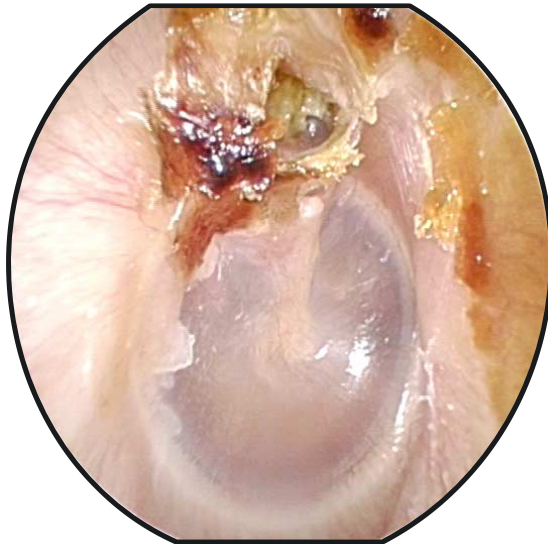


# Middle ear cholesteatoma

Surgical outcome and aspects of  
the innate immunity

**Johanna Westerberg**



Linköping University Medical Dissertations No.1747

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Johanna Westerberg



Faculty of Medicine and Health Sciences  
Department of Biomedical and Clinical Sciences  
Linköping University, Sweden

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To Lars, Lisa and Bror



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## **Abstract**

Cholesteatomas are bone destructive expansions of keratinizing squamous epithelium in the middle ear and temporal bone. Today, surgery is the only treatment. There are several controversies regarding cholesteatomas, including the definition, the pathogenesis and the surgical method. Intense efforts have been made searching for a comprehension of the cholesteatoma process at a cellular and molecular level. Recurrent infections and inflammation seem to be contributing factors for the cholesteatomas to expand. The innate immunity, essential to keep a healthy middle ear environment and to protect the middle ear from intruding pathogens, is therefore a matter of interest.

In this thesis, results are presented from a cohort of cholesteatoma surgeries in Östergötland from a 16-year period. A group of patients also filled in a questionnaire to assess changes in health-related quality of life (HRQoL) after surgery. According to the findings in this thesis, the residual and recurrence frequencies are low, and the hearing and HRQoL are improved in the majority of cases.

This thesis also presents an investigation of the innate immunity in ears with acquired cholesteatoma, in comparison to controls with healthy middle ears. The expression of mRNA of toll like receptors 2 and 4, participants of the Janus kinase/signal transducer and activator of transcription pathway, and nitric oxide synthases in middle ear mucosa, were investigated with quantitative polymerase chain reaction. An investigation of nitric oxide (NO) in the middle ear, with chemiluminescence measurements, is also presented. A derangement of the innate immune system is seen in ears with cholesteatoma, which supports the idea that the innate immunity participates in the cholesteatoma process, though the underlying mechanisms are still unclear. The suggestion of NO production in the middle ear sheds light on NOs possible participation in the healthy middle ear environment.

**List of scientific papers**

- I           **Johanna Westerberg**, Elina Mäki-Torkko and Henrik Harder  
Cholesteatoma surgery with the canal wall up technique with  
mastoid obliteration: results from primary surgery in 230  
consecutive cases  
*Acta Otolaryngol. 2018 May 138(5):452-457*
- II           **Johanna Westerberg**, Elina Mäki-Torkko and Henrik Harder  
The evaluation of canal wall up cholesteatoma surgery with the  
Glasgow Benefit Inventory  
*Eur Arch Otorhinolaryngol. 2020 Jan; 277(1):61-68*
- III           Cecilia Draxskog, Nele de Klerk, **Johanna Westerberg**, Elina  
Mäki-Torkko, Susanna Kumlien Georén, Lars Olaf Cardell  
Extensive qPCR analysis reveals altered gene expression in middle  
ear mucosa from cholesteatoma patients  
*PLoS ONE 2020 September 11, e0239161*  
<https://doi.org/10.1371/journal.pone.0239161>
- IV           **Johanna Westerberg**, Anna Granath, Cecilia Draxskog, Ellen  
Tideholm, Susanna Kumlien Georén, Eddie Weitzberg, Lars Olaf  
Cardell  
Nitric Oxide is part of the gas mixture in the human middle ear and  
reduced production may be related to chronic middle ear disease  
and acquired cholesteatoma  
*Manuscript*
- V           **Johanna Westerberg**, Ellen Tideholm, Krzysztof Piersiala,  
Cecilia Draxskog, Susanna Kumlien Georén, Elina Mäki-Torkko,  
Lars Olaf Cardell  
JAK/STAT Dysregulation with SOCS1 Overexpression in  
Acquired Cholesteatoma-adjacent Mucosa  
*Ahead of print, Otolology & Neurotology DOI:*  
*10.1097/MAO.0000000000002850*

**Abbreviations**

AC	air conduction
ACTB	beta actin
AOM	acute otitis media
BC	bone conduction
CAT	combined approach tympanoplasty
CCND2	cyclin D2
CES	Chronic Ear Survey
CD	cluster of differentiation
cDNA	complementary DNA
CI	cochlear implant
c-MYC	cellular myelocytomatosis oncogene
COM	chronic otitis media
CT	computed tomography
Ct	cycle threshold
CWD	canal wall down
CWU	canal wall up
dB	decibel
DC	dendritic cell
DNA	deoxyribonucleic acid
DW-MRI	diffusion-weighted magnetic resonance imaging
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBI	Glasgow Benefit Inventory
HL	hearing level
HRQoL	health-related quality of life
IL-7R $\alpha$	interleukin-7 receptor-alpha
iNOS	inducible nitric oxide synthase
JAK	Janus kinase
kHz	kilohertz
LPS	lipopolysaccharide
MA	mastoid antrum

mRNA	messenger ribonucleic acid
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
OME	otitis media with effusion
ORL	otorhinolaryngology
PORP	partial ossicular replacement prosthesis
PROM	patient-reported outcome measure
PTA	pure tone average
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TC	tympanic cavity
TGF- $\beta$ 1	transforming growth factor beta 1
TLR	toll-like receptor
TSLP	thymic stromal lymphopoietin
TORP	total ossicular replacement prosthesis

## **Introduction**

Cholesteatomas are often included in the group of middle ear diseases named chronic otitis media (COM); however, they should be understood as a separate entity. Cholesteatomas can be acquired, or less commonly, congenital. Acquired cholesteatomas can be further classified into a retraction pocket type or an uncommon non-retraction cholesteatoma type that occurs secondary to a tympanic membrane (TM) perforation, following trauma or iatrogenic causes. A recently presented definition of cholesteatoma is: “A mass formed by the keratinizing squamous epithelium in the tympanic cavity and/or mastoid and subepithelial connective tissue and by the progressive accumulation of keratin debris with/without a surrounding inflammatory reaction” (Yung et al. 2017).

Many attempts have been made to understand the pathogenesis of acquired cholesteatoma. The most established and generally accepted theory is that Eustachian tube dysfunction and poor middle ear ventilation lead to a retracted eardrum. The normal self-cleansing ability of the TM has a limited capacity that can be overloaded if the area of a retraction pocket is too large or if the skin has increased keratin production. Entrapment of keratin debris is a consequence.

Despite their histopathologically benign appearance, cholesteatomas cause local bone destruction and a range of complications if left without treatment. Although some patients vaguely experience symptoms, cholesteatomas may be associated with impaired hearing, infection from the ear canal with purulent ear discharge, and a sensation of fullness or pressure in the affected ear. There is also a risk for facial nerve palsy, vertigo, deafness, and even potentially lethal complications, such as meningitis and brain abscessation. The treatment is surgery. The two main surgical techniques are canal wall up (CWU) and canal wall down (CWD) with or without obliteration. The advantages and disadvantages of these surgical techniques have been debated for over 50 years (Palva 1993, Kerckhoffs *et al.* 2016).

## Background

### Middle ear cholesteatoma

#### History and description

The first description of the cholesteatoma process, given the name “steatome”, was probably made in 1683 by the French doctor Joseph Guichard Du Verney (Du Verney 1683). A French anatomist and pathologist, Jean Cruveilhier, described cholesteatoma as “a tumeur perlée” in 1829. Dr. Johannes Müller, a German anatomist, as reviewed by Ferlito (Ferlito 1993), proposed the name cholesteatoma in 1838. The term is incorrect as cholesteatomas are tumours (“-omas”), but they do *not* include either cholesterol (“chole-”) or fat (“-stea-”) (Jahn 1989). Dr. Müller described the appearance of cholesteatomas as a “layered pearly tumour of fat”, whereas Gray defined cholesteatomas in 1964 as “skin in the wrong place” (Gray 1964).

#### Anatomy and histology of the middle ear and mastoid

The TM has a unique role as a barrier to the outer world and is also integral in the function of the middle ear and hearing. The two parts, the pars flaccida or Sharpnell’s membrane (which in its medial portion is called the attic) and the pars tensa, differ in morphologic appearance. The fact that the pars flaccida contains connective tissue that is more elastic and loose than that in the pars tensa probably makes it predisposed to retractions in case of pressure changes in the middle ear (Stenfeldt et al. 2006).

The middle ear is also known as the tympanic cavity (TC). Its epithelial mucosal lining can be described as an extension and a modification of the respiratory epithelial lining of the airways. It consists of a simple squamous epithelium of low cuboidal cells with an underlying thin lamina propria that is adherent to the periosteum. A few goblet cells are occasionally seen in the TC. Near the orifice to the Eustachian tube, the epithelium is pseudostratified and columnar with ciliated cells interspersed with goblet cells (Sadé 1966, Lim 1979). The thin submucosal layer contains collagen fibres, fibroblasts, phagocytes, macrophages, mast cells, a few plasma cells and lymphocytes (Lim et al. 1973, Lim 1979).

The gas-containing middle ear communicates with the nasopharynx via the Eustachian tube. There is a concept of a united airway as both the Eustachian tube and middle ear are lined with ciliated airway epithelium and can be considered as an extension of the respiratory tract anatomically and physiologically. Episodes of acute otitis media (AOM), linked to viral infections of the upper airway as well as in a coexisting allergic rhinitis and asthma in the same patient, supports the united airway concept (Nguyen et al. 2004, Bachert et al. 2006).

#### Histopathology

In conformity with the skin, the cholesteatoma epithelium, also called matrix, contains a basal layer, a spinal layer, a granular layer and a lucid layer. The outermost layer is the perimatrix (lamina propria), an inflamed subepithelial connective tissue containing collagen fibres, fibrocytes, and inflammatory cells predominantly lymphocytes but also macrophages, plasma cells and neutrophil leucocytes (Lim and Saunders 1972, Dornelles *et al.* 2006). The middle cystic content contains keratin lamellae (Dornelles *et al.* 2006).

#### Epidemiology

In a Danish study, the incidence was 6.8 per 100 000 inhabitants per year (Britze et al. 2017), and in a Finish study, it was 9.2 per 100 000 inhabitants (Kempainen *et al.* 1999). A male predominance has been reported (Kempainen *et al.* 1999, Djurhuus *et al.* 2015). Bilateral disease is seen in approximately 4-15% of patients (Chalton and Stearns 1984, Kempainen *et al.* 1999). Epidemiological studies have suggested higher rates of cholesteatomas within areas of socioeconomic deprivation (Vikram et al. 2008, Khalid-Raja et al. 2014).

