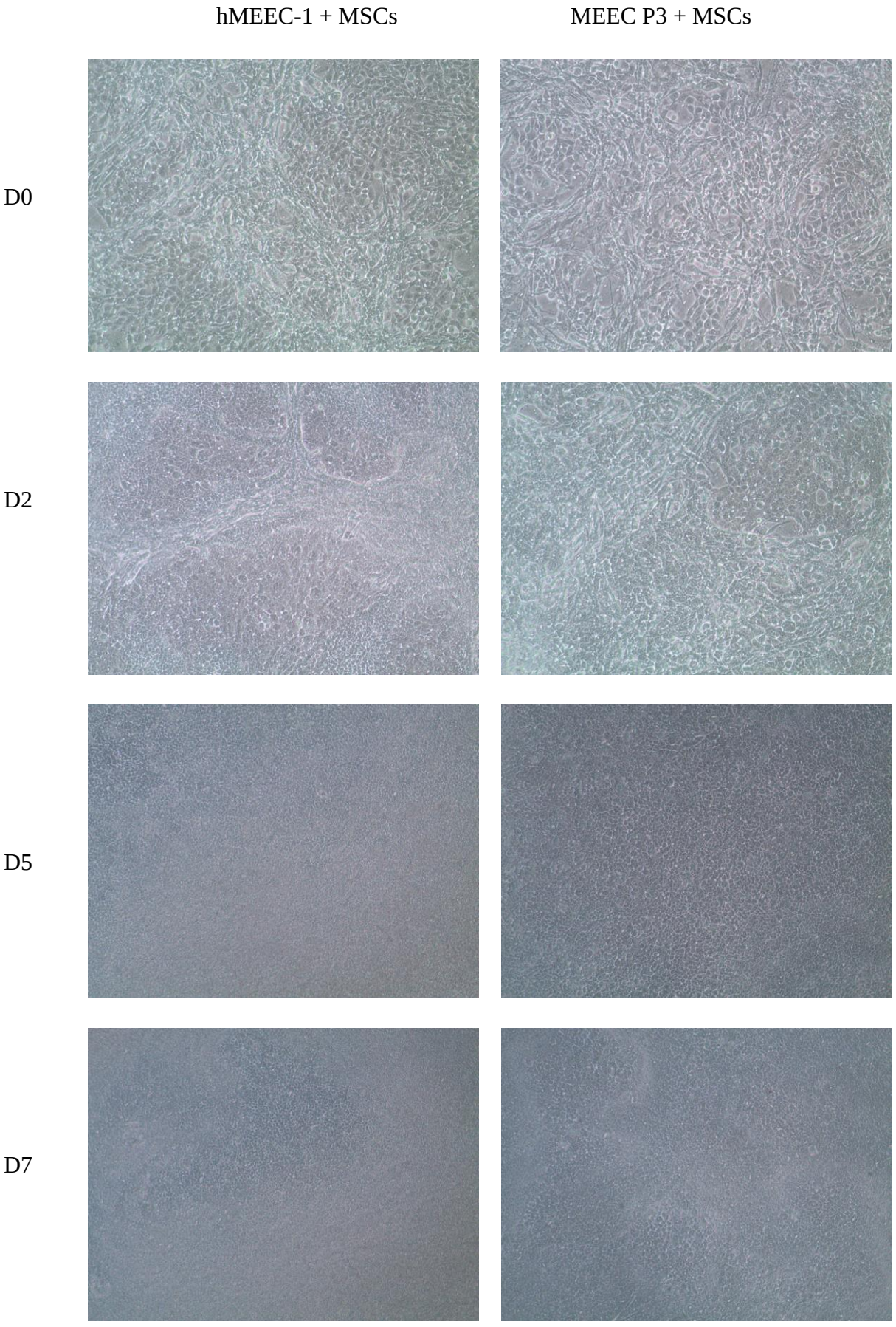


Figure 43 - Assessment of expression of PAFr on the surface of MEEC through immunofluorescence. Green labelling indicates the presence of PAFr through antibody binding, blue labelling indicates nuclei through DAPI. a) MEEC monolayer tagged with PAFr antibody conjugated with GFP-tagged secondary antibody, b) MEEC monolayer with DAPI, c) Merged construct of a) and b).

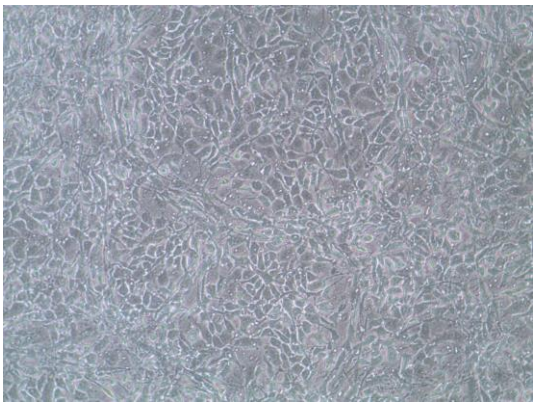
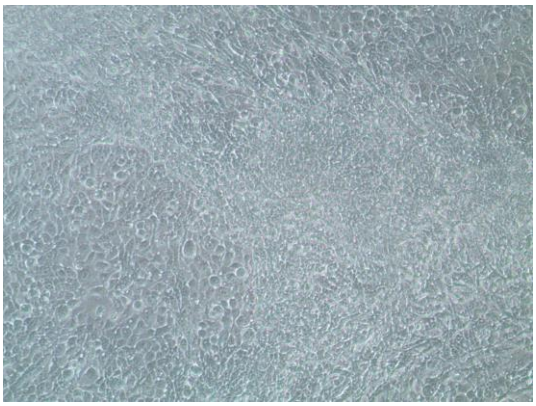
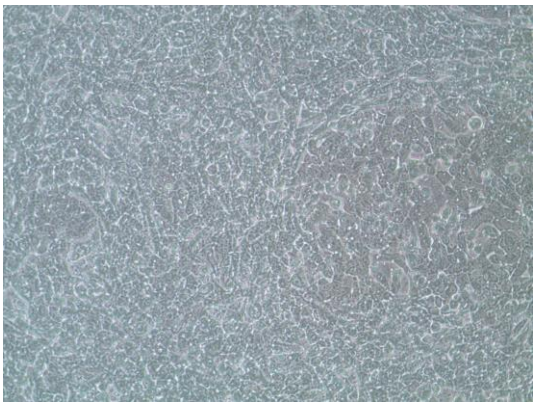
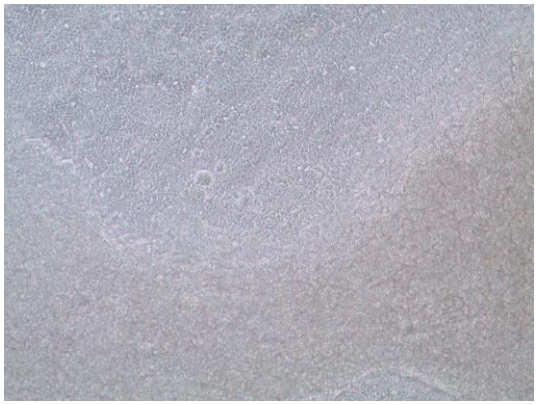
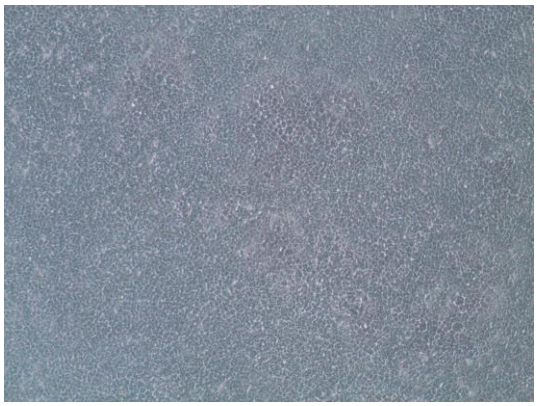
4.1.2. Air-liquid interface cell models

A functional 3D cell model that accurately represents the ME is essential in the research of the pathogenesis of OM. To evaluate the ability of hMEEC-1 and MEEC to produce a functional 3D model, cells were placed at ALI. The differentiation progress was followed through microscopic observation while end-point PCR and agarose gel electrophoresis were used to detect mRNA expression of specific differentiation markers at different timepoints to characterise the differentiation cells under different ALI culture conditions. When no expression of differentiation markers was found with the ME epithelial cells alone, alternative culture conditions were assessed for their ability to promote differentiation including a)

incubation with fibroblasts (NOF) in the basal chamber, b) addition of retinoic acid (RA) and/or $CaCl_2$, and c) incubation with ME MSC-like in the apical chamber (Figure 44).


hMEEC-1 + MSCs
MEEC P3 + MSCs
D0
D2
D5
D7

	hMEEC-1 + MSCs	MEEC P3 + MSCs
D9		
D12		
D14		

	MEEC P32	MEEC BMI-1 P28.4
D0		
D2		
D5		
D7		

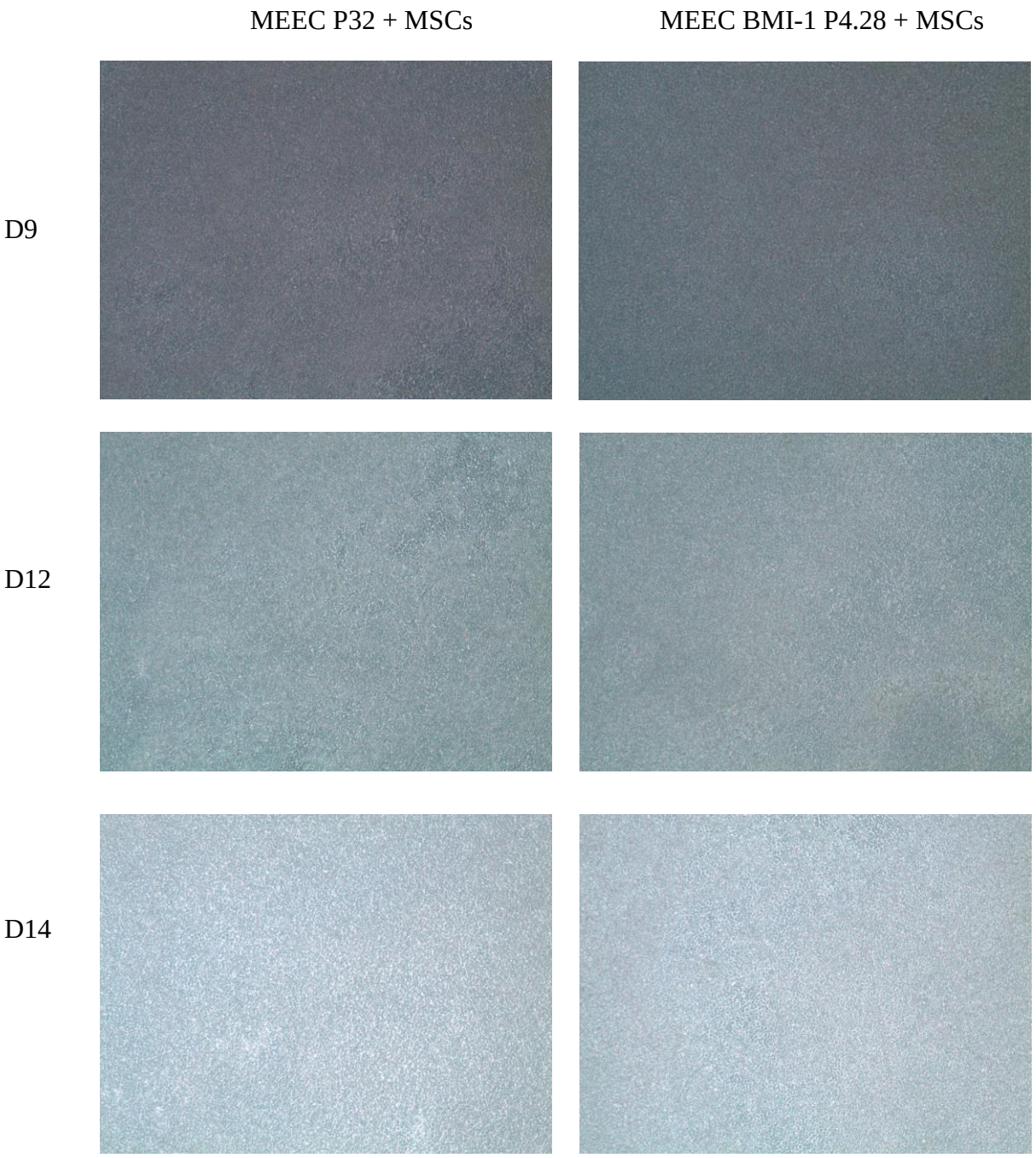


Figure 44 - Timeline of microscopical observations of ALI progress of hMEEC-1, MEEC P3, MEEC P32 and MEEC BMI-1 P4.28 co-cultured of MSC-like. Across all conditions, cells continued to proliferate indefinitely leading to multiple layers forming; this could contribute to nutrient starvation for the cells at the higher layers and lack of oxygen at the lower layers, leading to cell death. The effects of indefinite proliferation and cell death can be seen from day 5 onwards, where cell shapes are no longer distinguishable. This effect was seen across all tested conditions, including cells at different passage, co-cultured with fibroblasts and treated with $CaCl_2$.

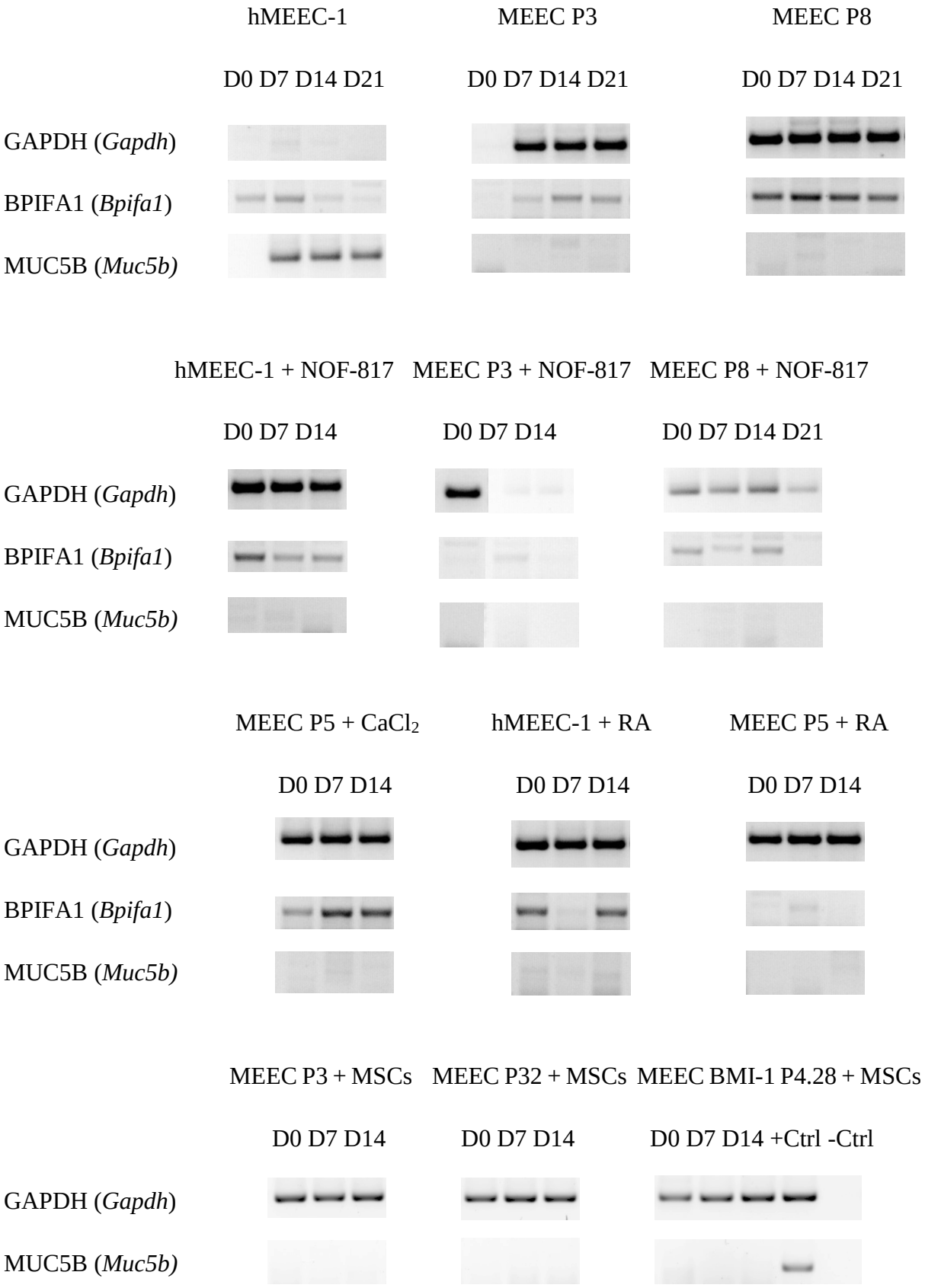


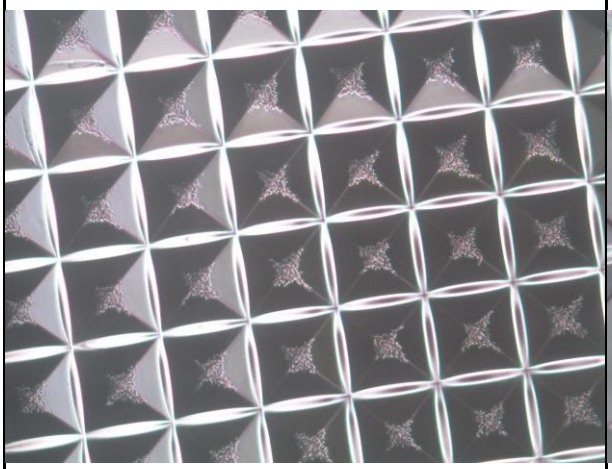
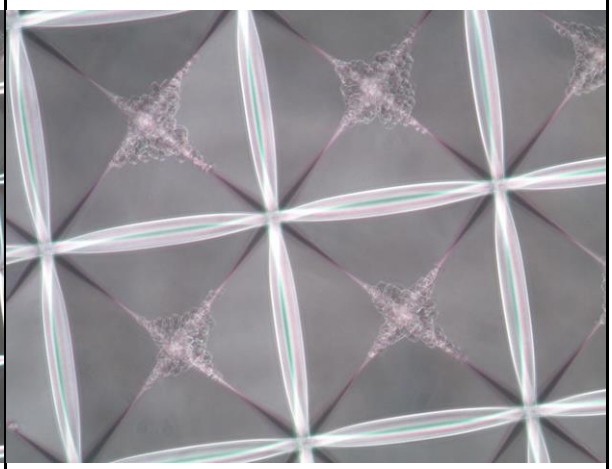
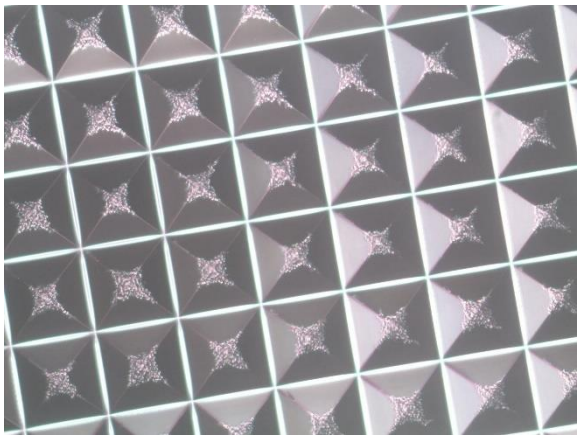
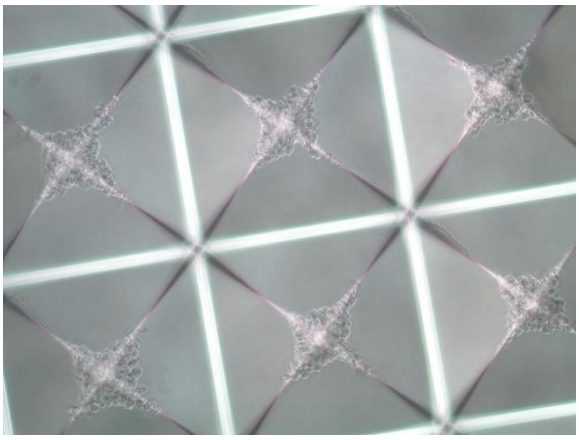
Figure 45 - hMEEC-1 and MEEC differentiation progress at ALI. mRNA was collected on D0, D7, D14 for all seeding conditions and D21 for ME cells at ALI alone and incubated with NOF817 in the basal chamber. Transcription of *Bpifa-1*, *Muc5ac*, *Muc5b* and *Tekt-1* was evaluated through end-point PCR.

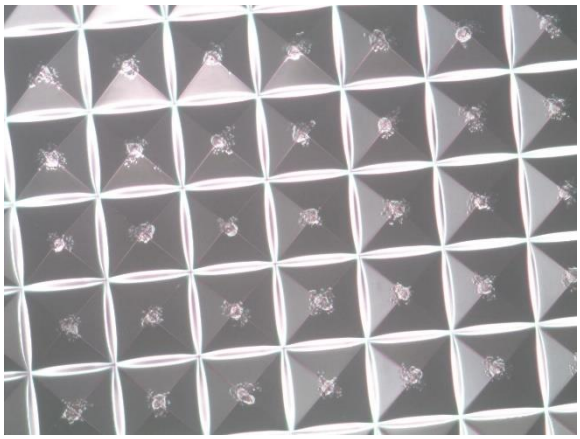
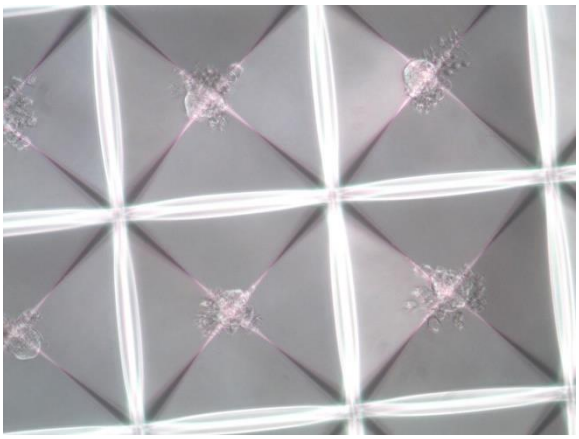
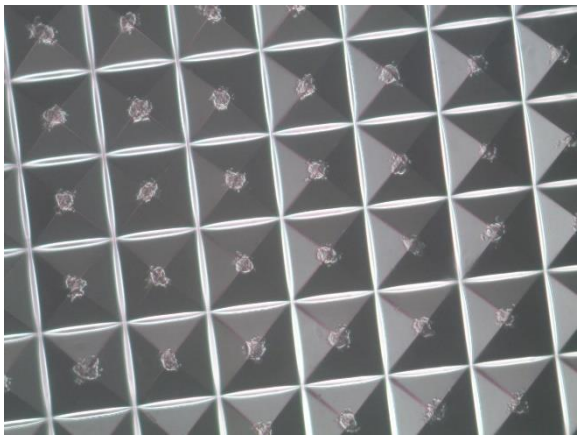
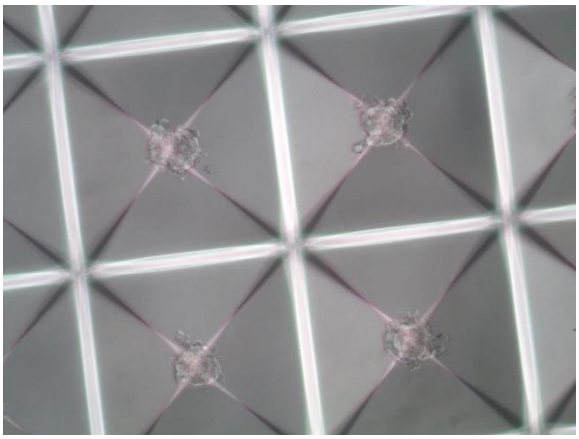
Transcription of *Bpifa-1*, *Muc5ac*, *Muc5b* and *Tekt-1* was evaluated. Although it is possible to detect transcription of *Bpifa1* across all ME cells and conditions at multiple timepoints, *Muc5b* transcription seems to be inconsistent, and mostly observed in hMEEC-1. No transcription of *Muc5ac* and *Tekt-1* was observed. Once again, *Muc5b* deficient expression by MEEC does not equate to an inability of these cells to express the gene, but rather the possibility of insufficient stimuli to promote transcription. However, absence of *Muc5b* expression allied to lack of *Muc5ac* expression suggests a lack of differentiation (Figure 45).

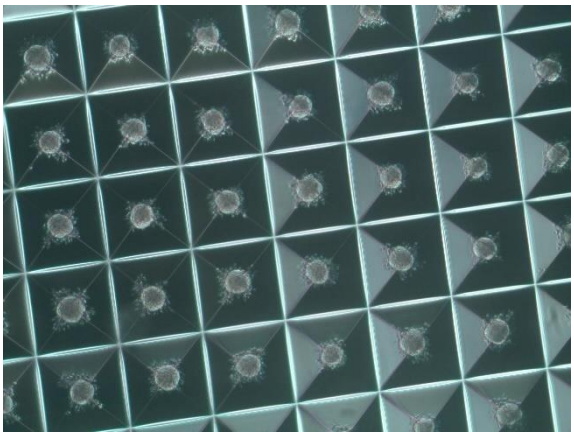
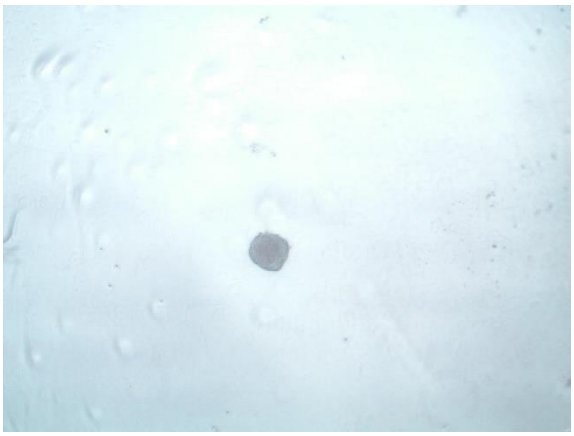
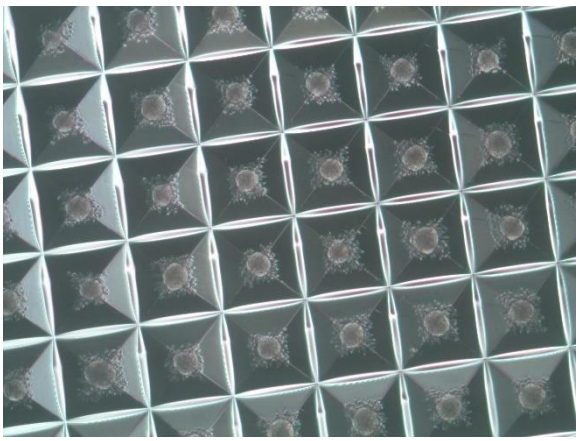
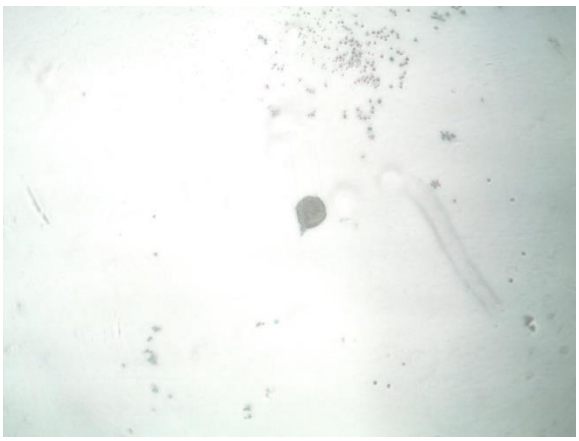
4.1.3. Apical-out airway organoid

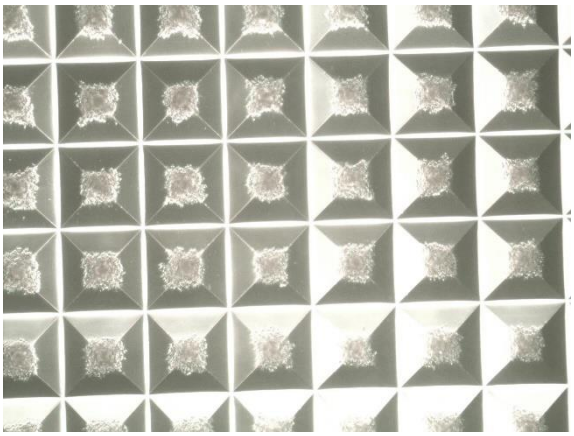
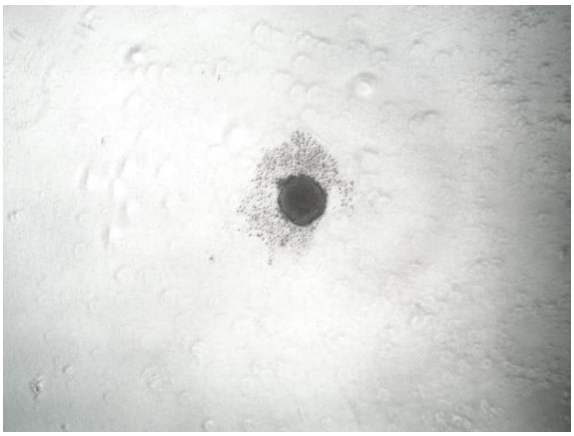
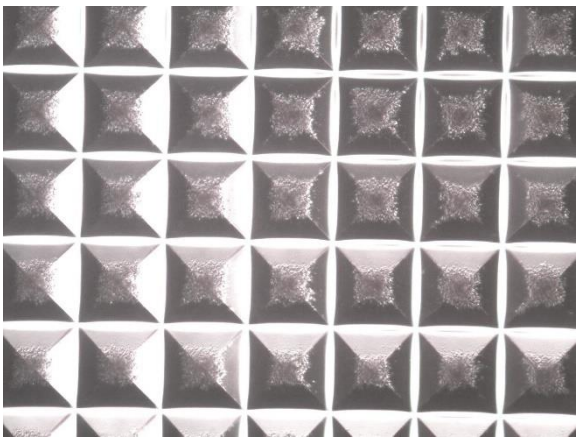
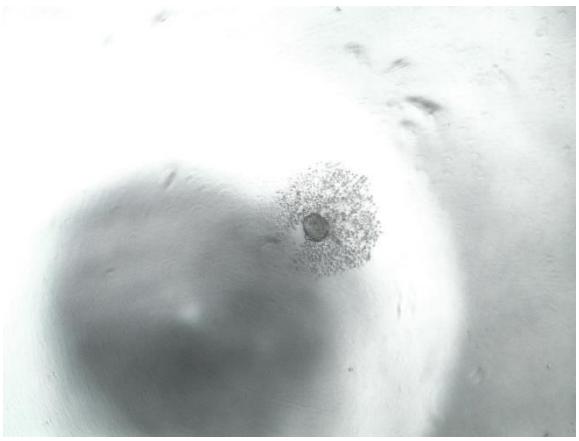
The ME epithelial cells did not undergo differentiation at ALI, which led to a new method to promote airway epithelium differentiation being assessed. PneumaCult™ Apical-Out Airway Organoid system (ApAO) is designed to generate ECM-free organoids that possess an apical side exposed to the environment. These airway organoids can replicate the polar organisation and functions of *in vivo* tissues, as previously shown by Stroulios *et al* (2022), using human bronchial epithelial cells. [258] ME cells were seeded onto specialised plates (AggreWell™400) that possess inverted pyramid sections allowing for the formation of individual spheroids, with low possibility of clumping during cell aggregation; centrifugation of the plates allows for even distribution of cells per section, leading to the formation of spheroids with similar number of cells and size.

