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Characterisation of Acquired Human Cholesteatoma in Ontogenetic Aspect

Summary of the Doctoral Thesis for obtaining the scientific degree "Doctor of Science (*PhD*)"

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Abbreviations used in the Thesis

AAI	Institute of Anatomy and Anthropology
BKUS	Children's Clinical University Hospital
ECM	extracellular matrix
HβD-2	human beta defensin-2
HβD-4	human beta defensin-4
IL-1α	interleukin-1 alfa
IL-10	interleukin-10
Ki-67	proliferation marker Kiel 67
MMPs	matrix metalloproteinases
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
NF-κβ	nuclear factor kappa beta
PCR	polymerase chain reaction
PSKUS	Pauls Stradiņš Clinical University Hospital
RSU	Rīga Stradiņš University
SHH	*Sonic hedgehog*
TIMPs	tissue inhibitors of metalloproteinases
TIMP-2	tissue inhibitor of metalloproteinases-2
TIMP-4	tissue inhibitor of metalloproteinases-4
VEGF	vascular endothelial growth factor

Introduction

An acquired cholesteatoma is a benign but locally aggressive formation in the middle ear. It consists of hyperproliferative keratinised epithelium (Bhutta et al., 2011). Although the formation is rare, with an average incidence in Europe of 7 per 100000 people a year, its complete surgical treatment is difficult and often a recurrence of cholesteatoma is expected (Britze et al., 2017). The most common complaints are conducive or mixed (conducive and sensorineural) hearing loss, otorrhea and, rarely, ear pain. Cholesteatoma can cause potentially life-threatening and quality-of-life decreasing complications such as meningitis, brain abscess, facial nerve paralysis and sigmoid sinus thrombosis (Kuo et al., 2015). An acquired cholesteatoma is characterised by chronic inflammation, rapid growth and destruction of surrounding tissue; therefore, the tissue factors that describe these processes were selected in our work: matrix metalloproteinases-2 and -9, tissue inhibitors of metalloproteinases-2 and -4, proliferation marker Ki-67, nuclear factor kappa beta, interleukin-1α and -10, human beta defensins-2 and-4, vascular endothelial growth factor and *Sonic hedgehog* gene protein.

The most studied tissue factors in cholesteatoma are matrix metalloproteinases (MMPs), because an association was found between MMP-2 and MMP-9 and bone destruction in the middle ear (Morales et al., 2007; Juhász et al., 2009).

The tissue inhibitor of metalloproteinase-4 (TIMP-4) restricts MMP-2 and MMP-9 (Givvimani et al., 2010). It has been investigated that an imbalance between MMPs and TIMPs can result in tissue degradation in cholesteatoma (Schönermark et al., 1996; Suchozebrska-Jesionek et al., 2008). However, the effects of TIMP-4 on cholesteatoma tissue have not been analysed so far.

One of the most characteristic features of cholesteatoma tissue is its rapid growth and continuous cell proliferation (Yeşilova et al., 2017). Ki-67 is the most widely used proliferation marker in cholesteatoma studies.

The nuclear factor kappa beta (NF-κβ) plays a role in many processes in the human body, mainly by regulating genes associated with inflammation, cell proliferation and apoptosis (Giuliani et al., 2018). In cholesteatoma pathogenesis, NF-κβ regulates cell proliferation and inflammatory processes (Schürmann et al., 2022; Byun et al., 2010). However, studies lack a complex view of the effects of NF-κβ on the various processes of this pathology.

Cholesteatoma maintain chronic inflammation, which manifests as recurrent purulent inflammation in the middle ear (Schürmann et al., 2022). Interleukin-1 alpha (IL-1 α) is a potent pro-inflammatory cytokine associated with aggressiveness of cholesteatoma (Bujía et al., 1996). Interleukin-10 (IL-10) is one of the most active inflammatory suppressant cytokines (Mosser and Zhang, 2008). It is believed that regulatory disturbances between pro- and anti-inflammatory cytokines could explain the local aggressiveness of cholesteatoma (Kuczkowski et al., 2011). Human beta-defensin-2 and -4 (HβD-2; HβD-4) are local antimicrobial peptides that are effective in combating bacteria that cause inflammation in cholesteatoma (Harder et al., 1997; García et al., 2001). However, there is little information on the activity of defensins in cholesteatoma tissue, as well as their interaction with cytokines. This is the first study to investigate HβD-4 in cholesteatoma tissue.

The growth of cholesteatoma is associated with an increased angiogenesis process and as one of the most active factors contributing to angiogenesis is vascular endothelial growth factor (VEGF) (Fukudome et al., 2013). However, the effects of VEGF on cholesteatoma tissue are still little studied and uncertain.

Gene studies are very relevant, therefore, this study looked at the relationship of the *Sonic hedgehog* (SHH) gene protein to cholesteatoma

tissue in an ontogenetic aspect. Although this is one of the first studies on SHH in cholesteatoma tissue and there is limited information about its significance, it is known that disorders of the SHH gene can cause external, internal and middle ear abnormalities (Chiang et al., 1996).