#### Clinical aspects and complications

Eardrum retractions, especially of the pars tensa, have the potential to recover spontaneously (Sadé *et al.* 1981, Cutajar *et al.* 2018), and based on clinical evidence, only a few attic retractions develop into cholesteatomas (Sudhoff and Tos 2000). There is no exact explanation for the reason why the retraction pocket develops into an expanding disease, and cholesteatomas often progress

insidiously. Initially, cholesteatomas are often asymptomatic, but secondary complications with functional impairment eventually appear. The bone erosive properties of cholesteatomas may affect the ossicles, the otic capsule and the temporal bone, leading to hearing loss with the risk of deafness and vertigo caused by a labyrinthine fistula. Facial palsy is not as common. Cholesteatoma creates a pathway for secondary external infections, with the risk of a chronic suppurative ear discharge. Even meningitis or brain abscessation may occur (Yorgancılar *et al.* 2013). Diagnosis of cholesteatoma is achieved by clinical examination and might be supported by high-resolution computed tomography (CT) and diffusion-weighted magnetic resonance imaging (DW-MRI) (Foer *et al.* 2010).

#### Congenital cholesteatoma

The distinction between congenital and acquired cholesteatoma was clarified in the 1960s (Derlacki and Clemis 1965). Congenital cholesteatoma was defined as an embryologic residue of epithelial tissue behind a normal TM. Levenson *et al.* established and modified these criteria and did not exclude cases with prior episodes of AOM, unless associated with perforation or otorrhea (Levenson *et al.* 1989).

#### Paediatric cholesteatoma

There are controversies in the literature whether paediatric cholesteatoma manifests differently from cholesteatoma in adults (Edfeldt *et al.* 2012). Paediatric cholesteatoma has been reported to have a higher recurrence rate and residual frequency and a more aggressive spread (Silvola and Palva 1999, Edfeldt *et al.* 2012, Jackson *et al.* 2018). In children, cholesteatoma is often more extensive at diagnosis, involving the entire mastoid and mesotympanum, and the status of the ossicular chain is often poorer due to more extensive inflammation and osteolysis (Iino *et al.* 1998, De Corso *et al.* 2006, Dornelles *et al.* 2006, Jackson *et al.* 2018). Treatment of paediatric cholesteatoma is challenging and includes the aspect of making the caregivers aware of the nature of the disease, the necessity for a long-term follow-up, the risk for revision surgery and/or aural rehabilitation (Trinidad *et al.* 2014, James 2018).

### Surgical treatment

Since the introduction of microsurgery for COM in the 1950s, there is a general agreement that the aims of cholesteatoma surgery are eradicating the disease, avoiding recurrence, and if possible, restoring auditory function (Smyth 1976, Walker *et al.* 2014). There is an ongoing debate about the pros and cons of “closed”/CWU/mastoidectomy with the canal wall preserved as opposed to “open”/CWD/mastoidectomy with removal of the bony canal. The CWD method is widely considered the “gold standard” procedure that provides good conditions for radicality but is accompanied by a risk for the need of frequent ear cleaning (performed by an otologist) and avoidance of contact with water (de Zinis *et al.* 2010). Hearing outcomes in CWD have been associated with worse results compared to CWU (Schraff and Strasnick 2006, Shirazi *et al.* 2006), although no significant differences were seen in meta-analyses (Harris *et al.* 2016, Kerckhoffs *et al.* 2016). In cases of CWD in children, reconstruction or obliteration of the mastoid has been recommended and can offer good healing and ameliorated hearing outcomes (Yung *et al.* 2007, Edfeldt *et al.* 2012). The obliteration technique was reported by Mosher (Mosher 1911). Palva modified the technique and described the use of an anteriorly based musculoperiosteal flap (Palva 1962, 1973). In the late 1970s, Feldmann described removal and reimplantation of the posterior ear canal (Feldmann 1978). Mercke concluded and described the technique of canal wall osteoplasty (Mercke 1987), which is a compromise between the CWD and CWU tympanoplasties (Babighian 2002). The CWU method shortens the time for healing and reduces the risk of development of a moist cavity, which is why it is suggested for most paediatric patients (Dodson *et al.* 1998). A criticism against the CWU technique is the association with a higher risk of residual and recurrent cholesteatomas (Kuo, Liao, *et al.* 2015, Harris *et al.* 2016, Kerckhoffs *et al.* 2016). Several surgeons have suggested an individualized solution (Lau and Tos 1989, Gocmen *et al.* 2003), which means that the surgeon has to be skilled to manage diverse perioperative situations. A minor proportion of cases are not suited for CWU due to anatomical and cholesteatoma expansion properties. In those cases, CWD is recommended.

### Health-related quality of life assessment of cholesteatoma surgery

The use of patient-related outcome measures (PROMs), are increasing, and according to Black *et al.*, essential to achieve high-quality health care (Black and Jenkinson 2009, Black 2013). The Glasgow Benefit Inventory (GBI), is a PROM that has been widely used in otorhinolaryngology (ORL) (Hendry *et al.* 2016), since first reported by Robinson *et al.* (Robinson *et al.* 1996). The GBI is an 18-item questionnaire, designed for post-interventional health-related quality of life (HRQoL) changes in ORL procedures. It has been validated and translated into several languages (Sanchez-Cuadrado *et al.* 2015, Aldriweesh *et al.* 2017, Dias-Vaz *et al.* 2018). A validation of the Swedish version of the GBI was recently published (Redfors *et al.* 2019). The GBI has the advantage of being single administered. It also includes both positive and negative scores, which could be clinically useful, and is useful in identifying predictors of benefit. Criticism regarding the GBI questionnaire is mainly that it is not disease specific, which makes it less sensitive to specific signs and symptoms. It does not give information of the current HRQoL but only the change (Weiss *et al.* 2020).

For assessment of HRQoL in cholesteatoma surgery, apart from the GBI questionnaire, other surveys have been used. The Chronic Ear Survey (CES) was introduced by Nadol *et al.* (Nadol *et al.* 2000). In the comparison between the two methods of CWU and CWD, assessed with the CES, both were associated with a good quality of life after cholesteatoma surgery (Quaranta *et al.* 2014). Hearing loss was the most frequently reported problem for the two techniques. Assessment of children with the five-item quality of life survey (COM-5), showed only small or moderate changes after cholesteatoma surgery although ear status was dramatically changed (Vlastos *et al.* 2009). The Korean implementation of the CES showed a moderate improvement in cholesteatoma surgery and highlighted factors affecting HRQoL, such as postoperative air conduction (AC) thresholds and the occurrence of complications (Choi *et al.* 2012).

### **Pathogenesis and theories of acquired cholesteatoma**

The pathogenesis of acquired cholesteatoma has been a subject of debate for decades, and several attempts have been made to understand the underlying mechanisms of the disease. Four established theories dominate the debate:

I: Metaplasia of the middle ear epithelium caused by infection was suggested by von Trötsch (von Trötsch 1864) who was the first to explain the possible epidermal origin of the disease. The metaplasia theory was well accepted among otologists in the 19<sup>th</sup> century and was supported by Ulrich 1917 and Sadé 1977 (Tos 1981).

II: The migration theory was independently proposed by Habermann and Bezold 1889. They proved that cholesteatomas could originate from the skin of the external auditory meatus and migrate through a tympanic perforation under the influence of chronic inflammation. Bezold also suggested that a retraction pocket could be a consequence of a Eustachian tube dysfunction (Semaan and Megerian 2006).

III: The basal cell hyperplasia or papillary proliferation theory was suggested by Lange in 1925 and supported by Rüedi; it explains that the occurrence of cholesteatoma is the result of an invasion of the subepithelial tissue by proliferative epithelial cells (Rüedi 1958, Soldati and Mudry 2001).

IV: In 1933, Wittmaack introduced the invagination or retraction pocket theory, which today is the most widely accepted among otologists (Olszewska *et al.* 2004).

In addition to the theories above, others have been suggested. Sudhoff and Tos combined the invagination and basal cell hyperplasia/proliferation theory to explain the formation of a retraction pocket cholesteatoma (Sudhoff and Tos 2000). Jackler *et al.* presented a theory on a squamous pouch drawn inwards by the interaction of opposing surfaces of the middle ear mucosa (Jackler *et al.* 2015). Hüttenbrink suggested a natural self-healing process in the retraction pocket where the stimulus of a mucosal inflammation in the middle ear participates in the development of the retraction of the TM and cholesteatoma process (Hüttenbrink 2019).

### Heredity

Cholesteatoma is a non-neoplastic lesion with a normal deoxyribonucleic acid (DNA) content (Desloge *et al.* 1997) and no inherent genetic instability is thought to be connected to the disease (Albino *et al.* 1998). Thus, heredity has not been thought to be involved in the pathogenesis of cholesteatoma, but family clusters have been demonstrated (Homøe and Rosborg 2007, Prinsley 2009). A positive association between having a family history of cholesteatoma and bilateral cholesteatoma has been reported. It has also been suggested that rare genetic variants may underlie the disease in some families (Collins *et al.* 2020).

### Eustachian tube dysfunction

Amongst the theories above, invagination or retraction of the TM caused by tubal dysfunction is the most widely accepted. Nonetheless, similar to other hypotheses, alone it cannot explain all the different properties of cholesteatoma disease. A number of otologists believe that the pathogenesis involves not one single explanatory factor but several (Kuo, Shiao, *et al.* 2015). Habitual sniffing is *one* important causative factor in cholesteatoma development (Magnuson 1978, Takizawa *et al.* 2013, Asawapittayanont *et al.* 2016). The underlying mechanism seems to be a closing failure of the Eustachian tube, which is seen to be frequently open or too easily opened causing the middle ear gases to be evacuated from the middle ear with forced sniffing (Magnuson 1981). Bunne *et al.* (Bunne *et al.* 1999) studied subjects with negative middle ear pressure and detected a preference amongst 19 (36%) of 53 of the sound perceived when the eardrum was retracted. This indicates that the motive for the patient to avoid sniffing is not easily altered.

### Acute otitis media and otitis media with effusion

AOM is a common diagnosis in children who are prone to ear infections due to anatomical and immunological factors (Howie *et al.* 1975, Prellner *et al.* 1989). Most children resolve otitis media with effusion (OME) spontaneously, but a subset of children have a lower rate of spontaneous healing (Ryding *et al.* 2005). Episodes of AOM and long-standing OME will cause structural changes and atrophy of the eardrum, which is a prerequisite to the development of retraction pockets (Tos 1988, Magnuson *et al.* 1996, Larsson *et al.* 2005).

### Biomolecular and immunological aspects

Currently, there is a growing body of scientific biomolecular studies investigating the ascribed properties of cholesteatoma, including its invasive, migratory, hyperproliferative, aggressive and recurrent tendencies (Olszewska *et al.* 2004, Yung *et al.* 2017). Individual events in the pathogenesis of cholesteatoma have been presented and described but are not able to explain the complete picture (Olszewska *et al.* 2004, Dornelles *et al.* 2009, Chen *et al.* 2016, Li *et al.* 2018). A selected part of the cholesteatoma-associated biomolecular research is presented below.

At least three areas of research at the molecular level have been investigated: properties linked to preneoplastic events, properties known from tissue repair and the effect of chronic or recurrent inflammation and infections in the ear. Cytokines can trigger keratinocytes into a hyperproliferative condition (Bujía *et al.* 1993, Alves *et al.* 2008), but there is no evidence of cholesteatomas being preneoplastic (Albino *et al.* 1998, Chae *et al.* 2000, Olszewska *et al.* 2004). Stimuli such as bacterial products can magnify the proliferative response. An upregulation of cellular myelocytomatosis oncogene (c-MYC) in cholesteatoma matrix compared to atheroma and normal skin from the ear canal has been described (Holly *et al.* 1995, Palkó *et al.* 2014).