Table 21 - Timeline of the development of ApAO models utilising hMEEC-1 and MEEC in AggreWell™400 plates. hMEEC-1 and MEEC were seeded into AggreWell™400 24-well plates and left to aggregate for 48 hours (aggregation period). Three different culture conditions were then assessed for the promotion of organoid differentiation. Firstly, half the spheroids were transferred to a 24 well plate pre-treated with anti-adherence solution. After 24 hours, fusion of spheroids was visible, and after 48 hours all the spheroids had fused together into a single clump. In the following conditions, cells were either left growing in the AggreWell™400 or plated into pre-treated 96 well plate at a density of 1 spheroid per well. In both cases, although the spheroids were able to maintain their shape 48 hours after the aggregation period, at 120 hours post aggregation there was visible cell shedding from the spheroids and loss of shape.

Seeding in AggreWell™400 24-well plates	
 hMEEC-1 (4x)	 hMEEC-1 (10x)
 MEEC (4x)	 MEEC (10x)

24 hours into aggregation period	
 hMEEC-1 4x	 hMEEC-1 10x
 MEEC 4x	 MEEC 10x

48 hours post aggregation period	
 hMEEC-1 in 24 well plate	 MEEC in 24 well plate
 hMEEC-1 in AggreWell™400 24-well plates	 hMEEC-1 in 96 well plate
 MEEC in AggreWell™400 24-well plates	 MEEC in 96 well plate

120 hours post aggregation period	
 hMEEC-1 in AggreWell™400 24-well plates	 hMEEC-1 in 96 well plate
 MEEC in AggreWell™400 24-well plates	 MEEC in 96 well plate

4.1.4. Senescence of primary middle ear cells and lifespan extension with BMI-1

Primary cells only have the capacity to undergo a limited number of passages and thus have a shorter lifespan than immortalised cells. As mentioned previously, Moon *et al* (2002) have reported that primary ME epithelial cells lose their phenotype around P4 or 5. [128] Thus, due to concerns of senescence in MEEC due to high passage or cell doubling, were evaluated the primary cells for production of β-Galactosidase, an enzyme that is overexpressed and accumulates in senescent cells. [259] In an attempt to extend the lifespan of the primary ME cells, they were transfected with a plasmid carrying *Bmi-1*, as previously described by Munye *et al* (2017). [252]

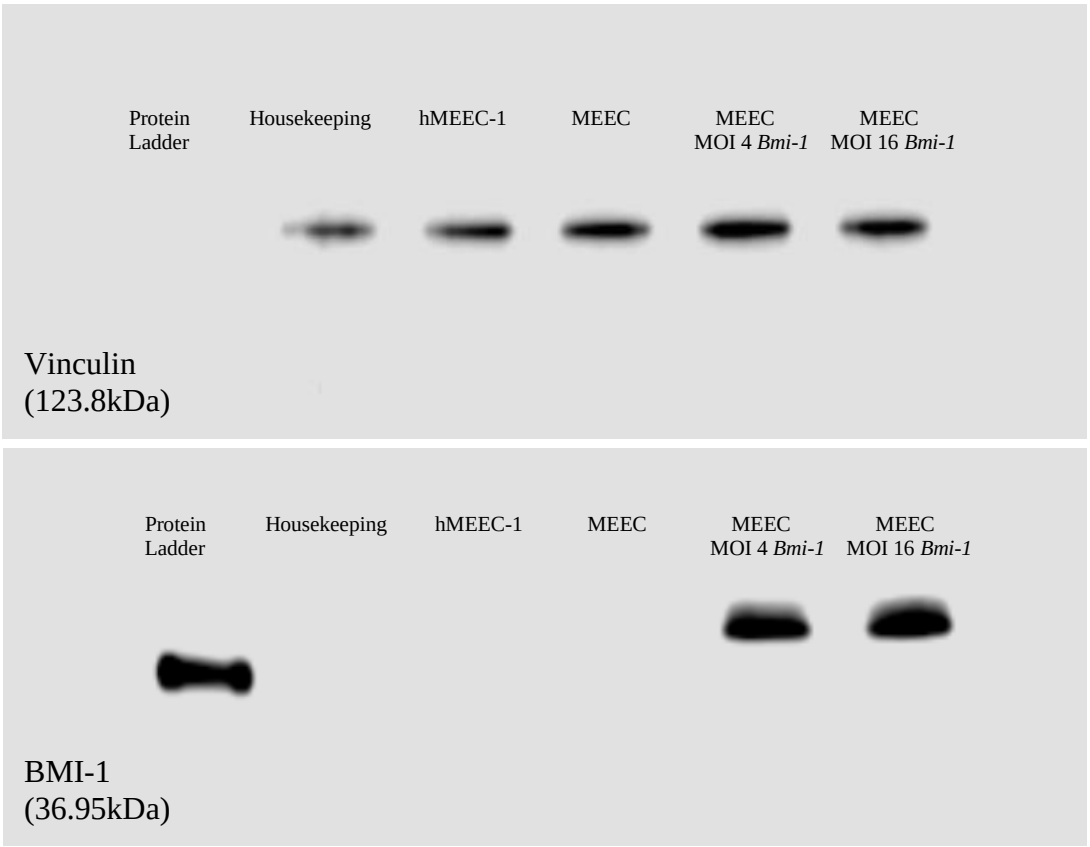


Figure 46 - Western blot to detect presence of BMI-1 in cells transfected with lentivirus particles carrying the gene, at an MOI of 4 or 16. Vinculin was utilised as housekeeping control.

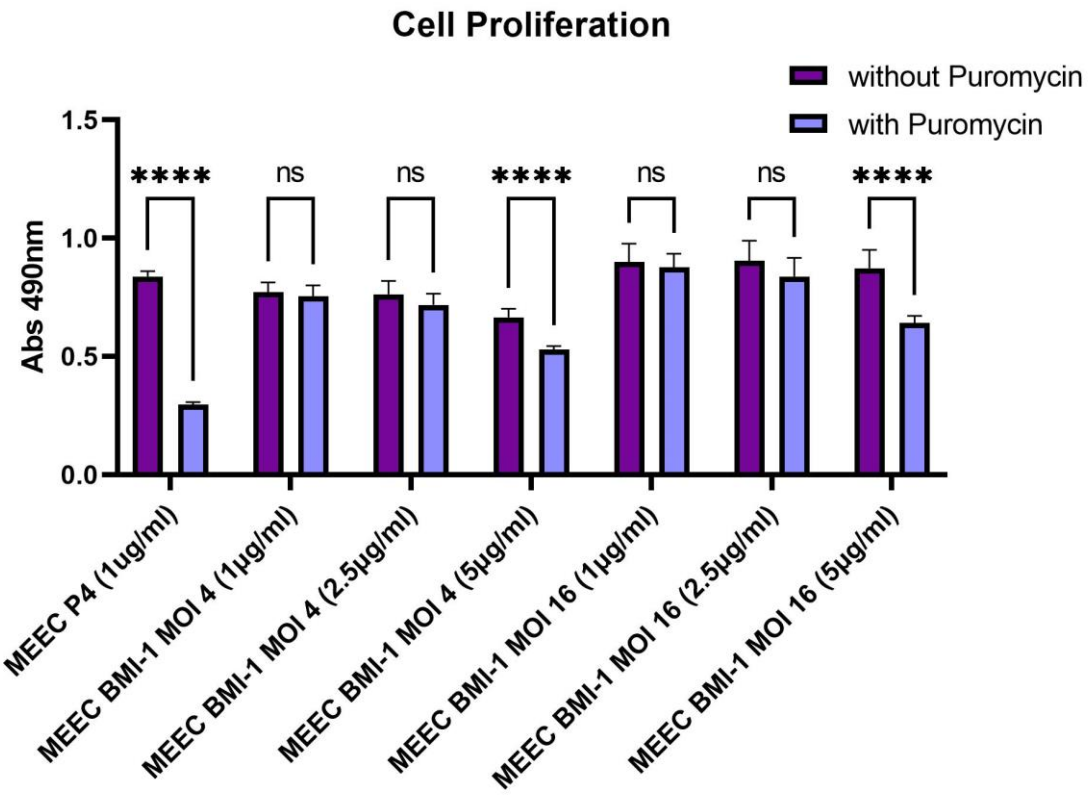


Figure 47 - Comparison of cell proliferation rates in response to puromycin treatment assessed using CellTiter 96® AQueous One Solution Cell Proliferation Assay. Cells overexpressing BMI-1 had previously been exposed to puromycin for 14 days. MEEC P4 (1µg/ml), MEEC BMI-1 MOI 4 and MOI 16 (5µg/ml) show significant changes in their proliferation levels when exposed to puromycin. (n=3, Two-way ANOVA)

To confirm effectiveness of the transfection with the plasmid carrying *Bmi-1* into MEEC, overexpression of BMI-1 was confirmed through Western blot. Furthermore, as the plasmid carries a cassette for puromycin resistance, cells were selected using to compound, and their proliferation in the presence and absence of puromycin was evaluated (Figure 46 and 47).

To quantify the cytotoxic effects of senescence and cisplatin treatment in ME epithelial cells, a lactate dehydrogenase (LDH) assay was performed. LDH is a cytosolic enzyme that is release into the medium upon damage to the cell membrane. Cisplatin is typically used as a chemotherapeutic drug that is highly toxic to cells and has been shown to induce senescence in normal cells (Figure 48). [260]

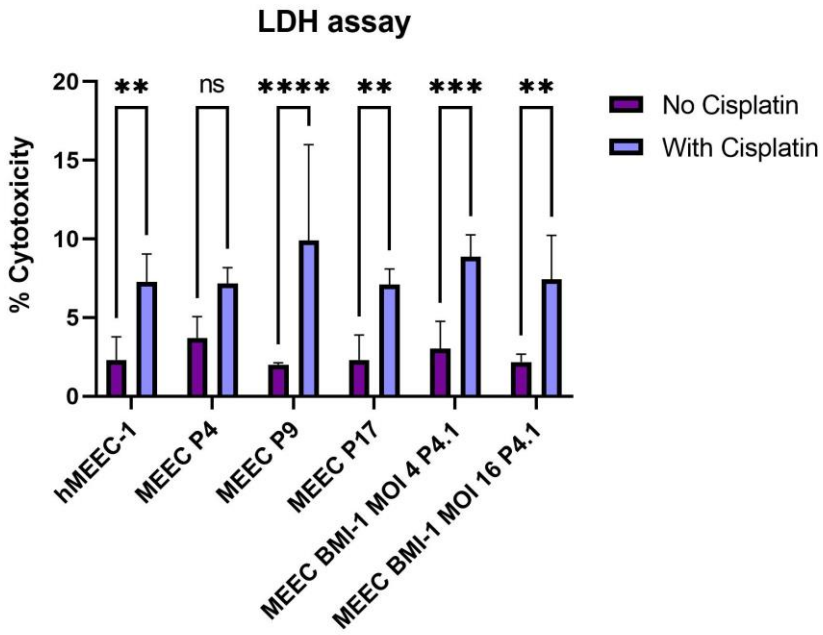


Figure 48 - LDH assay quantifying effects of cisplatin treatment in cell death. Cisplatin treatment increase cell death up to 10% at its most cytotoxic effect. (n=3, Two-Way ANOVA)

Cisplatin treatment significantly increased cell death in ME epithelial cells, except for those at P4. A 10% cytotoxicity rate indicates that at least 90% of cells are healthy enough and thus a senescence associated β-Galactosidase assay can be performed.

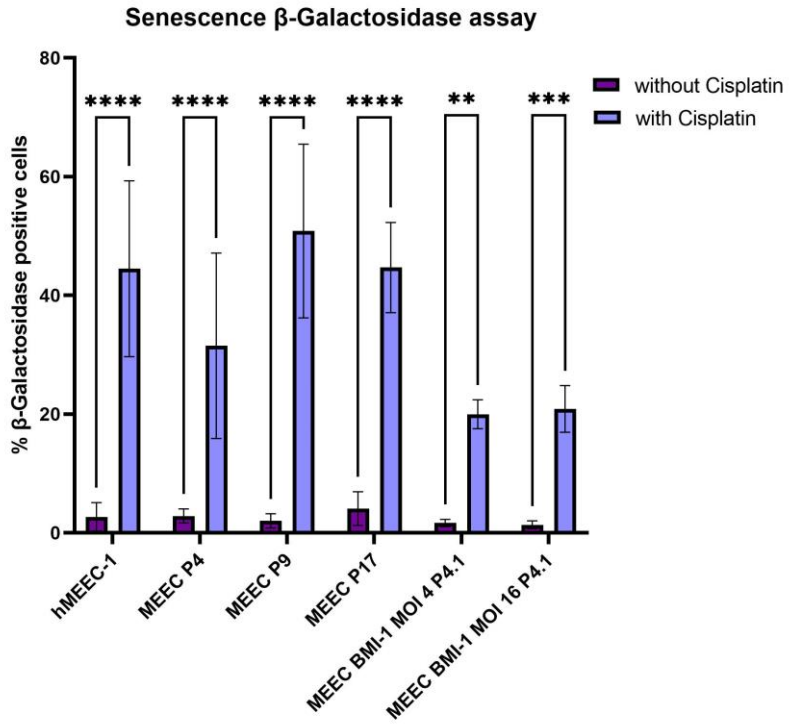


Figure 49 - Senescence β-Galactosidase assay to assess levels of senescence of MEEC at high passage. Comparison between cisplatin treated only and untreated only cells. (n=3, RM, One-Way ANOVA)

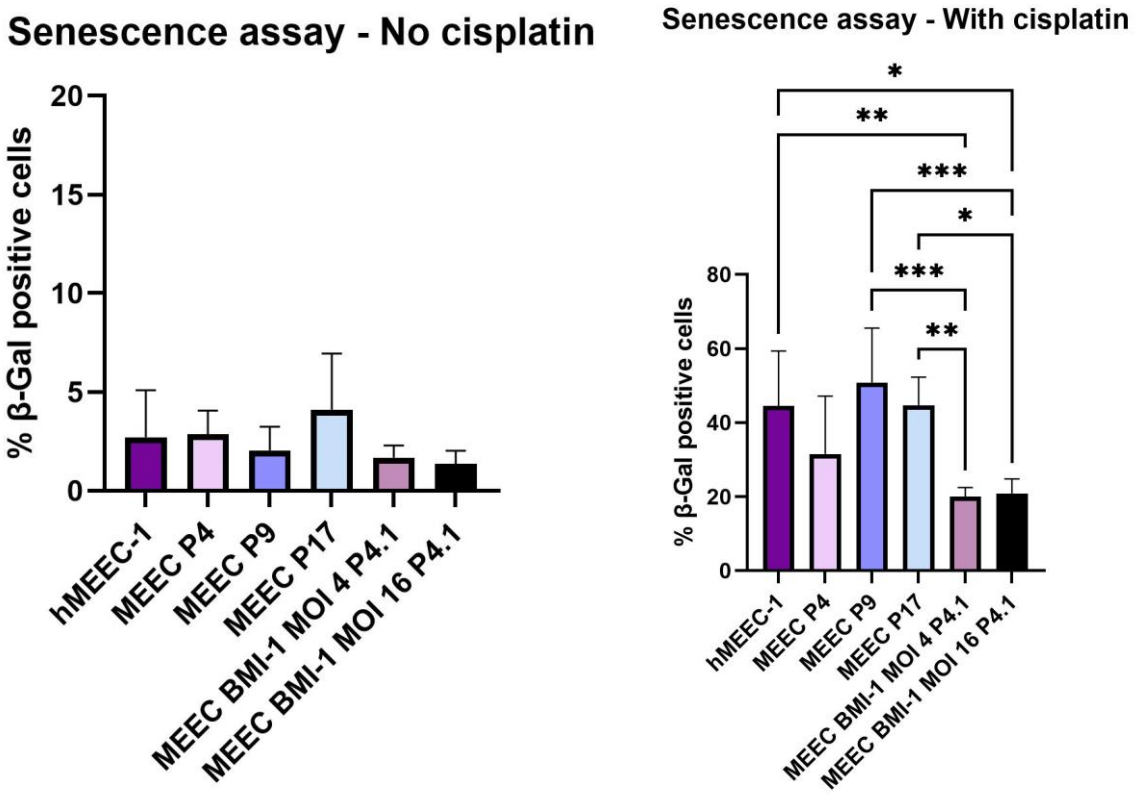


Figure 50 - Senescence β-Galactosidase assay to assess levels of senescence of MEEC at high passage. Comparison between cisplatin treated and untreated cells of the same type. (n=3, Two-Way ANOVA)

No significant senescence levels were observed in the untreated cells compared to the cells treated with cisplatin. Furthermore, untreated MEEC P17, cells at a relatively late passage, did not demonstrate significant senescence levels when compared to earlier passages. Interestingly, in cisplatin treated cells, MEEC that carried *Bmi-1* demonstrated higher resistance to the treatment than did hMEEC-1, MEEC P9 and P17 (Figure 49, 50 and 51).

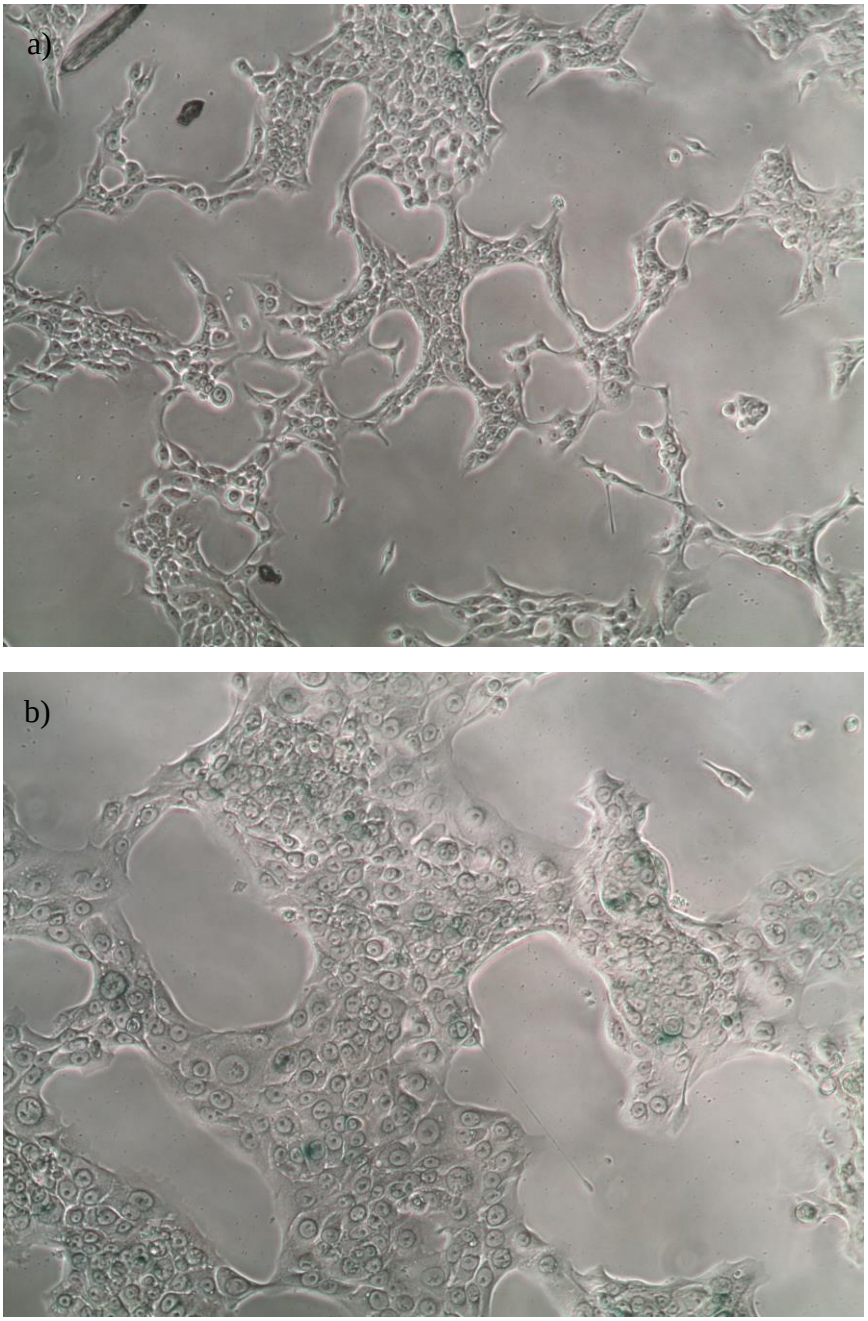


Figure 51 - Observation of cisplatin treated and untreated MEEC P4. Cisplatin treated cells are flatter and wider than untreated cells. Cisplatin induces senescence. Senescent cells stain blue due to the elevated presence of senescence-associated β-galactosidase. a) MEEC P4 without cisplatin b) MEEC P4 with cisplatin.

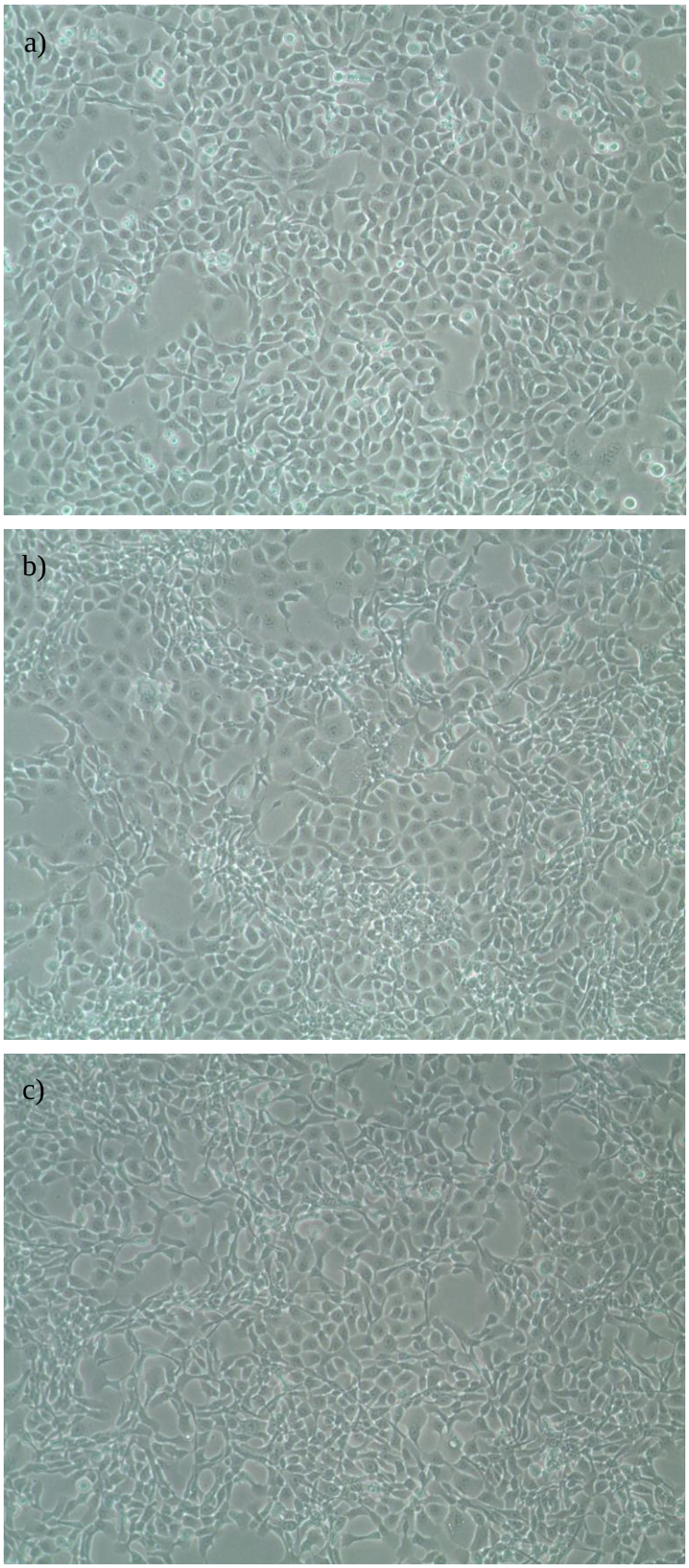


Figure 52 - Observation of MEEC at early passages, and comparison to BMI-1 transfected and non-transfected cells at late passage. a) MEEC P4 b) MEEC P35 c) MEEC BMI-1 P4.31

4.2. Discussion

4.2.1. Analysis of characterisation of hMEEC-1 and primary ME epithelial cells

Cell lines, such as hMEEC-1, are extremely useful for research and investigation of cell and molecular biology due to their extended lifespan and reduced variability, but often they do not accurately represent the *in vivo* cell phenotype and genotype, and thus molecular responses, which can be provided by primary cells. Furthermore, in the particular case of OM, a widely paediatric disease, a cell line such as hMEEC-1, obtained from an adult patient, may not accurately represent the response and development of the disease seen in paediatric cells. Despite this knowledge, hMEEC-1 has been popularly used in research associated with human OM.

Moon *et al* (2002) analysis of the immortalisation of hMEEC-1 included solely the immunolabelling of ME cells for von Wille-brandfactor VIII, an endothelial cell marker, pancytokeratin, an epithelial cell marker and vimentin, a mesenchymal cell marker. Unsurprisingly, no endothelial cell markers were present, but the authors also did not report presence of vimentin in both their primary ME epithelial cells and hMEEC-1, unlike our results. This suggests that changes to vimentin expression might have occurred at a later stage after the transformation. In agreement with our data is the expression of cytokeratin. [128] According to our results, the cell cultures isolated in this research project (MEEC and MixPop) present similar transcriptomic profiles to our analysis of hMEEC-1 (Figure 40). The collective expression of the above markers, particularly the epithelial cell markers, is a strong indicator that the cells isolated from the fetal middle ears are middle ear epithelial cells, and will provide a reliable tool in the understanding of the pathogenesis of OM. Further, I hypothesise that the cells will be able to differentiate into a pseudostratified epithelium and construct ALI-models that mimic the ME.

Despite their appearance, the MSC-like cells did not express *Nanog*, a stem cell marker. The strong transcription of *Kit* alongside *Krt5*, may indicate that, although these cells are not stem cells and may be further along their epithelial differentiation process, but still retain some pluripotency levels (Figure 41).

Furthermore, expression of PAFr in both hMEEC-1 and MEEC populations deems

these cells adequate for studies involving the role of this receptor in the pathogenesis of OM (Figure 42 and 43).

4.2.2. Analysis of Air-liquid interface and Apical-out airway organoid cell models

The ME cell population is a pseudostratified epithelium, with goblet and ciliated cells, as well as a squamous epithelium. In order to accurately investigate OM and its progression it is essential to have a representative and reproducible tool that is widely available. Despite being the only ME epithelium cell line available, there are no reports of hMEEC-1 functionality at ALI. Previously, models have been developed utilising primary murine ME cells, and human fetal ME cells, which limits the wide-scale reproducibility of the model. The models that have been previously constructed have either utilised ME cell populations from patients undergoing ear surgeries that had already been differentiated *in vivo* into the different subtypes found in the squamous and pseudostratified ME, but initially undifferentiated *in vitro*, or whole cell populations from the fetal ME that have not been selected as exclusively middle ear epithelial cells. Although it could be argued that maintaining the whole cell population would be beneficial for differentiation purposes as it would allow signalling from different subtypes to contribute to the process, the decision to pursue exclusive ME epithelial cell models was made in an attempt to ensure no fibroblast contamination would take place, as it would have potentially interfered with differentiation of epithelial models or limited the proliferation of epithelial cells. Exclusive epithelial populations could allow for a more specific investigation of the role of these cells during disease. Furthermore, observation of the epithelial populations from previous models appear to present initially with homogenous undifferentiated cell populations.

Unlike post-natal cells, fetal cells present high expansion rates, as observed in this project. Whereas Moon *et al* (2002) reported changes in the phenotype and proliferative ability of the primary ME cells that they later immortalised as early as passage 4 or 5, the MEEC recovered during this project seem to present high proliferative rates and minimal phenotypic changes at later passage. Based on these comparisons, fetal ME cells could prove to be a useful tool in the research of OM. [128,261] Furthermore, Mather *et al* (2021) have previously reported the ability of fetal ME cell populations to differentiate at ALI, producing an accurate mimic of

the ME. [142] However, although it was reported that exposure to air at regular humidity levels would be sufficient to induce differentiation in ME epithelial cells, these results could not be replicated in my research project. As seen in the timeline progression of the cells incubated with MSC-like, once exposed to air, hMEEC-1 and MEEC did not differentiate, but rather continue to proliferate, layering on top of each other, crowding the insert. Although it would be expected that these cells would be contact inhibited, evidence suggests that is not the case. It is noteworthy that even during cell expansion in flasks, the cells tended to detach from the surface after confluency, often requiring up to 2 passages per week. This lack of contact inhibition may be affecting the ability of cells to differentiate.

As PneumaCult ExPlus and ALI are proprietary media, their composition is unknown. Despite advertising from the company suggesting that ALI differentiation is facilitated with the use of these media, I was unable to reproduce this effect during my research project. Therefore, I tested additional conditions that could promote differentiation. In the airways mesenchymal cells, such as fibroblasts, modulate the epithelium function. The addition of fibroblasts to the basal chamber was an attempt of cell co-culture to promote differentiation in the apical chamber through the fibroblast secretion of soluble factors, as previously shown by Abs *et al* (2019). [262] Furthermore, Cozens *et al* (2018) and Ng-Blichfeldt *et al* (2018) have previously shown that RA also promotes differentiation of airway epithelium. [263,264] Calcium chloride channels have also been shown to modulate mucin secretion in the airways, and the supplement is widely used in the differentiation of models that utilise keratocytes, thus a condition with the addition of calcium chloride was also attempted. [265,266] Addition of MSC-like cells to the MEEC attempted to restore the initial population, MixPop, as the differential separation of cells could have hindered differentiation signalling, hence why the desired phenotype was not being obtained (Figure 44). However, the same proliferative and layering effect was reported across all conditions.