It should be noted that until now studies have mainly been conducted in the same age group compared to the tissues of the control group, as well as studies have affected a particular process. This is one of a small number of studies that compare the population of children and adults, as well as the control group, and evaluate several tissue factors in the same study that target different pathophysiological processes in cholesteatoma tissue, an approach that gives a greater understanding of disease development.

Aim of the Thesis

Complex characterisation and determination of relative amount and distribution of remodelling, proliferation, inflammation, local defence, angiogenetic factors and SHH gene protein of the cholesteatoma tissue in an ontogenetic aspect.

Tasks of the Thesis

1 Detect the expression of tissue remodelling factors (MMP-2; MMP-9, TIMP-2, TIMP-4) in cholesteatoma tissue from patients of different ages.
2 Detect the expression of tissue proliferation marker (Ki-67) in cholesteatoma tissue from patients of different ages.
3 Detect the expression of transcription factor NF-κβ in cholesteatoma tissue from patients of different ages.
4 Detect the expression of pro- and anti-inflammatory cytokines (IL-1α, IL-10) in cholesteatoma tissue from patients of different ages.
5 Detect the expression of human defensins (HβD-2 un HβD-4) in cholesteatoma tissue from patients of different ages.

6 Detect the expression of angiogenic factor (VEGF) in cholesteatoma tissue from patients of different ages.
7 Detect the expression of *Sonic hedgehog* gene protein (SHH) in cholesteatoma tissue from patients of different ages.
8 Detect the markers mentioned above in the patient material of the control group.
9 Perform statistical data analysis, compare study groups, and determine possible correlation of tissue factors in cholesteatoma tissue from patients of different ages and control group patient material.

Hypotheses of the Thesis

There are differences in the expression of remodelling, proliferation, inflammation, local defence, angiogenic factors and the *Sonic hedgehog* gene protein of children and adult cholesteatoma.

Novelty of the Thesis

Until now, selective tissue changes such as remodelling, proliferation, inflammation and local tissue defence have been studied sporadically and unrelated in patients with cholesteatoma. This study examines these tissue changes **together**. Twelve different tissue factors are used in the study which, based on the information available to us, is **the largest number of tissue factors** used as part of a **single study**. Additionally, we examined factors previously unexplored in the case of cholesteatoma, such as TIMP-4, HβD-4 and SHH. Combining these different tissue factors by analysing the correlations between them gives a much broader insight into the common and different morphological developments of cholesteatoma tissue in children and adults that are missing from studies that studied these tissue changes separately and in the past.

1 Materials and Methods

1.1 Subject material and patient grouping

Three groups were created to implement the study: control group, children and adult patient group.

1.1.1 Control group

The tissue of the control group was obtained from the historical collection of the Riga Stradiņš University (RSU) Institute of Anatomy and Anthropology (AAI). The control group consisted of the skin of the external ear meatus, which was obtained from the bodies of 7 deceased people.

The selection of control tissues was influenced by ethical considerations that prevented biopsies from the outer ear canal skin of healthy patients as this could pose a risk of complications in patients during the healing period.

The tissue from the outer ear passages was taken to the RSU AAI Morphology laboratory, where a routine histological examination was performed. After a primary examination of the tissue, tissue samples from 7 patients were included in the study and 3 patients were excluded from the study. Criteria for inclusion: patients without known skin disease; patients without known chronic inflammation of the middle ear. Exclusion criteria: insufficient external ear canal tissue material (missing epidermis or dermis layer after routine histological examination), signs of inflammation.

The use of control tissue in the study has been authorised by RSU Ethics Committee (No 2-PEK-4/475/2022; 29.10.2022.).

Characteristics of the patients of the control group

Material from the bodies of 7 deceased people, the skin of the outer ear canal, was used to form the control group. The group included 3 female, and 4 male aged 35 to 50, non-sensory and non-skin abnormalities.

The control group for comparison is valid for both the child and adult groups, since the child's skin resembles that of adults after the age of six (Stamatas et al., 2023). Therefore, skin derived from the bodies of deceased people is appropriate for use as a control group.

1.1.2 Paediatric patient group

The material was obtained for a group of paediatric patients at Children's Clinical University Hospital (BKUS) between November 2019 and June 2023. The tissue were obtained from patients during cholesteatoma surgeries. Samples were collected from 37 unique patients aged 5 to 17 years. A cholesteatoma was morphologically confirmed.

The tissue was further delivered to RSU AAI Morphology laboratory where routine histological evaluation was performed. After primary tissue evaluation (routine histological evaluation), 25 (15 male; 10 female) patients had their tissue evaluated as consistent with the study.

Criteria for inclusion: child's age (0–18 years); an acquired human cholesteatoma. Exclusion criteria: insufficient cholesteatoma tissue material not suitable for immunohistochemical treatment (a cholesteatoma matrix or perimatrix is missing when performing routine histological evaluation).

No harm was caused to patients' health during the collection of cholesteatoma tissue material due to surgery for cholesteatoma excision. The study was conducted in accordance with the 2013 Helsinki Declaration. The study was approved by RSU Ethics Committee (No 6-2/7/4; 5.09.2019.). Permission was obtained from the BKUS Education and Science department. The nature of the study was told to patients and the patient's parents, written consent was obtained from the patient's parents for participating in the study.