Wound healing is characterized by the phases of inflammation, proliferation and remodelling (Reinke and Sorg 2012). Cholesteatoma has been compared to a keratinocyte disease addressing a disturbed or chronic wound healing process that stopped in the stage of inflammation and proliferation (Albino *et al.* 1998, Huisman *et al.* 2008). Damage of the eardrum such as pressure induced invaginations, morphologic changes, or perforations in cholesteatomas could initiate a wound healing process (Albino *et al.* 1998). In the suggested theory, cholesteatomas never reach the remodelling phase due to a chronic inflammation. One important factor in wound healing is transforming growth factor beta (TGF $\beta$ ) (Roberts *et al.* 1986), which is overproduced in cholesteatoma perimatrix (Huisman *et al.* 2008). The multipotent growth factor TGF $\beta$ , participates in differentiation, proliferation, and apoptosis and regulates immune cell activation and inflammation (Kehrl *et al.* 1986, Christ *et al.* 1994).

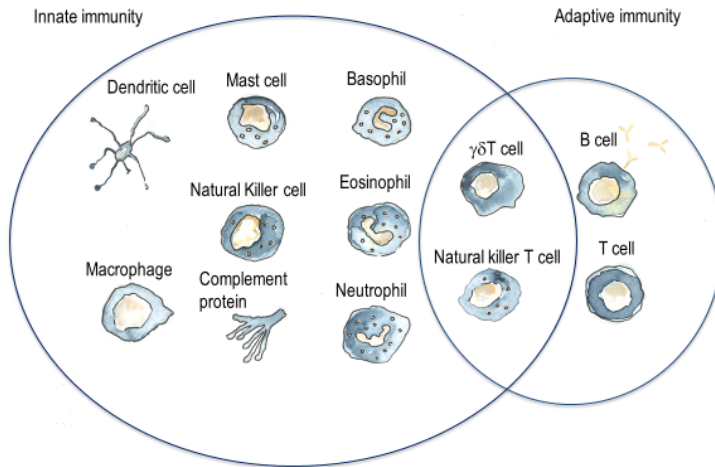
Increased production of TGF- $\beta$  has been linked to immune defects and autoimmune disorders (Caver *et al.* 1996).

An excessive immune response to inflammation may drive the proliferation and aggressiveness of cholesteatomas. When a retraction pocket is established, the keratinizing squamous epithelium and its debris provide a good culture medium for bacteria. An infection can easily develop in the deep part of the external auditory canal, leading to a chronic inflammation, which triggers an expansion of the disease and induces bone destruction (Sadé and Halevy 1974, Tos 1981, Si *et al.* 2015). Participation of both adaptive (Schilling *et al.* 1991, Hussein *et al.* 2010) and innate immunity (Kim *et al.* 2014, Leichtle *et al.* 2015) in cholesteatoma has been described, but their interaction in the pathogenesis and regulation of cholesteatoma has not been fully determined.

Moreover, several biomolecular studies have examined the underlying cause of bone erosion in cholesteatomas, and the mechanisms are controversial. Theories includes the effect of pressure necrosis (Chole *et al.* 1985), osteoclast activation (Hamzei *et al.* 2003, Imai *et al.* 2019), enzymatic digestion (Rezende *et al.* 2012) and inflammation (Si *et al.* 2015).

### Innate immunity

Innate immunity consists of a first line of defence against invading pathogens (i.e., non-self). Induction of the cells of innate immunity triggers signalling pathways and transcription of proinflammatory cytokines, as described and reviewed by several authors (Diamond *et al.* 2000, Medzhitov and Janeway 2000). As very few lymphocytes are found in the healthy middle ear, innate immunity presumably mediates the initial host response against infections (van der Baan *et al.* 1988).



**Figure 1.** Schematic illustration of innate and adaptive immunity.

### Toll-like receptors

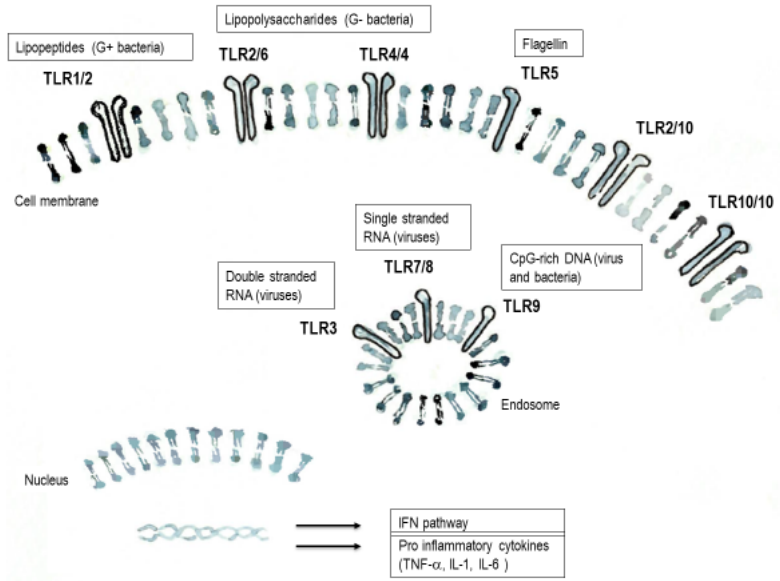
Included in innate immunity are toll-like receptors (TLRs), first described in *Drosophila*, which have an equivalent in humans (Lemaitre *et al.* 1996, Medzhitov *et al.* 1997). TLRs play a key role in the regulation of innate immunity for the clearance of invading microorganisms and activation and sensitization of the adaptive immune system (Schnare *et al.* 2001, Kumar *et al.* 2009, Ito *et al.* 2013). Today, 10 TLRs (TLR1-TLR10) have been described in humans (Chuang and Ulevitch 2001, Jarrossay *et al.* 2001, Matsumoto *et al.* 2002, Hayashi *et al.* 2003, Heil *et al.* 2004). TLR1, 2, 4, 5, 6 and 10 are located at the cellular surface, while TLR3, 7, 8, and 9 are found intracellularly on endosomes (Noh *et al.* 2020). TLRs are expressed on different immune cells, including macrophages, dendritic cells (DCs), and neutrophils, and also on mucosal epithelial and endothelial cells (Kawasaki and Kawai 2014, Tartey and Takeuchi 2017).

Several studies have implicated a relationship between deficiencies in TLR function and AOM and COM. In an animal model, TLR2 induction via TLR4 has been associated with the bacterial clearance and timely resolution of AOM (Leichtle *et al.* 2009). TLR2 responds to lipoproteins and lipoteichoic acid derived from Gram-positive bacteria, such as *Staphylococcus aureus* (Hashimoto *et al.* 2006), a common pathogen in COM (Xu *et al.* 2020). TLR 4 responds to lipopolysaccharides (LPS) of gram-negative bacteria (Poltorak *et al.* 2000). High levels of LPS have been reported to be correlated with bone resorption in cholesteatoma (Peek *et al.* 2003).

Granath *et al.* (Granath *et al.* 2011) investigated the middle ear mucosa from 47 patients with COM, including cholesteatoma. All subjects had an earlier history of infections or clinical signs of inflammation. Immunohistochemical staining for TLR3, TLR4, TLR5 and TLR7 was seen in the middle ear mucosa in ears with COM, and the COM group exhibited lower levels of *TLR4*, *TLR5*, and *TLR7* messenger ribonucleic acid (mRNA) than healthy ears. Conflicting data were presented by Hirai *et al.* who detected a higher expression of TLR2 and TLR4 in the middle ear mucosa in patients with COM including cholesteatoma (Hirai *et al.* 2013).

In addition to these investigations, other authors have searched for TLR expression mainly in cholesteatoma matrix, with a described upregulation of

*TLR2* and *TLR4* (Szczepański *et al.* 2006, Jesic *et al.* 2014, Lee *et al.* 2014, Si *et al.* 2015), *TLR3* (Szczepański *et al.* 2006, Lee *et al.* 2014) and *TLR5*, *TLR7* and *TLR10* (Lee *et al.* 2014).



**Figure 2.** Toll-like receptor (TLR) signalling pathways. TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface. TLR3, 7, 8 and 9 are expressed in endosomes. TLRs recognize structurally conserved molecules derived from microbes. When activated, adapter molecules are recruited and intracellular signalling lead to transcription of proteins participating in the inflammatory response.

## Nitric oxide

Nitric oxide (NO), known as a gas pollutant, is an inorganic free radical (unpaired electron) with the formula  $*N=O$  (abbreviated as NO). The small and short-lived NO molecule (with a half-life of only seconds) (Cocks *et al.* 1985) is involved in a range of cellular signalling processes (Whittle 1995). The NO molecule is so small that it can readily diffuse through membranes to reach the cell cytosol or target cell (Subczynski *et al.* 1996). It is synthesized by many cell types involved in immunity and inflammation, such as macrophages (Stuehr and Marletta 1985), mast cells, and neutrophils (Wright *et al.* 1989). NO is part of innate immunity and is characterized by its rapid recognition and response to invading microorganisms; furthermore, it may have a direct cytotoxic action against protozoa, fungi and bacteria (Malawista *et al.* 1992). A bactericidal effect of NO in low concentrations has been proposed against *Staphylococcus aureus* (Mancinelli and McKay 1983). NO is also a link between the innate and adaptive immune responses, which was previously reviewed (García-Ortiz and Serrador 2018), with versatile and complex involvement in the immune system (Bogdan *et al.* 2000). NO is synthesized from L-arginine, catalyzed by the rate controlling step of the enzyme nitric oxide synthase (NOS) which exists in three main isoforms: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). iNOS is upregulated when needed, whereas nNOS and eNOS are constitutively expressed (Dweik *et al.* 1998, Fleming *et al.* 1998, Hierholzer *et al.* 1998). iNOS in tissues may result in high and prolonged concentrations of NO, exerting proinflammatory effects, including vasodilation, oedema, cytotoxicity and mediation of cytokine activity. Conversely, low concentrations of NO produced by endothelial cells may serve protective and anti-inflammatory functions, preventing the adhesion and release of oxidants by activated neutrophils in the microvasculature (Stadler *et al.* 1991, Akerström *et al.* 2005, Connelly *et al.* 2005, Vo *et al.* 2005).

Sampling of NO has been performed both from the upper and lower human airways, including the paranasal sinuses, a major site of production (Kharitonov *et al.* 1994, Lundberg *et al.* 1995). The mucociliary system is suggested to be dependent on NO for its function (Lindberg *et al.* 1997a).