On a molecular level, MUC5AC and MUC5B are cell markers for goblet and secretory cells, whereas TEKT-1 is a cell marker for ciliation. Whereas *Muc5b* transcription is detected inconsistently, *Muc5ac* and *TEKT-1* transcription is not found, emphasising the lack of differentiation across conditions (Figure 45). However, Espahbodi *et al* (2019) have reported MUC5B expression in ME epithelial cells, including hMEEC-1, in 2D-cultures. [141] MUC5B expression increases upon stimulation, but basal levels of the mucin can be found early on, suggesting that MUC5B may not be a differentiation marker, and the presence of its transcription does not correlate with ALI differentiation. The detection of *Bpifa1* transcription

reinforces the origin of the cells as ME epithelium cells, as BPIFA1 is a common antimicrobial agent secreted by the airway epithelium. Its transcription, however, seems to be independent of stimuli or differentiation, as it is present as early as D0. It is noteworthy that *Bpifa1* transcription seems to diminish with time progression, however, it is possible that this effect is actually a result of cell death that likely follows the layering and uncontrolled proliferation of the cells, rather than a reduction in the transcription of the gene.

ApAO models have recently been used to assess respiratory cilia motility, viral infection and drug screening, by Wijesekara *et al* (2022) and Stroulios *et al* (2022), respectively. [258,267] Thus it was hypothesised that these innovative organoids could be utilised for the differentiation of other respiratory cells, such as the ME epithelium, and subsequently assessment of these organoids' response to infection. However, upon transferring the newly formed spheroids out of the AggreWell plates into 24 well plates, they would promptly clump into a large cluster. To avoid this, spheroids were transferred into a 96-well plate, at a density of 1 spheroid per well. However, instead of differentiating, spheroids would continuously shed cells, reducing the size of the spheroid until the structure disintegrated (Table 21).

This project did not achieve its goal of producing a ME epithelium model.

4.2.3. Analysis of senescence of primary middle ear cells and lifespan extension with BMI-1

Long cellular lifespans are a focal point in reproducibility of research and due to their naturally extended lifespan, fetal epithelial cells could provide a useful tool for investigation of cellular behaviour. Unlike previous studies, the MEEC collected for this project did not demonstrate evident phenotypical changes, and their proliferation rate was not affected beyond passage 4. As seen, MEEC at P17 did not present significant levels of senescence, compared to earlier passages and hMEEC-1. Due to time constraints, it was not possible to evaluate the levels of senescence of cells at P35, however, phenotypically, minor changes in the cell population were visible (Figure 52), potentially defining the limit for MEEC use to 30 passages.

Bmi-1 is an oncogene that mediates gene silencing through regulation of chromatin structures and the protein is vital for self-renewal of normal and cancer stem cells. [268] Although insertion of *Bmi-1* in MEEC might not directly lead to lifespan extension, its presence

positively correlated with resistance to senescence following cisplatin treatment (Figure 49 and 50). This effect has previously been reported in cancer patients undergoing chemotherapy treatment, where overexpression of BMI-1 inhibits cisplatin action. [269,270] Mechanistically, BMI-1 interacts with p53, leading to a molecular cascade that results in enhanced drug efflux and chemoresistance of cancer cells. Thus, although insertion of *Bmi-1* might not extend the cells proliferation time and capacity, it may contribute to maintaining healthy phenotypes and enabling usage of later passage cells in OM-related research.

In conclusion, MEEC expresses cell markers similar to those found in hMEEC-1, suggesting these cells might be a useful tool in the study of OM. Unlike hMEEC-1, MEEC have not been genetically modified, and they are expected to respond to stimuli differently. Although MEEC seem to offer naturally extended lifespans, MEEC overexpressing BMI-1 might provide more stable longevity, preventing defects in cellular replication and DNA damage. However, I did not evaluate the long-term effects of BMI-1 overexpression or its effects in response to stimuli. Surprisingly, MEEC did not differentiate at ALI, despite multiple condition attempts. The reason as to why this did not occur is unknown, but I speculate it is related to a lack of contact inhibition by the cells.

5. Chapter 5 - Intracellular invasion of middle ear epithelial cells by NTHi

This chapter will focus on the assessment of NTHi's susceptibility to antibiotics, sensitivity of ME epithelial cells to antibiotics and the ability of NTHi to invade ME epithelial cells. To the best of our knowledge, if proven, this is the first report of NTHi invading the intracellular space of ME epithelial cells.

The methods utilised in this chapter can be referred back to in Materials and Methods, section 2.2.2., 2.2.3., 2.2.4., 2.2.5., 2.3.4..

5.1. Results

5.1.1. Disk diffusion assays

NTHi is a common pathogen of the respiratory tract, and consequently a plethora of antibiotics have been investigated for their effectiveness against the bacterium. Disk diffusion assays are a common diagnostic tool, used to determine bacteria susceptibility to a variety of antibiotics. If susceptible, bacteria do not grow in the area surrounding the disk infused with antibiotic, and the diameter of that area (zone of inhibition) defines the level of bacteria susceptibility to the antibiotic.

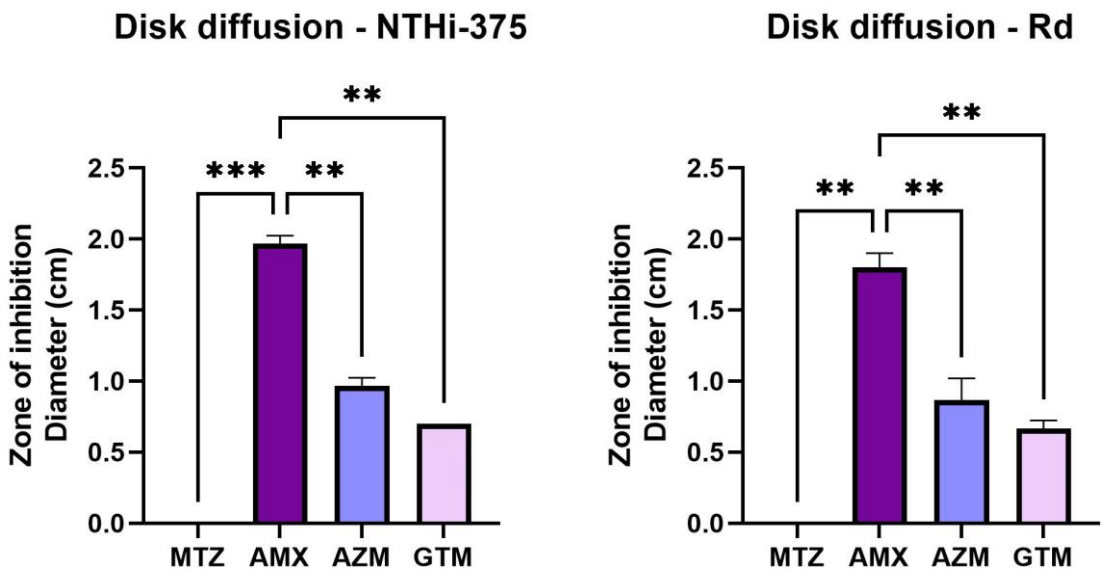


Figure 53 - NTHi-375 antibiotic susceptibility evaluation via disk diffusion assay. Blank disks loaded with 100µg/ml of antibiotics on chocolate agar plate streaked with NTHi-375 and NTHi Rd. MTZ: Metronidazole, AMX: amoxicillin, AZM: azithromycin, GTM: gentamicin. (n=3, RM One way ANOVA)

As seen, NTHi-375, an OM clinical isolate, and NTHi Rd, a laboratory strain, are susceptible to amoxicillin, azithromycin and gentamicin, at varying degrees, but not metronidazole. Amoxicillin is a beta-lactam antibiotic, that acts against both Gram-positive and negative bacteria, and it inhibits the biosynthesis of peptidoglycan and repair of the mucopeptide wall, by blocking the signalling of penicillin-binding proteins. Azithromycin is a macrolide antibiotic that inhibits bacterial protein synthesis by targeting the 50S subunit of the sensitive bacterial ribosome. Gentamicin is an aminoglycoside that binds to the 30s ribosomal subunit, impacting protein synthesis by blocking tRNA and mRNA's ability to bind. Metronidazole is a nitroimidazole antimicrobial that inhibits nucleic acid synthesis by nitroso radicals, disrupting bacterial DNA (Figure 53).

5.1.2. Minimal inhibitory concentration assay

Minimal inhibitory concentration (MIC) assays are used to define the optimal concentration of antimicrobial that will render the bacteria susceptible.

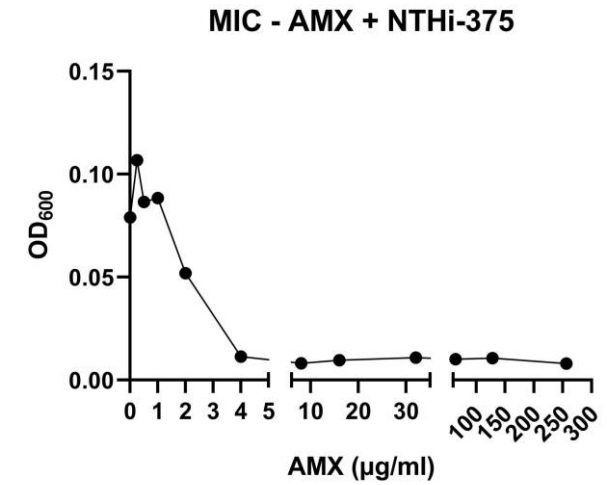


Figure 54 - MIC assay to assess NTHi-375 susceptibility to amoxicillin at various concentrations. NTHi-375 presents susceptibility to amoxicillin at 4µg/ml. (n=1)

NTHi-375 is susceptible to amoxicillin at concentrations as low as 4µg/ml, with growth being detected at concentrations of 2µg/ml of amoxicillin and lower. For the purposes of my study, and after a thorough literature review of NTHi growth and antibiotic protection assays, to ensure effective killing of bacteria a concentration of 200µg/ml was selected for further work (Figure 54).

5.1.3. Antibiotic selection with ME cells

To ensure antibiotic usage did not affect cell viability, hMEEC-1 were exposed to the three different antibiotics that NTHi presented susceptibility to in the disk diffusion assays, azithromycin, amoxicillin and gentamicin, at a concentration of 200µg/ml.

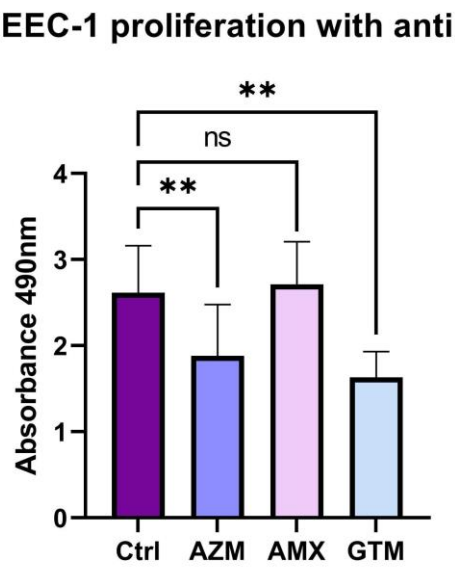


Figure 55 - Assessment of hMEEC-1 cell proliferation rates with different antibiotics (at a concentration of 200µg/ml) via CellTiter 96® AQueous One Solution Cell Proliferation Assay. hMEEC-1's survival was affected by the presence of azithromycin and gentamicin, but not amoxicillin. MTZ: Metronidazole, AMX: amoxicillin, AZM: azithromycin, GTM: gentamicin. (n=3, RM One way ANOVA).

Azithromycin and gentamicin negatively impacted cell viability and proliferation, however amoxicillin did not affect hMEEC-1 proliferation (Figure 55).

5.1.4. Antibiotic protection assays utilising NTHi-375

Antibiotic protection assays, or invasion assays, assess the number of bacteria that are able to invade the cells, evading the immune system and establishing intracellular infections, which are not easily targeted by antimicrobials. Invasion assays provide both information on the number of bacteria that adhere to the cell surface and the number of bacteria that invade the cell.

Initially, interactions between NTHi-375 and hMEEC-1 and MEEC were evaluated. To understand the role of PAFr in NTHi invasion, ABT-491, a PAFr antagonist, was added as a test condition, to block interactions between the bacteria and the receptor.

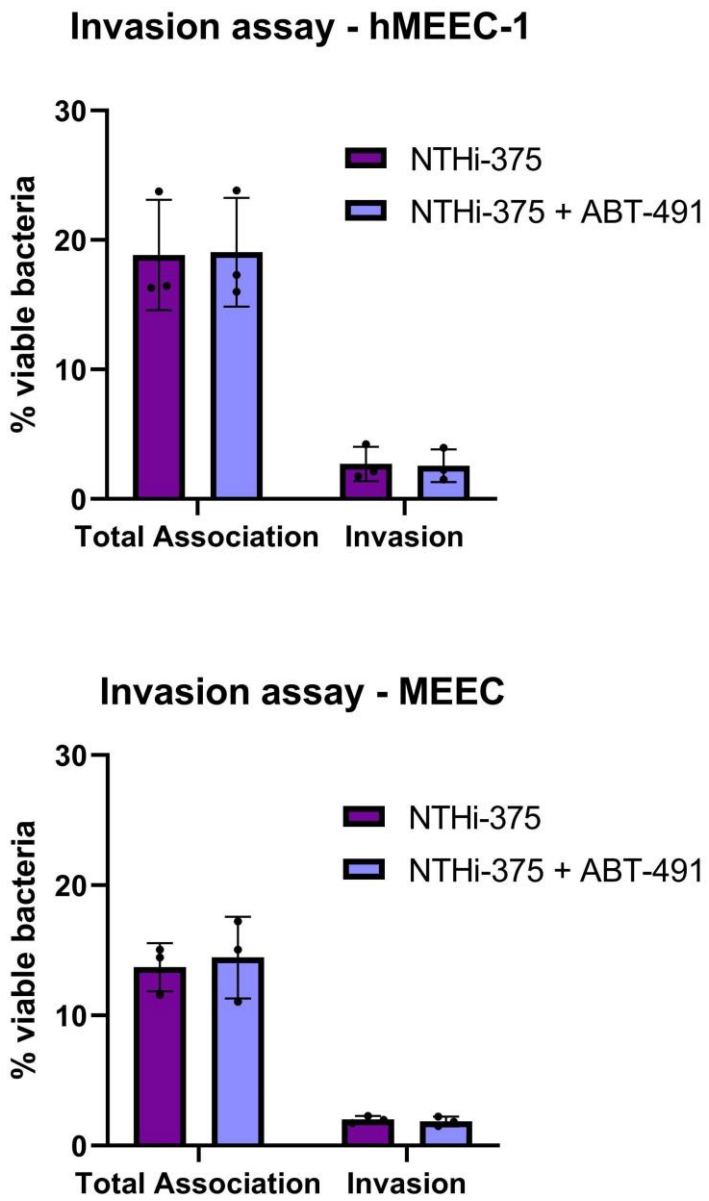


Figure 56 - Assessment of NTHi-375 MOI 100 invasion levels in infected hMEEC-1 and MEEC, treated with and without ABT-491. NTHi-375 invades ME epithelial cells, but invasion and adhesion are not affected by the presence of ABT-491. (n=3, Unpaired T-test).

To my knowledge, this is the first report of NTHi invading human middle ear epithelial cells. Although invasion is reported at high levels in both hMEEC-1 and MEEC (approximately 2%), no differences in invasion levels were observed in NTHi-375 adhesion or invasion between ABT-491 treated and untreated cells (Figure 56).

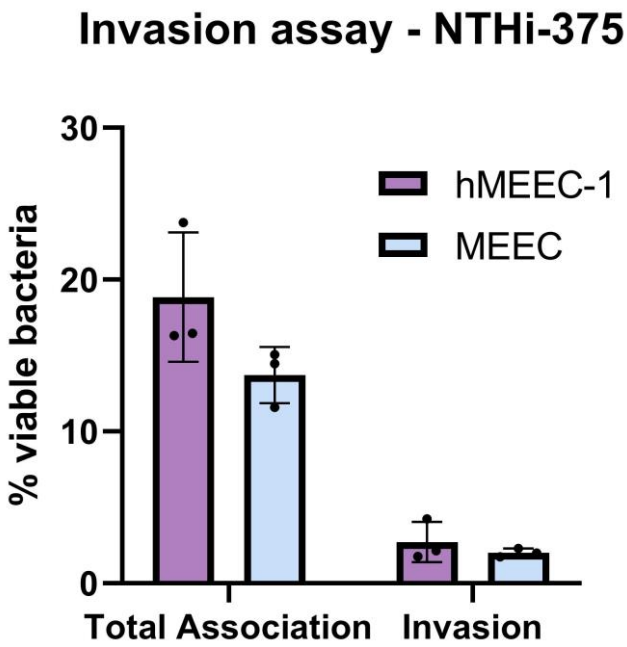


Figure 57 - Comparison of NTHi-375 MOI 100 invasion levels in infected hMEEC-1 and MEEC. NTHi-375 invades ME primary fetal cells and cell line at similar levels. (n=3, unpaired t-test)

Furthermore, no significant difference is seen between NTHi-375's ability to adhere or invade hMEEC-1 and MEEC (Figure 57).

As Iuchi *et al* (2019) had previously demonstrated that ABT-491 is effective in reducing NTHi invasion levels of tracheal epithelial cells, Detroit 562, my expectations were of a similar result, but this was not demonstrated with our ABT-491 treated ME epithelial cells. [271] In order to confirm and attempt to reproduce the result of Iuchi *et al* (2019), I carried out invasion assays on lower respiratory tract epithelial cells using KT cells, a bronchial epithelial cell line.

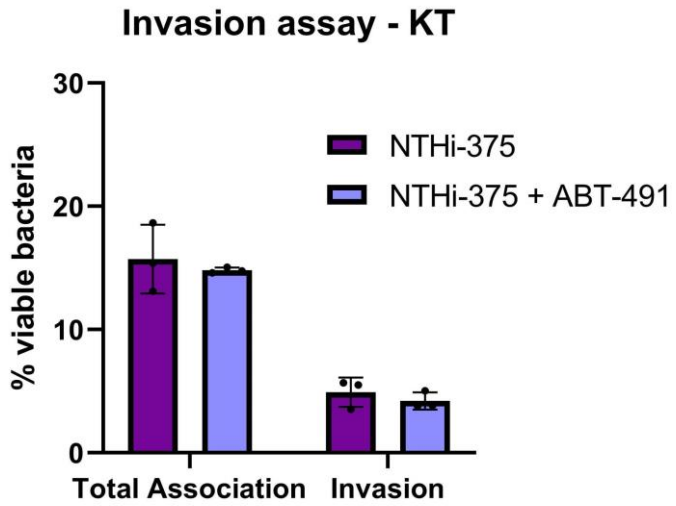


Figure 58 - Assessment of NTHi-375 MOI 100 invasion levels in infected KT cells, treated with and without ABT-491. NTHi-375 invades bronchial epithelial cells, but, again, invasion and adhesion are not affected by the presence of ABT-491. (n=3, Unpaired T-test).

Once again, no significant differences were observed in NTHi-375's ability to adhere and invade ABT-491 treated and untreated KT cells (Figure 58).

5.1.5. Antibiotic protection assays utilising Rd, H446, H457 and H491

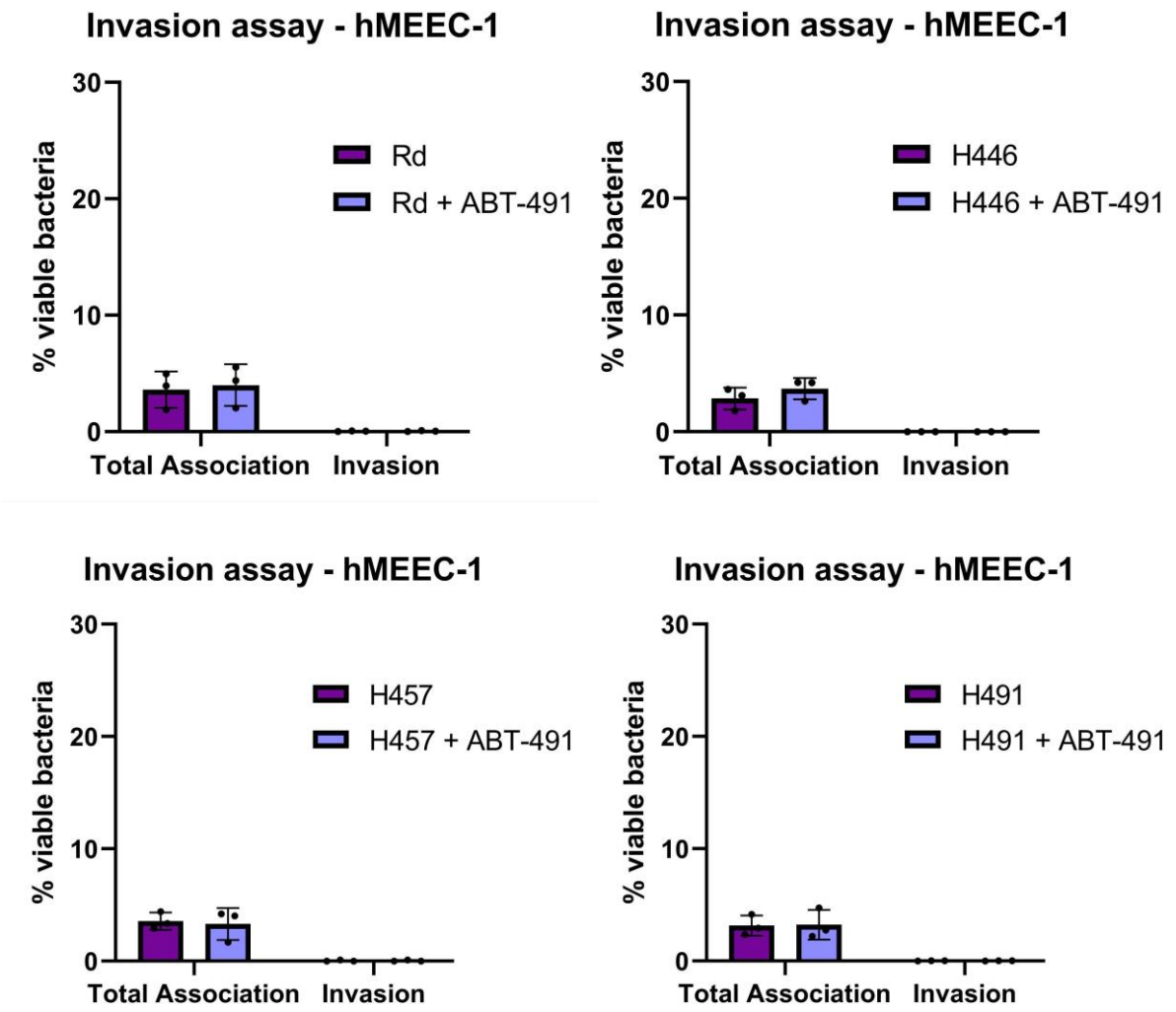


Figure 59 - Assessment of Rd, H446, H457, H491 MOI 100 invasion levels in infected hMEEC-1, treated with and without ABT-491. Rd and PCho strains do not invade ME epithelial cells, and adhesion is not affected by ABT-491 treatment. (n=3, Unpaired T-test).

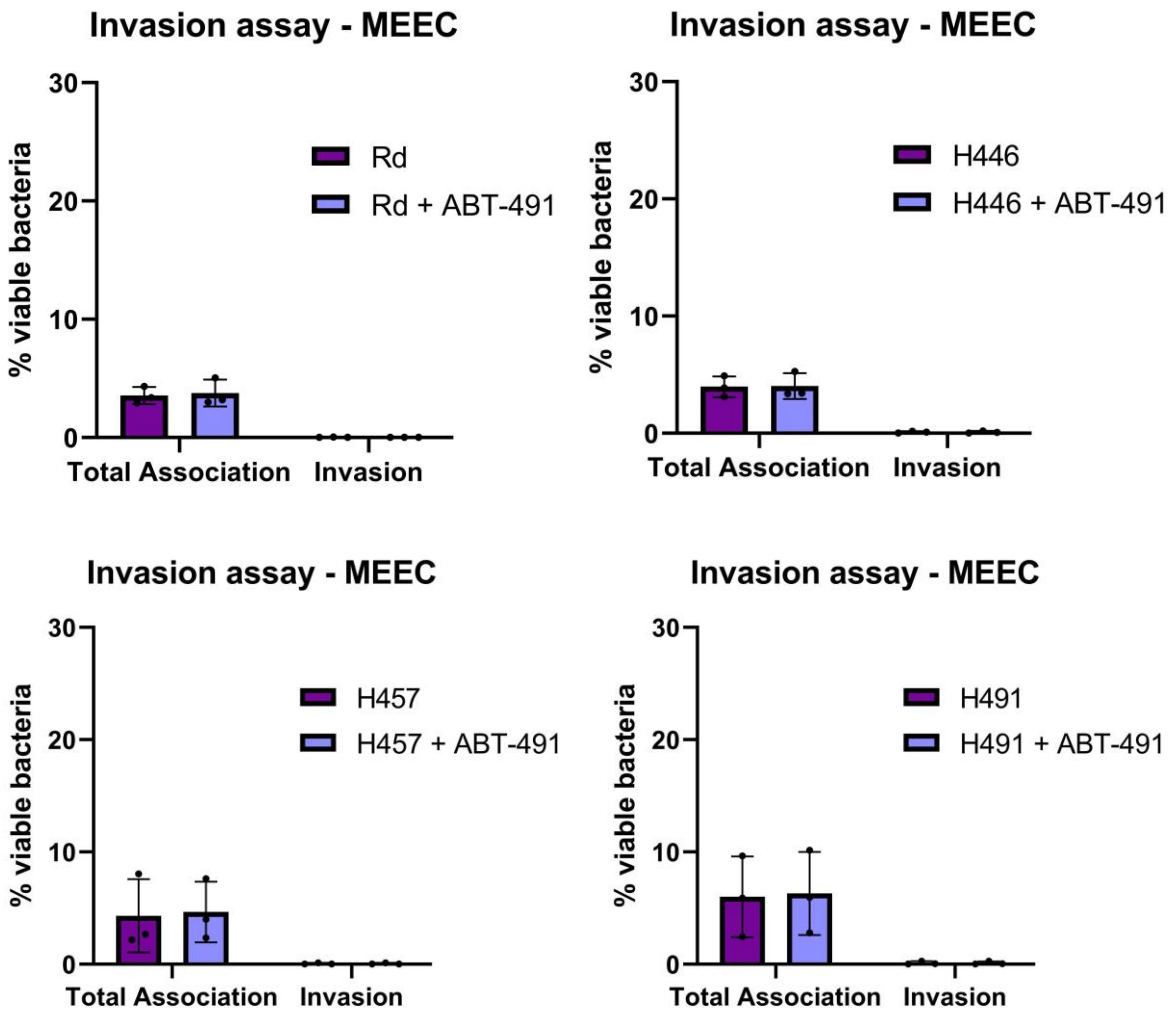


Figure 60 - Assessment of Rd, H446, H457, H491 MOI 100 invasion levels in infected MEEC, treated with and without ABT-491. Rd and PCho strains do not invade ME epithelial cells, and adhesion is not affected by ABT-491 treatment. (n=3, Unpaired T-test).

Unlike NTHi-375, Rd, H446, H457 and H491 did not invade either hMEEC-1 or MEEC. Furthermore, adhesion levels were lower than those seen with NTHi-375, at about 4-5% across all laboratory strains. Adhesion was not affected by ABT-491 treatment (Figure 59 and 60).

5.2. Discussion

5.2.1. Analysis of antibiotic use in non-typeable *Haemophilus influenzae* and middle ear epithelial cells

Azithromycin, amoxicillin and gentamicin show antimicrobial activity against NTHi-375. Azithromycin and amoxicillin are commonly used to treat middle ear infections, strep throat and pneumonia, whereas gentamicin is used to treat a wider range of bacterial infections, typically caused by Gram-negative coccobacilli, such as endocarditis, pelvic inflammatory disease, meningitis, pneumonia and urinary tract infections. Further, gentamicin has also been used to treat otitis externa and chronic suppurative OM. Metronidazole, however, is commonly used to treat anaerobic bacterial infections, such as gastrointestinal or reproductive system infections, and so antimicrobial activity against NTHi-375 would not be expected (Figure 53).

Krause *et al* (1982) reported that, in a study involving 19 patients diagnosed with COME between the ages of 1 to 12 years old, oral administration of amoxicillin at a dosage of 15mg/kg led to a mean peak level of 5.6μg/ml of amoxicillin in the ME effusions, thus it is positive to report that NTHi-375 susceptibility to amoxicillin at 4μg/ml would have presented clinically relevant results. [272] However, the decision to work with a high amoxicillin concentration, 200μg/ml, is due to the fact that whereas MIC testing took place over a period of days, the incubation in the antibiotic protection assay took place for an hour, and thus killing of all external bacteria in a short period of time was ensured by increased concentrations of the antibiotic (Figure 54).

hMEEC-1 were sensitive to azithromycin and gentamicin but not amoxicillin. As amoxicillin is reconstituted in PBS and widely used in the treatment of OM, it was predicted that it would not present a cytotoxic effect. hMEEC-1's sensitivity to azithromycin may result from the fact that the antibiotic is reconstituted utilising DMSO, a chemical solvent that has been reported to be cytotoxic at concentrations greater than 1%. Gentamicin's impact on cell viability was unexpected, as the antibiotic can be utilised to treat respiratory tract infections and was reconstituted with PBS. However, previous research has highlighted gentamicin as an ototoxic, vastly affecting inner ear hair cells and causing vestibular damage. This effect could extend to cells within the middle ear cavity. Therefore, all infection work was conducted

utilising amoxicillin (Figure 55).