The paediatric patient group was defined according to Section 3, Paragraph 1 of the Law on "Protection of Children's Rights of Latvia": "*A child*

shall mean a person under the age of 18, with the exception of those declared by law to be of legal age or married before the age of 18".

The characteristics of paediatric patients can be found in Table 1.1.

Table 1.1

Characteristics of paediatric patients

No	Code	Gender	Age (Years)	Diagnosis; Treatment
1	BH 24	Female	5	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
2	BH 25	Male	5	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
3	BH 23	Male	6	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
4	BH 19	Male	6	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
5	BH 21	Female	8	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
6	BH 3	Female	9	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
7	BH 6	Male	10	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
8	BH 1	Male	11	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
9	BH 11	Female	12	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
10	BH 10	Male	13	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
11	BH 32	Female	13	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty

Table 1.1 continued

No	Code	Gender	Age (Years)	Diagnosis; Treatment
12	BH 30	Male	14	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
13	BH 34	Male	14	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
14	BH 12	Female	15	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
15	BH 14	Female	15	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
16	BH 18	Male	15	Otitis media chronica bilateralis cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty – left side
17	BH 27	Male	15	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
18	BH 2	Male	16	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
19	BH 7	Female	16	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
20	BH 8	Female	16	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
21	BH 20	Female	16	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
22	BH 5	Male	17	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
23	BH 29	Male	17	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
24	BH 35	Male	17	Otitis media chronica dextra cum cholesteatoma; *Canal wall-up* surgery, tympanoplasty
25	BH 37	Male	17	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty

1.1.3 Adult patient group

The material was obtained for a group of adult patients at P. Stradiņš Clinical University Hospital (PSKUS) between November 2019 and June 2023. The studied tissue was obtained from patients during cholesteatoma surgeries. Samples were collected from 35 unique patients between the ages of 19 and 75. A cholesteatoma was morphologically confirmed.

The tissue was further delivered to RSU AAI Morphology laboratory where routine histological evaluation was performed. After primary tissue evaluation (routine histological evaluation), 25 (11 male; 14 female) patients had their tissue evaluated as consistent with the study.

Inclusion criteria: adult age (> 18 years); an acquired cholesteatoma. Exclusion criteria: insufficient cholesteatoma tissue material not suitable for immunohistochemical treatment (a cholesteatoma matrix or perimatrix is missing when performing routine histological evaluation).

No harm was caused to patients' health during the collection of cholesteatoma tissue material. The study was conducted in accordance with the 2013 Helsinki Declaration. The study was approved by RSU Ethics Committee (No 6-2/7/4; 5.09.2019.). Permission was obtained from the PSKUS Education and Science department. The nature of the study was told to patients, written consent was obtained from the patient for participating in the study. Patients were encrypted to ensure patient data was protected.

The characteristics of adult patients can be found in Table 1.2.

Table 1.2

Characteristics of adult patients

No	Code	Gender	Age (Years)	Diagnosis; Treatment
1	PH 16	Male	19	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
2	PH 15	Female	22	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
3	PH 3	Male	23	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
4	PH 18	Female	24	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
5	PH 12	Male	26	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
6	PH 30	Male	26	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
7	PH 23	Female	27	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
8	PH 6	Female	28	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
9	PH 7	Female	31	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
10	PH 25	Female	32	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
11	PH 35	Female	34	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
12	PH 4	Male	38	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
13	PH 8	Male	38	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty

Table 1.2 continued

No	Code	Gender	Age (Years)	Diagnosis; Treatment
14	PH 13	Male	39	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
15	PH 20	Male	39	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
16	PH 22	Female	40	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
17	PH 24	Female	41	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
18	PH 28	Male	41	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
19	PH 17	Female	45	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
20	PH 2	Male	46	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
21	PH 31	Male	50	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
22	PH 1	Female	58	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
23	PH 33	Female	70	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
24	PH 29	Female	74	Otitis media chronica sinistra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty
25	PH 5	Female	75	Otitis media chronica dextra cum cholesteatoma; *Canal wall-down* surgery, tympanoplasty

1.2 Morphological methods

1.2.1 Acquisition and preparation of tissue for staining

Acquisition of control tissue

The control tissue, the skin of the outer ear passage, was derived from the tissue of deceased people. Tissue necropsy was performed from a deeper area of the outer ear passage using small forceps. Tissue samples of approximately 3x3 mm were obtained and placed in *Stefanini* solution and delivered to the RSU AAI Morphology laboratory.

Acquisition of cholesteatoma tissue from children and adults

Adult and children cholesteatoma tissue were obtained during cholesteatoma surgeries at BKUS and PSKUS. During surgery, the evacuated cholesteatoma tissues (as large a tissue material as possible, or at least 5 mm in diameter) were immediately placed in *Stefanini* solution. The patients were not subjected to additional manipulation during the operations. There was also no additional harm to the patients who participated in the study – all patients underwent surgery of the same magnitude as any cholesteatoma surgery.