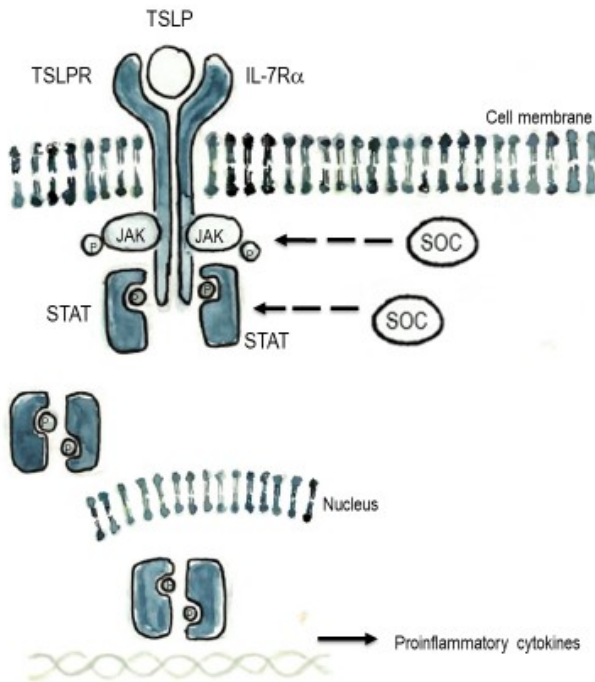
### Thymic stromal lymphopoietin

The cytokine thymic stromal lymphopoietin (TSLP) is an important participant in the innate and adaptive immune response as well as in inflammation. Initially, TSLP was known to activate and promote growth of B cells (Levin *et al.* 1999). Currently, it has been established that TSLP may also promote functions on DCs, T cells, mast cells, basophils and other granulocyte populations (Reche *et al.* 2001, Soumelis *et al.* 2002, Allakhverdi *et al.* 2007, Ziegler and Artis 2010), when activated, contributes to an inflammatory response and participates in the pathogenesis of allergic disease. TSLP is mainly produced by epithelial cells, such as keratinocytes and the airway epithelium, at barrier surfaces (Soumelis *et al.* 2002, Liu *et al.* 2007). The release of TSLP can be triggered by bacteria, viruses, cigarette smoke, skin barrier injury, and endogenous triggers, such as proinflammatory cytokines (Varricchi *et al.* 2018). The functional receptor for TSLP in humans and activation of the complex of the TSLP-receptor and interleukin-7 receptor alpha (IL-7R $\alpha$ ) lead to phosphorylation of signal transducer and activator of transcription (STAT) 5 and 3. TSLP signals via the Janus kinase (JAK)/STAT signalling pathway (Reche *et al.* 2001).

Expression of *TSLP* has been found in the middle ear mucosa in eosinophilic otitis media (Miura *et al.* 2018). In these cases, the TSLP expression was seen (close to the Eustachian tube), which also supports the idea of the concept of “one airway-one disease”. High expression of *TSLP* has also been detected in epithelial nasal mucosa in allergic rhinitis (Kamekura *et al.* 2009).

### Janus kinase/signal transducer and activator of transcription pathway

The JAK/STAT pathway is the principal signalling mechanism for a wide array of cytokines and growth factors in humans. It is one of the primary pathways regulating the innate and adaptive immune response (Ghoreschi *et al.* 2009), but it also stimulates cell proliferation, differentiation, cell migration, and apoptosis (O’Shea *et al.* 2002). JAKs are found in the cytosol and are intimately associated with cytokine receptors. Phosphorylated JAKs both phosphorylate the receptor and create a docking site for STATs. The activated STATs enter the nucleus and promotes transcription (Yamaoka *et al.* 2004). Seven mammalian STATs has been described (Yamamoto *et al.* 1994, Zhong *et al.* 1994, Liu *et al.* 1995), including STAT 1-6, with STAT 5 having two isoforms: 5A and 5B.



**Figure 3.** The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway. JAKs are stimulated by a variety of ligands, amongst them, thymic stromal lymphopoietin (TSLP). Upon binding of TSLP to the high-affinity heteromeric complex composed of TSLP receptor chain and interleukin-7 receptor alpha, associated JAKs will become activated, and phosphorylate STATs, that translocate into the nucleus, where it binds to specific DNA sequences to regulate gene transcription. The negative regulator suppressor of cytokine signalling (SOCS), are negative feedback-loop regulators of JAK/STAT signalling.

STAT5 is expressed in most cell types and activated by a variety of cytokines, each with a defined function (Ihle 1996). The potential involvement of the JAK/STAT pathway in the pathogenesis in cholesteatoma has been investigated in earlier publications, but it is still largely unknown, and conflicting results have been found (Eskiizmir *et al.* 2014, Liu *et al.* 2014). An overexpression of STAT3 (Liu *et al.* 2014), in contrast to a deficiency in JAK/STAT signalling (Eskiizmir *et al.* 2014), was found in cholesteatoma matrix compared to skin from the ear canal.

#### Suppressor of cytokine signalling

Suppressor of cytokine signalling (SOCS) were described by Starr *et al.* (Starr *et al.* 1997) as being responsible for the switch off mechanism of cytokine signal transduction. The SOCS proteins were seen to inhibit the activity of JAKs and reduce phosphorylation of receptors and STATs, i.e., suppressing transduction and ensuing biological responses. Currently, there are eight mammalian SOCS that have been described: SOCS1-7 and cytokine-inducible Src homology 2 protein (Whyte *et al.* 2011).

**Table 1.** Overview of studies included in the thesis. Design, data collection, subjects, outcome measures, methods for analysis and statistics. In **Papers III-V**, mucosa specimens were collected during the following surgeries: tympanoplasty for acquired middle ear cholesteatoma (chol) or chronic middle ear disease (COM) including acquired cholesteatoma, cochlear implantation (CI) for sensorineural hearing loss and translabyrinthine removal of vestibular schwannoma (skull base). The mucosa samples were collected from the tympanic cavity (TC) in COM and cholesteatoma ears, from the TC during skull base surgery and from the mastoid antrum (MA) during either skull base or CI surgery.

Paper	I	II	III	IV	V
<b>Design</b>	Retrospective chart review	Retrospective chart review and questionnaire study	Case-control study	Case-control study	Case-control study
<b>Data collection (Years)</b>	1994-2009	2005-2009	2011-2016	2010-2016	2011-2016
<b>Subjects</b>	Primary cholesteatoma surgical events n=230	Primary cholesteatoma surgical events n=47	chol n=30 controls: MA (CI+skull base) n=37 TC n=25	*Gas analysis middle ear n=11 *Gas analysis controls n=11 **COM n=48 **controls: MA (CI) n=45 **chol n=26 **controls: MA (CI) n=15	chol n=26 controls: MA (CI+skull base) n=27
<b>Outcome measures</b>	Surgical outcome and hearing results	Surgical outcome, hearing results and GBI scores as a measure for change in HRQoL after surgery	mRNA expression of <i>TLR2</i> and <i>TLR4</i> in cholesteatoma ears and controls  Preferable reference genes in middle ear mucosa, validation with analysis of <i>c-MYC</i>	*Occurrence of NO in the middle ear  **mRNA expression of <i>NOS</i> in COM and cholesteatoma ears and controls	mRNA expression of <i>TSLP</i> , <i>IL-7R<math>\alpha</math></i> , <i>JAK1</i> , <i>JAK2</i> , <i>JAK3</i> , <i>STAT5A</i> , <i>STAT5B</i> , <i>SOC1</i> , <i>CD3</i> , <i>CD19</i> , <i>CCND2</i> and <i>TGF-<math>\beta</math>1</i> in mucosa in cholesteatoma ears and controls
<b>Methods for analysis</b>	Medlog® database program, IBM® SPSS® Statistics version 23, Microsoft Excel	Medlog® database program, IBM® SPSS® Statistics version 23, Microsoft Excel	qPCR GraphPad Prism version 8 software for windows Microsoft Excel	*chemiluminescence **qPCR GraphPad Prism version 8 software for windows Microsoft Excel	qPCR GraphPad Prism version 8 software for windows Microsoft Excel
<b>Statistics</b>	Descriptive statistics, one-way ANOVA, Tukey's post hoc test, paired t-test	Descriptive statistics, Linear regression analysis	Descriptive statistics, D'Agostino-Pearson omnibus normality test, one-way ANOVA with Holm-Sidak post-test, one-way Kruskal-Wallis test with Dunn's post test	Descriptive statistics, Mann-Whitney test, Wilcoxon's test	Descriptive statistics, Mann-Whitney test, Spearman's rank correlation

Abbreviations: GBI=Glasgow Benefit Inventory, HRQoL=health-related quality of life, TLR2 and -4=toll-like receptor 2 and -4, c-MYC=cellular myelocytomatosis oncogene, NO=nitric oxide, NOS=nitric oxide synthase, TSLP=thymic stromal lymphopoietin, IL-7R $\alpha$ =interleukin-7 receptor alpha, JAK1, JAK2, JAK3=Janus kinase 1,-2 and-3, STAT5A and -5B=signal transducer and activator of transcription 5A and -5B, SOCS1=suppressor of cytokine signalling-1, CD3 and -19=cluster of differentiation 3 and -19, CCND2=cyclin D2, TGF- $\beta$ 1=transforming growth factor beta 1, qPCR=quantitative polymerase chain reaction. \*chemiluminescence study, \*\*qPCR analysis

## **Aims of the thesis**

The thesis contains two parts. In the first part, including **Papers I and II**, the aim was to investigate the outcome of cholesteatoma surgery. In the second part, **Papers III-V**, the aim was primarily to investigate the occurrence of the innate immunity in middle ears with acquired cholesteatoma.

### **Paper I**

The aim of the study was to evaluate the surgical and hearing outcomes of primary cholesteatoma surgery with CWU, which involved combined approach tympanoplasty (CAT) with obliteration.

### **Paper II**

As a complement to **Paper I**, the aim was to examine the outcome of cholesteatoma surgery from the patient perspective.

### **Paper III**

In this paper, the aim was to investigate the expression of TLR2 and TLR4 in the middle ear mucosa to understand the participation of innate immunity in acquired cholesteatoma. The aim was also to investigate reference genes in the middle ear mucosa.

### **Paper IV**

The aim was to assess if NO is part of the gas mixture in the middle ear. The second aim was to identify the prerequisites for local NO production in the middle ear and if NOSs are related to COM, with an emphasis on acquired cholesteatoma.

### **Paper V**

The aim was to investigate the participation of the innate immunity, with focus on the JAK/STAT pathway, in ears with acquired middle ear cholesteatoma compared with healthy.

## Material and Methods

### Papers I and II

#### Study populations

In **Paper I**, our clinical database Medlog® was used to include 230 consecutively performed primary cholesteatoma surgical events (CWU comprising CAT with obliteration) from January 1994 to December 2009. Pre- and post-operative audiograms had continuously been registered in the Medlog® database. As 98 (43%) of the cases were referrals from other counties, some data were completed retrospectively after requisition of audiograms and patient files from other hospitals. Our initial intention was a follow-up period of 5 years, but as clinical data of hearing and healing were difficult to obtain, we limited our follow-up period to 3 years.

In **Paper II**, a subgroup of 47 adults from the study group in **Paper I** who had surgery from January 2005 to December 2009 was analysed to assess changes in HRQoL after performed cholesteatoma surgery. The patients completed a Swedish version of the GBI questionnaire in 2012.

#### Surgical technique

The surgical technique of CWU with obliteration was used in all surgical events included in **Papers I** and **II**. Surgery was performed by senior surgeons or junior surgeons supervised by a senior colleague.

#### Audiometry

Pure tone audiometry in **Papers I** and **II** was performed with clinical audiometers regularly calibrated according to international standards on all participants (ISO 389-1. 1998). Pure tone AC and bone conduction (BC) hearing thresholds were established according to relevant standards described in the Swedish group of audiology method book, SAME (Almqvist *et al.* 2004). The pure tone average (PTA) used in the statistical analysis was based on threshold levels at frequencies of 0.5, 1, 2 and 3 kHz according to guidelines from the Committee on Hearing and Equilibrium of the American Academy of Otolaryngology-Head and Neck Surgery (Monsell 1995). A hearing level (HL) of 30 dB was

used as the level for socially adequate hearing (Browning *et al.* 1991). An AC threshold exceeding the maximum output level of clinical audiometers were registered as 130 dB HL, whereas BC thresholds exceeding the output level of the audiometer were registered as missing, as recommended by the British Society of Audiology (British Society of Audiology recommendation, Descriptors for pure-tone audiograms 1988).