5.2.2. Analysis of antibiotic protection assays utilising NTHi-375

NTHi possesses multiple mechanisms of infection of respiratory epithelial cells. One of these infection mechanisms relies on NTHi's PCho, a mimic of PAF expressed by bacteria that inhibit the human mucosa, and its ability to bind to PAFr, a surface receptor expressed on epithelial cells, and utilise it as a means to invade ME epithelial cells. The interactions between PAFr and PCho are common in the respiratory tract, thus the same could be possible in the ME. Whereas Pang *et al* (2008) have shown that the presence of PCho on NTHi's surface delayed clearance from the lungs, Kadowaki *et al* (2021), Hong *et al* (2007) and Fujita *et al* (2009) have shown that PCho's presence is associated with increased NTHi persistence in mice, chinchillas and children with OM. [191,273–275] Multiple mechanisms of action highlighting PCho's role in prolonged infection have been identified. Clark *et al* (2012) have linked PCho's presence to evasion of antibody tagging. As PCho is a positively charged molecule that alters the physical properties of the membrane, it reduces antibody accessibility and binding to NTHi's outer membrane. [224] Antibody binding elicits an immune response, rendering the bacteria susceptible to antibody-dependent classical pathway complement-mediated killing, thus if antibody binding is affected, so is bacterial killing. Furthermore, Hong *et al* (2007) and Krishnamurthy *et al* (2014), have shown that although PCho is not essential for biofilm formation, its presence leads to increased mass, thickness and maturation *in vivo* and *in vitro*. [276,277]

Biofilms are matrix-enclosed bacterial populations that adhere to each other and surfaces, forming a robust structure that presents antimicrobial resistance. This resistance may rise from different mechanisms, the antimicrobials might not be able to diffuse through the biofilm matrix or might be inactivated by polysaccharides present within the matrix. Biofilm formation also alters cellular growth rate. Slowing growth rates limits the uptake of antimicrobials present in the matrix, reducing their effectiveness. However, Puig *et al* (2014) noted that these studies were only performed on laboratory bacterial strains, and so tested the hypothesis on clinical isolates. Their research did not find a link between PCho presence and increased maturation of biofilm *in vitro*. [193]

To attest to the relevance of PAFr in ME epithelial cell invasion by NTHi, cells were treated with ABT-491, to block potential interactions between PAFr and PCho. Surprisingly, ABT-491 had no effect on adhesion or invasion of NTHi with hMEEC-1, MEEC or KT cells (Figure 56 and 58). As ABT-491 was added to the cell culture as part of the blocking solution (2% BSA in unsupplemented media), the effectiveness of ABT-491 binding was considered, however previous work by Albert *et al* (1997) presented strong evidence that ABT-491 is effective in binding to PAFr even in blood, demonstrating that the presence of high concentrations of proteins and other serum factors only slightly affects the ability of ABT-491 to interact with PAFr. [278] Thus an inefficient interaction between PAFr and ABT-491 was probably not the reason for the lack of difference in invasion levels between treated and untreated cells. This result is not consistent with previous studies, which showed that invasion of epithelial cells by bacterial species expressing high levels of PCho, including NTHi, is affected by ABT-491. However, as NTHi-375 is a clinical isolate with no induced modifications to its phasevarion, the PCho levels may be variable, and the bacterium might not need to rely on PAFr-PCho interactions to invade epithelial cells. NTHi can also invade epithelial cells through macropinocytosis, lipid raft-mediated endocytosis and clathrin-mediated endocytosis, or by binding its other adhesins (Hap, Protein D, and Protein E) to the respective cell receptors.

As previously mentioned, since expression levels of PCho are heavily reliant on the bacteria's phasevarion, NTHi-375 expression of PCho could be varied or inconsistent and of less relevance if the bacteria possess other mechanisms of invasion. To exclude variability caused by NTHi phasevarion, strains with varying levels of PCho expression were selected for invasion assays, including H446, H457, H491 and Rd, which is a laboratory strain, and the wild type of strains H446, H457 and H491. Previous reports show that the position of PCho on the NTHi LOS can impact binding to other proteins, such as C-reactive protein, thus both H457 and H491 were used in my study.

It is interesting to note that NTHi invades adult and paediatric cells similarly. Although Minami *et al* (2017) have identified that the ME microbiome of healthy children and adults is significantly different, their study did not allow for an analysis down to the species level. [83] Their research did, however, conclude that the *Proteobacteria* phylum is the most abundant phylum found in the healthy ME; NTHi is a part of this phylum. Furthermore, Jinhage *et al* (2021) and Revai *et al* (2008) have noted that the upper respiratory tract bacterial microbiome

is similar to the low levels of ME bacterial load. [34,279] In the upper respiratory tract, NTHi is carried by 20-50% of healthy children and 20-30% of healthy adults, and it is more likely to be found in the microbiome of children suffering from URTI than adults. Based on the findings by Jinhage *et* al (2021) and Revai *et al* (2008) it could be speculated that similar proportions of the bacterium from the nasopharynx are found in the ME, and higher representation of NTHi in the ME could be a contributing factor in the higher incidences of OM in children compared to adults. This hypothesis could be supported by the data collected during this project, as my results suggests that at similar starting MOIs, NTHi adherence and invasion rates are similar in ME cells from children and adults (Figure 57). Although, I have previously argued the need for *in vitro* OM-related research to be conducted in paediatric ME cells, intracellular invasion by NTHi does not seem to be altered by this fact, under these experimental conditions.

5.2.3. Analysis of antibiotic protection assays utilising Rd, H446, H457 and H491

Regardless of cell type none of the bacterial strains H446, H457, H491 or the wild-type, Rd, demonstrated invasive activity due to the "laboratory nature" of the strains. Laboratory strains are essential for a microbiologists' understanding of bacterial genetics and molecular processes, as they maintain genotypic and phenotypic characteristics, enhancing reproducibility, since they are less prone to mutations and environmental pressures. However, these strains often lack the ability to mimic the infectivity and pathogenesis seen in clinical isolates.

In addition to the lack of invasion, the adhesion levels reported for the laboratory strains were also considerably lower than that seen with NTHi-375. Although it has previously been reported by Lysenko *et al* (2002) that the position of PCho on NTHi can affect binding to different proteins, it was not possible, using these strains, to confirm the effect of PCho on binding to ME epithelial cell surface, due to their low virulence. [255] As previously mentioned, NTHi can utilise multiple mechanisms of invasion and similar numbers of viable bacteria have been shown to cross cells with or without functional PAFr being available. Branger *et al* (2004) used a NTHi pneumonia mouse model to demonstrate that the infection levels were not affected between PAFR−/− and WT mice. [280] Orihuela *et al* (2009) also showed that pneumococcal adherence is similar in PAFR−/− and WT mice. [281] Interestingly, McCullers and Rehg (2002)

showed that in mouse models with upregulated levels of PAFr, infection with *S. pneumoniae* and blocking of the receptor, actually led to increased bacterial titres in the lungs, showcasing alternative infection pathways for PCho-expressing bacteria. [282]

The potential for PCho to adhere to paediatric and adult cells differently was also considered. However, again there are no significant differences between bacteria expressing different levels of PCho and their ability to adhere to fetal or adult cells.

My study did not allow to address the efficacy of ABT-491 as a non-antibiotic antimicrobial, that should promote blockage of bacterial binding, prevent intracellular bacterial invasion and potentially bacterial persistence, and consequently lead to modulation of immune response. However, future studies should consider utilisation of alternative non-antibiotic antimicrobials as means of prevention and possibly treatment of OM. Molecules that target NTHi virulence factors, such as its surface receptors, preventing binding to host, or its phasevarion, targeting the bacteria's ability to adapt to the immune system's response, could facilitate bacterial clearance.

6. Chapter 6 - Middle ear epithelial cells response to infection by NTHi

This chapter will focus on the response of ME epithelial cells to infection by whole live bacteria, NTHi-375, H446, H457, H491 and Rd.

The methods utilised in this chapter can be referred back to in Materials and Methods, section 2.2.2., 2.2.6., 2.3.1., 2.3.2., 2.3.3., 2.3.9., 2.3.10..

6.1. Results

6.1.1. ME epithelium protein transcription in response to NTHi-375 infection

OM, as one of the most common paediatric diseases, has been widely investigated, however understanding of the disease process is still lacking. Reliable and accurate ME models are not widely available, and an extensive proportion of our current knowledge has resulted from animal models *in vivo*, which may not accurately translate into human therapeutics. Further, a significant number of studies focus on later stages of the disease, instead of its onset, by analysing ME effusion of paediatric patients or the bullae of animal models a number of days post-infection. Finally, a large volume of the work that has been conducted *in vitro*, in human cells, has extensively used hMEEC-1, which may not accurately represent paediatric *in vivo* responses; the *in vitro* research that evaluated primary fetal or paediatric ME cell responses describes only a limited number of factors at play.

The response of hMEEC-1 and MEEC to infection by NTHi-375, Rd and the PCho variants was evaluated at different timepoints, by assessing the transcription of *Muc5b*, through end-point PCR, and *Il-6* and *Il-8*, through qPCR.

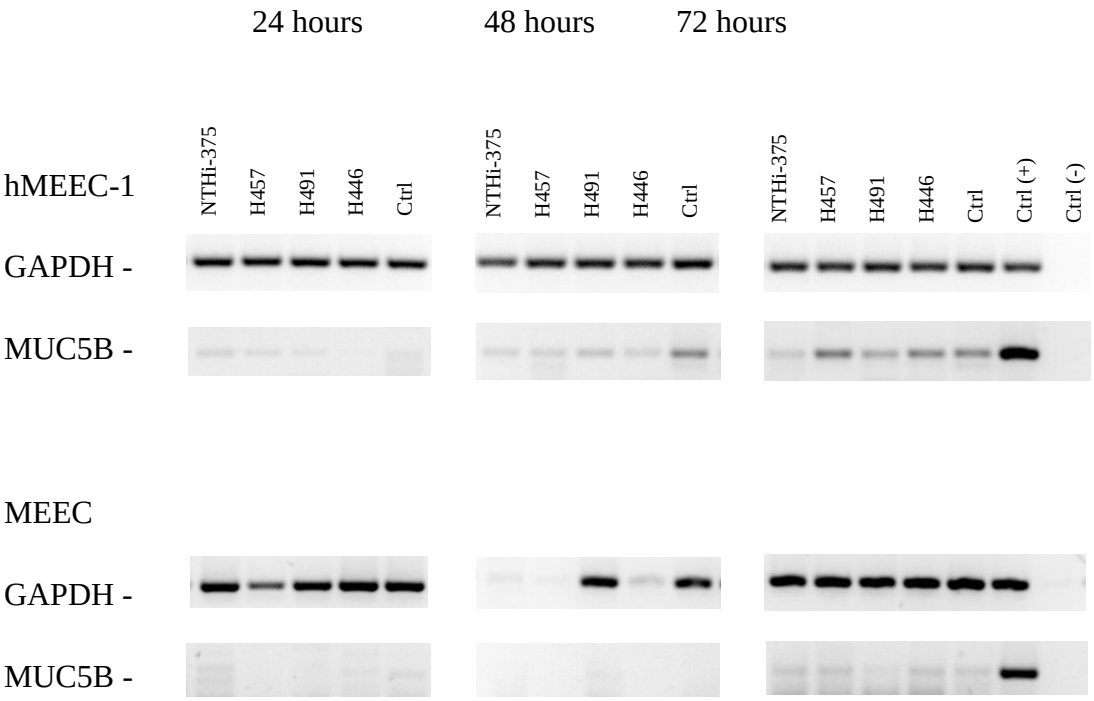


Figure 61 - hMEEC-1 and MEEC response to infection by NTHI-375, Rd and PCho strains, at 24, 48 and 72 hours. Transcription of *Muc5b* was evaluated through end-point PCR. NTHi-375, H457, H491, H446 is mRNA extracted from cells infected with bacterial strains, Ctrl is mRNA extracted from uninfected cells, Ctrl (+) is mRNA extracted from dissected fetal labial gland primary epithelial cells and Ctrl (-) is the negative control.

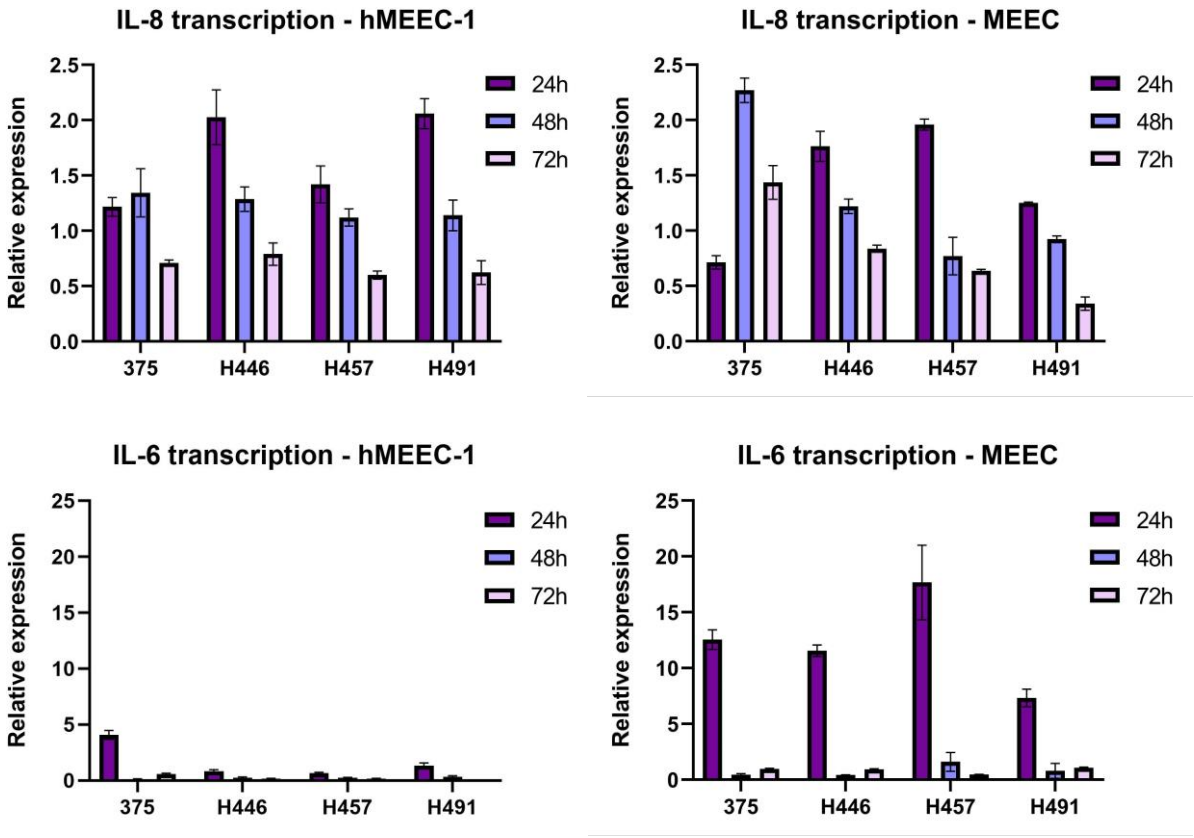


Figure 62 - hMEEC-1 and MEEC response to infection by NTHI-375, Rd and PCho strains (MOI 100), at 24, 48 and 72 hours. Transcription of *Il-8* and *Il-6* was evaluated through qPCR. qPCR relative expression was standardised to uninfected control, at 48 hours. (ct threshold = 0.04) (n=1)

MUC5B is transcribed in the ME epithelium, regardless of NTHi infection. The weaker transcription signal in MEEC at 48 hours was due to poor DNA quality, as extracted mRNA extraction was of low quality and quantity (Figure 61).

In both hMEEC-1 and MEEC, H446, H457 and H491 trigger peak IL-8 transcription at 24 hours (relative expression is 0.5 to 1 times more than untreated control), followed by a decrease in transcript levels at subsequent time points. However, the peak in transcription of IL-8 mRNA, following NTHi-375 infection, occurred at 48 hours. IL-6 transcription peaked at 24 hours, across all strains with both cell types. Although IL-8 transcription presents similar relative levels in stimulated cells to their respective controls, IL-6 transcription seems to be intensified in MEEC (Figure 62).

6.1.2. ME epithelium cytokine expression in response to NTHi-375 infection

After transcription levels for IL-6 and IL-8 had been assessed, it was necessary to also assess protein expression levels of hMEEC-1 and MEEC cells in response to infection with NTHi-375. Cells were infected with NTHi-375 at MOI of 10 and 100, and supernatants were collected for ELISA analysis at 1, 18 and 42 hours post-infection.

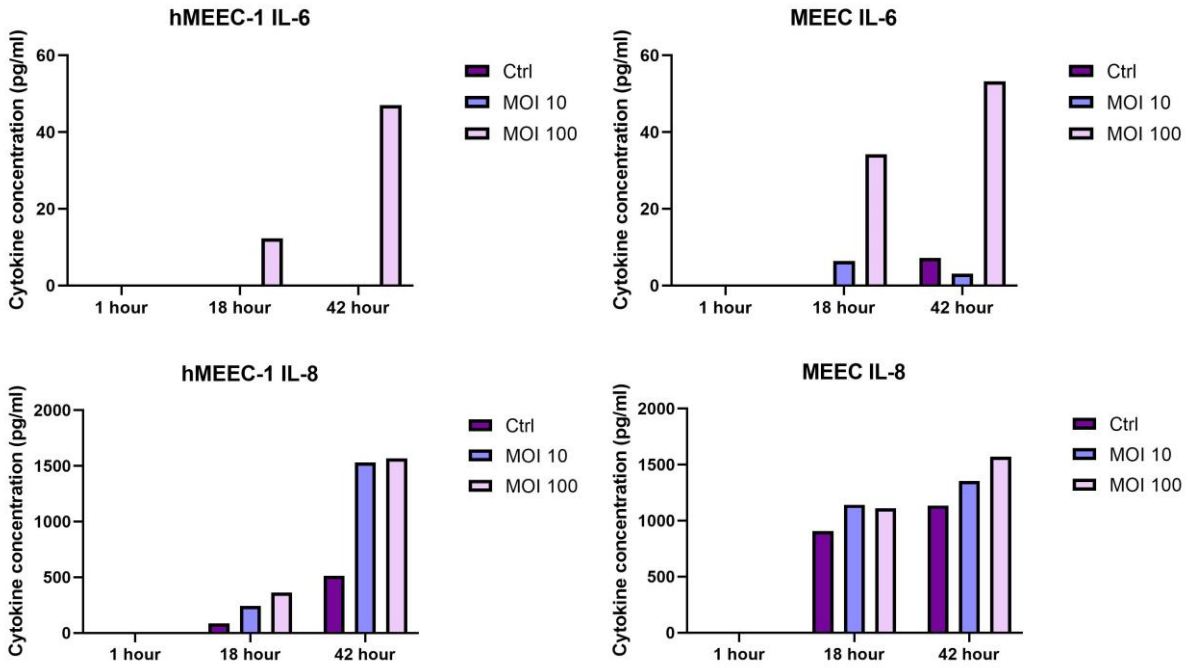


Figure 63 - hMEEC-1 and MEEC response to infection by NTHI-375, at MOI 10 and 100, at 1, 18 and 42 hours. Expression of IL-6 and IL-8 was evaluated through ELISA. (n=1)

Neither epithelial cell types expressed proinflammatory cytokines early, at 1 hour. IL-8 is expressed by both MEEC and hMEEC-1 in higher concentrations than IL-6. IL-6 and IL-8 were expressed by both hMEEC-1 and MEEC at 18 and 42 hours, following infection with NTHi-375 at MOI 100. As IL-6 expression was not triggered by NTHi-375 at an MOI of 10, future infections were conducted using MOI 100 (Figure 63).

Cytokine arrays were used to assess a wide plethora of immune effectors that might play a role in the pathogenesis and trigger of human OM with NTHi.

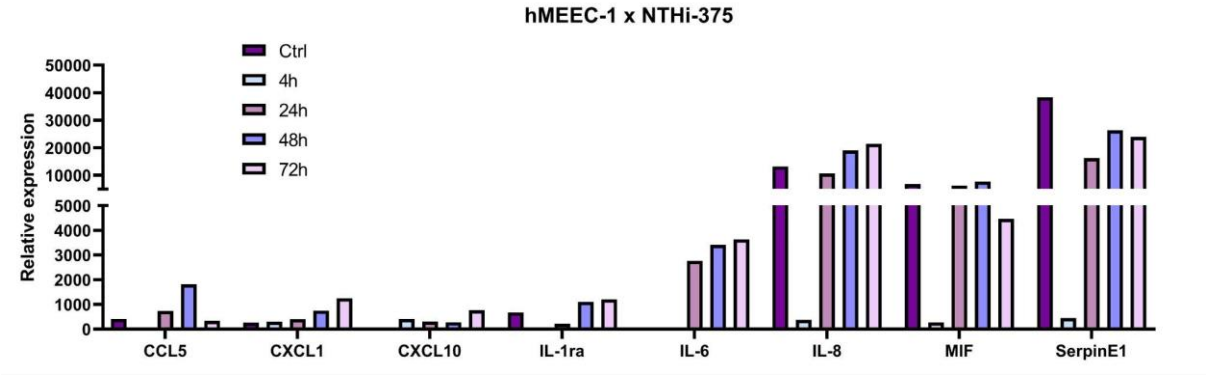


Figure 64 - hMEEC-1 response to infection by NTHI-375, at MOI 100, at 4, 24, 48 and 72 hours. Expression of cytokines was evaluated through Proteome Profiler Human Cytokine Array Kit. (n=1)

NTHi-375 infection of hMEEC-1 increased expression of basal levels of CCL5, CXCL1 and IL-1ra, and triggered expression of CXCL10 and IL-6. Peak expression of CCL5 and SerpinE1 by infected hMEEC-1 was seen at 48 hours, whereas all other cytokines increased chronologically (Figure 64).

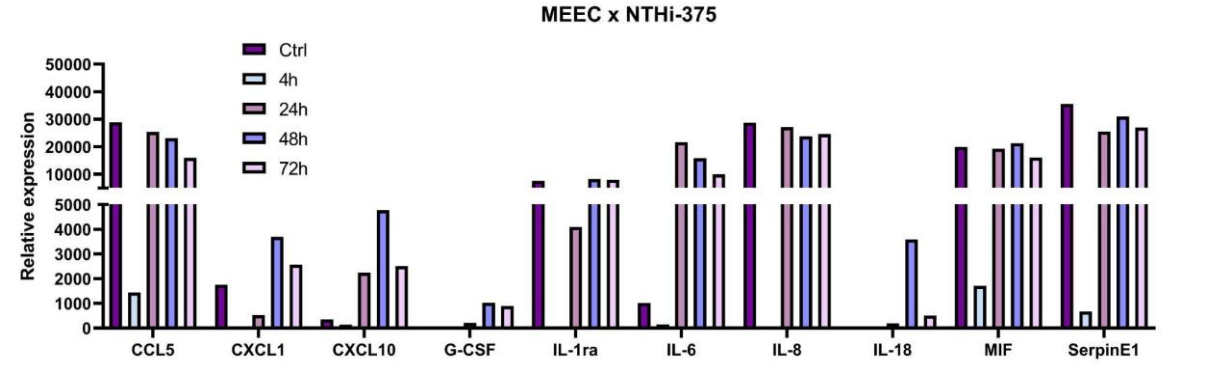


Figure 65 - MEEC response to infection by NTHI-375, at MOI 100, at 4, 24, 48 and 72 hours. Expression of cytokines was evaluated through Proteome Profiler Human Cytokine Array Kit. (n=1)

NTHi-375 infection of MEEC increased the expression of basal levels of CXCL1, CXCL10, and IL-6, and triggered the expression of G-CSF and IL-18. Unlike hMEEC-1, CCL5 in MEEC did not appear to be affected by invasion. Infected MEEC expression of CXCL1, CXCL10, G-CSF and IL-18 peaked at 48 hours, whereas CCL5 and IL-6 decreased

chronologically post 24 hours. The decrease in concentration at 72 hours seen in almost all cytokines could be due to cell death or protein degradation, or a combined effect of both factors. LDH assays could be used in future to determine whether the effect was related to cell death (Figure 65).

Both hMEEC-1 and MEEC present basal expression of a variety of cytokines at 48 hours, namely IL-1ra, IL-8, MIF, SerpinE1. The levels of these cytokines in infected cells were similar to the basal expression at all time points, except 4 hours, and this was probably due to the protein only just starting to accumulate in the supernatant.

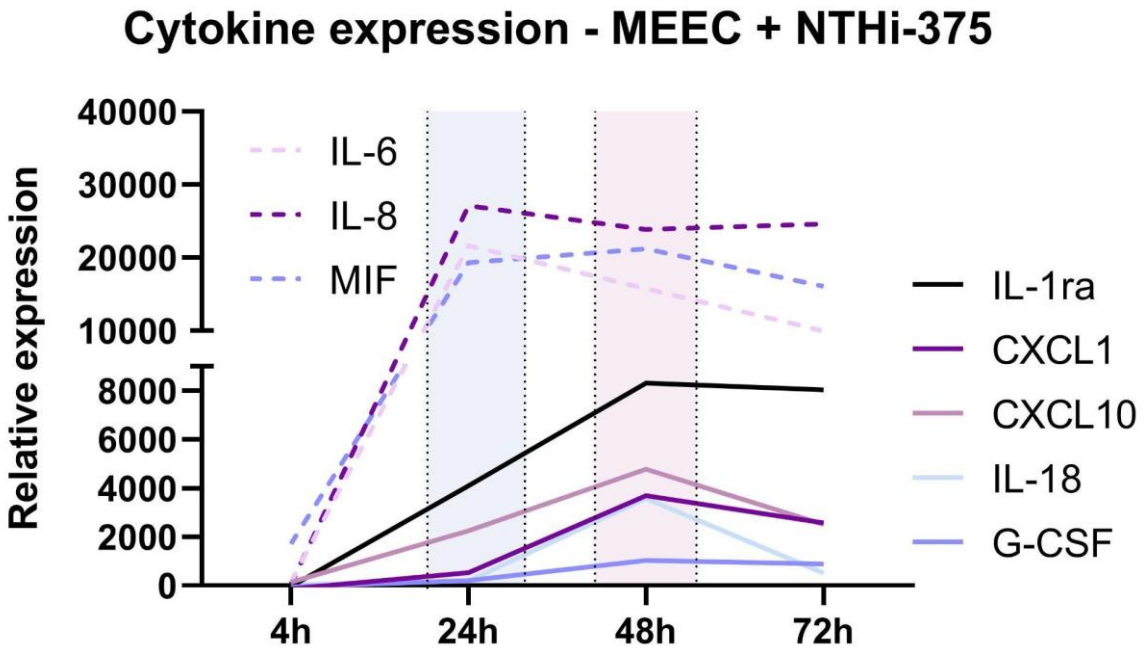


Figure 66 - Cytokine expression of potentially relevant OM cytokines over time.

6.1.3. ME epithelium cytokine expression in response to NTHi H446, H457, H491, Rd infection

The final stage of the evaluation of the response of ME epithelium to infection by NTHi was to assess differences in the response of cells infected by clinical isolates versus those infected by laboratory strains. PCho is an immunomodulatory protein, utilised by bacteria to reduce the immune response to infection. Bacterial PCho is a mimic of human

phosphorylcholine, an essential component of the eukaryotic membrane phospholipid, phosphatidylcholine. Bacteria expressing PCho utilise it to affect immune recognition and epithelial cell adhesion increasing bacterial adhesion to the epithelial cell surface thus presenting increased colonisation and delayed clearance of the respiratory tract. Furthermore, PCho reduces antibody binding to the bacterial surface and susceptibility to a variety of antimicrobials, as it alters the physical properties of the membrane. Although it is possible that intracellular bacteria use PCho to modify the activity of host proteins, to date PCho-mediated immune modulation has only been reported in parasitical infections. [283]

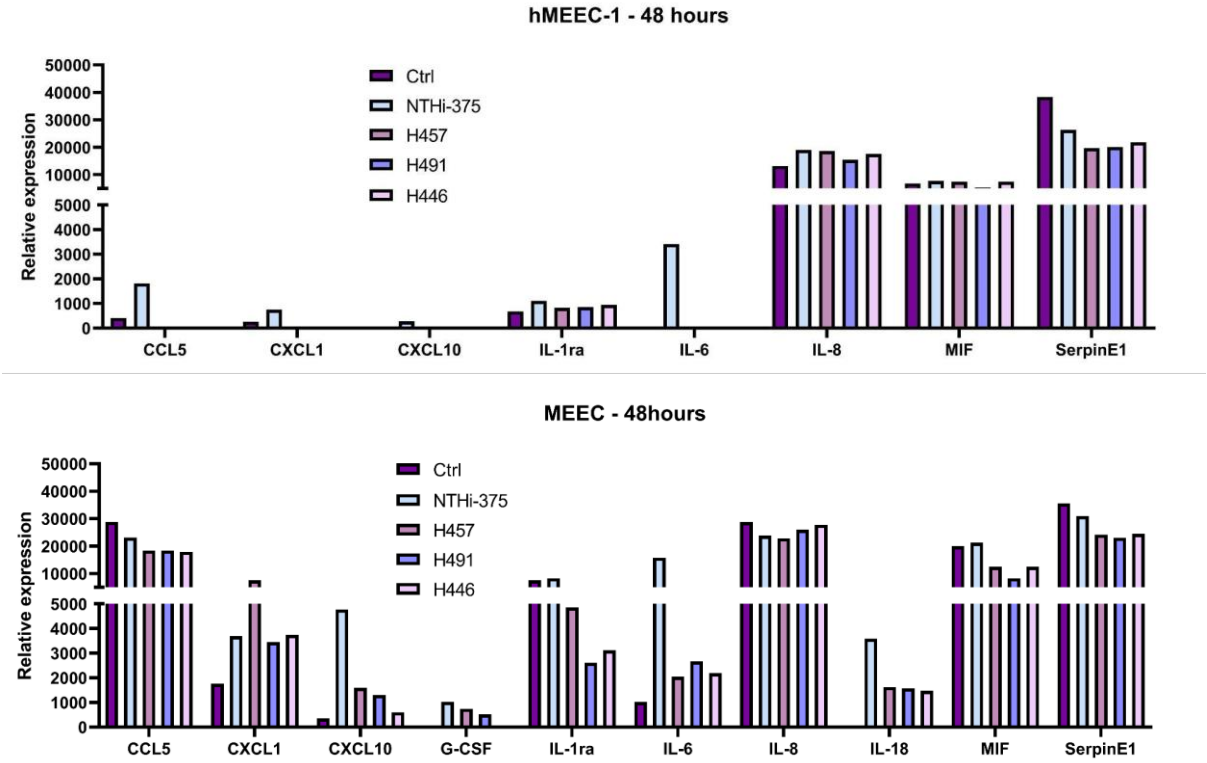


Figure 67 - hMEEC-1and MEEC response to infection by NTHI-375, Rd and PCho strains, at MOI 100, at 4, 24, 48 and 72 hours. Expression of cytokines was evaluated through Proteome Profiler Human Cytokine Array Kit. (n=1)

hMEEC-1 infected with NTHi-375 and PCho strains presented similar levels of expression of IL-1ra, IL-8, MIF and SerpinE-1. However, only hMEEC-1 infected with NTHi-375 expressed CCL5, CXCL1, CXCL10 and IL-6. In MEEC-infected cells, CCL5 expression was not affected by NTHi strain. Surprisingly, infection with H457 induced the highest expression of CXCL1, suggesting an interesting interaction between PCho locked in this

particular position and higher levels of the cytokine. Levels of CXCL1 were similar in NTHi-375, H491 and H446 infections. NTHi-375 infection induced the highest level of CXCL10 expression, whereas H457 and H491 infection had similar levels, and the lowest levels were expressed by H446 infected cells. NTHi-375, H457 and H491, but not H446, induced production of G-CSF suggesting a potential role for PCho in expression and activation of G-CSF. Surprisingly, IL-1ra expression was reduced following infection with PCho strains, but once again H457 induced the highest level of cytokine expression. Although IL-6 and IL-18 were induced by all strains, there was a visible difference in expression levels between the clinical isolates and laboratory strains. IL-8 expression was similar following infection by all strains, however, PCho strains caused a slight reduction in the expression of MIF and SerpinE1 (Figure 67).