Tissue fixation and preparation for staining

The tissues obtained by biopsy were immediately fixed in *Stefanini's* solution (Stefanini et al., 1967). The fixing solution is made from 20 g of paraformaldehyde, 150 ml of picric acid, 425 ml of *Sorensen* phosphate buffer (pH 7.2) and 425 ml of distilled water. Paraformaldehyde was dissolved in distilled water and buffer and picric acid was added. This solution was loaded into Ependorf-type tubes containing biopsy tissue material. The biopsy material was stored in a refrigerator (+4° C) until it was delivered to the RSU AAI Morphology laboratory where further tissue processing was performed.

The tissue material was rinsed with *Tyrode's* solution for 24 h after being taken to the RSU morphology laboratory (136.9 mM NaCl, 2.68 nM KCl, 1.8 mM $CaCl_2$ $2H_2O$, 1.05 mmol/l $MgCl_2$ $6H_2O$, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4 H_2O, 5.5 mM glucose in distilled water). The fixed material was dehydrated using alcoholic solutions according to the following scheme: 70° alcoholic solution for 30 minutes, 80° alcoholic solution for 1–2 hours, 96° alcoholic solution for 3–4 hours, 96° alcoholic solution for 24 hours. The tissue samples were degreased for 30 minutes in xylene I and 30 minutes in xylene II and then kept for one hour in paraffin I and 2 hours in paraffin II. Then they were invested in molten paraffin, creating blocks of paraffin. From paraffin blocks with semi-automatic rotation microtome (*Leica* RM2245, *Leica biosystems Richmond Inc*., United States), 3–4 μm thin tissue cuts were made and placed on slides (*HistoBond ® +, Paul Marienfeld GmbH & Co. KG*, Germany). The slides were then placed for drying at the thermostat at 56º C for 20–60 minutes. Further processing was performed according to routine histological staining or immunohistochemistry.

1.2.2 Routine histology staining method

Routine histological staining underwent with haematoxylin and eosin. Tissue cuts dried in the thermostat were deparaffinised in xylene I (5 minutes) and xylene II (5 minutes). Further the tissue was rehydrated with different concentrations of alcohols following the following regimen: 96º alcohol solution for 3 minutes; 96º alcohol solution for 3 minutes; 70º alcohol solution for 3 minutes. Furthermore, tissue sections were stained with haematoxylin (code 05-M06002, *Mayer's haematoxylin, bio Optica Milano S.p.A*., Italy) and eosin (code 05-B10003, *alcoholic solution of Eosin Y, bio Optica Milano S.p.A*., Italy). Subsequent dyeing was followed by rinsing with running water and dehydration with different concentrations of alcohol: 70º alcohol solution for 3 minutes; 96º alcohol solution for 3 minutes; 96º alcohol solution for 3 minutes.

And clarification with xylene and carboxylene. The preparation was completed by filling with histological glue (code 6900002, *Paul Marienfeld GmbH & Co. KG*, Germany) and coating the samples with cover glasses (*Carl Roth GmbH + Co*, Germany). In micropreparations, acidophilic structures coloured pink, and basophils coloured blue or purple (Lillie et al., 1976; Fischer et al., 2008).

Micropreparations were analysed with a light microscope (*Leica* DM500RB, *Leica Biosystems Richmond Inc*., United States) and microphotography was captured with a digital camera (*Leica* DC 300F, *Leica microsystem AG*, Germany).

1.2.3 Immunohistochemical (biotin-streptavidin) assay method and reagents

The tissue sections were stained using the biotin-streptavidin method (Ozola and Pilmane, 2023). Fixation of tissue samples, preparation of material for placement in paraffin blocks and preparation of cuts on slides were performed according to the schema described in the previous chapter (see Chapter 1.2.2). The tissue cuts, dried at the thermostat, were deparaffinised in xylene I (5 minutes) and xylene II (5 minutes). The tissue dehydration was then performed with different concentrations of alcohols following the following regimen: 96º alcohol solution for 3 minutes; 96º alcohol solution for 3 minutes; 70º alcohol solution for 3 minutes. The deparaffinised tissue was placed in a holder and rinsed twice for 5 minutes with the *TRIS* buffer solution (code 2022X02692, *Diapath S.p.A.,* Italy), then boiled in the *EDTA* buffer solution (code 2020X19334, *Diapath S.p.A*., Italy) in the microwave for 20 minutes. They were cooled to 65º C and washed twice for 5 minutes In *TRIS* buffer solution and blocked endogenous peroxidase activity for 10 minutes with 3 % peroxide solution (code 925B-02, *cell MarqueTM*, United States). Further rinsing was performed with *TRIS* buffer solution twice for 5 minutes. A blocking serum was used for 20 minutes to reduce background colouring. All tissue

samples to be analysed were treated with the primary antibody for one hour (the antibody information is shown in Table 1.3). All antibodies used in the study were diluted with antibody diluent (code 938B-05, *cell MarqueTM*, United States).