#### Glasgow Benefit Inventory

In **Paper II**, the 18-item post intervention questionnaire GBI was sent to the patients in a prepaid self-addressed envelope. Written informed consent was received from the participants or the guardian if the patient was under the age of 18 years. Non-respondents were contacted twice to be reminded of the study.

#### **Papers III-V**

##### Quantitative polymerase chain reaction

The nature of quantitative polymerase chain reaction (qPCR), which was used in **Papers III-V**, is high sensitivity, enabling detection of specific mRNA sequences in samples even if only a few copies are present. In **Papers III-V**, the qPCR method was used to perform an instant analysis of the presence or absence of a specific DNA sequence. In the qPCR method, reverse transcriptase generates complementary DNA (cDNA) from extracted ribonucleic acid (RNA), which reflects RNA expression of a defined DNA sequence of a particular gene. The cDNA was amplified by qPCR in an automated system and then quantitatively measured with the help of a fluorescent dye in real time. The gene expression was assessed using the cycle threshold (Ct) method.

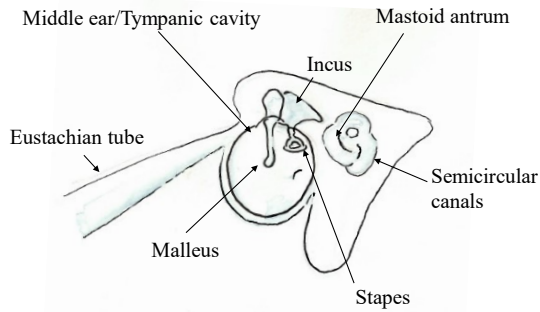
##### Reference genes

Reference genes, also called housekeeping genes, are constitutive genes required for basic cellular functions. They are expressed in all tissues and cell types (Zhu *et al.* 2008). In qPCR, reference genes are used as internal controls in the analysis of the relative expression of the gene of interest. Important features of reference genes are their stable expression in tissues in levels above background and their ability to avoid influence by experimental procedures. Amongst others, two reference genes frequently and generally used as normalizers are

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta actin (*ACTB*). Reported problems and criticism against their utilization have though been described. *GAPDH* is criticized because its levels of expression vary. Lin et al. (Lin and Redies 2012) showed in animal studies that neither *GAPDH* nor *ACTB* mRNA was expressed in all cell types at a high level; instead, expression levels were low in some organs. Moreover, the usage of two or more reference genes for normalization and to carefully choose reference genes suitable for the analysed tissue is recommended (Vandesompele *et al.* 2002, Radonić *et al.* 2004). Thus, the solution is to identify a suitable reference gene for the specific cells or tissue being analysed. In **Papers III-V**, the reference genes *GAPDH* and *ACTB* were used as internal controls.

#### Mucosa sampling

In **Papers III-V**, mucosa samples from the TC were obtained at cholesteatoma surgery. Our aim was to avoid matrix in the samples, and the biopsies were thus taken with caution distant from the cholesteatoma epithelium. As controls, mucosa biopsies were obtained from the mastoid antrum (MA), in the area of the lateral semicircular canal, in cochlear implant (CI) surgery. Additionally, mucosa biopsies from the MA and TC were obtained at skull base surgery during a translabyrinthine approach in cases of vestibular schwannoma (**Papers III and V**). Specimens were collected at Linköping University Hospital between February 2011 and December 2016, and for **Paper IV**, they were also collected from Karolinska Institutet, Stockholm, Sweden. Mucosal biopsies were placed in an RNA stabilization reagent, RNA-later (Qiagen), and stored at -70 or -80°C until analysis at the Karolinska Institutet, Stockholm, or the ENT-laboratory Malmö, Sweden, see **Paper IV**. Exclusion criteria for all subjects in **Papers IV-V** were systemic autoimmune disease and/or systemic corticosteroid therapy, congenital cholesteatoma or non-retraction pocket cholesteatoma. Exclusions in the translabyrinthine group in **Paper V** were history of middle ear infections and neurofibromatosis type II.



**Figure 4.** Schematic drawing of the middle ear/tympanic cavity, mastoid antrum, auditory ossicles (malleus, incus and stapes), the Eustachian tube and the semicircular canals.

### Paper III

In **Paper III**, a total of 162 mucosa samples from middle ears with acquired cholesteatoma and from controls were collected. Due to the low yield and purity, some of the samples were excluded, and finally 92 samples were used for cDNA synthesis and qPCR analysis. Fourteen different candidate reference genes were analysed with qPCR. Three different software tools, NormFinder, geNorm and BestKeeper, were used for studying the stability in gene transcription (Vandesompele *et al.* 2002, Andersen *et al.* 2004, Pfaffl *et al.* 2004). NormFinder was used for intergroup variation analysis. For validation, the expression of the highest and lowest ranked reference genes was related to the expression of *c-MYC* in middle ear mucosa from ears with cholesteatoma compared to controls.

The expression of *TLR2* and *TLR4* mRNA was analysed in mucosa from thirty ears with cholesteatoma and compared to thirty-seven control samples from the MA in CI and skull base surgery. Control samples were also obtained from the TC in twenty-five control samples from skull base surgery. Thus, two sites of control tissue were used for comparison (MA and TC).

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## **Paper IV**

The study of **Paper IV** contains three integrated elements. In the first part, fifteen individuals were addressed for chemiluminescence measurements of NO from the ear canal. Eleven of these measurements could be used in further analysis. Gas from the middle ear was sampled from the ear canal in immediate vicinity of a one-sided ear drum perforation or a grommet opening. The contralateral healthy ears were used as controls.

### Chemiluminescence

In **Paper IV**, the levels of NO in the middle ear were measured by chemiluminescence. The method is based on the reaction of NO with ozone (O<sub>3</sub>) to produce excited-state nitrogen dioxide (NO<sub>2</sub>\*). When it is returning to the normal state (NO), the emission of a photon/light can be measured. Reviewed by Bates, the chemiluminescent reaction is highly sensitive and specific for assessment of NO in the gas phase (Bates 1992).

### Nitric oxide synthase

In the second part of **Paper IV**, analysis with qPCR using the SYBR green technique for detection of NOSs mRNA was performed in 48 pooled samples from the TC in patients with COM. Forty-five mucosa samples from CI surgeries were used as controls. In the third part of **Paper IV**, 26 samples from the TC in ears with acquired cholesteatoma were analysed with qPCR using the dual-labelled probe technique. Fifteen mucosa samples from CI surgeries were used as controls.

## **Paper V**

In **Paper V**, 26 middle ear mucosa samples from ears with acquired cholesteatoma and 27 samples from the MA in healthy controls (CI surgery and skull base surgery) were analysed. The laboratory part in **Paper V** is in conformity with those in **Papers III** and **IV**, performed with qPCR. It was possible to use the same mucosa samples for analysis of the investigated genes in **Papers III-V**.

## **Statistics**

In **Papers I-II**, statistical analyses were performed using IBM® SPSS® (version 23), Microsoft Excel and Medlog® database program. In **Papers III-V**, GraphPad Prism version 8.0, software for Windows (GraphPad Software, Inc.), was used.

In **Paper I**, one-way ANOVA, Tukey's post hoc test and paired t-tests were used.

In **Paper II**, a linear regression analysis was performed.

In **Paper III**, the datasets were analysed for a Gaussian distribution using the D'Agostino-Pearson omnibus normality test. If a Gaussian distribution could be assumed, one-way ANOVA with the Holm-Sidak post-test was used. If a Gaussian distribution could not be assumed, a one-way Kruskal-Wallis test with Dunn's post-tests was used.

In **Paper IV**, the Mann-Whitney test for non-parametric data was used.

Wilcoxon's test for non-parametric data was used for paired observations.

In **Papers IV-V**, the Mann-Whitney test and Spearman's rank correlation for non-parametric data was used.

## **Ethical approval and considerations**

All studies in this thesis complied with the Declaration of Helsinki (World Medical Association 2013). Ethical approval for the papers in the thesis was obtained from the Ethical Review Board in Linköping (DNR 2011/88-31). A complementary ethical approval for **Paper IV** was obtained from the Ethical Review Board in Stockholm (DNR 2009/677-32).

In **Papers I** and **II** data that were already recorded in the Medlog® database were used. The patients were informed by a letter and asked for agreement for inclusion in **Paper I**. In **Paper II**, patients who did not return a filled in questionnaire and a signed approval of participation were contacted by telephone and asked for their participation without persuasion. Complementary information, specifically designed for children under the age of 18 years, was used for inclusion in **Papers III-V**. All participants received appropriate information about the surgical procedures, the aim of the studies and that the participation was voluntary with the possibility to cancel their participation any time. A written consent was obtained from the included patients or guardians. The participants or guardians could not profit from and were not compensated economically or otherwise compensated for their participation. The code keys were kept separate from the questionnaires and was only available for the research team. This means that the results were anonymised to respect the integrity of all participants. In **Papers III-V** mucosa biopsies were obtained during planned ear and skull base surgery. Biopsies from the TC and MA are impossible to reach without microsurgery and must be carefully harvested to prevent any complications for the patient. Only experienced ear surgeons were involved in the biopsy procedure. The size of the biopsies was 2-3 mm. To our knowledge, no complications were caused from the sampling of mucosa.

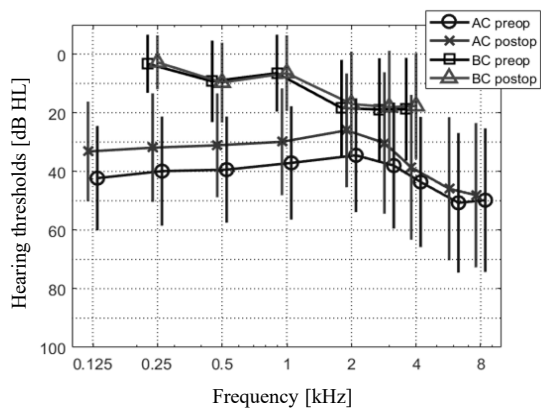
## Results

### Paper I

In the consecutive group of 230 included surgeries with a follow-up period of 3 years, the residual frequency was 1% (three cases), and the recurrence rate was 8% (eighteen cases). In 56 (24%) cases, revision surgery was performed, mostly due to ossicle reconstructions. A second look or a postoperative DW-MRI was not performed. The hearing results were based on data from 219 cases. Hearing data were excluded in seven cases as no improvement could be expected. In addition, in four cases, pure tone audiometry data were missing. One-year follow-up audiometry was obtained in 98% of the included cases. Hearing was improved overall one and three years postoperatively. The one-year postoperative results are shown in Figure 5. No case of a total postoperative hearing loss was detected. There was a significant improvement of PTA one- and three-years postoperatively. The proportion of individuals with socially adequate hearing (PTA of 30dB or better) increased from 41% to 64% one year postoperatively, and to 60% 3 years postoperatively. In cases of a TORP, indicating stapes erosion and a more severe disease, the mean PTA was 35dB, at one- and three-years postoperatively. In cases of a partial ossicular replacement prosthesis (PORP), when disease was less severe, the PTA was 28dB and 30dB, at 1 and 3 years postoperatively.