6.2. Discussion

6.2.1. Analysis of ME epithelium protein transcription in response to NTHi-375 infection

In the respiratory tract, epithelial cells play a central role in maintaining homeostasis and triggering an effective immune response against external stimuli. The mucociliary system provides active clearance of pathogens and pollutants through the activity of beating cilia and the properties of mucin. Epithelial cells secrete a variety of factors that contribute to both proinflammatory, facilitating pathogen clearance or killing, and anti-inflammatory immune responses, that dampen the inflammation levels, preventing damage to the host, and allowing a return to homeostasis. As the pseudostratified ME epithelium is very similar to the respiratory epithelium, it was necessary to perform a broad evaluation of which immune factors play a role in the pathogenesis of OM.

MUC5AC and MUC5B are produced by secretory epithelial cells in the airways and submucosal glands, they are antimicrobial mediator mucins that contribute to the host defence of the respiratory epithelial surface. MUC5AC expression is associated with specialised secretory populations that are not represented in the 2D models where NTHi infections were conducted, due to a lack of epithelial differentiation, as no MUC5AC transcription was seen with either hMEEC-1 or MEEC. Transcription of MUC5B was apparent in both hMEEC-1 and

MEEC, strengthening the argument that the MEEC recovered for the purposes of my study through dissected fetal tissue are ME epithelial cells (Figure 61).

IL-6 and IL-8 are pro-inflammatory cytokines that are commonly seen in early immune responses to infection and tissue injury. IL-6 regulates the activity of monocytes and activates the NFκB pathway. IL-8 has chemotactic activity, recruiting monocytes to the site of infection. While transcription of IL-8 appears to be constitutive, that of IL-6 is trigger dependent. The lower IL-8 transcription levels seen at 24 hours could be related to rapid mRNA translation into protein, thus less mRNA available to be quantified, rather than a delayed response to infection (Figure 62). Further repeats would have to be performed in order to ascertain a trend regarding the levels of IL-6 mRNA transcription levels in MEEC and the effect of PCho position in the LOS.

6.2.2. Analysis of ME epithelium cytokine expression in response to NTHi infection

The work in this chapter expanded that of Espahbodi *et al* (2021). Unlike their study, which used bacterial lysate or cytokine (IL-1β and TNF-α) stimulation, my study resorted to live bacteria stimulation. My study also investigated a wider range of cytokines, for longer periods of time of up to 72 hours, rather than 4 hours. Whereas Espahbodi *et al* (2021) focused on the upregulation of TNF-α, IL-1β, IL-6, IL-8, IL-10 and MUC5B mRNA transcription in response to the stimuli mentioned above, in hMEEC-1 and primary epithelial cell populations of paediatric patients with and without rAOM or OME, the focus of my study was the analysis of a wider range of cytokine expression on the onset of AOM *in vitro*. [141]

Despite hMEEC-1 and MEEC cells presenting with similar levels of NTHi-375 adhesion and invasion, the profiles of cytokine expression were different (Figure 64 and 65). Compared to hMEEC-1, MEEC demonstrated increased expression of all cytokines detected (CCL5, CXCL1, CXCL10, IL-1ra, IL-6, IL-8, MIF and SerpineE1), including cytokines not found during hMEEC-1 infection (G-CSF and IL-18). This enhanced cytokine expression by primary cells agrees with the findings of Espahbodi *et al* (2021), highlighting the fact that although cell lines offer extended lifespans, an increased number of proliferations and are vital to ensure reproducibility, they do not always represent *in vivo* systems as accurately as primary

cells do. Furthermore, Espahbodi *et al* (2021) detected mRNA transcription of IL-6 and IL-8, which my study was also able to detect, on both an mRNA and protein level. Interestingly, their study reports increased transcription of both *Il-6* and *Il-8* in response to stimuli, agreeing with my findings on the increased mRNA levels of both cytokines upon infection, however, in my study, this effect is not reflected on IL-8 at a protein level. Espahbody *et al* (2021) also detected transcription of *Tnf-α*, *Il-1β*, and *Il-10*, which was not detected during my study on a protein level.

The cytokines reported above are involved in a variety of cell signalling pathways. CCL5 (or RANTES) is a pro-inflammatory chemokine involved in maintaining inflammation, and it is abundantly expressed by epithelial cells. It is constitutively expressed in a number of tissues, such as the rat retina and brain, and human gut and adipose tissue. [284–287] CCL5 binds to CCR5, eliciting activation of various downstream responses: PI3K/AKT, NF-κB, HIF-α, RAS-ERK-MEK, JAK-STAT and TGF-β-smad. These lead to the recruitment of leukocytes, T-cells and NK cells to the site of inflammation. However, Nokso-Koivisto *et al* (2014) concluded that a CCL5 single nucleotide polymorphism (SNP) (−403, rs2107538), which significantly reduces the levels of CCL5 expression, did not correlate with a predisposition to OM. [288] This SNP has previously shown to have physiological relevance in protection against SARS-CoV-2, HIV-1 and benign prostatic hyperplasia. [289–291] The evidence in my study also suggests that CCL5 does not play a role in the onset of OM, as expression is found in both infected and non-infected MEEC, at similar levels cells. Schousboe *et al* (2001) analysed human paediatric ME effusions and hypothesized that CCL5 was an important chemotactic factor associated with OME. However, their study did not include an analysis of normal human ME, thus it is hard to determine if CCL5 plays a role in OME or whether the expression levels found by Schousboe *et al* in diseased samples are similar to control levels, as found my study. [292] Nevertheless, OME is a borderline phenotype of COME - the main distinction being frequency rate - thus, CCL5 could play a role in the induction of COM, particularly due to its role in the activation of HIF-α, a hypoxia regulator. Hypoxia is a common feature of COM, and Cheeseman *et al* (2011) have previously demonstrated that the HIF-VEGF pathways are essential to development of COM in *Junbo* and *Jeff* mice mutants, both well-established chronic OM animal models. Their research showed that treatment with VEGF inhibitors reduced fluid presence in the mice bullae and HIF-VEGF pathway inhibitors led to inactivation of HIF-α and attenuation of inflammatory pathways. [122] Furthermore, Vikhe *et al* (2019) found that CCL5 is significantly upregulated in the bullae fluid of *Junbo* mice infected with NTHi

when compared to non-infected mice. [293] Therefore, although CCL5 does not seem to play a central role in the onset of OM, its role in COM requires further investigation, particularly in the context of hypoxia, as it may be key for the restoration of homeostasis.

CXCL1 (or GRO1) is also a proinflammatory chemokine, responsible for the recruitment of neutrophils. CXCL1 is expressed by epithelial cells, as well as macrophages and neutrophils, but it has previously been shown that expression is not constitutive. [294] CXCL1 binds to CXCR2, a chemokine receptor expressed on the surface of neutrophils and epithelial cells. This triggers a cellular response that induces calcium release, activation of the Ras/MAPK, and PI3K signalling cascades. As demonstrated by Trune *et al* (2015), CXCL1 expression is upregulated in the mice ME in response to heat-killed *H. influenzae*, which I have also shown in the response of human ME cells to NTHi infection. [295] Previously, CXCL1 expression in mice was shown to be inhibited by corticosteroid action. [294] Although corticosteroids were previously abundantly used in the treatment of OM, recent studies have found no link between the administration of corticosteroids and improved recovery times, highlighting the importance of looking at the immune response and disease as a whole, and not its individual components. [69,70]

CXCL10 (or IP-10) is a chemokine expressed by epithelial cells, but not in a constitutive manner. [297,298] CXCL10 binds to CXCR3, which presents 2 isoforms, A, which contributes to chemotaxis and proliferation of multiple cell types, and B, which inhibits migration and proliferation while inducing apoptosis and is predominantly expressed in macrophages, lymphocytes and NK cells. Activated CXCR3-A triggers ERK1/2, p38/MAPK, JNK and PI3-kinase/Akt signalling pathways. Binding of CXCL10 to CXCR3 is involved in the chemotaxis of leukocytes, T-cells and NK cells, as well as modulation of T-cell responses and NK cell migration. [299] In mice, CXCL10 has been shown to be expressed in early responses to infection to live and heat-killed NTHi, however, its role has not been explored further with regard to the onset of OM. [300,301] Furthermore, Bhutta *et al* (2020), in an analysis of paediatric ME effusions, reported that expression of CXCL10 was associated with serous COM fluids rather than mucoid, but Surendran *et al* (2015) found that in the ME effusions of otitis prone, where they present repeated AOM episodes despite tympanostomy and antibiotic treatment, and non-otitis prone children, who have infrequent AOM episodes, between the ages of 6 to 9 months old, the levels of CXCL10 during an AOM episode were not significantly different between the two groups, highlighting that CXCL10 role may be critical for COM but not AOM or rAOM. [78,302]

G-CSF (or CSF3) is a growth factor that regulates the production, proliferation, survival and maturation of granulocytes, such as neutrophils; it can also be expressed by epithelial cells. [303] Interaction between G-CSF and its receptor triggers a downstream cascade that involves the JAK/STAT, PI3K/AKT and MAPK/ERK pathways. Patel *et al* (2009) have previously demonstrated that respiratory syncytial virus infection induces G-CSF expression during AOM. [304] Vikhe *et al* (2019) showed that G-CSF was also expressed in COM, by analysing the bullae fluid of NTHi-infected and non-infected *Junbo* mice; their results concluded that, in COM, G-CSF expression levels were not significantly different. [293] Combined with the data from my study I hypothesise that G-CSF plays a role in the onset of OM in infections by PCho expressing bacteria.

IL-1ra is an anti-inflammatory cytokine, produced in epithelial cells, that acts as an antagonist for IL-1, modulating its immune and inflammatory response, by competitively binding to the IL-1 receptor. IL-1ra possesses 4 different isoforms that can be secreted or released upon cell death. [305] Absence of IL-1ra has been linked to severe systemic and local inflammation, due to disruption of IL-1 regulation. [306,307] Zielnik-Jurkiewicz and Stankiewicz-Szymczak (2016) evaluated the expression of IL-1ra in ME effusions and washes of children with and without COME. Their study found that there were no significant differences in IL-1ra expression in children with COME compared to children without. The findings of my research project also suggest that IL-1ra does not play a fundamental role in the infection of ME cells by NTHi. [308] However, this does not dismiss the role of IL-1ra in reestablishment of homeostasis. As COME is characterized by dysregulation of inflammation, leading to exacerbation of proinflammatory cytokines, it is possible that IL-1ra expression is affected by this dysregulation, by not having its expression altered. It has previously been shown, in mice, that administration of IL-1ra is beneficial and decreases inflammation in a variety of conditions, such as rheumatoid arthritis, septic shock, inflammatory bowel disease, and leukemia. [309] Thus the role of IL-1ra as a therapeutic agent should be investigated, potentially in combination with antimicrobials.

IL-6 is a pro-inflammatory cytokine involved in a variety of processes, secreted by epithelial cells in response to microbial triggers. IL-6 binds to the IL-6 receptor system, inducing homodimerization of gp130, leading to downstream signalling of the JAK-STAT3 and the JAK-SHP-2-MAPK pathways. IL-6 is key to the development of the innate and adaptive immune response, stimulating synthesis of acute phase proteins, and participating in maturation and differentiation of T-cells, thus playing a role in the resolution of inflammation.

[310] IL-6 has been found in ME effusions of children with OME and spontaneously perforated AOM, both of which are borderline phenotypes for COME and CSOM, respectively. [311,312] In the lung, in tracheobronchial epithelial cells, Chen *et al* (2003) demonstrated that expression of IL-6 is required for the production and expression of MUC5B/5AC. MUC5AC and MUC5B are important antimicrobial mediators in the respiratory tract, however, in my study, the levels of MUC5B transcription detected appeared to be dependent on stimuli as amplification was barely detectable in monolayers/3D models. [313]

IL-8 (or CXCL8) is a chemokine also produced by epithelial cells and binds to CXCR1 and CXCR2, with differing affinities, triggering activation of p38 MAPK, ERK1/2 and Akt. IL-8 induces chemotaxis of granulocytes, as well as phagocytosis. [314] Previous analysis of ME fluid by Leibovitz *et al* (2000), obtained through tympanocentesis, linked IL-8 to AOM and COM as higher IL-8 concentrations were noted in bacterial AOM than in children with negative ME effusion cultures, and antibiotic treatment was shown to decrease IL-8 expression as early as 72 hours post treatment. [315] Furthermore, Patel *et al* (1996) also saw increased IL-8 expression in AOM with synergistic viral and bacterial infections, when compared to bacterial infections alone. [316] Maxwell *et al* (1994) also showed COME ME effusions from children presented with higher expression of IL-8, and that IL-8 expression correlated with IL-1β and TNF-α levels in ME effusions. However, these studies did not include control groups that analysed basal expression of the cytokine in healthy ME. [317] Serban *et al* (2021) evaluated IL-8 expression in ME effusions and washes of healthy individuals and those suffering from CSOM with or without cholesteatoma, and their study found that IL-8 was expressed at significantly higher levels in patients with CSOM than healthy control groups. Their study, however, did not focus on paediatric OM, as the age range of patients was between 9 and 54 years old, whereas the previously discussed studies focused on patients up to 80 months old. [320] Furthermore, my study did not find a link between IL-8 expression and infection of ME cells with NTHi, suggesting that IL-8 does not play a central role in the onset of OM.

IL-18 is a pro-inflammatory cytokine expressed by epithelial cells that promotes T helper type 1 and 2 responses and synthesis of IFN-γ. IL-18 is a member of the IL-1 family, that like IL-1β is synthesized as an inactive precursor, however IL-18 precursor is constitutively expressed in endothelial cells and epithelial cells. IL-18 binds to IL-18Rα, expressed in most cells, and co-receptor IL-18Rβ, expressed by T-cells and dendritic cells. Binding of IL-18 to IL-18Rα alone does not elicit a proinflammatory signal. Following assembly of the complex with TIR-1, MyD88 is engaged, leading to expression of NFκB, in a

manner identical to IL-1. [319] Both IL-18 and IL-1β are induced at the earliest stages of the innate immune response and stimulate a downstream cascade of proinflammatory cytokines. However, IL-1 is effective at the level of picograms/ml, whereas IL-18 requires ng/ml. Analysis by Liu *et al* (2021) of ME effusions from children associated IL-1β and IL-18 with OME, however, once again, this study did not include a healthy ME control. Furthermore, their study found a significant correlation between cytokine expression and duration of OME, with the cytokine levels decreasing overtime but, surprisingly, no differences were found in IL-1β and IL-18 expression from serous and mucoid ME effusions. The authors hypothesised that the ME of patients presenting with mucoid ME effusions would have undergone a more intense remodelling of the ME than that of patients presenting with serous ME effusions [320] Although Liu *et al* (2021) did not investigate microbiology cultures from the effusions, Enoksson *et al* (2020) study did. Their study found an elevated abundance of NTHi in the ME effusions of OM-patients which correlated with increased IL-18 expression, aligning with our findings. [321]

Macrophage migration inhibitory factor (MIF) is a constitutively expressed pro-inflammatory mediator cytokine, that induces upregulation of TLR4 and sustains inflammation by inhibiting p53-dependent apoptosis in macrophages. MIF binds to CD74, activating a ERK1/ERK2/MAPK downstream cascade. [322] MIF expression has been shown to be significantly higher in ME effusions of children than those of adults, but a link between OME and MIF expression has not been established as a control group of healthy ME was not included. [323] However, in mice, LPS-induced AOM results in significantly different levels of MIF in the ME bullae between treated and non-treated mice. Furthermore, Zhang *et al* (2014) noted that blocking the activity of MIF reduced inflammation in AOM, alleviating symptoms and improving hearing levels. [324] Our results suggest there is no difference in expression MIF between NTHi-infected and non-infected ME cells, however, it is still possible that inhibition of MIF activity may aid quicker resolution of ME infection, which could be a potential therapeutic target that requires investigation.

SerpinE1 (or PAI-1) is a serine protease inhibitor, expressed by epithelial cells and associated with wound healing and airway epithelium remodelling but whose role in infection is not yet fully understood. [325] While Bhutta *et al* (2020) associated CXCL10 with serous ME fluids, their data suggested higher expressions of SerpinE1 in mucoid fluid. However, Esposito *et al* (2015) did not find a correlation between a polymorphism for SerpineE1, which is strongly associated with the presence of the protein, and increased susceptibility to rAOM. [78,326] My data suggests that there is no link between the expression of SerpinE1 and infection of ME

cells.

Unlike previous studies that analysed ME fluids from patients, TNF-α, IL-1β, IL10, and IL17a were not detected in this data set. The reason as to why these cytokines were not identified in the *in vitro* ME epithelium monolayers utilised in my study to assess response to NTHi-infection is that most are cytokines that are only expressed in specific subsets of the ME cell population and not represented in the monolayers. For example, IL-10 is highly expressed in leukocytes and lymphocytes, whereas TNF-α and IL-1β is expressed mostly in monocytes, such as macrophages. IL-17a is mainly produced by specialised T-helper cells while IL-10 is an anti-inflammatory cytokine that limits the immune response to pathogens preventing damage to host tissues, IL-1β is involved in cell proliferation, differentiation and apoptosis and TNF-α expression leads to necrosis or apoptosis. IL-17a promotes bacterial clearance by recruiting neutrophils and inducing apoptosis. Expression of IL-1β, TNF-α and IL-8 has been shown to be bacterial load dependent. Interestingly, the expression of TNF-α and IL-17a is not as consistent across all ME effusions of children affected by NTHi invasion, as IL-6 and IL-8. Furthermore, lack of differentiated cells, such as goblet and ciliated cells, could also impact the presence of some of the proteins that would be expected to be found in response to OM, namely MUC5AC and MUC5B. Middle ear effusions and washes recovered from the ME of patients represent a complete recollection of the immune response and all the interactions between the different cell populations in the ME, which is why their analysis has been such a popular method of trying to understand the pathogenesis of otitis media.

Unexpectedly, there are a number of pathways where the cytokines secreted by the ME epithelial cells interact. The cytokines found in my study mainly relate to proinflammatory pathways, triggered in response to bacterial stimuli (Figure 68).

hMEEC-1 MEEC

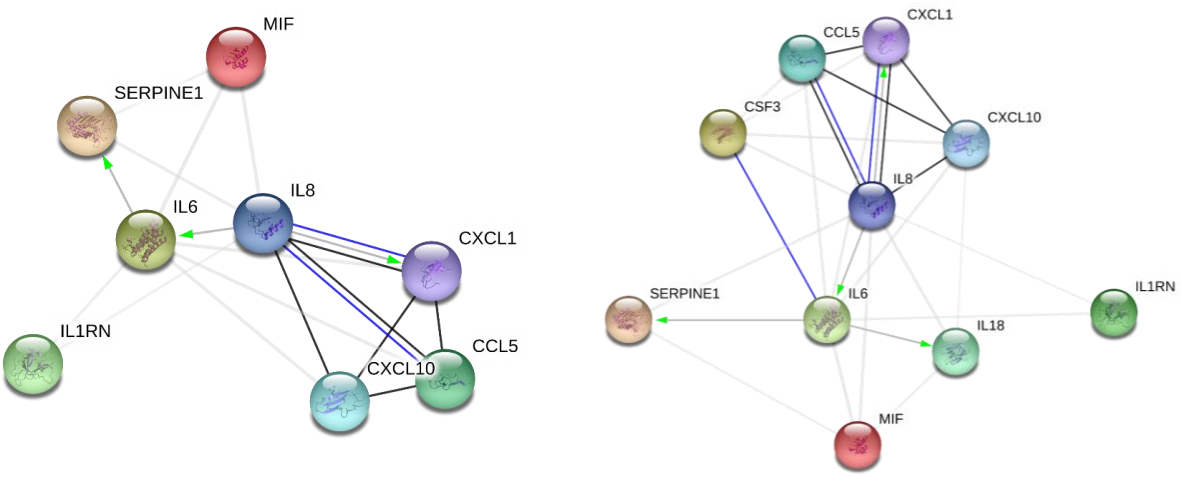


Figure 68 - Interactions of cytokines. Green: activation. Blue line: binding (protein-protein interactions related to signalling pathways). Black line: biochemical reaction (G-protein couple complex dissociation). CXCL1, CXCL10, CCL5, GCS-F, IL-8, IL-6, IL-18, SERPINE1 and IL-1RN are commonly expressed in response to bacterial stimuli. IL-8, IL-6, CCL5, CXCL1, CXCL10 and SERPINE1 regulate leukocyte recruitment and chemotaxis. IL-8 expression leads to activation of IL-6 and CXCL1. IL-6 expression leads to activation of SERPINE 1 and IL-18. CSF-3 also known as G-CSF. Adapted from String (http://version10.string-db.org/).

In conclusion, at early stages of NTHi infection a variety of pathways are engaged, eliciting a number of downstream responses, leading to cytokine secretion and inflammation. Through an interplay of the cytokines secreted by ME epithelium, the ultimate goal of these early effectors is to recruit innate immune cells that will contribute to the clearance of the pathogen.

After understanding the cytokine interactions in the context of NTHi infection, it was necessary to evaluate the temporality of an NTHi infection and onset of AOM. Hernandez *et al* (2015) evaluated the transcriptome of an AOM episode, by analysing the transcriptomic and histological profile of 60–90-day old mouse middle ears in response to an inoculation with a paediatric OM clinical isolate, NTHi 3655, and PBS, up to 7 days. Their study identified 3 stages to an AOM episode: the early induction phase that occurs 3-6 hours post NTHi inoculation, where transcription of interleukins and chemokines, such as IL-6, CXCL1, CXCL10 is first activated, as well as growth-factors, such as G-CSF and TNF-induced genes. In this initial stage, genes activated by the NFκB, JAK/STAT and cell apoptosis pathways are the most represented. Their histological work also identified the onset of neutrophil infiltration

and loss of the epithelial layer and the differentiation phenotype at this time point, recognising it as a key early event in AOM. This initial phase is followed by an intermediate bactericidal phase that occurs 24 hours post inoculation. Genes expressed at this stage are heavily involved in inflammation, chemotaxis, phagocytosis, neutrophil activation, cytotoxicity and mucin production, including MUC5AC and MUC5B, and ROS production. Further, the authors also reported that genes responsible for ciliary morphogenesis and organisation were found to be downregulated. In their histological analysis at this stage, ME effusion was reported, and neutrophil infiltration peaked. The last phase, the resolution phase, was identified by day 5 to 7, where they were able to identify that most genes had returned to pre-inoculation levels, but anti-bacterial surveillance, leukocyte and lymphocyte markers were still present, suggesting a shift from innate immunity to adaptive immune and development of immune "memory". [300]

Our findings followed a similar chronological pattern to that seen by Hernandez *et al* (2015), however, responses might be slightly delayed as the *in vitro* models are limited through a lack of immune cells and positive feedback loops (Figure 66). A response amplification is often created by resident neutrophils, basophils and macrophages, and I studied protein expression rather than gene expression, which may not necessarily happen simultaneously. At 24 hours, I identified peaks in expression of IL-6, IL-8 and MIF, suggesting an interleukin signalling network activation, which is also the first event identified by Hernandez *et al* (2015). Between 24- and 48-hours expression of cytokines involved in the recruitment and activation of neutrophils and macrophages, namely CXCL1 and CXCL10 was noted with peak expression s at 48 hours. Furthermore, peak expression of IL-18, an interleukin that enhances lymphocyte cytotoxic effects, was seen at that time which agrees with the findings of Hernandez *et al* (2015). Expression of G-CSF, a growth factor for granulocytes that regulates production of leukocytes in the bone marrow, also slightly increased around this time point, from 24 hours, to assist in the recruitment of innate immune cells, and potentially demonstrating epithelial cell "frustration" due to lack of immune cell assistance. Post 48 hours, the expression of IL-1ra, an interleukin with anti-inflammatory properties that reduces the inflammatory effects of IL-1, could be the bridge in our *in vitro* model between the intermediate stage and the final stage, the resolution phase. Although the response of hMEEC-1 follows a similar temporal pattern, increased expression levels and more cytokines were identified in the response of MEEC. This evidence suggests that fetal primary cells are strong tools for the study of the pathogenesis of OM.

As a caveat, MEEC increased cytokine expression could also be due to a number of

other factors. For example, at the time of isolation and immortalisation, the patient from which hMEEC-1 were isolated did not have middle ear disease, but their previous ME disease history is unknown and the possibility of having experienced OM is not unlikely. MEEC are fetal cells which have previously never encountered NTHi infection. Thus, the enhanced expression effect seen on MEEC could be due to lack of cell training, as the hMEEC-1 cells might have encountered OM episodes previously. Furthermore, expression of certain cytokines at high levels in MEEC controls could be explained by the protein being constitutively expressed, high stress due to confluence or, given the cells' primary nature, the possibility of mycoplasma contamination. Nonetheless, I believe that the effects seen between hMEEC-1 and MEEC expression are due to MEEC being more responsive to stimuli than hMEEC-1.

6.2.3. Analysis of ME epithelium cytokine expression in response to NTHi H446, H457, H491, Rd infection

The binding of PCho to PAFr has been reported to be dual purpose in function as it allows the bacteria to adhere to and invade epithelial cells, thus providing physical protection from the immune system effectors that patrol and act on the extracellular space, but also allows for evasion of the immune response through modulation of cellular responses.

PAF is a host-derived inflammatory phospholipid whose expression is commonly upregulated as a consequence of pro-inflammatory cytokine production. In the airways *S. pneunomiae* and *H. influenzae* expressing PCho esterase (PCe) and glycerophosphodiester phosphodiesterase (GlpQ, also known as Protein D), respectively, hydrolyse PAF. These esterases are also expressed in species that express PCho and are responsible for remodelling the bacterial cell wall by hydrolysing PCho. Although the enzymes are commonly used by the bacteria to hydrolyse their own PCho, Hergott *et al* (2015) determined that they can be utilised to subvert PAF-mediated neutrophil phagocytosis and bacterial clearance. [327,328] PCho also alters host recognition of the bacteria, due to its ability to alter membrane properties, decreasing membrane accessibility and permeability. Strains that express PCho have shown resistance to killing by antimicrobial peptides, such as LL37/hCAP18, and reduced antibody binding. [226]

Despite its extremely successful evasion mechanisms, PCho can still be detected by the immune system, eliciting an immune response. Weiser *et al* (1998) found that C-reactive

protein (CRP) bound to PCho, initiating a classical complement pathway and increasing complement mediated-killing. Langereis *et al* (2019) expanded on this knowledge, by showing that PCho-specific IgM and, to a lesser extent, CRP is dependent on the level of PCho expressed by the bacteria. High expression of PCho contributes to strong complement-mediated killing. Importantly, Langereis *et al* (2019) noted that CRP in antibody depleted serum had limited contributions to NTHi complement-mediated killing. [329,330]

Although the role of PCho in airway infection has previously been described little is known regarding its role in ME infections. Hong *et al* (2007) have found decreased levels of early inflammation and strongly established biofilm communities at days 7 and 14 in the chinchilla' ME infected with an NTHi strain expressing PCho. This study also demonstrated that infections with the isogenic strain lacking PCho, early inflammation was high, bacterial counts were decreased, and no biofilms were observed on day 14. This suggests the immune evasion function of PCho is also an advantage in the colonisation of the ME. [276]

My study investigated the effect of the presence or absence of PCho and found that PCho presence (H457/H491) or absence (H446) had little impact on the expression of cytokines (Figure 67). The expression level differences of IL-6, IL-18 and CXCL10 following infection with a clinical isolate or laboratory strains was probably due to the lack of infectivity of the laboratory strains, including their inability to invade cells. This finding suggests that intracellular bacterial invasion promotes exacerbated expression of IL-6, IL-18 and CXCL10. Interestingly, presence of PCho seems to trigger expression of G-CSF, as the cytokine is not present when cells are infected with H446. Both PAF and G-CSF possess hematopoietic functions, thus it could be hypothesised that detection of PAF, or mimics of it such as PCho, trigger the expression of proteins associated with hematopoietic functions. Furthermore, the position of PCho may affect the expression of CXCL1 and IL-1ra, but no other substantial effects were demonstrated.

Unfortunately, due to the low virulence of the PCho-variant strains used in my study, no conclusions can be drawn regarding the role of PCho in invasion and cell response.

In conclusion, MEEC should be considered as powerful tools to propel the study of OM, as they have the potential to differentiate into representative models of the ME epithelium, as shown by Mather *et al* (2021), and, as shown in my study, mimic *in vitro* representative

responses of cytokines found in *in vivo* ME effusions in response to NTHi infection.

7. Chapter 7 - Final Discussion

7.1. Summary

OM is a disease that affects a large number of children and adults worldwide. When left untreated, OM carries long-term, high morbidity consequences, but the most concerning is conductive hearing loss. This typically occurs in the later stages of OM, whether that is in the form of rAOM or COME, where ME effusions fill the ME cavity and prevent free movement of the ME ossicles, or CSOM, where the rupture of the tympanic membrane leads to the inability of the ME ossicles to transmit sound vibrations along the ME cavity. In order to understand OM, it is important to have a reliable *in vitro* model of the ME that can mimic the *in vivo* environment. At the moment, accurate human ME models have only been achieved through ALI seeding of whole cell populations recovered from the ME of fetal, paediatric and adult individuals, and the mechanisms that lead to differentiation of the epithelium in the ME have not yet been understood. My study attempted to elucidate the ME epithelium differentiation process by selecting ME epithelial cells from fetal MEs and providing different stimuli that could elicit differentiation at ALI.

Further, in order to understand progression of OM into COM, we need a better understanding of the aetiology of the disease including triggers for the onset of OM and the importance of various factors leading to AOM. Current research tools are not appropriate for the study of OM. A significant volume of the work done in the study of human OM relies on hMEEC-1, an adult cell line, that does not accurately represent the middle ear, as shown in my study, due its lack of ability to differentiate at ALI, its lack of ability to reproduce cellular responses to infection to the same degree as fetal primary ME epithelial cells. On the other hand, fetal ME cells might present as an alternative tool for investigating OM, as these seem to naturally possess longer lifespans, when compared to ME cells recovered from paediatric patients that have already undergone differentiation. Fetal ME cell lifespan can also potentially be extended through the introduction of proto-oncogenes, such as Bmi-1. Additionally, the primary fetal epithelial cells utilised in my study responded to infection in a manner that was in agreement with the current literature on OM, by expressing cytokines commonly associated with OM, in response to infection by one of the most common otopathogens, NTHi.