HiDef DetectionTM HRP Polymer (code 954D-30, *cell MarqueTM*, United States) was used for antibodies (obtained from a mouse or rabbit). After incubating the primary antibody and rinsing tissue samples in the *TRIS* buffer (three times for 5 minutes), a *HiDef DetectionTM* reaction amplifier (code 954D-31, *cell MarqueTM*, United States) was used at room temperature for 10 minutes. The preparations were then rinsed with *TRIS* buffer solution (three times for 5 minutes), followed by the addition of the *HiDef DetectionTM HRP polymer marker* (code 954D-32, *cell MarqueTM*, United States) and incubation at room temperature for 10 minutes. After incubation, the samples were re-rinsed with *TRIS* buffer solution (three times for 5 minutes). The tissue was then treated with the *DAB* substrate chromogenic system (code 957D-30, *cell MarqueTM*, United States) and incubated at room temperature for up to 10 minutes to achieve brown colouration of the positive structures. The samples were subsequently rinsed with running water and dyed with haematoxylin (code 05-M06002, *Mayer's Hematoxylin, bio Optica Milano S.p.A*., Italy) for 2 minutes. In the end, the preparations were dehydrated with an increased concentration (70º to 96º) of alcohol and clarified with carboxylene and xylene. Histological glue *Pertex* ® (code 00801-EX, *HistoLab*, Sweden) was then applied and the samples were coated with cover glasses.

Micropreparations were analysed with a light microscope (*Leica* DM500RB, *Leica Biosystems Richmond Inc*., United States) and microphotography was captured with a digital camera (*Leica* DC 300F, *Leica microsystem AG,* Germany). The positive control was performed on the tissue according to the antibody companies, which always show a positive

reaction. Negative control was achieved by excluding the primary antibody and replacing it with antibody diluent.

Table 1.3.

Antibodies used in the study

No	Antibody	Code	Origin	Working dilution	Manufacturer
1	MMP-2	sc-53630	Mouse Monoclonal	1:100	*Santa Cruz Biotechnology, Inc., Santa Cruz, CA*, USA
2	MMP-9	sc-10737	Rabbit Polyclonal	1:100	*Santa Cruz Biotechnology, Inc., Santa Cruz, CA*, USA
3	TIMP-2	sc-21735	Mouse Monoclonal	1:100	*Santa Cruz Biotechnology, Inc., Santa Cruz, CA*, USA
4	TIMP-4	sc-30076	Rabbit Polyclonal	1:100	*Santa Cruz Biotechnology, Inc., Santa Cruz, CA*, USA
5	Ki-67	1325506A	Rabbit Polyclonal	1:100	*Cell Marque, Rocklin, CA*, USA
6	NF-κβ	Sc-109	Rabbit Polyclonal	1:200	*Santa Cruz Biotechnology, Inc., Santa Cruz, CA*, USA
7	IL-1α	sc-9983	Mouse Monoclonal	1:50	*Santa Cruz Biotechnology, Inc., Santa Cruz, CA*, USA
8	IL-10	ab34843	Rabbit Polyclonal	1:400	*Abcam, Cambridge*, UK
9	HβD-2	sc-20798	Rabbit Polyclonal	1:200	*Santa Cruz Biotechnology, Inc., Santa Cruz, CA*, USA
10	HβD-4	sc-59496	Mouse Monoclonal	1:50	*Santa Cruz Biotechnology, Inc., Santa Cruz, CA*, USA
11	VEGF	orb191500	Rabbit Polyclonal	1:100	*Biorbyt Ltd.*, UK

Table 1.3 continued

No	Antibody	Code	Origin	Working dilution	Manufacturer
12	SHH	LS-C49806	Rabbit Polyclonal	1:100	*LifeSpan BioSciences, Inc., Seattle, WA,* USA

Abbreviations: MMP-2 – matrix metalloproteinase-2; MMP-9 – matrix metalloproteinase-9; TIMP-2 – tissue inhibitor of metalloproteinases-2; TIMP-4 – tissue inhibitor of metalloproteinases-4; Ki-67 – *Kiel*-67; NF-κβ – nuclear factor kappa beta; IL-1α – interleukin-1 alfa; IL-10 – Interleukin-10; HβD-2 – human beta defensin-2; HβD-4 – human beta defensin-4; VEGF – vascular endothelial growth factor; SHH – *Sonic hedgehog* gene protein.

1.3 Data processing methods

1.3.1 Visual evaluation of data

A semiquantitative counting method (Pilmane et al., 2021) was used in the immunohistochemical tissue analysis to assess the relative number of factor-positive cells. The relative number of positive structures was determined in five random visual fields, and the average number from these visual fields was used as the final result. The micropreparations were evaluated by two independent morphologists. The legend and explanation of the semi-quantitative counting method can be found in Table 1.4.