### Paper II

Thirty-four (72%) out of 47 patients returned a filled in questionnaire. The overall GBI scores showed an improvement in HRQoL after cholesteatoma surgery. Twenty-nine (85%) patients benefitted from surgery, while 1 (3%) had no change, and 4 (12%) expressed deterioration. In the respondent group, the mean PTA for AC was 47 dB preoperatively compared to 29 dB at 1 year and 33 dB at 3 years postoperatively. In the non-respondent group, the PTA for AC was 39 dB preoperatively compared to 38 dB at 1 year and 42 dB at 3 years postoperatively. There were no cases of residual or recurrent cholesteatomas in either group. The results for the respondent and non-respondent groups are shown in Table 2.



**Figure 5.** Preoperative and one-year postoperative hearing based on pure tone thresholds averaged over the frequencies 0.5, 1, 2 and 3 kHz (PTA 0.5-3 kHz). N=207. Symbols and error bars are means and 95% confidence intervals. From the study group (n=230), seven patients were excluded from the hearing outcome analysis as improvement was not expected. Only data where pre- and postoperative PTA was documented were included in the calculations.

**Table 2.** Demographic data and results of the Glasgow Benefit Inventory (GBI) scores and hearing in respondents (n=34), and non-respondents (n=13). Pure tone average (PTA) is defined as the mean value of hearing thresholds at frequencies 0.5, 1, 2 and 3 kHz. Gain is calculated as preoperative (pre-op) minus postoperative (po) mean PTA. The interaural difference is calculated as the difference of mean PTA hearing level between the right and left ear.

### Respondents

Pt No	GBI score				Operated ear	Gender	Age yrs	Yrs since op yrs	Preoperative hearing PTA ≤30 dB pre-op Y/N	Postoperative hearing				
	Total score	General score	Social score	Physical score						R/L	F/M	PTA ≤30 dB 1-yr po Y/N	Mean PTA AC 1-yr po	Mean PTA AC 1-yr po contralateral ear
1	-28	-42	0	0	L	M	39	3	N*	N	44	10	34	25
2	-8	-13	0	0	R	M	36	4	N	Y	20	0	20	15
3	-6	-4	0	-17	R	F	47	5	N	N	36	14	23	8
4	-5	-50	-50	-117	L	F	25	5	Y	Y	19	0	19	5
5	0	-25	0	0	R	F	76	6	N	N	66	28	39	11
6	3	0	0	17	R	M	25	4	Y	Y	13	3	10	16
7	3	4	0	0	R	M	73	5	N	N	MD	MD	MD	MD
8	3	-29	50	-17	R	F	55	6	N	Y	26	8	19	23
9	8	8	0	17	R	F	15	6	N	Y	15	6	9	29
10	8	8	0	17	R	M	42	7	Y	Y	20	33	13	-3
11	11	13	0	17	L	F	66	7	Y	Y	24	14	10	-9
12	11	13	0	17	R	M	61	4	Y	Y	25	30	5	5
13	14	13	17	17	R	F	35	5	Y	Y	19	8	11	9
14	17	25	0	0	R	M	34	7	N	Y	16	10	6	36
15	17	33	17	-50	L	F	43	7	N	N	34	54	20	11
16	17	25	0	0	R	M	33	6	N	N	60	13	48	6
17	18	13	0	0	L	F	64	7	N	N	36	21	15	9
18	20	29	0	0	R	M	60	6	N	N	33	20	13	18
19	22	33	0	0	R	M	16	7	N*	N	33	10	23	11
20	24	29	0	-33	L	F	56	7	N	N	31	14	18	20
21	25	25	0	50	R	M	22	7	N	Y	25	38	13	16
22	25	21	17	50	L	M	53	3	N	Y	28	24	4	50
23	25	38	0	0	R	F	29	7	Y	N	31	MD	MD	-5
24	26	21	0	17	L	M	74	6	N	N	35	19	16	16
25	28	38	17	0	L	M	44	5	Y	Y	15	9	6	4
26	29	25	17	0	L	F	67	5	N	N	101	29	72	-10
27	31	29	0	67	R	M	43	7	N	Y	28	20	8	21
28	31	4	0	50	R	F	36	7	N	N	38	MD	MD	61
29	38	38	0	17	L	F	58	7	N	Y	26	21	5	23
30	47	58	50	0	R	F	33	6	N	Y	30	33	3	29
31	50	67	0	33	L	M	16	6	N	Y	13	10	3	20
32	56	83	0	0	L	F	33	4	N	Y	11	4	8	49
33	75	79	83	50	L	F	17	4	Y	Y	1	-5	6	14
34	75	79	67	67	L	F	54	4	N	Y	20	1	19	20

## Non respondents

Pt No	GBI score				Operated ear	Gender	Age	Yrs since op	Preoperative hearing	Postoperative hearing				
	Total score	General score	Social score	Physical score						R/L	F/M	yrs	yrs	PTA $\leq$ 30 dB pre-op Y/N
1					R	F	79	7	N	N	71	31.25	40	24
2					R	F	23	4	N	N	33.75	-2.5	36	12.5
3					R	F	39	3	Y	Y	15	6.25	9	6.25
4					L	M	21	7	N*	N	81	3.75	78	15
5					L	F	44	6	N	N	36	28.75	7.5	24
6					L	F	18	6	Y	Y	24	3.75	20	-1.25
7					L	F	23	6	N	Y	29	6.25	23	8.75
8					L	F	23	6	Y	Y	20	8.75	11	-10
9					L	M	32	5	N	Y	21	18.75	2	22
10					L	M	73	5	N	N	49	27.5	21	-3.75
11					L	M	26	3	Y	Y	15	7.4	8	13.75
12					L	M	28	3	Y	MD	MD	MD	MD	MD
13					R	F	40	7	Y	Y	20	47.5	27.5	10

Abbreviations: R=right, L=left, F=female, M=male, Y=yes, N=no, yrs=years, MD=missing data, AC=air conduction, contralat=contralateral. \*labyrinthine fistula of semicircular canal

### Paper III

*TLR4* was downregulated in cholesteatoma ears (n=30) in relation to control mucosa samples from the MA (n=37) but not in relation to controls from the TC (n=25). No significant difference in *TLR2* expression was found between the two control sites, MA and TC, or compared with the mucosa from cholesteatoma ears.

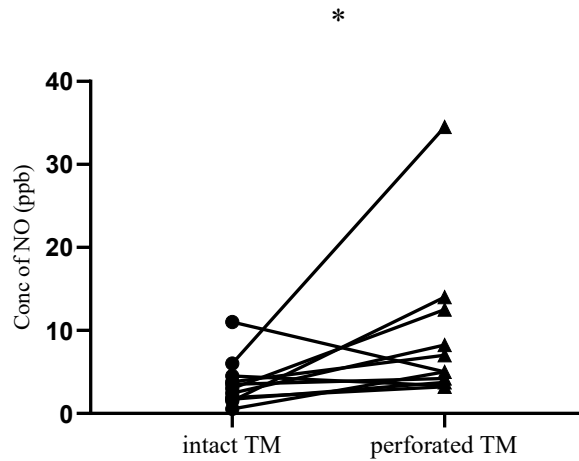
Out of 14 different candidates for reference genes, *ACTB* and *GAPDH* were the most stably expressed in qPCR analysis of middle ear mucosa. The relative expression of *c-MYC* mRNA confirmed the suitability of these reference genes.

### Paper IV

NO was detected in eleven samples, collected from the immediate vicinity of a perforation or grommet opening communicating with the middle ear. There was a statistically significantly higher level of NO in the ears with a TM perforation or a grommet than in the contralateral ear canals with intact TMs, see Figure 6. In the second part of the study, pooled middle ear mucosa samples from ears with COM (n=48) showed lower *eNOS* expression than controls (n=45). *iNOS* was also lower in the COM group than in the control group, but the difference was not statistically significant. In the third part of the study, the expression of *nNOS* in the cholesteatoma group (n=26) was lower than that in the control group (n=15).

### Paper V

Upregulation of *SOCS1* and downregulation of *STAT5B* were seen in the middle ear mucosa in cholesteatoma ears (n=26) compared to controls (n=27) from the MA at CI and skull base surgery. Furthermore, overexpression of *IL-7R $\alpha$* , cluster of differentiation (*CD3*) and cyclin D2 (*CCND2*) was seen in cholesteatoma ears compared to controls. A correlation was found between the level of *SOCS1* and *IL-7R $\alpha$*  expression (p=0.0259). The remaining significantly altered genes did not correlate with each other.



**Figure 6.** Concentration of nitric oxide (NO) in parts per billion (ppb) in the middle ear by chemiluminescence measurements from 11 cases with perforated tympanic membrane (TM) or grommet, and 11 controls with intact TM. Measurements were made close to a grommet opening or ear drum perforation. The contralateral ear with intact eardrum served as the control. (\* $p < 0.05$ )

## Discussion

### Surgical and hearing outcomes

Residual and recurrence rates were comparable to, or low, in comparison with other studies. (Edfeldt *et al.* 2012, 2013, Gaillardin *et al.* 2012, Møller *et al.* 2020, van der Toom *et al.* 2020). Hearing results were also in line with findings from other authors (Yung *et al.* 2007, Suzuki *et al.* 2014).

### Surgery

In **Paper I**, we excluded 18 (5%) CWD cases from our initial cohort. In another 6 cases we had the intention of performing a CWU but the ear canal had to be removed due to an unfavourable anatomy. This exemplifies an individualized management of the cholesteatoma cases at our department. However, our standard surgical method is a CWU with obliteration. Our surgical philosophy is similar to that of Edfeldt *et al.* (Edfeldt *et al.* 2012, 2013) and focuses on meticulous dissection for radicality in a one-stage surgery. Other similarities are the usage of autologous bone and cartilage at reconstruction, obliteration of the mastoid and epitympanic spaces, and use of a silastic sheeting in selected cases to avoid adhesions of the ear drum to the promontory. There are advantages to ensure eradication of the disease and anatomical reconstruction in one-stage surgery. It is desirable to avoid unnecessary revision surgeries and to avoid the strategy of leaving adjustments to be performed at a second look. Compatible with a second look, 56 (24%) of the cases in the study group ended up with a revision surgery. Amongst those, one residual cholesteatoma was found. Our strategy was to reconstruct the attic and lateral attic wall as well as obliterate the space between the facial nerve and medial edge of the bony ear canal, in order to prevent recurrent cholesteatomas. For obliteration, an anteriorly based vascularized flap together with cartilage was used.

### Hearing

A secondary goal in cholesteatoma surgery is to restore, or to maintain hearing as best as possible. In cases of an extensive disease and bone erosion caused by the cholesteatoma, hearing restoration might be difficult or even impossible to achieve.

There is no consensus in how to report hearing outcome in cholesteatoma surgery, which makes it difficult to do comparisons with other studies (Yung *et al.* 2007). In **Paper I**, the proportion of individuals with socially adequate hearing (PTA of 30dB or better), is presented. The definition of socially adequate or socially serviceable hearing can be discussed, as there is a lack of consensus. The level of 30dB or better was used in earlier studies on hearing results after otosurgery (Yung *et al.* 2007, Kisilevsky *et al.* 2010, Kuo *et al.* 2012).

Postoperative hearing results are most dependent on the preoperative hearing (Stankovic 2008), and the possibility to preserve the ossicular chain or to perform a reconstruction with a PORP which is beneficial for hearing restoration compared to a TORP (Edfeldt *et al.* 2012). The majority of cases (n=148, 64%), in **Paper I** was reconstructed with a PORP. The same method was used in studies with comparative hearing results (Edfeldt *et al.* 2012, 2013, van Dinther *et al.* 2015). No patient suffered a total hearing loss postoperatively, which we interpret as the surgical method being atraumatic.