In my study, I have also documented for the first time, that NTHi invades ME epithelial

cells, in a similar manner to that seen in the respiratory epithelium. Invasion is a key component of NTHi's infection process as it allows for the bacteria to form intracellular reservoirs, extending infection time, and hampering the bactericidal action of immune system. My study, however, was not able to elucidate the mechanisms behind the process of invasion, as it was not possible to show that this process is surface receptor-mediated.

Finally, OM presents a massive financial burden system to healthcare systems, as it is the leading cause for paediatric appointments and paediatric antibiotic prescription. As OM treatment is heavily reliant on the use of antibiotics, it presents a concern in the context of the growing emergence of antibiotic resistant bacteria. Thus, there is an urgent need to find antimicrobial alternatives to treat OM, or factors that allow for the prevention of onset or development into chronic infection. As NTHi relies heavily on its phasevarion to evade the immune system and antibiotic treatments, the phasevarion and its virulent components, such as the PCho, a bacterial LOS decoration that binds to human PAFr, present as potential targets for therapeutic agents that could benefit treatment alternatives and resolution of disease and should be investigated further. In my study, due to technical difficulties with the obtention of NTHi PCho-locked clinical isolates I was not able to investigate the role of ABT-491, a PAFr antagonist, to the extent initially planned, as I wanted to evaluate the effects of ABT-491 on the ability of PCho lockedON NTHi strains to invade ME epithelial cells.

7.2. Future work

Future work should include:

- Seeding a whole cell ME population directly from culture expanded after dissection into ALI and evaluate the expression of differentiation markers. As this method is most similar to Mather *et al* (2021), it should lead to cell differentiation.
    - Test IL-1β's ability to promote cell ME epithelium differentiation, as Choi *et al* (2005) reported that IL-1β promotes ciliogenesis of human ME epithelial cells. [331]
    - Once model is established and is reproducible, macrophages should be incorporated into the model, as that would further mimic the ME cell

population.

- Repeat antibiotic protection assays with PCho-locked clinical isolates, such as Hong *et al* (2007) NTHi-2019. [332] Unlike laboratory strains, clinical isolates have higher virulence, as seen with NTHi-375. While PCho-lockON strains would be expected to have higher invasion rates than wild-type bacteria, PCho-lockOFF strains should present lower invasion rates.
    - Assess binding of ABT-491 to PAFr or edit PAFr out of cells.
- Infect MEEC BMI-1 cells and assess differences in the response compared to MEEC.
- Infect differentiated ALI models with PCho-locked NTHi clinical isolates and evaluate response to infection and assess differences in the response to infection by 2D and 3D models.
    - Analysis of phasevarion changes during infection in wild-type strains, through whole genome sequencing.
    - Assessment of replication/growth rates of NTHi intracellularly and consequences of bacterial reinfection in the phasevarion.
    - Elucidate method of invasion, if it is either through macropinocytosis or receptor-mediated endocytosis, potentially through electron microscopy.
    - Infection with other otopathogens, such as *M. catarrhalis* and *S. pneumoniae*.

Bibliography

1. Plack CJ. *The Sense of Hearing*. 3rd ed. Routledge; 2018. doi:10.4324/9781315208145
2. Brownell WE. HOW THE EAR WORKS - NATURE'S SOLUTIONS FOR LISTENING. *Volta Rev*. 1997;99(5):9-28.
3. Davis H, Richard Silverman S. *Hearing and Deafness*. 3rd ed. Holt, Rinehart and Winston https://search.library.uq.edu.au/permalink/f/12kerkf/61UQ_ALMA2198231080003131
4. Tysome JR, Sudhoff H. The Role of the Eustachian Tube in Middle Ear Disease. In: Lloyd SKW, Donnelly NP, eds. *Advances in Oto-Rhino-Laryngology*. Vol 81. S. Karger AG; 2018:146-152. doi:10.1159/000485581
5. Oliver ER, Kesser BW. Embryology of Ear (General). In: Kountakis SE, ed. *Encyclopedia of Otolaryngology, Head and Neck Surgery*. Springer Berlin Heidelberg; 2013:743-749. doi:10.1007/978-3-642-23499-6_531
6. Anthwal N, Thompson H. The development of the mammalian outer and middle ear. *J Anat*. 2016;228(2):217-232. doi:10.1111/joa.12344
7. Takechi M, Kitazawa T, Hirasawa T, et al. Developmental mechanisms of the tympanic membrane in mammals and non-mammalian amniotes: Development of the tympanic membrane. *Congenit Anom*. 2016;56(1):12-17. doi:10.1111/cga.12132
8. Thompson H, Tucker AS. Dual Origin of the Epithelium of the Mammalian Middle Ear. *Science*. 2013;339(6126):1453-1456. doi:10.1126/science.1232862
9. Tucker AS, Dyer CJ, Romero JMF, Teshima THN, Fuchs JC, Thompson H. Mapping the distribution of stem/progenitor cells across the middle ear during homeostasis and inflammation. *Development*. Published online January 1, 2017:dev.154393. doi:10.1242/dev.154393
10. Van Waegeningh HF, Ebbens FA, Van Spronsen E, Oostra RJ. Single origin of the epithelium of the human middle ear. *Mech Dev*. 2019;158:103556. doi:10.1016/j.mod.2019.103556
11. Milmoe NJ, Tucker AS. Craniofacial transitions: the role of EMT and MET during head development. *Development*. 2021;148(4):dev196030. doi:10.1242/dev.196030
12. Miyanaga S, Morimitsu T. Prussak's space: chronological development and routes of aeration. *Auris Nasus Larynx*. 1997;24(3):255-264. doi:10.1016/S0385-8146(96)00023-5
13. Ryan AF, Nasamran CA, Pak K, et al. Single-Cell Transcriptomes Reveal a Complex Cellular Landscape in the Middle Ear and Differential Capacities for Acute Response to Infection. *Front Genet*. 2020;11:358. doi:10.3389/fgene.2020.00358
14. Lim DJ, Shimada T. Secretory Activity of Normal Middle Ear Epithelium: Scanning and Transmission Electron Microscopic Observations. *Ann Otol Rhinol Laryngol*. 1971;80(3):319-329. doi:10.1177/000348947108000304
15. Shimada T, Lim DJ. Distribution of Ciliated Cells in the Human Middle Ear: Electron and Light Microscopic Observations. *Ann Otol Rhinol Laryngol*. 1972;81(2):203-211. doi:10.1177/000348947208100205
16. Graves GO, Edwards LF. THE EUSTACHIAN TUBE: A REVIEW OF ITS DESCRIPTIVE, MICROSCOPIC, TOPOGRAPHIC AND CLINICAL ANATOMY. *Arch Otolaryngol - Head Neck Surg*. 1944;39(5):359-397. doi:10.1001/archotol.1944.00680010374001
17. Lim DJ. Functional Morphology of the Lining Membrane of the Middle Ear and Eustachian Tube: An Overview. *Ann Otol Rhinol Laryngol*. 1974;83(11_suppl):5-26. doi:10.1177/0003489474083S1102
18. Hentzer E. Ultrastructure of the Middle Ear Mucosa. *Ann Otol Rhinol Laryngol*.

1976;85(2_suppl):30-35. doi:10.1177/00034894760850S208
19. Lim DJ, Klainer A. CELLULAR REACTIONS IN ACUTE OTITIS MEDIA - SCANNING AND TRANSMISSION ELECTRON MICROSCOPY: *The Laryngoscope*. 1971;81(11):1772-1786. doi:10.1288/00005537-197111000-00003
20. Ali MY. Histology of the human nasopharyngeal mucosa. *J Anat*. 1965;99(Pt 3):657-672.
21. Bernstein JM, Hayes ER, Ishikawa T, Tomasi TB, Herd JK. Secretory otitis media: a histopathologic and immunochemical report. *Trans - Am Acad Ophthalmol Otolaryngol Am Acad Ophthalmol Otolaryngol*. 1972;76(5):1305-1318.
22. Veltri RW, Sprinkle PM. Serous Otitis Media: Immunoglobulin and Lysozyme Levels in Middle Ear Fluids and Serum. *Ann Otol Rhinol Laryngol*. 1973;82(3):297-301. doi:10.1177/000348947308200305
23. Sade J. Ciliary Activity and Middle Ear Clearance. *Arch Otolaryngol - Head Neck Surg*. 1967;86(2):128-135. doi:10.1001/archotol.1967.00760050130002
24. Luo W, Yi H, Taylor J, et al. Cilia distribution and polarity in the epithelial lining of the mouse middle ear cavity. *Sci Rep*. 2017;7(1):45870. doi:10.1038/srep45870
25. Ryan AF, Sharp PA, Harris JP. Lymphocyte circulation to the middle ear. *Acta Otolaryngol (Stockh)*. 1990;109(3-4):278-287. doi:10.3109/00016489009107444
26. Freijd A, Hammarström L, Persson MA, Smith CI. Plasma anti-pneumococcal antibody activity of the IgG class and subclasses in otitis prone children. *Clin Exp Immunol*. 1984;56(2):233-238.
27. Palva T, Taskinen E, Lehtinen T, Ramsay H, Bjorksten F, Hackman P. Mast Cells and Histamine in Adenoid Tissue and Middle Ear. *Acta Otolaryngol (Stockh)*. 1991;111(2):349-353. doi:10.3109/00016489109137399
28. Suenaga S, Kodama S, Ueyama S, Suzuki M, Mogi G. Mucosal Immunity of the Middle Ear: Analysis at the Single Cell Level: *The Laryngoscope*. 2001;111(2):290-296. doi:10.1097/00005537-200102000-00019
29. Swarr DT, Morrisey EE. Lung Endoderm Morphogenesis: Gasping for Form and Function. *Annu Rev Cell Dev Biol*. 2015;31(1):553-573. doi:10.1146/annurev-cellbio-100814-125249
30. Sade J, Ar A. Middle ear and auditory tube: Middle ear clearance, gas exchange, and pressure regulation. *Otolaryngol Head Neck Surg*. 1997;116(4):499-524. doi:10.1016/S0194-5998(97)70302-4
31. Mogre DA. The Middle Ear Cleft a "Miniature Lung": from Development to Disease. *Glob J Otolaryngol*. 2017;4(4). doi:10.19080/GJO.2017.04.555641
32. Firat A, Onerci-Celebi O, Tuncel A, Ergun M, Hayran M. Microscopic study of human nasal cavity microanatomy using semi-thin resin embedding and methylene blue staining. *J Histotechnol*. 2019;42(1):13-18. doi:10.1080/01478885.2018.1550848
33. Scherzad A, Hagen R, Hackenberg S. Current Understanding of Nasal Epithelial Cell Mis-Differentiation. *J Inflamm Res*. 2019; 12:309-317. doi:10.2147/JIR.S180853
34. Jinhage M, Hermansson A, Gisselsson-Solén M. Nasopharyngeal cultures in children with AOM – A retrospective study on bacteriological findings and impact on management. *Int J Pediatr Otorhinolaryngol*. 2021;149:110848. doi:10.1016/j.ijporl.2021.110848
35. Mansini AP, Peixoto E, Jin S, Richard S, Gradilone SA. The Chemosensory Function of Primary Cilia Regulates Cholangiocyte Migration, Invasion, and Tumor Growth. *Hepatol Baltim Md*. 2019;69(4):1582-1598. doi:10.1002/hep.30308
36. Schilder AGM, Bhutta MF, Butler CC, et al. Eustachian tube dysfunction: consensus statement on definition, types, clinical presentation and diagnosis. *Clin Otolaryngol*. 2015;40(5):407-411. doi:10.1111/coa.12475
37. Bluestone C, Doyle W. Anatomy and physiology of eustachian tube and middle ear

related to otitis media. *J Allergy Clin Immunol*. 1988;81(5):997-1003. doi:10.1016/0091-6749(88)90168-6
38. Rovers MM, Schilder AG, Zielhuis GA, Rosenfeld RM. Otitis media. *The Lancet*. 2004;363(9407):465-473. doi:10.1016/S0140-6736(04)15495-0
39. Bhutta MF, Thornton RB, Kirkham LAS, Kerschner JE, Cheeseman MT. Understanding the aetiology and resolution of chronic otitis media from animal and human studies. *Dis Model Mech*. 2017;10(11):1289-1300. doi:10.1242/dmm.029983
40. Leichtle A, Lai Y, Wollenberg B, Wasserman SI, Ryan AF. Innate Signaling in Otitis Media: Pathogenesis and Recovery. *Curr Allergy Asthma Rep*. 2011;11(1):78-84. doi:10.1007/s11882-010-0158-3
41. Aslıer M, Aslıer NGY, Ercan İ, Keskin S. Clustering upper airway physicals, otitis media with effusion and auditory functions in children. *Auris Nasus Larynx*. 2022;49(2):195-201. doi:10.1016/j.anl.2021.07.001
42. Palacios SD, Pak K, Rivkin AZ, et al. Role of p38 Mitogen-Activated Protein Kinase in Middle Ear Mucosa Hyperplasia during Bacterial Otitis Media. *Infect Immun*. 2004;72(8):4662-4667. doi:10.1128/IAI.72.8.4662-4667.2004
43. Qureishi A, Lee Y, Belfield K, Birchall J, Daniel M. Update on otitis media & prevention and treatment. *Infect Drug Resist*. Published online January 2014:15. doi:10.2147/IDR.S39637
44. Liese JG, Silfverdal SA, Giaquinto C, et al. Incidence and clinical presentation of acute otitis media in children aged <6 years in European medical practices. *Epidemiol Infect*. 2014;142(8):1778-1788. doi:10.1017/S0950268813002744
45. Kaur R, Morris M, Pichichero ME. Epidemiology of Acute Otitis Media in the Postpneumococcal Conjugate Vaccine Era. *Paediatrics*. 2017;140(3):e20170181. doi:10.1542/peds.2017-0181
46. Teele DW, Klein JO, Rosner B, Greater Boston Otitis Media Study Group. Epidemiology of Otitis Media During the First Seven Years of Life in Children in Greater Boston: A Prospective, Cohort Study. *J Infect Dis*. 1989;160(1):83-94. doi:10.1093/infdis/160.1.83
47. Tonnaer EL, Mylanus EA, Mulder JJ, Curfs JH. Detection of Bacteria in Healthy Middle Ears During Cochlear Implantation. *Arch Otolaryngol Neck Surg*. 2009;135(3):232. doi:10.1001/archoto.2008.556
48. Bhutta. Evolution and Otitis Media: A Review, and a Model to Explain High Prevalence in Indigenous Populations. *Hum Biol*. 2015;87(2):92. doi:10.13110/humanbiology.87.2.0092
49. DeLacy J, Dune T, Macdonald JJ. The social determinants of otitis media in Aboriginal children in Australia: are we addressing the primary causes? A systematic content review. *BMC Public Health*. 2020;20(1):492. doi:10.1186/s12889-020-08570-3
50. Coleman A, Wood A, Bialasiewicz S, Ware RS, Marsh RL, Cervin A. The unsolved problem of otitis media in indigenous populations: a systematic review of upper respiratory and middle ear microbiology in indigenous children with otitis media. *Microbiome*. 2018;6(1):199. doi:10.1186/s40168-018-0577-2
51. Kong K, Coates HLC. Natural history, definitions, risk factors and burden of otitis media. *Med J Aust*. 2009;191(S9). doi:10.5694/j.1326-5377.2009.tb02925.x
52. Mackenzie GA, Leach AJ, Carapetis JR, Fisher J, Morris PS. Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. *BMC Infect Dis*. 2010;10(1):304. doi:10.1186/1471-2334-10-304
53. World Health Organisation. 1 in 4 people projected to have hearing problems by 2050. Published March 2, 2021. https://www.who.int/news/item/02-03-2021-who-1-in-4-people-projected-to-have-hearing-problems-by-2050

54. World Health Organisation. Deafness and Hearing. Published February 27, 2023. https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss
55. Brophy-Williams S, Jarosz K, Sommer J, Leach AJ, Morris PS. Preventative and medical treatment of ear disease in remote or resource-constrained environments. *J Laryngol Otol*. 2019;133(1):59-72. doi:10.1017/S0022215119000057
56. Leskinen K, Jero J. Acute complications of otitis media in adults: Complications of otitis media in adults. *Clin Otolaryngol*. 2005;30(6):511-516. doi:10.1111/j.1749-4486.2005.01085.x
57. Kacmarynski DSF, Levine SC, Pearson SE, Maisel RH. Complications of Otitis Media Before Placement of Tympanostomy Tubes in Children. *Arch Otolaryngol Neck Surg*. 2004;130(3):289. doi:10.1001/archotol.130.3.289
58. Penido NDO, Chandrasekhar SS, Borin A, Maranhão ASDA, Gurgel Testa JR. Complications of otitis media – a potentially lethal problem still present. *Braz J Otorhinolaryngol*. 2016;82(3):253-262. doi:10.1016/j.bjorl.2015.04.007
59. Smith JA, Danner CJ. Complications of Chronic Otitis Media and Cholesteatoma. *Otolaryngol Clin North Am*. 2006;39(6):1237-1255. doi:10.1016/j.otc.2006.09.001
60. Leach AJ, Homøe P, Chidziva C, et al. Panel 6: Otitis media and associated hearing loss among disadvantaged populations and low to middle-income countries. *Int J Pediatr Otorhinolaryngol*. 2020;130:109857. doi:10.1016/j.ijporl.2019.109857
61. Monasta L, Ronfani L, Marchetti F, et al. Burden of Disease Caused by Otitis Media: Systematic Review and Global Estimates. Moormann AM, ed. *PLoS ONE*. 2012;7(4):e36226. doi:10.1371/journal.pone.0036226
62. Suzuki HG, Dewez JE, Nijman RG, Yeung S. Clinical practice guidelines for acute otitis media in children: a systematic review and appraisal of European national guidelines. *BMJ Open*. 2020;10(5):e035343. doi:10.1136/bmjopen-2019-035343
63. Lode H. Safety and Tolerability of Commonly Prescribed Oral Antibiotics for the Treatment of Respiratory Tract Infections. *Am J Med*. 2010;123(4):S26-S38. doi:10.1016/j.amjmed.2010.02.004
64. Schilder AGM, Chonmaitree T, Cripps AW, et al. Otitis media. *Nat Rev Dis Primer*. 2016;2(1):16063. doi:10.1038/nrdp.2016.63
65. Paul T. Russell, Christopher A. Chu. Effects of Common Topical Otic Preparations on the Morphology of Isolated Cochlear Outer Hair Cells. *Acta Otolaryngol (Stockh)*. 2001;121(2):135-139. doi:10.1080/000164801300043208
66. Masaki M, Wright CG, Lee DH, Meyerhoff WL. Effects of Otic Drops on Chinchilla Tympanic Membrane. *Arch Otolaryngol - Head Neck Surg*. 1988;114(9):1007-1011. doi:10.1001/archotol.1988.01860210073019
67. Wooltorton E. Ototoxic effects from gentamicin ear drops. *CMAJ Can Med Assoc J J Assoc Medicale Can*. 2002;167(1):56.
68. Marom T, Shefer G, Tshori S, Mingelgrin S, Pitaro J. Antibiotic prescription policy for acute otitis media: do we follow the guidelines? *J Antimicrob Chemother*. 2021;76(10):2717-2724. doi:10.1093/jac/dkab250
69. Francis NA, Waldron CA, Cannings-John R, et al. Oral steroids for hearing loss associated with otitis media with effusion in children aged 2–8 years: the OSTRICH RCT. *Health Technol Assess*. 2018;22(61):1-114. doi:10.3310/hta22610
70. Ranakusuma RW, Pitoyo Y, Safitri ED, et al. Systemic corticosteroids for acute otitis media in children. Cochrane Acute Respiratory Infections Group, ed. *Cochrane Database Syst Rev*. 2018;2018(3). doi:10.1002/14651858.CD012289.pub2
71. Shaikh N, Dando EE, Dunleavy ML, et al. A Cost-Utility Analysis of 5 Strategies for the Management of Acute Otitis Media in Children. *J Pediatr*. 2017;189:54-60.e3. doi:10.1016/j.jpeds.2017.05.047

72. Spurling GK, Del Mar CB, Dooley L, Clark J, Askew DA. Delayed antibiotic prescriptions for respiratory infections. Cochrane Acute Respiratory Infections Group, ed. *Cochrane Database Syst Rev*. 2017;2022(8). doi:10.1002/14651858.CD004417.pub5
73. Hoberman A, Paradise JL, Rockette HE, et al. Shortened Antimicrobial Treatment for Acute Otitis Media in Young Children. *N Engl J Med*. 2016;375(25):2446-2456. doi:10.1056/NEJMoa1606043
74. Santos-Cortez RLP, Bhutta MF, Earl JP, et al. Panel 3: Genomics, precision medicine and targeted therapies. *Int J Pediatr Otorhinolaryngol*. 2020;130:109835. doi:10.1016/j.ijporl.2019.109835
75. Yang R, Sabharwal V, Okonkwo OS, et al. Treatment of otitis media by transtympanic delivery of antibiotics. *Sci Transl Med*. 2016;8(356). doi:10.1126/scitranslmed.aaf4363
76. Kurabi A, Beasley KA, Chang L, McCann J, Pak K, Ryan AF. Peptides actively transported across the tympanic membrane: Functional and structural properties. Sokolowski B, ed. *PLOS ONE*. 2017;12(2):e0172158. doi:10.1371/journal.pone.0172158
77. Kurabi A, Schaerer D, Noack V, et al. Active Transport of Peptides Across the Intact Human Tympanic Membrane. *Sci Rep*. 2018;8(1):11815. doi:10.1038/s41598-018-30031-6
78. Bhutta MF, Lambie J, Hobson L, et al. Transcript Analysis Reveals a Hypoxic Inflammatory Environment in Human Chronic Otitis Media With Effusion. *Front Genet*. 2020;10:1327. doi:10.3389/fgene.2019.01327
79. Rosenfeld RM, Bhaya MH, Bower CM, et al. Impact of Tympanostomy Tubes on Child Quality of Life. *Arch Otolaryngol Neck Surg*. 2000;126(5):585. doi:10.1001/archotol.126.5.585
80. Paradise JL, Feldman HM, Campbell TF, et al. Effect of Early or Delayed Insertion of Tympanostomy Tubes for Persistent Otitis Media on Developmental Outcomes at the Age of Three Years. *N Engl J Med*. 2001;344(16):1179-1187. doi:10.1056/NEJM200104193441601
81. Pawar D. Common post-operative complications in children. *Indian J Anaesth*. 2012;56(5):496. doi:10.4103/0019-5049.103970
82. Hoffman HJ, Daly KA, Bainbridge KE, et al. Panel 1: Epidemiology, Natural History, and Risk Factors. *Otolaryngol Neck Surg*. 2013;148(S4). doi:10.1177/0194599812460984
83. Minami SB, Mutai H, Suzuki T, et al. Microbiomes of the normal middle ear and ears with chronic otitis media. *The Laryngoscope*. 2017;127(10). doi:10.1002/lary.26579
84. Block SL, Hedrick J, Harrison CJ, et al. Community-Wide Vaccination with the Heptavalent Pneumococcal Conjugate Significantly Alters the Microbiology of Acute Otitis Media. *Pediatr Infect Dis J*. 2004;23(9):829-833. doi:10.1097/01.inf.0000136868.91756.80
85. Casey JR, Pichichero ME. Changes in Frequency and Pathogens Causing Acute Otitis Media in 1995–2003. *Pediatr Infect Dis J*. 2004;23(9):824-828. doi:10.1097/01.inf.0000136871.51792.19
86. Soysal A, Gönüllü E, Yıldız I, et al. Impact of the 13-valent pneumococcal conjugate vaccine on the incidences of acute otitis media, recurrent otitis media and tympanostomy tube insertion in children after its implementation into the national immunization program in Turkey. *Hum Vaccines Immunother*. 2020;16(2):445-451. doi:10.1080/21645515.2019.1656021
87. Slack M, Esposito S, Haas H, et al. *Haemophilus influenzae* type b disease in the era of conjugate vaccines: critical factors for successful eradication. *Expert Rev Vaccines*. 2020;19(10):903-917. doi:10.1080/14760584.2020.1825948
88. Sigurdsson S, Eythorsson E, Hrafnkelsson B, Erlendsdóttir H, Kristinsson KG,

Haraldsson Á. Reduction in All-Cause Acute Otitis Media in Children <3 Years of Age in Primary Care Following Vaccination With 10-Valent Pneumococcal Haemophilus influenzae Protein-D Conjugate Vaccine: A Whole-Population Study. *Clin Infect Dis*. 2018;67(8):1213-1219. doi:10.1093/cid/ciy233
89. Wu PW, Huang CC, Chao WC, Sun CC, Chiu CH, Lee TJ. Impact of influenza vaccine on childhood otitis media in Taiwan: A population-based study. Goldstein E, ed. *PLOS ONE*. 2018;13(1):e0190507. doi:10.1371/journal.pone.0190507
90. Bowatte G, Tham R, Allen K, et al. Breastfeeding and childhood acute otitis media: a systematic review and meta-analysis. *Acta Paediatr*. 2015;104:85-95. doi:10.1111/apa.13151
91. Casselbrant ML, Mandel EM, Fall PA, et al. The Heritability of Otitis Media: A Twin and Triplet Study. *JAMA*. 1999;282(22):2125. doi:10.1001/jama.282.22.2125
92. Geng R, Wang Q, Chen E, Zheng QY. Current Understanding of Host Genetics of Otitis Media. *Front Genet*. 2020;10:1395. doi:10.3389/fgene.2019.01395
93. Santos-Cortez RLP, Chiong CM, Frank DN, et al. FUT2 Variants Confer Susceptibility to Familial Otitis Media. *Am J Hum Genet*. 2018;103(5):679-690. doi:10.1016/j.ajhg.2018.09.010
94. Santos-Cortez RLP, Chiong CM, Reyes-Quintos MRT, et al. Rare A2ML1 variants confer susceptibility to otitis media. *Nat Genet*. 2015;47(8):917-920. doi:10.1038/ng.3347
95. Van Ingen G, Li J, Goedegebure A, et al. Genome-wide association study for acute otitis media in children identifies FNDC1 as disease contributing gene. *Nat Commun*. 2016;7(1):12792. doi:10.1038/ncomms12792
96. Carpinelli MR, Kruse EA, Arhatari BD, et al. Mice Haploinsufficient for Ets1 and Fli1 Display Middle Ear Abnormalities and Model Aspects of Jacobsen Syndrome. *Am J Pathol*. 2015;185(7):1867-1876. doi:10.1016/j.ajpath.2015.03.026
97. Tian C, Harris BS, Johnson KR. Ectopic Mineralization and Conductive Hearing Loss in Enpp1asj Mutant Mice, a New Model for Otitis Media and Tympanosclerosis. Heymann D, ed. *PLOS ONE*. 2016;11(12):e0168159. doi:10.1371/journal.pone.0168159
98. Crompton M, Purnell T, Tyrer HE, et al. A mutation in Nischarin causes otitis media via LIMK1 and NF-κB pathways. Justice MJ, ed. *PLOS Genet*. 2017;13(8):e1006969. doi:10.1371/journal.pgen.1006969
99. Deniffel D, Nuyen B, Pak K, et al. Otitis Media and Nasopharyngeal Colonization in *ccl3* $^{-/-}$ Mice. Payne SM, ed. *Infect Immun*. 2017;85(11):e00148-17. doi:10.1128/IAI.00148-17
100. Leichtle A, Hernandez M, Ebmeyer J, et al. CC Chemokine Ligand 3 Overcomes the Bacteriocidal and Phagocytic Defect of Macrophages and Hastens Recovery from Experimental Otitis Media in TNF−/− Mice. *J Immunol*. 2010;184(6):3087-3097. doi:10.4049/jimmunol.0901167
101. Mulay A, Akram K, Williams D, et al. An *in vitro* model of murine middle ear epithelium. *Dis Model Mech*. Published online January 1, 2016:dmm.026658. doi:10.1242/dmm.026658
102. Roy MG, Livraghi-Butrico A, Fletcher AA, et al. Muc5b is required for airway defence. *Nature*. 2014;505(7483):412-416. doi:10.1038/nature12807
103. Mulay A, Hood DW, Williams D, et al. Loss of the homeostatic protein BPIFA1, leads to exacerbation of otitis media severity in the Junbo mouse model. *Sci Rep*. 2018;8(1):3128. doi:10.1038/s41598-018-21166-7
104. Barré-Sinoussi F, Montagutelli X. Animal models are essential to biological research: issues and perspectives. *Future Sci OA*. 2015;1(4):fso.15.63. doi:10.4155/fso.15.63
105. Mitchell RB, Pereira KD, Lazar RH. Fat graft myringoplasty in children – a safe and successful day-stay procedure. *J Laryngol Otol*. 1997;111(2):106-108.