Table 1.4

Criteria for listing immunohistochemical factor-positive structures

Grading scale	Conversion into figures for statistical analysis	Explanation of Grading Scale
0	0	No positive structures in the visual field
0/+	0.5	Occasional positive structures in the visual field
+	1	Few positive structures in the visual field
+/++	1.5	Few-to-moderate number of positive structures in the visual field
++	2	Moderate number of positive structures in the visual field

Table 1.4 continued

Grading scale	Conversion into figures for statistical analysis	Explanation of Grading Scale
++/+++	2.5	Moderate-to-numerous positive structures in the visual field
+++	3	Numerous positive structures in the visual field
+++/++++	3.5	Numerous-to-abundant structures in the visual field
++++	4	An abundance of positive structures in the visual field

1.3.2 Statistical methods

The study data was analysed using descriptive statistical methods (Teibe, 2007). Because the factor assessment is an ordinal data and does not correspond to the normal distribution (following the Shapiro-Wilk test and the histogram visual evaluation), nonparametric tests were used in the calculations.

The statistical differences between the study groups (child, adult and control) were determined by the Kruskal-Wallis test (Barton and Peat, 2014), which was selected because it includes a Bonferroni correction that does not exceed the alpha error 5 % if three and more comparator groups are used in the calculations.

A Spearman rank correlation coefficient (Forthofer et al., 2007) was used to assess the correlation between two different factors within the same group. The interpretation of the calculated correlations can be found in Table 1.5.

Results were considered statistically significant with a value of $p < 0.05$. Statistical analysis of the data was performed using the statistical software IBM SPSS (*Statistical Product and Service Solutions*) version 25.0 (*IBM company*, Chicago, Illinois, United States).

Table 1.5

***Spearman rank* correlation explanation**

r_s (*Spearman's rho*)	Explanation
0.0–0.2	a very weak correlation,
0.2–0.4	a weak correlation,
0.4–0.6	a moderately strong correlation
0.6–0.8	a strong correlation
0.8–1.0	a very strong correlation

2 Results

2.1 Routine histological finding

The tissue of the **control group** – the skin of the outer ear canal of 7 deceased people – showed an unaltered and non-inflammatory epidermis and subepithelial connective tissue.

It was a stratified squamous epithelium, distinguished by five layers: *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum*, and *stratum corneum* – all layers were in accordance with the generally accepted norm.

In the papillary layer of the dermis, loose unformed connective tissues and cells (fibroblasts, macrophages, extravascular leukocytes) were observed, but in the reticular layer of the dermis, blood vessels and cells were fewer and connective tissue was denser (see Figure 2.1).

No significant differences were observed between the tissue materials of **children and adult cholesteatoma** in routine histology examinations. For all the cholesteatoma tissue samples included in the study, all 3 layers were distinguishable: (1) The cystic layer was a large mass of anucleated keratinocytes that can normally be assimilated to the stratum corneum of unchanged skin. In the case of cholesteatoma, this layer is more pronounced. (2) Matrix – stratified squamous epithelium, capable of distinguishing the same layers as the unaltered skin epithelium in the control group – the difference is that, in the case of cholesteatoma, these layers of epithelium were hyperproliferated. (3) Perimatrix – subepithelial connective tissue, or granulation tissue, containing many inflammatory cells (white blood cells, lymphocytes, plasma cells, giant cells), collagen fibres and fibroblasts. The slides showed many small blood vessels. All tissue slides were characterised by severe infiltration of inflammatory cells. This layer can be compared to that of the unchanged skin dermis. In the case of cholesteatoma, unlike unaltered skin, the connective tissue here is looser, with severe infiltration of inflammatory cells and more vascularised (see Figure 2.2).

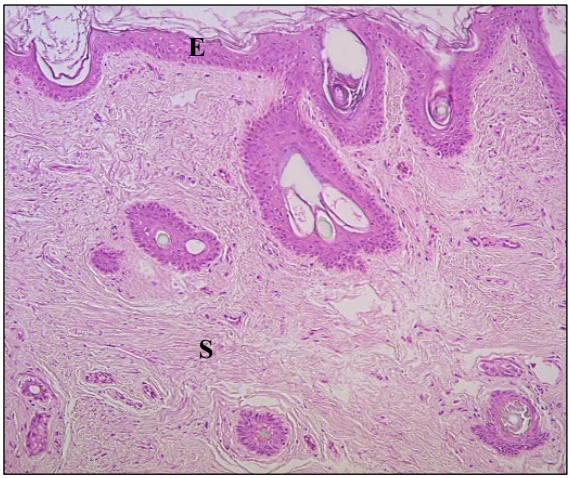

Figure 2.1 **Human unaltered outer ear canal skin, control tissue**

E – stratified squamous epithelium
above which is visible stratum corneum;
S – subepithelial connective tissues
without any signs of inflammation.
Hematoxylin and eosin × 200