#### Inclusion and representation of the patient population

Patients were included from a medical database, Medlog®, in which all otosurgical performances are recorded consecutively by the otosurgeons in the county of Östergötland. The Medlog® database was available from 1994 in Linköping and from 2002 in Norrköping, which explains the difference in the years of inclusion between the sites.

The study group was based on a consecutive series of cholesteatoma surgeries, including ten cases (4%) with labyrinthine fistulas and the necessity of total ossicular replacement prosthesis (TORP) in 60 (26%) ears as a result of an eroded stapes. Thus, we believe that the study group is representative of evaluation of cholesteatoma surgery in general and does not only represent “easy cases”. The number of perioperative infections was 52 (23%) (unpublished data). The inclusion criteria for primary cholesteatoma surgery and exclusion of revision surgeries facilitate comparison to other similar cohorts.

### Follow-up

A long-term follow-up period is of great importance in cholesteatoma surgery as recurrence and residual disease may be revealed after several years (Silvola and Palva 2000, Ajalloueyan 2006, Møller *et al.* 2020). The choice of a three-year follow-up period in our study was based on the fact that a five-year follow-up period resulted in too much incomplete data. The follow-up rate in **Paper I** for hearing results and clinical status was 98% one year postoperatively and 87% three years postoperatively. Thus, in a worst-case scenario, there is a possibility of a one-year postoperative residual frequency of 3%. However, this scenario is not probable as the joint clinics of Linköping and Norrköping are the only caregivers for cholesteatomas for the inhabitants in the county of Östergötland. Linköping is the site of referrals in this region; thus, we believe that residual and recurrent cholesteatomas eventually would have come to our knowledge. During the time of the study we did not perform a postoperative DW-MRI to detect cases of residual, as the technique was not available.

### Interpretation of results

Key factors for our surgical outcome included a standardised surgical method, the skills of the surgeons, the supervision of a senior surgeon when needed, and probably the allowance for the surgeons to have a reasonable surgical time at their disposal. The fact that several surgeons (three senior and mainly two junior surgeons) were involved in the study permits generalisation regarding the used method (CWU).

In a wider aspect, the surgery for cholesteatoma is an important factor but not the only factor influencing the long-term outcomes of hearing and normalised ear anatomy. Good ventilation of the middle ear prevents recurrent disease, and columella failure. It is known that failure of Eustachian tube closure and sniffing behaviour can cause negative pressure in the middle ear, leading to retraction of the eardrum (Magnuson 1981, Shibata *et al.* 2015). The complexity of the psychological factors of patients with sniffing behaviour is described by Bunne *et al.* (Bunne *et al.* 1999). It seems that it is of importance for the clinician to be aware of the phenomena of sniffing and to be aware of cases that could lead to recurrent retractions.

Surgical results in paediatric patients are considered poorer than those of adults (Silvola and Palva 1999), but some reports have challenged this opinion (Edfeldt *et al.* 2012, Trindade *et al.* 2014). Our choice was to present data without separating children from adults. The three residual cholesteatomas found in our study were diagnosed among adults.

### **Health-related quality of life assessment of cholesteatoma surgery**

#### Glasgow Benefit Inventory scores

The GBI was administered once. Compared to pre- and postoperative questionnaire strategies, the single administration comes with some advantages; it reduces the effect of participants dropping out during the study and does not allow participants to change their performance or to be influenced by surrounding factors between two performances.

A majority of patients in Paper II described an improvement of HRQoL after cholesteatoma surgery, and the overall GBI scores were comparable to the results of Robinson *et al.* (Robinson *et al.* 1996). However, 4 patients expressed a deterioration, which, in one case was not easy to explain as the hearing results were improved and the anatomy was normalised after surgery. The fact that preoperative symptoms in cholesteatoma can be very discrete makes it even more difficult to achieve postoperative improvement of HRQoL despite good surgical results from the point of view of the surgeon. Smyth (Smyth and Patterson 1985) discuss the importance of a final interaural difference, postoperative AC thresholds (averaged from 0.5-4kHz), and gain in the paper “Results of middle ear reconstruction; Do patients and surgeons agree”. In theory, these factors were possible predictive measures for the outcome of the GBI assessment. But we could not detect any statistically significant correlation of hearing results and GBI scores, which was surprising. It is a challenge for the surgeon to perform cholesteatoma surgery to improve patient HRQoL; thus, the patients’ expectations of surgery must be included in the preoperative discussion. Although the questions in the GBI questionnaire address a specific intervention, the interpretation of these questions may be found to be difficult, especially when a longer time has passed since the surgical event.

### Response rate and generalisation

It is a known dilemma that an adequate response rate in patient questionnaire studies is difficult to achieve. A high survey response rate is of importance to avoid a potential non-respondent bias (Compton *et al.* 2019). The difficulty of reaching a high response rate and the trend towards its decline in public health research has been highlighted in earlier studies (Groves and Peytcheva 2008, Compton *et al.* 2019). In a meta-analysis on studies using the GBI questionnaire, surveys with a response rate over 50% were included (Hendry *et al.* 2016). Earlier HRQoL surveys in COM-related surgical performances using the GBI reached a 29-100% response rate (Dornhoffer *et al.* 2008, Kurien *et al.* 2013, Bernardeschi *et al.* 2017, Uluyol *et al.* 2017). The response rate in **Paper II** was 72%, which was equal to that in the study by Robinson *et al.* (Robinson *et al.* 1996). Amongst the respondents and non-respondents, there was a fairly even distribution of the genders and ages of the patients, although the non-respondent group was younger, with a mean age of 36 years and a median of 28 years, than the respondent group, with a mean age of 44 years and a median of 43 years. Both groups had cases of labyrinthine fistulas as well as TORPs, as an indication of a severe disease. It is therefore our assumption that not only “easy cases” were included in the respondent group. However, our results indicate deteriorated hearing in the non-respondent group (n=13) one and three years postoperatively, but the number of measured cases was low, and only nine measurements were taken at the three-year postoperative audiograms, making it difficult to draw conclusions. The question regarding whether the results of the GBI questionnaire in **Paper II** could be generalised but not easily answered. In favour of generalisation is the well-defined surgical method used, the cohort of consecutive surgical performances at two surgical sites, Linköping and Norrköping, and the participation of several surgeons. This means that the results of the GBI questionnaire in **Paper II** are most probably applicable in a similar setting. In a different context, such as in the study of Nepali patients with COM by Maile *et al.* (Maile *et al.* 2015), parameters such as cultural and social differences must be taken into account.

### Innate immunity

The qPCR laboratory parts of the thesis in **Papers III-V** investigated the involvement of innate immunity in acquired middle ear cholesteatoma. The role of innate immunity in the middle ear is exemplified in the resistance against and fast recovery from middle ear infections (Leichtle *et al.* 2011). Its detailed involvement in ear pathology is still incompletely understood (Lee *et al.* 2019).

#### Toll-like receptors

To analyse the expression of TLRs in the MA and the TC, we compared mucosa samples from these sites collected at translabyrinthine skull base surgery for vestibular schwannomas. Our findings confirmed expression in both the MA and TC for *TLR1-9* in pairwise controls. Some of the TLRs, including *TLR5*, *TLR6*, *TLR7* and *TLR9*, showed very low expression, which was not easy to explain. These are unpublished data. Based on these analyses, we considered MA a suitable control site for *TLR* expression of the middle ear mucosa. Mucosa from the MA in CI patients has previously been used as a control tissue (Granath *et al.* 2011, Hirai *et al.* 2013). The expression of *TLR3*, *TLR4*, *TLR5* and *TLR7*, and downregulation of *TLR4*, *TLR5* and *TLR7* in the middle ear mucosa in ears with COM compared to healthy middle ears was detected by Granath *et al.* (Granath *et al.* 2011). The investigated group in the study by Granath *et al.* was composed of a variety of COM phenotypes and initiated our interest in analysing *TLR* expression specifically in middle ears in the presence of cholesteatoma. The expression of TLRs in the human middle ear mucosa in cholesteatoma ears was also investigated by Hirai *et al.* (Hirai *et al.* 2013). In contrast to the results of Granath *et al.*, significantly higher expression of *TLR 2* and *TLR 4* with immunohistochemical staining in 7 patients with COM and 5 patients with middle ear cholesteatoma was described.

In our study, *TLR2* and *TLR4* were detected in healthy middle ears as well as in ears with acquired cholesteatoma. No difference in the expression of *TLR2* was seen, but there was a downregulation of *TLR4* in cholesteatoma ears compared to the control tissue from the MA but not compared to controls from the TC. These conflicting results mean that we cannot conclude that a difference in the expression was clearly identified although the findings of a *TLR4* derangement in cholesteatoma ears are in line with the findings of the study by Granath *et al.*

(Granath *et al.* 2011). The difference in expression of *TLR4* mRNA between the MA and TC in healthy middle ears is difficult to explain. In theory, either control group might be influenced by factors that are currently unknown. A limitation of our study is that the findings of *TLR* mRNA were not confirmed at the protein level or with proteins participating in the TLR signalling pathway. Such analysis would be of interest because post-transcriptional factors might influence the final expression. The fact that the *TLR4* expression differed in the MA and TC augments the interest for further investigations.

#### Nitric oxide and nitric oxide synthase

Measurements of NO in the middle ear is challenging because of the nature of its short half-life of only seconds in its gaseous form. The middle ear compartment is an inaccessible site for measurements, and analytic precision and avoiding contamination must be regarded. To the best of our knowledge, NO has not been previously measured in the human middle ear compartment. The detected higher levels of NO in ears with a perforated ear drum than in controls indicate the participation of NO in the gas mixture of the middle ear.

The chemiluminescence method provides a high sensitivity and specificity when measuring NO (Archer 1993, Shapiro *et al.* 2019). Therefore, this method has frequently been used in earlier research concerning NO in the airways and sinuses (Alving *et al.* 1993, Lindberg *et al.* 1997b). An advantage for the NO measurements described in **Paper IV** is that the chemiluminescence analyser was already in use at the Karolinska Institutet in Stockholm. A possible source of error is that the measured levels of NO could be a contamination or originate from the Eustachian tube. In earlier reports, the partial pressure of the middle ear gases resembled the partial pressure of gases in local tissues (Hergils and Magnuson 1990, Sadé and Luntz 1993). Studies on dogs revealed a change in middle ear pressure with different ventilation without interference from the passage of gas through the Eustachian tube (Buckingham *et al.* 1985). The differences in the concentrations of NO in ears with one-sided perforations or grommets compared to the contralateral ear canals also indicate that the measured NO originates from the middle ear and is not a contamination.

This supports our suggestion of the measured NO belonging to the middle ear gas mixture. Patients served as their own controls, which minimizes an effect of individual variation.

NOS was found in ears with COM and acquired cholesteatomas as well as in mucosa controls from CI patients. Constitutive *NOSs*, *eNOS* and *nNOS* was downregulated in COM and cholesteatoma ears. This indicates that NO can be produced in the middle ear, which corroborates earlier experimental, animal and human studies (Forséni *et al.* 2001, Morineau *et al.* 2001, Andersson *et al.* 2002). *eNOS* was downregulated in the middle ear mucosa in COM ears but not in cholesteatoma ears. The difference in the results could be explained by the fact that the COM group included a heterogeneity of diagnoses and pooled samples.