doi:10.1017/S002221510013659X
106. Rye MS, Bhutta MF, Cheeseman MT, et al. Unraveling the genetics of otitis media: from mouse to human and back again. *Mamm Genome*. 2011;22(1-2):66-82. doi:10.1007/s00335-010-9295-1
107. Tyrer HE, Crompton M, Bhutta MF. What Have We Learned from Murine Models of Otitis Media? *Curr Allergy Asthma Rep*. 2013;13(5):501-511. doi:10.1007/s11882-013-0360-1
108. Bhutta MF. Mouse Models of Otitis Media: Strengths and Limitations. *Otolaryngol Neck Surg*. 2012;147(4):611-614. doi:10.1177/0194599812449986
109. Hardisty RE, Erven A, Logan K, et al. The Deaf Mouse Mutant Jeff (Jf) is a Single Gene Model of Otitis Media. *J Assoc Res Otolaryngol*. 2003;4(2):130-138. doi:10.1007/s10162-002-3015-9
110. MacArthur CJ, Hefeneider SH, Kempton JB, Parrish SK, McCoy SL, Trune DR. Evaluation of the mouse model for acute otitis media. *Hear Res*. 2006;219(1-2):12-23. doi:10.1016/j.heares.2006.05.012
111. Davidoss NH, Varsak YK, Santa Maria PL. Animal models of acute otitis media – A review with practical implications for laboratory research. *Eur Ann Otorhinolaryngol Head Neck Dis*. 2018;135(3):183-190. doi:10.1016/j.anorl.2017.06.013
112. Kuijpers W, Van Der Beek JMH, Jap PHK, Tonnaer ELG. The structure of the middle ear epithelium of the rat and the effect of Eustachian tube obstruction. *Histochem J*. 1984;16(8):807-818. doi:10.1007/BF01002787
113. Cayé-Thomasen P, Tos M. Histopathologic differences due to bacterial species in acute otitis media. *Int J Pediatr Otorhinolaryngol*. 2002;63(2):99-110. doi:10.1016/S0165-5876(01)00641-3
114. Daniel HJ, Brinn JE, Fulghum RS, Barrett KA. Comparative Anatomy of Eustachian Tube and Middle Ear Cavity in Animal Models for Otitis Media. *Ann Otol Rhinol Laryngol*. 1982;91(1):82-89. doi:10.1177/000348948209100118
115. Hellström S, Stenfors LE. The pressure equilibrating function of pars flaccida in middle ear mechanics. *Acta Physiol Scand*. 1983;118(4):337-341. doi:10.1111/j.1748-1716.1983.tb07280.x
116. Hermansson A, Emgrd P, Prellner K, Hellström S. A rat model for pneumococcal otitis media. *Am J Otolaryngol*. 1988;9(3):97-101. doi:10.1016/S0196-0709(88)80013-9
117. Prellner K, Hermansson A, White P, Melhus Å, Briles D. Immunization and Protection in Pneumococcal Otitis Media Studied in a Rat Model. *Microb Drug Resist*. 1999;5(1):73-82. doi:10.1089/mdr.1999.5.73
118. Watanabe N, DeMaria TF, Lewis DM, Mogi G, Lim DJ. Experimental otitis media in chinchillas. II. Comparison of the middle ear immune responses to S pneumoniae types 3 and 23. *Ann Otol Rhinol Laryngol Suppl*. 1982;93:9-16.
119. Bakaletz LO. Chinchilla as a robust, reproducible and polymicrobial model of otitis media and its prevention. *Expert Rev Vaccines*. 2009;8(8):1063-1082. doi:10.1586/erv.09.63
120. Murrah KA, Pang B, Richardson S, et al. Nonencapsulated Streptococcus pneumoniae causes otitis media during single-species infection and during polymicrobial infection with nontypeable Haemophilus influenzae. *Pathog Dis*. 2015;73(5). doi:10.1093/femspd/ftu011
121. Kubinyecz O, Vikhe PP, Purnell T, Brown SDM, Tateossian H. The Jeff Mouse Mutant Model for Chronic Otitis Media Manifests Gain-of-Function as Well as Loss-of-Function Effects. *Front Genet*. 2020;11:498. doi:10.3389/fgene.2020.00498
122. Cheeseman MT, Tyrer HE, Williams D, et al. HIF–VEGF Pathways Are Critical for Chronic Otitis Media in Junbo and Jeff Mouse Mutants. Avraham KB, ed. *PLoS Genet*. 2011;7(10):e1002336. doi:10.1371/journal.pgen.1002336

123. Hood D, Moxon R, Purnell T, et al. A new model for NTHi middle ear infection in the *Junbo* mutant mouse. *Dis Model Mech*. Published online January 1, 2015:dmm.021659. doi:10.1242/dmm.021659
124. Khomtchouk KM, Kouhi A, Xia A, et al. A novel mouse model of chronic suppurative otitis media and its use in preclinical antibiotic evaluation. *Sci Adv*. 2020;6(33):eabc1828. doi:10.1126/sciadv.abc1828
125. Dewan KK, Taylor-Mulneix DL, Campos LL, et al. A model of chronic, transmissible Otitis Media in mice. Philpott DJ, ed. *PLOS Pathog*. 2019;15(4):e1007696. doi:10.1371/journal.ppat.1007696
126. Langhans SA. Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol*. 2018;9:6. doi:10.3389/fphar.2018.00006
127. Ueyama S, Jin S, Ueyama T, Rhim JS, Lim DJ. Immortalization of Rat Middle Ear Epithelial Cells by Adeno 12-SV40 Hybrid Virus. *Ann Otol Rhinol Laryngol*. 2001;110(2):132-141. doi:10.1177/000348940111000208
128. Moon SK, Brackmann DE, Chun YM, et al. Immortalization of Normal Adult Human Middle Ear Epithelial Cells Using a Retrovirus Containing the E6/E7 Genes of Human Papillomavirus Type 16. *Ann Otol Rhinol Laryngol*. 2002;111(6):507-517. doi:10.1177/000348940211100606
129. Tsuchiya K, Kim Y, Ondrey FG, Lin J. Characterization of a temperature-sensitive mouse middle ear epithelial cell line. *Acta Otolaryngol (Stockh)*. 2005;125(8):823-829. doi:10.1080/00016480510031533
130. Schousboe LP, Blegvad S, Ovesen T. Quantification of in vitro growth and synthesis in rabbit middle ear epithelial cells. *Eur Arch Otorhinolaryngol*. 1996;253(7). doi:10.1007/BF00168490
131. Nakamura A, Lim DJ, DeMaria TF, Van Blitterswijk CA. Primary Culture of Chinchilla Middle Ear Epithelium. *Ann Otol Rhinol Laryngol*. 1991;100(9):774-782. doi:10.1177/000348949110000916
132. Nakamura A, DeMaria TF, Arya G, Lim DJ, Van Blitterswijk C. Serial Culture and Characterization of the Chinchilla Middle Ear Epithelium. *Ann Otol Rhinol Laryngol*. 1991;100(12):1024-1031. doi:10.1177/000348949110001212
133. Amesaral R, Kim Y, Sano S, Harada T, Juhn SK. Primary cultures of middle ear epithelial cells from chinchillas. *Eur Arch Otorhinolaryngol*. 1992;249(3). doi:10.1007/BF00183493
134. Herman P, Friedlander G, Huy PT, Amiel C. Ion transport by primary cultures of Mongolian gerbil middle ear epithelium. *Am J Physiol-Ren Physiol*. 1992;262(3):F373-F380. doi:10.1152/ajprenal.1992.262.3.F373
135. Mulay A, Chowdhury MMK, James CT, Bingle L, Bingle CD. The transcriptional landscape of the cultured murine middle ear epithelium *in vitro*. *Biol Open*. 2021;10(4):bio056564. doi:10.1242/bio.056564
136. Nell MJ, Op't Hof BM, Koerten HK, Grote JJ. Effect of Endotoxin on Cultured Human Middle Ear Epithelium. *ORL*. 1999;61(4):201-205. doi:10.1159/000027671
137. Choi JY, Kim CH, Lee WS, Kim HN, Song KS, Yoon JH. Ciliary and Secretory Differentiation of Normal Human Middle Ear Epithelial Cells. *Acta Otolaryngol (Stockh)*. 2002;122(3):270-275. doi:10.1080/000164802753648141
138. Choi JY, Cho K nam, Yoo K han, Shin J hyun, Yoon J heon. Retinoic Acid Depletion Induces Keratinizing Squamous Differentiation in Human Middle Ear Epithelial Cell Cultures. *Acta Otolaryngol (Stockh)*. 2003;123(4):466-470. doi:10.1080/0036554021000028097
139. Buchman CA, Fregien N. Influenza A Virus Infection of Human Middle Ear Cells In Vitro: *The Laryngoscope*. 2000;110(10):1739-1744. doi:10.1097/00005537-200010000-

00034
140. Chen Y, Val S, Krueger A, et al. Human primary middle ear epithelial cell culture: A novel in vitro model to study otitis media. *Laryngoscope Investig Otolaryngol*. 2019;4(6):663-672. doi:10.1002/lio2.319
141. Espahbodi M, Samuels TL, McCormick C, et al. Analysis of Inflammatory Signaling in Human Middle Ear Cell Culture Models of Paediatric Otitis Media. *The Laryngoscope*. 2021;131(2):410-416. doi:10.1002/lary.28687
142. Mather MW, Verdon B, Botting RA, et al. Development of a physiological model of human middle ear epithelium. *Laryngoscope Investig Otolaryngol*. 2021;6(5):1167-1174. doi:10.1002/lio2.661
143. Epa AP, Thatcher TH, Pollock SJ, et al. Normal Human Lung Epithelial Cells Inhibit Transforming Growth Factor-β Induced Myofibroblast Differentiation via Prostaglandin E2. Ding Q, ed. *PLOS ONE*. 2015;10(8):e0135266. doi:10.1371/journal.pone.0135266
144. Ishikawa S, Ishimori K, Ito S. A 3D epithelial–mesenchymal co-culture model of human bronchial tissue recapitulates multiple features of airway tissue remodeling by TGF-β1 treatment. *Respir Res*. 2017;18(1):195. doi:10.1186/s12931-017-0680-0
145. Yoshikawa M, Kojima H, Yaguchi Y, Okada N, Saito H, Moriyama H. Cholesteatoma Fibroblasts Promote Epithelial Cell Proliferation through Overexpression of Epiregulin. Slominski AT, ed. *PLoS ONE*. 2013;8(6):e66725. doi:10.1371/journal.pone.0066725
146. Schürmann M, Oppel F, Shao S, et al. Chronic inflammation of middle ear cholesteatoma promotes its recurrence via a paracrine mechanism. *Cell Commun Signal*. 2021;19(1):25. doi:10.1186/s12964-020-00690-y
147. Neeff M, Biswas K, Hoggard M, Taylor MW, Douglas R. Molecular Microbiological Profile of Chronic Suppurative Otitis Media. Munson E, ed. *J Clin Microbiol*. 2016;54(10):2538-2546. doi:10.1128/JCM.01068-16
148. Haas J, Braun S, Wutzler P. Burden of influenza in Germany: a retrospective claims database analysis for the influenza season 2012/2013. *Eur J Health Econ*. 2016;17(6):669-679. doi:10.1007/s10198-015-0708-7
149. Stockmann C, Ampofo K, Hersh AL, et al. Seasonality of Acute Otitis Media and the Role of Respiratory Viral Activity in Children. *Pediatr Infect Dis J*. 2013;32(4):314-319. doi:10.1097/INF.0b013e31827d104e
150. Hullegie S, Schilder AGM, Marchisio P, et al. A Strong Decline in the Incidence of Childhood Otitis Media During the COVID-19 Pandemic in the Netherlands. *Front Cell Infect Microbiol*. 2021;11:768377. doi:10.3389/fcimb.2021.768377
151. Torretta S, Cantoni B, Bertolozzi G, et al. Has Otitis Media Disappeared during COVID-19 Pandemic? A Fortuitus Effect of Domestic Confinement. *J Clin Med*. 2021;10(13):2851. doi:10.3390/jcm10132851
152. Iannella G, Magliulo G, Lechien JR, et al. Impact of COVID-19 pandemic on the incidence of otitis media with effusion in adults and children: a multicenter study. *Eur Arch Otorhinolaryngol*. 2022;279(5):2383-2389. doi:10.1007/s00405-021-06958-4
153. Lasminingrum L, Mahdiani S, Dermawan A, Husnayain MM. Impact of the COVID-19 pandemic on the volume of chronic suppurative otitis media surgeries. *Ann Med Surg*. 2022;79. doi:10.1016/j.amsu.2022.103921
154. Haapanen M, Renko M, Artama M, et al. Tympanostomies and tonsillar surgery in children during the COVID -19 pandemic in Finland. *Laryngoscope Investig Otolaryngol*. 2021;6(4):878-884. doi:10.1002/lio2.622
155. Yatsyshina S, Mayanskiy N, Shipulina O, et al. Detection of respiratory pathogens in paediatric acute otitis media by PCR and comparison of findings in the middle ear and nasopharynx. *Diagn Microbiol Infect Dis*. 2016;85(1):125-130. doi:10.1016/j.diagmicrobio.2016.02.010

156. Ngo CC, Massa HM, Thornton RB, Cripps AW. Predominant Bacteria Detected from the Middle Ear Fluid of Children Experiencing Otitis Media: A Systematic Review. Reid S, ed. *PLOS ONE*. 2016;11(3):e0150949. doi:10.1371/journal.pone.0150949
157. Hullegie S, Venekamp RP, Van Dongen TMA, et al. Prevalence and Antimicrobial Resistance of Bacteria in Children With Acute Otitis Media and Ear Discharge: A Systematic Review. *Pediatr Infect Dis J*. 2021;40(8):756-762. doi:10.1097/INF.0000000000003134
158. Kolbe AR, Castro-Nallar E, Preciado D, Pérez-Losada M. Altered Middle Ear Microbiome in Children With Chronic Otitis Media With Effusion and Respiratory Illnesses. *Front Cell Infect Microbiol*. 2019;9:339. doi:10.3389/fcimb.2019.00339
159. Hall-Stoodley L, Hu FZ, Gieseke A, et al. Direct Detection of Bacterial Biofilms on the Middle-Ear Mucosa of Children With Chronic Otitis Media. *JAMA*. 2006;296(2):202. doi:10.1001/jama.296.2.202
160. Thornton RB, Rigby PJ, Wiertsema SP, et al. Multi-species bacterial biofilm and intracellular infection in otitis media. *BMC Pediatr*. 2011;11(1):94. doi:10.1186/1471-2431-11-94
161. Bair KL, Campagnari AA. Moraxella catarrhalis Promotes Stable Polymicrobial Biofilms With the Major Otopathogens. *Front Microbiol*. 2019;10:3006. doi:10.3389/fmicb.2019.03006
162. Anwar S, Acharya S, Thapa S, Rabadi J, Mobarakai N. Carbapenemase producing Klebsiella pneumoniae (KPC) meningitis from chronic otitis media. *IDCases*. 2020;22:e00963. doi:10.1016/j.idcr.2020.e00963
163. Byrd MS, Pang B, Hong W, et al. Direct Evaluation of Pseudomonas aeruginosa Biofilm Mediators in a Chronic Infection Model. McCormick BA, ed. *Infect Immun*. 2011;79(8):3087-3095. doi:10.1128/IAI.00057-11
164. Gaikwad UN, Arora R, Gade N, et al. Corynebacterium diphtheriae: An emerging cause of chronic suppurative otitis media. *Int J Infect Dis*. 2016;45:173. doi:10.1016/j.ijid.2016.02.409
165. Kurono Y, Tomonaga K, Mogi G. Staphylococcus epidermidis and Staphylococcus aureus in Otitis Media With Effusion. *Arch Otolaryngol - Head Neck Surg*. 1988;114(11):1262-1265. doi:10.1001/archotol.1988.01860230056023
166. Mansoor T, Musani MA, Khalid G, Kamal M. Pseudomonas aeruginosa in chronic suppurative otitis media: sensitivity spectrum against various antibiotics in Karachi. *J Ayub Med Coll Abbottabad JAMC*. 2009;21(2):120-123.
167. McGOVERN FH, Khuri AA. Chronic Otitis Media and Mastoiditis Due to Proteus Vulgaris (Bacillus Proteus). *Arch Otolaryngol - Head Neck Surg*. 1958;67(4):403-409. doi:10.1001/archotol.1958.00730010413004
168. Sengupta M. Corynebacterium amycolatum : An Unexpected Pathogen in the Ear. *J Clin Diagn Res*. Published online 2015. doi:10.7860/JCDR/2015/15134.7002
169. Senior BW, Sweeney G. The association of particular types of Proteus with chronic suppurative otitis media. *J Med Microbiol*. 1984;17(2):201-205. doi:10.1099/00222615-17-2-201
170. Ralte Z, Naina P, Amladi A, John M, Anndan S, Varghese AM. Determination of Biofilm-Forming Capacity of Otopathogens Isolated from Discharging Ears in Children with Chronic Otitis Media. *Indian J Med Microbiol*. 2019;37(3):442-445. doi:10.4103/ijmm.IJMM_19_404
171. Yadav MK, Chae SW, Go YY, Im GJ, Song JJ. In vitro Multi-Species Biofilms of Methicillin-Resistant Staphylococcus aureus and Pseudomonas aeruginosa and Their Host Interaction during In vivo Colonization of an Otitis Media Rat Model. *Front Cell Infect Microbiol*. 2017;7. doi:10.3389/fcimb.2017.00125

172. Girois SB, Chapuis F, Decullier E, Revol BGP. Adverse effects of antifungal therapies in invasive fungal infections: review and meta-analysis. *Eur J Clin Microbiol Infect Dis*. 2006;25(2):138-149. doi:10.1007/s10096-005-0080-0
173. Keller LE, Bradshaw JL, Pipkins H, McDaniel LS. Surface Proteins and Pneumolysin of Encapsulated and Nonencapsulated Streptococcus pneumoniae Mediate Virulence in a Chinchilla Model of Otitis Media. *Front Cell Infect Microbiol*. 2016;6. doi:10.3389/fcimb.2016.00055
174. Yadav MK, Vidal JE, Go YY, Kim SH, Chae SW, Song JJ. The LuxS/AI-2 Quorum-Sensing System of Streptococcus pneumoniae Is Required to Cause Disease, and to Regulate Virulence- and Metabolism-Related Genes in a Rat Model of Middle Ear Infection. *Front Cell Infect Microbiol*. 2018;8:138. doi:10.3389/fcimb.2018.00138
175. Singh B, Al-Jubair T, Voraganti C, et al. Moraxella catarrhalis Binds Plasminogen To Evade Host Innate Immunity. Payne SM, ed. *Infect Immun*. 2015;83(9):3458-3469. doi:10.1128/IAI.00310-15
176. Tan A, Li WS, Verderosa AD, et al. Moraxella catarrhalis NucM is an entry nuclease involved in extracellular DNA and RNA degradation, cell competence and biofilm scaffolding. *Sci Rep*. 2019;9(1):2579. doi:10.1038/s41598-019-39374-0
177. Armbruster CE, Hong W, Pang B, et al. Indirect Pathogenicity of *Haemophilus influenzae* and *Moraxella catarrhalis* in Polymicrobial Otitis Media Occurs via Interspecies Quorum Signaling. McDaniel LS, ed. *mBio*. 2010;1(3):e00102-10. doi:10.1128/mBio.00102-10
178. Foxwell AR, Kyd JM, Cripps AW. Nontypeable *Haemophilus influenzae* : Pathogenesis and Prevention. *Microbiol Mol Biol Rev*. 1998;62(2):294-308. doi:10.1128/MMBR.62.2.294-308.1998
179. Clementi CF, Murphy TF. Non-Typeable Haemophilus influenzae Invasion and Persistence in the Human Respiratory Tract. *Front Cell Infect Microbiol*. 2011;1. doi:10.3389/fcimb.2011.00001
180. Swords WE. Nontypeable Haemophilus influenzae biofilms: role in chronic airway infections. *Front Cell Infect Microbiol*. 2012;2. doi:10.3389/fcimb.2012.00097
181. Starner TD, Zhang N, Kim G, Apicella MA, McCray PB. *Haemophilus influenzae* Forms Biofilms on Airway Epithelia: Implications in Cystic Fibrosis. *Am J Respir Crit Care Med*. 2006;174(2):213-220. doi:10.1164/rccm.200509-1459OC
182. Devaraj A, Justice SS, Bakaletz LO, Goodman SD. DNABII proteins play a central role in UPEC biofilm structure: Structure of UPEC biofilm depends on DNABII proteins. *Mol Microbiol*. 2015;96(6):1119-1135. doi:10.1111/mmi.12994
183. Das J, Mokrzan E, Lakhani V, et al. Extracellular DNA and Type IV Pilus Expression Regulate the Structure and Kinetics of Biofilm Formation by Nontypeable *Haemophilus influenzae*. Parsek MR, ed. *mBio*. 2017;8(6):e01466-17. doi:10.1128/mBio.01466-17
184. Kress-Bennett JM, Hiller NL, Eutsey RA, et al. Identification and Characterization of msf, a Novel Virulence Factor in Haemophilus influenzae. Roop RM, ed. *PLOS ONE*. 2016;11(3):e0149891. doi:10.1371/journal.pone.0149891
185. Osgood R, Salamone F, Diaz A, Casey JR, Bajorski P, Pichichero ME. Effect of pH and oxygen on biofilm formation in acute otitis media associated NTHi clinical isolates: pH and Oxygen Effect on AOM NTHi Isolates. *The Laryngoscope*. 2015;125(9):2204-2208. doi:10.1002/lary.25102
186. Fox KL, Dowideit SJ, Erwin AL, Srikhanta YN, Smith AL, Jennings MP. Haemophilus influenzae phasevarions have evolved from type III DNA restriction systems into epigenetic regulators of gene expression. *Nucleic Acids Res*. 2007;35(15):5242-5252. doi:10.1093/nar/gkm571
187. Brockman KL, Jurcisek JA, Atack JM, Srikhanta YN, Jennings MP, Bakaletz LO. ModA2 Phasevarion Switching in Nontypeable *Haemophilus influenzae* Increases the Severity of

Experimental Otitis Media. *J Infect Dis*. 2016;214(5):817-824. doi:10.1093/infdis/jiw243
188. Kaur R, Casey JR, Pichichero ME. Relationship With Original Pathogen in Recurrence of Acute Otitis Media After Completion of Amoxicillin/Clavulanate: Bacterial Relapse or New Pathogen. *Pediatr Infect Dis J*. 2013;32(11):1159-1162. doi:10.1097/INF.0b013e31829e3779
189. Krueger A, Val S, Pérez-Losada M, et al. Relationship of the Middle Ear Effusion Microbiome to Secretory Mucin Production in Paediatric Patients With Chronic Otitis Media. *Pediatr Infect Dis J*. 2017;36(7):635-640. doi:10.1097/INF.0000000000001493
190. Kerschner JE, Tripathi S, Khampang P, Papsin BC. *MUC5AC* Expression in Human Middle Ear Epithelium of Patients With Otitis Media. *Arch Otolaryngol Neck Surg*. 2010;136(8):819. doi:10.1001/archoto.2010.123
191. Pang B, Winn D, Johnson R, et al. Lipooligosaccharides Containing Phosphorylcholine Delay Pulmonary Clearance of Nontypeable *Haemophilus influenzae*. *Infect Immun*. 2008;76(5):2037-2043. doi:10.1128/IAI.01716-07
192. West-Barnette S, Rockel A, Swords WE. Biofilm Growth Increases Phosphorylcholine Content and Decreases Potency of Nontypeable *Haemophilus influenzae* Endotoxins. *Infect Immun*. 2006;74(3):1828-1836. doi:10.1128/IAI.74.3.1828-1836.2006
193. Puig C, Marti S, Hermans PWM, et al. Incorporation of Phosphorylcholine into the Lipooligosaccharide of Nontypeable Haemophilus influenzae Does Not Correlate with the Level of Biofilm Formation *In Vitro*. Camilli A, ed. *Infect Immun*. 2014;82(4):1591-1599. doi:10.1128/IAI.01445-13
194. Langereis JD, Cremers AJH, Vissers M, Van Beek J, Meis JF, De Jonge MI. Nontypeable Haemophilus influenzae Invasive Blood Isolates Are Mainly Phosphorylcholine Negative and Show Decreased Complement-Mediated Killing That Is Associated with Lower Binding of IgM and CRP in Comparison to Colonizing Isolates from the Oropharynx. Payne SM, ed. *Infect Immun*. 2019;87(2):e00604-18. doi:10.1128/IAI.00604-18
195. Pickering JL, Prosser A, Corscadden KJ, et al. Haemophilus haemolyticus Interaction with Host Cells Is Different to Nontypeable Haemophilus influenzae and Prevents NTHi Association with Epithelial Cells. *Front Cell Infect Microbiol*. 2016;6. doi:10.3389/fcimb.2016.00050
196. Atack JM, Winter LE, Jurcisek JA, Bakaletz LO, Barenkamp SJ, Jennings MP. Selection and Counterselection of Hia Expression Reveals a Key Role for Phase-Variable Expression of Hia in Infection Caused by Nontypeable Haemophilus influenzae. *J Infect Dis*. 2015;212(4):645-653. doi:10.1093/infdis/jiv103
197. Atack JM, Day CJ, Poole J, et al. The HMW2 adhesin of non-typeable Haemophilus influenzae is a human-adapted lectin that mediates high-affinity binding to 2-6 linked N-acetylneuraminic acid glycans. *Biochem Biophys Res Commun*. 2018;503(2):1103-1107. doi:10.1016/j.bbrc.2018.06.126
198. Su YC, Mukherjee O, Singh B, et al. *Haemophilus influenzae* P4 Interacts With Extracellular Matrix Proteins Promoting Adhesion and Serum Resistance. *J Infect Dis*. 2016;213(2):314-323. doi:10.1093/infdis/jiv374
199. Fink DL, Buscher AZ, Green B, Fernsten P, St. Geme JW. The Haemophilus influenzae Hap autotransporter mediates microcolony formation and adherence to epithelial cells and extracellular matrix via binding regions in the C-terminal end of the passenger domain. *Cell Microbiol*. 2003;5(3):175-186. doi:10.1046/j.1462-5822.2003.00266.x
200. Ronander E, Brant M, Janson H, Sheldon J, Forsgren A, Riesbeck K. Identification of a novel Haemophilus influenzae protein important for adhesion to epithelial cells. *Microbes Infect*. 2008;10(1):87-96. doi:10.1016/j.micinf.2007.10.006
201. Ikeda M, Enomoto N, Hashimoto D, et al. Nontypeable Haemophilus influenzae exploits the interaction between protein-E and vitronectin for the adherence and invasion to

bronchial epithelial cells. *BMC Microbiol*. 2015;15(1):263. doi:10.1186/s12866-015-0600-8
202. Hallström T, Blom AM, Zipfel PF, Riesbeck K. Nontypeable Haemophilus influenzae protein E binds vitronectin and is important for serum resistance. *J Immunol Baltim Md 1950*. 2009;183(4):2593-2601. doi:10.4049/jimmunol.0803226
203. Kubiet M, Ramphal R, Weber A, Smith A. Pilus-mediated adherence of Haemophilus influenzae to human respiratory mucins. *Infect Immun*. 2000;68(6):3362-3367. doi:10.1128/IAI.68.6.3362-3367.2000
204. Janson H, Carlén B, Cervin A, et al. Effects on the Ciliated Epithelium of Protein D–Producing and –Nonproducing Nontypeable *Haemophilus influenzae* in Nasopharyngeal Tissue Cultures. *J Infect Dis*. 1999;180(3):737-746. doi:10.1086/314921
205. Ahrén IL, Williams DL, Rice PJ, Forsgren A, Riesbeck K. The Importance of a β-Glucan Receptor in the Nonopsonic Entry of Nontypeable *Haemophilus influenzae* into Human Monocytic and Epithelial Cells. *J Infect Dis*. 2001;184(2):150-158. doi:10.1086/322016
206. Ketterer MR, Shao JQ, Hornick DB, Buscher B, Bandi VK, Apicella MA. Infection of Primary Human Bronchial Epithelial Cells by *Haemophilus influenzae* : Macropinocytosis as a Mechanism of Airway Epithelial Cell Entry. Kozel TR, ed. *Infect Immun*. 1999;67(8):4161-4170. doi:10.1128/IAI.67.8.4161-4170.1999
207. Zimmerman GA, McIntyre TM, Prescott SM, Stafforini DM. The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis: *Crit Care Med*. 2002;30(Supplement):S294-S301. doi:10.1097/00003246-200205001-00020
208. Heuer HO. Involvement of platelet-activating factor (PAF) in septic shock and priming as indicated by the effect of hetrazepinoic PAF antagonists. *Lipids*. 1991;26(12):1369-1373. doi:10.1007/BF02536569
209. Muñoz-Cano RM, Casas-Saucedo R, Valero Santiago A, Bobolea I, Ribó P, Mullol J. Platelet-Activating Factor (PAF) in Allergic Rhinitis: Clinical and Therapeutic Implications. *J Clin Med*. 2019;8(9):1338. doi:10.3390/jcm8091338
210. Pałgan K, Bartuzi Z. Platelet activating factor in allergies. *Int J Immunopathol Pharmacol*. 2015;28(4):584-589. doi:10.1177/0394632015600598
211. Patel PS, Kearney JF. CD36 and Platelet-Activating Factor Receptor Promote House Dust Mite Allergy Development. *J Immunol*. 2017;199(3):1184-1195. doi:10.4049/jimmunol.1700034
212. Yost CC, Weyrich AS, Zimmerman GA. The platelet activating factor (PAF) signaling cascade in systemic inflammatory responses. *Biochimie*. 2010;92(6):692-697. doi:10.1016/j.biochi.2010.02.011
213. O'Toole R, Dhar Shukla S, Fairbairn R, et al. An antagonist of the platelet-activating factor receptor inhibits adherence of both nontypeable Haemophilus influenzae and Streptococcus pneumoniae to cultured human bronchial epithelial cells exposed to cigarette smoke. *Int J Chron Obstruct Pulmon Dis*. 2016;Volume 11:1647-1655. doi:10.2147/COPD.S108698
214. Van Der Sluijs KF, Van Elden LJR, Nijhuis M, et al. Involvement of the platelet-activating factor receptor in host defense against *Streptococcus pneumoniae* during postinfluenza pneumonia. *Am J Physiol-Lung Cell Mol Physiol*. 2006;290(1):L194-L199. doi:10.1152/ajplung.00050.2005
215. Rijneveld AW, Weijer S, Florquin S, et al. Improved Host Defense against Pneumococcal Pneumonia in Platelet-Activating Factor Receptor–Deficient Mice. *J Infect Dis*. 2004;189(4):711-716. doi:10.1086/381392
216. Garcia CC, Russo RC, Guabiraba R, et al. Platelet-Activating Factor Receptor Plays a Role in Lung Injury and Death Caused by Influenza A in Mice. Fouchier RAM, ed. *PLoS*