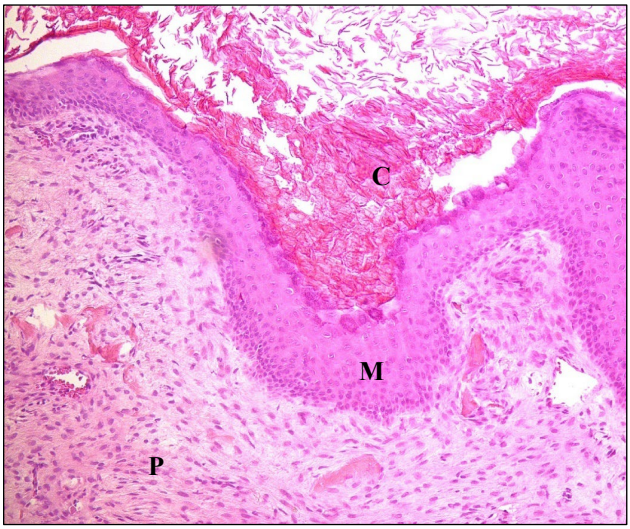

Figure 2.2 **Adult cholesteatoma**

C – a cystic layer consisting of anucleated epithelial cells;
M – matrix, which is a hyperproliferative
stratified squamous epithelium;
P – perimatrix, which is subepithelial connective tissue,
or granulating tissue, and consists of a variety of
inflammatory cells and loose unformed connective tissue.
The material was obtained from a 58-year-old woman.
Hematoxylin and eosin, × 200

2.2 Characterisation of immunohistochemical markers in control and patient groups

2.2.1 Matrix metalloproteinases and tissue inhibitors of metalloproteinases

Matrix metalloproteinase-2

In the skin epithelium of the **control group**, in general, an occasional (0/+) number of MMP-2 positive cells in the visual field were observed. A few (+) MMP-2 positive cells in the visual field was detected in connective tissue.

In **paediatric cholesteatoma**, generally, a few to moderate (+/++) number of MMP-2 positive cells were observed in the matrix. Whereas, in the perimatrix, the median number were a few (+) positive cells in the visual field.

In the matrix and perimatrix of **adult cholesteatoma**, generally, a few to moderate (+/++) MMP-2 positive cells were observed in the visual field (see Figure 2.3).

No statistically discernible differences were observed in the relative amount of MMP-2 positive cells between the children and adult cholesteatoma groups in both the matrix and the perimatrix. Although the relative number of MMP-2 positive cells in both groups was higher than in the control group, it did not achieve statistically significant differences (see Tables 2.1, 2.2, 2.3).

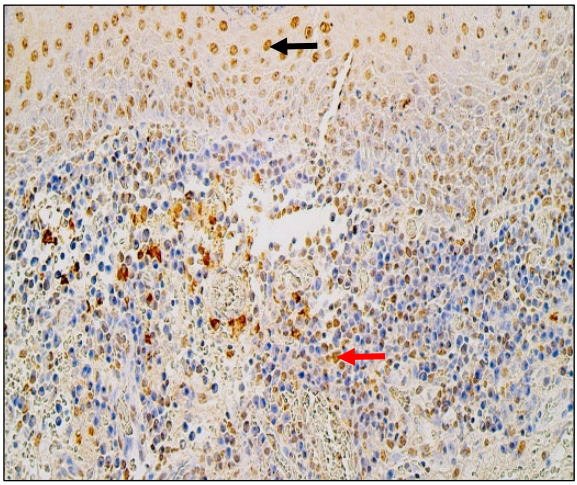

Figure 2.3 **Moderate (++) MMP-2 positive cells in the matrix** (black arrow) **and a few to moderate (+/++) factor positive cells in perimatrix** (red arrow)

Adult cholesteatoma The material obtained from the cholesteatoma of a 27-year-old woman. MMP-2 IHC × 250

Matrix metalloproteinase-9

In general, a few (+) MMP-9 positive cells were observed in the skin epithelium of the **control group**, while in connective tissue, the group median was occasional (0/+) MMP-9 positive cells in the visual field.

In the **paediatric cholesteatoma** matrix, the median number of MMP-9 positive cells were occasional (0/+) in the visual field, but the relative number of positive cells observed in the perimatrix were less than occasional (0–0/+) (see Figure 2.4).

In general, an occasional (0/+) relative number of MMP-9 positive cells were observed in the matrix and perimatrix of **adult cholesteatoma**.

No statistically discernible differences were observed between the relative amount of MMP-9 in children and adult cholesteatoma's matrix and perimatrix. Although the relative amount of MMP-9 in the epithelium and connective tissue of the control group was slightly higher than in both groups of patients, no statically significant differences were observed between the control group and the two patient groups (see Tables 2.1, 2.2, 2.3).

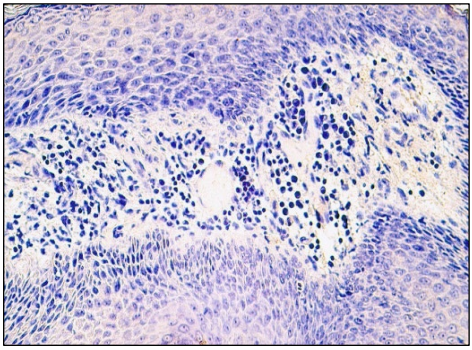

Figure 2.4 **Absence of MMP-9 positive cells (0) in the matrix and perimatrix**

Child cholesteatoma. The material obtained from the cholesteatoma of a 14-year-old boy. MMP-9 IHC × 250

Tissue inhibitor of metalloproteinases-2

In general, a few to moderate (+/++) TIMP-2 positive cells were observed in the skin epithelium of the **control group** and a few (+) factor-positive cells in the connective tissue.

In the matrix and perimatrix of the **paediatric cholesteatoma**, an occasional (0/+) relative number of TIMP-2 positive cells were observed within the group (see Figure 2.5).