Lindberg *et al.* (Lindberg *et al.* 1997b) found decreased levels of NO in chronic sinusitis, possibly due to a decreased number and function of ciliated cells. Similar to our results of lower constitutive *NOSs* in COM and cholesteatoma, NO seems to be an important mediator involved in the mucosal defence mechanisms both in the sinus and the middle ear. This is also in line with the concept of a united airway. In earlier reports, findings of increased *iNOS* expression were seen in cholesteatoma matrix with skin as controls (Çatlı *et al.* 2014, Lee *et al.* 2014). These reports differed from ours as they investigated the epithelial layer in cholesteatoma, whereas our investigations focused on the middle ear mucosa.

Thymic stromal lymphopoietin and the Janus kinase/signal transducer and activator of transcription pathway

Similar to **Papers III** and **IV**, our focus in **Paper V** was to identify the presence of innate immunity expression in cholesteatoma ears compared to healthy middle ears. Our intention was to follow the signalling pathway and its interactive components (*TSLP*, *IL-7R $\alpha$* , *JAK/STAT*, *SOCS1*). This idea was expanded to include analysis of the gene expression of *CD3* (T cells), *CD19* (B cells), *CCND2* and *TGF- $\beta$ 1*. Our main findings were that two important players of the JAK/STAT pathway are significantly altered in ears with cholesteatoma.

A downregulation of *STAT5B* and overexpression of *SOCS1* was presented. Only a few studies have previously showed interest in the JAK/STAT pathway in cholesteatomas. Downregulation of JAK1, JAK2 and JAK3, and STAT1, STAT2, STAT4 and STAT5 in cholesteatoma matrix (Eskiizmir *et al.* 2014) and increased expression of STAT3 and IL-6 in cholesteatomas have previously been reported (Liu *et al.* 2014). In these immunohistochemical studies, the cholesteatoma matrix was investigated with the skin from the ear canal as control.

A local inflammatory reaction in the ear is thought of as a contributing factor to the pathogenesis of cholesteatoma (Olszewska *et al.* 2004, Si *et al.* 2015). TSLP, known as a pleiotropic cytokine with a wide range of effects on cells of the innate and adaptive immune system, could be a participant in such an inflammatory reaction. Theoretically, TSLP, in line with upregulation in acute and chronic atopic dermatitis (Soumelis *et al.* 2002), could also be a possible link to hyperkeratosis, possibly during infectious conditions in cholesteatoma ears. The expression of TSLP and the involved genes in the JAK/STAT pathway would be assumed to follow the same pattern in ears with cholesteatoma as in dermatitis. However, in **Paper V**, no statistically significant difference was seen in the relative mRNA expression of *TSLP* in cholesteatoma ears compared to controls. The number of cases with a macroscopically reactive mucosa with ongoing inflammation or infection at the time for sample collection was low (6 (23%) out of 26 cases). A Kruskal-Wallis test for non-parametric data showed no significant differences of *TSLP* expression between the inflamed or infected cholesteatoma ears compared to healthy controls ( $p=0.0918$ ) (Unpublished data). Instead we detected an overexpression of *IL-7R $\alpha$* , *CD3* and *CCND2* in cholesteatoma ears. In theory, an upregulation of the IL-7 pathway might explain these findings, as the IL-7R $\alpha$  is also used by IL-7. The IL-7R $\alpha$  is predominantly expressed on the cells from the lymphocyte lineage (Mazzucchelli and Durum 2007), and it is known that T cells (CD3) are represented in cholesteatoma perimatrix (Dornelles *et al.* 2009). This theory must be further examined and could not be confirmed because we did not analyse the expression of IL-7.

There was also a correlation between the expression of *SOCS1* and *IL-7R $\alpha$* . In previous findings, *SOCS1* (and *SOCS2*), coprecipitated with *IL-7R $\alpha$*  in human cytotoxic T cells, but its relevance is not known (Ghazawi *et al.* 2016). *CCDN2* is important for the G1 to S phase transition in the cell cycle and could be an indication for cell division activity.

The cholesteatoma process resembles defect wound healing and a key factor in wound healing is TGF- $\beta$ . An upregulation of TGF- $\beta$  in the stroma of cholesteatoma but not in the matrix has been described (Huisman *et al.* 2008). In our data, we did not see any significant difference in expression of *TGF- $\beta$*  mRNA in mucosa from ears with acquired cholesteatoma compared to healthy controls.

Our findings support the idea that the JAK/STAT pathway participates in the acquired middle ear cholesteatoma process. However, a limitation in **Paper V** is the lack of verification of gene expression at a corresponding protein level. The result of higher levels of *IL-7R $\alpha$*  and *CD3* could theoretically be an effect of the presence of T cells and activation of adaptive immunity, but further studies must be conducted to confirm this hypothesis.

#### Reference genes in the middle ear

*ACTB* and *GAPDH* were shown to be optimal genes for the normalisation of target gene expression in qPCR, when analysing middle ear mucosa. Compared to other candidate reference genes *ACTB* and *GAPDH* were the most stably expressed in middle ear mucosa samples. Earlier studies supports the idea to include two reference genes in the qPCR analysis (Vandesompele *et al.* 2002, Radonić *et al.* 2004). This was applied in **Papers III-V** where *ACTB* and *GAPDH* were used.

#### Validation of reference genes with cellular myelocytomatosis oncogene

Expression of cellular myelocytomatosis oncogene (*c-MYC*) mRNA was used for the validation of the highest (*ACTB* and *GAPDH*) compared to the lowest ranked reference genes. The expression of *c-MYC* is associated with certain tumours, such as squamous cell carcinomas of the head and neck, but it is also

expressed in normal tissues (Jack *et al.* 1986). It participates in the regulation of cell proliferation as well as in differentiation, apoptosis and transformation (Shichiri *et al.* 1993). C-MYC occurs in several different cell lines and in different vertebrate species, which indicates its evolutionary stable expression (Persson *et al.* 1984). An expression of c-MYC in the middle ear tissue could thus be expected. In **Paper III**, different results of expression of *c-MYC* were seen using different reference genes. When using *GAPDH* and *ACTB* as reference genes, *c-MYC* showed increased expression in ears with acquired cholesteatoma in the MA but not in the TC compared with controls, which is difficult to explain. According to our findings, the reference genes *ACTB* and *GAPDH* seem to be specifically suitable for middle ear mucosa analysis with qPCR, which is valuable and useful knowledge for future research.

#### **Methodological considerations**

In **Paper IV**, one limitation was that the qPCR analyses were performed at different laboratories (Malmö and Stockholm) using different qPCR equipment for SYBR green detection and the dual-labelled probe technique. In our defence, the recommendations from the manufacturer for RNA-later (Qiagen, Germany) were followed, and it seems unlikely that the different qPCR techniques would have a significant effect on the results as the qPCR method is well established and enables generalisation and comparison between analyses (Tajadini *et al.* 2014). The setting for the surgical sampling of tissue was equal between the two sites of Stockholm and Linköping, in terms of preoperative evaluation, anaesthesia, surgical techniques and the use of preoperative antibiotics.

## Conclusions

The following conclusions are drawn based on the findings in the studies of this thesis:

- CWU, comprising CAT with obliteration, is a safe surgical method for treatment of cholesteatoma. Hearing and change in HRQoL are improved in a majority of cases after cholesteatoma surgery with the described method.
- The investigated parts of innate immunity, including the relative mRNA expression of *TLR2*, *TLR4*, *NOSs*, and participants of the *JAK/STAT* pathway, are present in the healthy middle ear and in ears with acquired cholesteatoma.
- Derangement of *TLR4* as well as the *JAK/STAT* pathway and *NOSs*, supports the idea of a role of the innate immune system in the cholesteatoma process.
- NO could be a part of the gas mixture in the middle ear.
- Findings of *NOSs* in the middle ear mucosa suggest the possibility of a local NO production.
- A lower relative mRNA expression of constitutive *NOSs* in ears with acquired cholesteatoma, suggest that NO is a part of the maintenance of a healthy middle ear environment.
- *ACTB* and *GAPDH* are suitable reference genes for the middle ear mucosa.

### **Future perspectives**

Studies with long-term follow-up must be conducted to ensure that the applied surgical method in cholesteatoma is durable.

Additional HRQoL measurements are important as a complement to the hearing and healing outcomes after cholesteatoma surgery. Qualitative studies could be a complement. Paediatric patients should be included in the HRQoL measurements.

To deepen the knowledge about the pathogenesis, further biomolecular research may clarify the multifactorial process of acquired middle ear cholesteatoma development. Biomolecular findings must then be related to the clinic.

Studies in the field of the united airways concerning the middle ear are lacking. Physiologic and biomolecular studies are yet to be performed to augment our understanding of this topic.

To clarify the participation of NO in the middle ear, further studies are warranted to replicate and to extend our findings.

## Populärvetenskaplig sammanfattning

Kolesteatom, är en sjukdom som drabbar örat och som även kallas pärlcysta. Avstötta hudceller ansamlas i en indragen del av trumhinnan och hudansamlingen expanderar långsamt och bildar en ofarlig tumör. Hörselnedsättning och en illaluktande flytning från örat är vanliga symtom och allvarigare komplikationer kan uppstå till följd av bendedstruktion och infektion i örat och skallbenet. Behandlingen är kirurgi. Få sjukdomar som kolesteatom är så omgärdade av oenigheter. Bland annat diskuteras vilken kirurgisk metod som är bäst lämpad, och vad som orsakar uppkomsten. Ett undertryck i mellanörat och en dåligt fungerande örontrumpet är en del av, men inte hela förklaringen. Återkommande infektioner och inflammation i örat bidrar till kolesteatomets utbredning. Huruvida det medfödda immunförsvaret har en roll vid kolesteatom är ännu okänt.

I denna avhandling redovisas läknings och hörselresultat vid kolesteatomkirurgi från patienter som opererats i Östergötland under en 16-års period. En grupp patienter har fått skatta sin förändrade hälsorelaterade livskvalité efter kirurgin. Vi har också undersökt förekomst av det medfödda immunförsvaret i öron med kolesteatom jämfört med friska. Slemhinneprover har tagits från öron med kolesteatom, och från friska kontroller. Man har sedan jämfört uttryck av gener som deltar i det medfödda immunförsvaret. Bl a har vi undersökt förekomst av sk toll-like receptorer (TLR), thymic stromal lymphopoietin (TSLP) och dess signalväg i cellen via JAK/STAT signaltransduktionsvägen. Vi har också undersökt förekomst av kväveoxid (NO) i mellanörat.

Sammanfattningsvis ses goda läknings och hörselresultat hos en majoritet. Våra resultat står sig bra i jämförelse med andra internationella studier. En majoritet av de tillfrågade patienterna upplevde en förbättrad hälsorelaterad livskvalité efter kirurgin. I "kolesteatomöron" sågs ett lägre uttryck av TLR4, delar av JAK/STAT signaltransduktionsvägen var påverkad, och enzymer viktiga för produktion av NO var nedreglerade. Det medfödda immunförsvaret verkar således vara påverkat i öron med kolesteatom, men de bakomliggande mekanismerna behöver undersökas ytterligare. Vidare verkar NO finnas i mellanörat och teoretiskt sett kan NO vara delaktig i att hålla örat friskt. Detta är oss veterligen ej påvisat tidigare, och öppnar för nya insikter om mellanörat.

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Department of Biomedical and Clinical Sciences, Division of Sensory Organs and  
Communication

Linköping University  
SE-581 83 Linköping, Sweden

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