Pathog. 2010;6(11):e1001171. doi:10.1371/journal.ppat.1001171
217. Palur Ramakrishnan AVK, Varghese TP, Vanapalli S, Nair NK, Mingate MD. Platelet activating factor: A potential biomarker in acute coronary syndrome? *Cardiovasc Ther*. 2017;35(1):64-70. doi:10.1111/1755-5922.12233
218. Faden H, DeCastro G, Brodsky L, et al. Arachidonic Acid Metabolites in Middle Ear Effusions of Children. *Ann Otol Rhinol Laryngol*. 1991;100(7):589-592. doi:10.1177/000348949110000714
219. Ogura M, Furukawa M, Tada N, Ikeda H, Yamashita T. Platelet-activating factor (PAF) induced cytokine production and otitis media with effusion (OME) in the rat middle ear. *Auris Nasus Larynx*. 2008;35(3):338-343. doi:10.1016/j.anl.2007.09.009
220. Tachibana F, Shimada T, Hori Y, Wada Y, Ishitani Y, Koike Y. Platelet-Activating Factor and Leukotrienes in Acute Otitis Media, Secretory Otitis Media, and Chronic Otitis Media on the Acute Excerbation. *Auris Nasus Larynx*. 1996;23(1):20-25. doi:10.1016/S0385-8146(96)80004-6
221. Swords WE, Buscher BA, Ver Steeg Ii K, et al. Non-typeable Haemophilus influenzae adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. *Mol Microbiol*. 2000;37(1):13-27. doi:10.1046/j.1365-2958.2000.01952.x
222. Clementi CF, Håkansson AP, Murphy TF. Internalization and Trafficking of Nontypeable Haemophilus influenzae in Human Respiratory Epithelial Cells and Roles of IgA1 Proteases for Optimal Invasion and Persistence. McCormick BA, ed. *Infect Immun*. 2014;82(1):433-444. doi:10.1128/IAI.00864-13
223. Swords WE, Ketterer MR, Shao J, Campbell CA, Weiser JN, Apicella MA. Binding of the non-typeable Haemophilus influenzae lipooligosaccharide to the PAF receptor initiates host cell signalling. *Cell Microbiol*. 2001;3(8):525-536. doi:10.1046/j.1462-5822.2001.00132.x
224. Clark SE, Snow J, Li J, Zola TA, Weiser JN. Phosphorylcholine allows for evasion of bactericidal antibody by Haemophilus influenzae. *PLoS Pathog*. 2012;8(3):e1002521. doi:10.1371/journal.ppat.1002521
225. Fernández-Calvet A, Rodríguez-Arce I, Almagro G, et al. Modulation of Haemophilus influenzae interaction with hydrophobic molecules by the VacJ/MlaA lipoprotein impacts strongly on its interplay with the airways. *Sci Rep*. 2018;8(1):6872. doi:10.1038/s41598-018-25232-y
226. Lysenko ES, Gould J, Bals R, Wilson JM, Weiser JN. Bacterial Phosphorylcholine Decreases Susceptibility to the Antimicrobial Peptide LL-37/hCAP18 Expressed in the Upper Respiratory Tract. Tuomanen EI, ed. *Infect Immun*. 2000;68(3):1664-1671. doi:10.1128/IAI.68.3.1664-1671.2000
227. Chao W, Olson MS. Platelet-activating factor: receptors and signal transduction. *Biochem J*. 1993;292 (Pt 3)(Pt 3):617-629. doi:10.1042/bj2920617
228. Leipziger J, Nitschke R, Greger R. Regulation of the Intracellular Calcium Concentration in Epithelial Cells. *Kidney Blood Press Res*. 1996;19(3-4):148-150. doi:10.1159/000174062
229. Furukawa M, Kubo N, Yamashita T. Biochemical evidence of platelet-activating factor (paf) in human middle ear effusions. *The Laryngoscope*. 1995;105(2):188-191. doi:10.1288/00005537-199502000-00015
230. Diven WF, Burckart GJ, Alper CM, Jaffe R, Evans RW, Doyle WJ. Expression of Acute Otitis Media after Receptor Blockade of Platelet Activating Factor, Thromboxane, and Leukotrienes in the Chinchilla. *Ann Otol Rhinol Laryngol*. 1998;107(3):199-206. doi:10.1177/000348949810700303
231. Karasen RM, Sutbeyaz Y, Ozdemir H, Aktan B, Gundogdu C. Effect of Web 2170 BS,

Platelet Activating Factor Receptor Inhibitor, in the Guinea PIG Model of Middle Ear Inflammation. *Ann Otol Rhinol Laryngol*. 2000;109(6):549-553. doi:10.1177/000348940010900604
232. Yokota S ichi, Okabayashi T, Yoto Y, Hori T, Tsutsumi H, Fujii N. Fosfomycin suppresses RS-virus-induced Streptococcus pneumoniae and Haemophilus influenzae adhesion to respiratory epithelial cells via the platelet-activating factor receptor: Fosfomycin suppresses bacterial adhesion. *FEMS Microbiol Lett*. 2010;310(1):84-90. doi:10.1111/j.1574-6968.2010.02049.x
233. Nogues JC, Pérez-Losada M, Preciado D. Review of otitis media microbiome studies: What do they tell us? *Laryngoscope Investig Otolaryngol*. 2020;5(5):936-940. doi:10.1002/lio2.460
234. Lin J, Hafrén H, Kerschner J, et al. Panel 3: Genetics and Precision Medicine of Otitis Media. *Otolaryngol Neck Surg*. 2017;156(S4). doi:10.1177/0194599816685559
235. Parker D, Prince A. Innate Immunity in the Respiratory Epithelium. *Am J Respir Cell Mol Biol*. 2011;45(2):189-201. doi:10.1165/rcmb.2011-0011RT
236. Mittal R, Kodiyan J, Gerring R, et al. Role of innate immunity in the pathogenesis of otitis media. *Int J Infect Dis*. 2014;29:259-267. doi:10.1016/j.ijid.2014.10.015
237. Yang D, Liu Z hua, Tewary P, Chen Q, De La Rosa G, Oppenheim J. Defensin Participation in Innate and Adaptive Immunity. *Curr Pharm Des*. 2007;13(30):3131-3139. doi:10.2174/138161207782110453
238. Lee HY, Andalibi A, Webster P, et al. Antimicrobial activity of innate immune molecules against Streptococcus pneumoniae, Moraxella catarrhalis and nontypeable Haemophilus influenzae. *BMC Infect Dis*. 2004;4:12. doi:10.1186/1471-2334-4-12
239. Jones EA, McGillivary G, Bakaletz LO. Extracellular DNA within a Nontypeable Haemophilus influenzae-Induced Biofilm Binds Human Beta Defensin-3 and Reduces Its Antimicrobial Activity. *J Innate Immun*. 2013;5(1):24-38. doi:10.1159/000339961
240. McGillivary G, Bakaletz LO. The Multifunctional Host Defense Peptide SPLUNC1 Is Critical for Homeostasis of the Mammalian Upper Airway. DeLeo FR, ed. *PLoS ONE*. 2010;5(10):e13224. doi:10.1371/journal.pone.0013224
241. Gakhar L, Bartlett JA, Penterman J, et al. PLUNC Is a Novel Airway Surfactant Protein with Anti-Biofilm Activity. Kreindler JL, ed. *PLoS ONE*. 2010;5(2):e9098. doi:10.1371/journal.pone.0009098
242. Preciado D, Goyal S, Rahimi M, et al. MUC5B Is the Predominant Mucin Glycoprotein in Chronic Otitis Media Fluid. *Pediatr Res*. 2010;68(3):231-236. doi:10.1203/PDR.0b013e3181eb2ecc
243. Jecker P, Pabst R, Westermann J. The Mucosa of the Middle Ear and Eustachian Tube in the Young Rat: Number of Granulocytes, Macrophages, Dendritic Cells, NK Cells and T and B Lymphocytes in Healthy Animals and During Otitis Media. *Acta Otolaryngol (Stockh)*. 1996;116(3):443-450. doi:10.3109/00016489609137871
244. Val S, Poley M, Brown K, et al. Proteomic Characterization of Middle Ear Fluid Confirms Neutrophil Extracellular Traps as a Predominant Innate Immune Response in Chronic Otitis Media. Palaniyar N, ed. *PLOS ONE*. 2016;11(4):e0152865. doi:10.1371/journal.pone.0152865
245. Seppanen E, Tan D, Corscadden KJ, et al. Evidence of functional cell-mediated immune responses to nontypeable Haemophilus influenzae in otitis-prone children. Borrow R, ed. *PLOS ONE*. 2018;13(4):e0193962. doi:10.1371/journal.pone.0193962
246. Ebmeyer J, Furukawa M, Pak K, et al. Role of mast cells in otitis media. *J Allergy Clin Immunol*. 2005;116(5):1129-1135. doi:10.1016/j.jaci.2005.07.026
247. Massa HM, Spann KM, Cripps AW. Innate Immunity in the Middle Ear Mucosa. *Front Cell Infect Microbiol*. 2021;11:764772. doi:10.3389/fcimb.2021.764772

248. Si Y, Zhang ZG, Chen SJ, et al. Attenuated TLRs in middle ear mucosa contributes to susceptibility of chronic suppurative otitis media. *Hum Immunol*. 2014;75(8):771-776. doi:10.1016/j.humimm.2014.05.009
249. Tong HH, Lambert G, Li YX, et al. Deletion of the Complement C5a Receptor Alleviates the Severity of Acute Pneumococcal Otitis Media following Influenza A Virus Infection in Mice. Metzger DW, ed. *PLoS ONE*. 2014;9(4):e95160. doi:10.1371/journal.pone.0095160
250. He Y, Scholes MA, Wiet GJ, Li Q, Clancy C, Tong HH. Complement activation in paediatric patients with recurrent acute otitis media. *Int J Pediatr Otorhinolaryngol*. 2013;77(6):911-917. doi:10.1016/j.ijporl.2013.03.004
251. Robledo-Avila FH, Ruiz-Rosado JDD, Partida-Sanchez S, Brockman KL. A Bacterial Epigenetic Switch in Non-typeable Haemophilus influenzae Modifies Host Immune Response During Otitis Media. *Front Cell Infect Microbiol*. 2020;10:512743. doi:10.3389/fcimb.2020.512743
252. Munye MM, Shoemark A, Hirst RA, et al. BMI-1 extends proliferative potential of human bronchial epithelial cells while retaining their mucociliary differentiation capacity. *Am J Physiol-Lung Cell Mol Physiol*. 2017;312(2):L258-L267. doi:10.1152/ajplung.00471.2016
253. McKay TR, Camarasa MV, Iskender B, et al. Human feeder cell line for derivation and culture of hESc/hiPSc. *Stem Cell Res*. 2011;7(2):154-162. doi:10.1016/j.scr.2011.04.005
254. Fleischmann RD, Adams MD, White O, et al. Whole-Genome Random Sequencing and Assembly of *Haemophilus influenzae* Rd. *Science*. 1995;269(5223):496-512. doi:10.1126/science.7542800
255. Lysenko E, Richards JC, Cox AD, et al. The position of phosphorylcholine on the lipopolysaccharide of Haemophilus influenzae affects binding and sensitivity to C-reactive protein-mediated killing. *Mol Microbiol*. 2000;35(1):234-245. doi:10.1046/j.1365-2958.2000.01707.x
256. Hood DW, Makepeace K, Deadman ME, et al. Sialic acid in the lipopolysaccharide of Haemophilus influenzae: strain distribution, influence on serum resistance and structural characterization. *Mol Microbiol*. 1999;33(4):679-692. doi:10.1046/j.1365-2958.1999.01509.x
257. Hellner K, Mar J, Fang F, Quackenbush J, Münger K. HPV16 E7 oncogene expression in normal human epithelial cells causes molecular changes indicative of an epithelial to mesenchymal transition. *Virology*. 2009;391(1):57-63. doi:10.1016/j.virol.2009.05.036
258. Stroulios G, Brown T, Kondro D, et al. Generation of apical-out airway organoids from human primary airway epithelial cells. In: *Airway Cell Biology and Immunopathology*. European Respiratory Society; 2022:116. doi:10.1183/23120541.LSC-2022.116
259. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-βgal) activity, a biomarker of senescent cells in culture and in vivo. *Nat Protoc*. 2009;4(12):1798-1806. doi:10.1038/nprot.2009.191
260. Olszewska A, Borkowska A, Granica M, et al. Escape From Cisplatin-Induced Senescence of Hypoxic Lung Cancer Cells Can Be Overcome by Hydroxychloroquine. *Front Oncol*. 2022;11:738385. doi:10.3389/fonc.2021.738385
261. Chalphin AV, Fauza DO. Fetal Tissue. In: *Reference Module in Biomedical Sciences*. Elsevier; 2018:B9780128012383658000. doi:10.1016/B978-0-12-801238-3.65843-9
262. Abs V, Bonicelli J, Kacza J, Zizzadoro C, Abraham G. Equine bronchial fibroblasts enhance proliferation and differentiation of primary equine bronchial epithelial cells co-cultured under air-liquid interface. Tesfaigzi Y, ed. *PLOS ONE*. 2019;14(11):e0225025. doi:10.1371/journal.pone.0225025

263. Cozens D, Grahame E, Sutherland E, Taylor G, Berry CC, Davies RL. Development and optimization of a differentiated airway epithelial cell model of the bovine respiratory tract. *Sci Rep*. 2018;8(1):853. doi:10.1038/s41598-017-19079-y
264. Ng-Blichfeldt JP, Schrik A, Kortekaas RK, et al. Retinoic acid signaling balances adult distal lung epithelial progenitor cell growth and differentiation. *EBioMedicine*. 2018;36:461-474. doi:10.1016/j.ebiom.2018.09.002
265. Huang F, Zhang H, Wu M, et al. Calcium-activated chloride channel TMEM16A modulates mucin secretion and airway smooth muscle contraction. *Proc Natl Acad Sci*. 2012;109(40):16354-16359. doi:10.1073/pnas.1214596109
266. Mahanty S, Dakappa SS, Shariff R, et al. Keratinocyte differentiation promotes ER stress-dependent lysosome biogenesis. *Cell Death Dis*. 2019;10(4):269. doi:10.1038/s41419-019-1478-4
267. Wijesekara P, Yadav P, Perkins LA, et al. Engineering rotating apical-out airway organoid for assessing respiratory cilia motility. *iScience*. 2022;25(8):104730. doi:10.1016/j.isci.2022.104730
268. Bhattacharya R, Banerjee Mustafi S, Street M, Dey A, Dwivedi SKD. Bmi-1: At the crossroads of physiological and pathological biology. *Genes Dis*. 2015;2(3):225-239. doi:10.1016/j.gendis.2015.04.001
269. Wu Z, Min L, Chen D, et al. Overexpression of BMI-1 Promotes Cell Growth and Resistance to Cisplatin Treatment in Osteosarcoma. Wang H, ed. *PLoS ONE*. 2011;6(2):e14648. doi:10.1371/journal.pone.0014648
270. Chen MK, Zhou JH, Wang P, et al. BMI1 activates P-glycoprotein via transcription repression of miR-3682-3p and enhances chemoresistance of bladder cancer cell. *Aging*. 2021;13(14):18310-18330. doi:10.18632/aging.203277
271. Iuchi H, Ohori J, Kyutoku T, Ito K, Kurono Y. Role of phosphorylcholine in Streptococcus pneumoniae and nontypeable Haemophilus influenzae adherence to epithelial cells. *Auris Nasus Larynx*. 2019;46(4):513-519. doi:10.1016/j.anl.2018.11.003
272. Krause PJ, Owens NJ, Nightingale CH, Klimek JJ, Lehmann WB, Quintiliani R. Penetration of Amoxicillin, Cefaclor, Erythromycin-Sulfisoxazole, and Trimethoprim-Sulfamethoxazole into the Middle Ear Fluid of Patients with Chronic Serous Otitis Media. *J Infect Dis*. 1982;145(6):815-821. doi:10.1093/infdis/145.6.815
273. Kadowaki Y, Hirano T, Fujita K, et al. Phase variation with altering phosphorylcholine expression of nontypeable Haemophilus influenzae affects bacteria clearance and mucosal immune response in the middle ear and nasopharynx. *Auris Nasus Larynx*. 2021;48(1):57-64. doi:10.1016/j.anl.2020.07.001
274. Hong KJ, Wickstrum JR, Yeh HW, Parmely MJ. Toll-Like Receptor 2 Controls the Gamma Interferon Response to *Francisella tularensis* by Mouse Liver Lymphocytes. *Infect Immun*. 2007;75(11):5338-5345. doi:10.1128/IAI.00561-07
275. Fujita K, Hirano T, Kodama S, Suzuki M. Prognostic impact of phosphorylcholine expression in nontypeable Haemophilus influenzae in otitis media with effusion. *Acta Otolaryngol (Stockh)*. 2009;129(8):832-838. doi:10.1080/00016480802468195
276. Hong W, Mason K, Jurcisek J, Novotny L, Bakaletz LO, Swords WE. Phosphorylcholine Decreases Early Inflammation and Promotes the Establishment of Stable Biofilm Communities of Nontypeable *Haemophilus influenzae* Strain 86-028NP in a Chinchilla Model of Otitis Media. *Infect Immun*. 2007;75(2):958-965. doi:10.1128/IAI.01691-06
277. Krishnamurthy A, Kyd J. The roles of epithelial cell contact, respiratory bacterial interactions and phosphorylcholine in promoting biofilm formation by Streptococcus pneumoniae and nontypeable Haemophilus influenzae. *Microbes Infect*. 2014;16(8):640-647. doi:10.1016/j.micinf.2014.06.008
278. Albert DH, Magoc TJ, Tapang P, et al. Pharmacology of ABT-491, a highly potent

platelet-activating factor receptor antagonist. *Eur J Pharmacol*. 1997;325(1):69-80. doi:10.1016/S0014-2999(97)00109-X
279. Revai K, Mamidi D, Chonmaitree T. Association of Nasopharyngeal Bacterial Colonization during Upper Respiratory Tract Infection and the Development of Acute Otitis Media. *Clin Infect Dis*. 2008;46(4):e34-e37. doi:10.1086/525856
280. Branger J, Wieland CW, Florquin S, et al. PLATELET-ACTIVATING FACTOR RECEPTOR-DEFICIENT MICE SHOW AN UNALTERED CLEARANCE OF NONTYPEABLE HAEMOPHILUS INFLUENZAE FROM THEIR RESPIRATORY TRACT: *Shock*. 2004;22(6):543-547. doi:10.1097/01.shk.0000142818.91693.73
281. Orihuela CJ, Mahdavi J, Thornton J, et al. Laminin receptor initiates bacterial contact with the blood brain barrier in experimental meningitis models. *J Clin Invest*. 2009;119(6):1638-1646. doi:10.1172/JCI36759
282. McCullers JA, Rehg JE. Lethal Synergism between Influenza Virus and *Streptococcus pneumoniae:* Characterization of a Mouse Model and the Role of Platelet-Activating Factor Receptor. *J Infect Dis*. 2002;186(3):341-350. doi:10.1086/341462
283. Clark SE, Weiser JN. Microbial Modulation of Host Immunity with the Small Molecule Phosphorylcholine. Maurelli AT, ed. *Infect Immun*. 2013;81(2):392-401. doi:10.1128/IAI.01168-12
284. Duncan DAS, McLaughlin WM, Vasilakes N, Echevarria FD, Formichella CR, Sappington RM. Constitutive and Stress-induced Expression of CCL5 Machinery in Rodent Retina. *J Clin Cell Immunol*. 2017;08(03). doi:10.4172/2155-9899.1000506
285. Lanfranco MF, Mocchetti I, Burns MP, Villapol S. Glial- and Neuronal-Specific Expression of CCL5 mRNA in the Rat Brain. *Front Neuroanat*. 2018;11:137. doi:10.3389/fnana.2017.00137
286. Mazzucchelli L, Hauser C, Zgraggen K, et al. DIFFERENTIALIN SITU EXPRESSION OF THE GENES ENCODING THE CHEMOKINES MCP-1 AND RANTES IN HUMAN INFLAMMATORY BOWEL DISEASE. *J Pathol*. 1996;178(2):201-206. doi:10.1002/(SICI)1096-9896(199602)178:2<201::AID-PATH440>3.0.CO;2-4
287. Skurk T, Mack I, Kempf K, Kolb H, Hauner H, Herder C. Expression and Secretion of RANTES (CCL5) in Human Adipocytes in Response to Immunological Stimuli and Hypoxia. *Horm Metab Res*. 2009;41(03):183-189. doi:10.1055/s-0028-1093345
288. Nokso-Koivisto J, Chonmaitree T, Jennings K, Matalon R, Block S, Patel JA. Polymorphisms of Immunity Genes and Susceptibility to Otitis Media in Children. Lee YL, ed. *PLoS ONE*. 2014;9(4):e93930. doi:10.1371/journal.pone.0093930
289. Liu H, Chao D, Nakayama EE, et al. Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression. *Proc Natl Acad Sci*. 1999;96(8):4581-4585. doi:10.1073/pnas.96.8.4581
290. Pati A, Nayak N, Sarangi S, et al. *CCL5* rs2107538 Variant Is Associated With Protection Against SARS-CoV-2 Infection and Related Mortality: A Population-Based Study. *J Infect Dis*. 2022;225(10):1865-1866. doi:10.1093/infdis/jiac026
291. Pang Y, Li H, Gong Y, et al. Association of CCL2, CCR2 and CCL5 genetic polymorphisms with the development and progression of benign prostatic hyperplasia. *Oncol Rep*. Published online February 6, 2019. doi:10.3892/or.2019.7002
292. Schousboe LP, Rasmussen LM, Ovesen T. RANTES in otitis media with effusion: Presence, role and correlation with cytokines and microbiology. *APMIS*. 2001;109(6):441-446. doi:10.1034/j.1600-0463.2001.090606.x
293. Vikhe PP, Purnell T, Brown SDM, Hood DW. Cellular content plays a crucial role in Non-typeable *Haemophilus influenzae* infection of preinflamed *Junbo* mouse middle ear. *Cell Microbiol*. 2019;21(1):e12960. doi:10.1111/cmi.12960
294. Egesten A, Eliasson M, Olin AI, et al. The proinflammatory CXC-chemokines GRO-

α/CXCL1 and MIG/CXCL9 are concomitantly expressed in ulcerative colitis and decrease during treatment with topical corticosteroids. *Int J Colorectal Dis*. 2007;22(12):1421-1427. doi:10.1007/s00384-007-0370-3
295. Trune DR, Kempton B, Hausman FA, Larrain BE, MacArthur CJ. Correlative mRNA and protein expression of middle and inner ear inflammatory cytokines during mouse acute otitis media. *Hear Res*. 2015;326:49-58. doi:10.1016/j.heares.2015.04.006
296. Deng ZW, Denkinger DJ, Peterson KE, Deuel TF, Kawahara RS. Glucocorticoids Negatively Regulate the Transcription of KC, the Mouse Homolog of MGSA/GRO. *Biochem Biophys Res Commun*. 1994;203(3):1809-1814. doi:10.1006/bbrc.1994.2397
297. Schroepf S, Kappler R, Brand S, et al. Strong overexpression of CXCR3 axis components in childhood inflammatory bowel disease: *Inflamm Bowel Dis*. 2010;16(11):1882-1890. doi:10.1002/ibd.21312
298. Kaplan G, Luster AD, Hancock G, Cohn ZA. The expression of a gamma interferon-induced protein (IP-10) in delayed immune responses in human skin. *J Exp Med*. 1987;166(4):1098-1108. doi:10.1084/jem.166.4.1098
299. Liu M, Guo S, Hibbert JM, et al. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. *Cytokine Growth Factor Rev*. Published online July 2011:S1359610111000293. doi:10.1016/j.cytogfr.2011.06.001
300. Hernandez M, Leichtle A, Pak K, Webster NJ, Wasserman SI, Ryan AF. The transcriptome of a complete episode of acute otitis media. *BMC Genomics*. 2015;16(1):259. doi:10.1186/s12864-015-1475-7
301. MacArthur CJ, Hausman F, Kempton JB, Choi D, Trune DR. Otitis Media Impacts Hundreds of Mouse Middle and Inner Ear Genes. Sokolowski B, ed. *PLoS ONE*. 2013;8(10):e75213. doi:10.1371/journal.pone.0075213
302. Surendran N, Nicolosi T, Kaur R, Pichichero ME. Peripheral blood antigen presenting cell responses in otitis-prone and non-otitis-prone infants. *Innate Immun*. 2016;22(1):63-71. doi:10.1177/1753425915616267
303. Dwivedi P, Greis KD. Granulocyte colony-stimulating factor receptor signaling in severe congenital neutropenia, chronic neutrophilic leukemia, and related malignancies. *Exp Hematol*. 2017;46:9-20. doi:10.1016/j.exphem.2016.10.008
304. Patel JA, Nair S, Grady J, et al. Systemic Cytokine Response Profiles Associated With Respiratory Virus-Induced Acute Otitis Media. *Pediatr Infect Dis J*. 2009;28(5):407-411. doi:10.1097/INF.0b013e318194b7c6
305. Gabay C, Lamacchia C, Palmer G. IL-1 pathways in inflammation and human diseases. *Nat Rev Rheumatol*. 2010;6(4):232-241. doi:10.1038/nrrheum.2010.4
306. Arend WP. The balance between IL-1 and IL-1Ra in disease. *Cytokine Growth Factor Rev*. 2002;13(4-5):323-340. doi:10.1016/S1359-6101(02)00020-5
307. Aksentijevich I, Masters SL, Ferguson PJ, et al. An Autoinflammatory Disease with Deficiency of the Interleukin-1–Receptor Antagonist. *N Engl J Med*. 2009;360(23):2426-2437. doi:10.1056/NEJMoa0807865
308. Zielnik-Jurkiewicz B, Stankiewicz-Szymczak W. Evaluation of the Interleukin-1 Receptor Antagonist and Immunoregulatory Interleukin-10 in the Middle Ear in Chronic Otitis Media With Effusion in Children With and Without Atopy. *Clin Exp Otorhinolaryngol*. 2016;9(2):104-108. doi:10.21053/ceo.2015.00129
309. Arend WP. Interleukin 1 receptor antagonist. A new member of the interleukin 1 family. *J Clin Invest*. 1991;88(5):1445-1451. doi:10.1172/JCI115453
310. Rose-John S, Winthrop K, Calabrese L. The role of IL-6 in host defence against infections: immunobiology and clinical implications. *Nat Rev Rheumatol*. 2017;13(7):399-409. doi:10.1038/nrrheum.2017.83
311. Yellon RF, Leonard G, Marucha P, et al. Demonstration of Interleukin 6 in Middle Ear

Effusions. *Arch Otolaryngol - Head Neck Surg*. 1992;118(7):745-748. doi:10.1001/archotol.1992.01880070075014
312. Skovbjerg S, Roos K, Nowrouzian F, et al. High cytokine levels in perforated acute otitis media exudates containing live bacteria. *Clin Microbiol Infect*. 2010;16(9):1382-1388. doi:10.1111/j.1469-0691.2010.03083.x
313. Chen Y, Thai P, Zhao YH, Ho YS, DeSouza MM, Wu R. Stimulation of Airway Mucin Gene Expression by Interleukin (IL)-17 through IL-6 Paracrine/Autocrine Loop. *J Biol Chem*. 2003;278(19):17036-17043. doi:10.1074/jbc.M210429200
314. Wang Y, Wang W, Wang L, Wang X, Xia J. Regulatory mechanisms of interleukin-8 production induced by tumour necrosis factor-α in human hepatocellular carcinoma cells. *J Cell Mol Med*. 2012;16(3):496-506. doi:10.1111/j.1582-4934.2011.01337.x
315. Leibovitz E. Interleukin 8 in middle ear fluid during acute otitis media: correlation with aetiology and bacterial eradication. *Arch Dis Child*. 2000;82(2):165-168. doi:10.1136/adc.82.2.165
316. Patel JA, Sim T, Chonmaitree T, et al. Role of Leukotriene B4 and Interleukin-8 in Acute Bacterial and Viral Otitis Media. *Ann Otol Rhinol Laryngol*. 1996;105(12):968-974. doi:10.1177/000348949610501207
317. Maxwell KS, Fitzgerald JE, Burleson JA, Leonard G, Carpenter R, Kreutzer DL. Interleukin-8 Expression in Otitis Media: *The Laryngoscope*. 1994;104(8):989???995. doi:10.1288/00005537-199408000-00013
318. Serban R, Filip C, Radulescu L, et al. IL-1α, IL-6 and IL-8 serum values in patients with chronic suppurative otitis media. *Exp Ther Med*. 2021;22(5):1226. doi:10.3892/etm.2021.10660
319. Dinarello CA, Novick D, Kim S, Kaplanski G. Interleukin-18 and IL-18 Binding Protein. *Front Immunol*. 2013;4. doi:10.3389/fimmu.2013.00289
320. Liu S, Guo L, Chen M, et al. Evaluation of Caspase-1, Interleukin-1β, and Interleukin-18, in the Middle Ear Effusion in Children With Otitis Media With Effusion. *Front Pediatr*. 2021;9:732973. doi:10.3389/fped.2021.732973
321. Enoksson F, Ruiz Rodriguez A, Peno C, et al. Niche- and Gender-Dependent Immune Reactions in Relation to the Microbiota Profile in Paediatric Patients with Otitis Media with Effusion. Bäumler AJ, ed. *Infect Immun*. 2020;88(10):e00147-20. doi:10.1128/IAI.00147-20
322. Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. *Nat Rev Immunol*. 2003;3(10):791-800. doi:10.1038/nri1200
323. Kariya S, Okano M, Aoji K, et al. Role of Macrophage Migration Inhibitory Factor in Otitis Media with Effusion in Adults. *Clin Vaccine Immunol*. 2003;10(3):417-422. doi:10.1128/CDLI.10.3.417-422.2003
324. Zhang J, Xu M, Zheng Q, Zhang Y, Ma W, Zhang Z. Blocking macrophage migration inhibitory factor activity alleviates mouse acute otitis media in vivo. *Immunol Lett*. 2014;162(1):101-108. doi:10.1016/j.imlet.2014.07.013
325. Iwaki T, Urano T, Umemura K. PAI-1, progress in understanding the clinical problem and its aetiology. *Br J Haematol*. 2012;157(3):291-298. doi:10.1111/j.1365-2141.2012.09074.x
326. Esposito S, Marchisio P, Orenti A, et al. Genetic Polymorphisms of Functional Candidate Genes and Recurrent Acute Otitis Media With or Without Tympanic Membrane Perforation. *Medicine (Baltimore)*. 2015;94(42):e1860. doi:10.1097/MD.0000000000001860
327. Iovino F, Brouwer MC, Van De Beek D, Molema G, Bijlsma JJE. Signalling or binding: the role of the platelet-activating factor receptor in invasive pneumococcal disease: PAFR and invasive pneumococcal disease. *Cell Microbiol*. 2013;15(6):870-881.

doi:10.1111/cmi.12129
328. Hergott CB, Roche AM, Naidu NA, Mesaros C, Blair IA, Weiser JN. Bacterial exploitation of phosphorylcholine mimicry suppresses inflammation to promote airway infection. *J Clin Invest*. 2015;125(10):3878-3890. doi:10.1172/JCI81888
329. Langereis JD, Van Der Pasch ES, De Jonge MI. Serum IgM and C-Reactive Protein Binding to Phosphorylcholine of Nontypeable *Haemophilus influenzae* Increases Complement-Mediated Killing. Payne SM, ed. *Infect Immun*. 2019;87(8):e00299-19. doi:10.1128/IAI.00299-19
330. Weiser JN, Pan N, McGowan KL, Musher D, Martin A, Richards J. Phosphorylcholine on the Lipopolysaccharide of Haemophilus influenzae Contributes to Persistence in the Respiratory Tract and Sensitivity to Serum Killing Mediated by C-reactive Protein. *J Exp Med*. 1998;187(4):631-640. doi:10.1084/jem.187.4.631
331. Choi JY, Kim JY, Kim CW, et al. IL-1β promotes the ciliogenesis of human middle ear epithelial cells: possible linkage with the expression of mucin gene 8. *Acta Otolaryngol (Stockh)*. 2005;125(3):260-265. doi:10.1080/00016480410022985
332. Hong W, Pang B, West-Barnette S, Swords WE. Phosphorylcholine Expression by Nontypeable *Haemophilus influenzae* Correlates with Maturation of Biofilm Communities In Vitro and In Vivo. *J Bacteriol*. 2007;189(22):8300-8307. doi:10.1128/JB.00532-07