The median number of TIMP-2 positive cells in the **adult cholesteatoma** group, both matrix and perimatrix, was an occasional (0/+).

No statistically significant differences were observed between the children and adult cholesteatoma in the relative numbers of TIMP-2 positive cells in the matrix or perimatrix. There was a **tendency for statistically significant increase** in the relative amount of TIMP-2 positive cells in the epithelium of the control group compared to the cholesteatoma matrix of children ($p = 0.082$). No statistically significant differences were observed when comparing the control group with the adult cholesteatoma group, but the relative number of TIMP-2 positive cells was higher in the control group (see Tables 2.1, 2.2, 2.3).

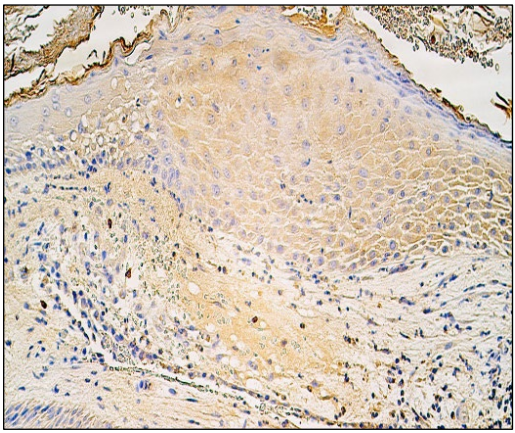

Figure 2.5 **Moderate (++) TIMP-2 positive cells in the matrix and a few (+) factor positive cells in the perimatrix** (arrow)

Child cholesteatoma. The material obtained from the cholesteatoma of a 14-year-old boy. TIMP-2 IHC × 250

Tissue inhibitor of metalloproteinases-4

In the epithelium of the **control group**, on average, moderate to numerous (++/+++) TIMP-4 positive cells were observed. In the connective tissue, the group median was moderate (++) TIMP-4 positive cells.

On average, in the matrix of **paediatric cholesteatoma** were observed a moderate to numerous (++/+++) TIMP-4 positive cells, while in the perimatrix – a moderate (++) TIMP-4 positive cells (see Figure 2.6).

On average, a moderate to numerous (++/+++) TIMP-4 positive cells were observed in the **adult cholesteatoma** group both in the matrix and in the perimatrix.

In all groups, the relative amount of TIMP-4 positive cells was similar and no statistically discernible differences were observed between the control, paediatric and adult cholesteatoma groups (see Tables 2.1, 2.2, 2.3).

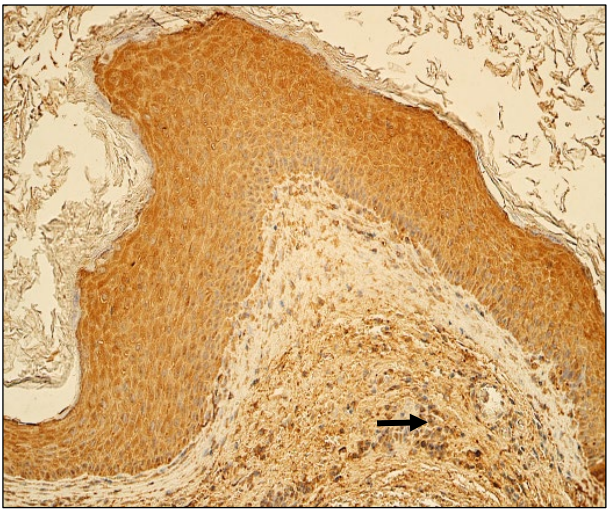

Figure 2.6 **Numerous to abundant (+++/++++) TIMP-4 positive cells in the matrix and moderate (++) factor positive cells** (arrow) **in the perimatrix**

Child cholesteatoma. The material obtained from the cholesteatoma of a 13-year-old boy. TIMP-4 IHC × 200

Table 2.1

Statistical differences in the numbers of remodelling factors between the cholesteatoma groups of children and adults

Tissue marker		Kruskal-Wallis	p value
Children group	Adult group		
MMP-2 matrix	MMP-2 matrix	0.415	> 0.999
MMP-2 perimatrix	MMP-2 perimatrix	0.986	0.972
MMP-9 matrix	MMP-9 matrix	−0.365	> 0.999
MMP-9 perimatrix	MMP-9 perimatrix	1.290	0.591
TIMP-2 matrix	TIMP-2 matrix	0.576	> 0.999
TIMP-2 perimatrix	TIMP-2 perimatrix	0.958	> 0.999
TIMP-4 matrix	TIMP-4 matrix	−0.205	> 0.999
TIMP-4 perimatrix	TIMP-4 perimatrix	0.159	> 0.999

Abbreviations: MMP-2 – matrix metalloproteinase-2; MMP-9 – matrix metalloproteinase-9; TIMP-2 – tissue inhibitor of metalloproteinases-2; TIMP-4 – tissue inhibitor of metalloproteinases-4

Table 2.2

Statistical differences in the numbers of remodelling factors between the control group and children group

Tissue marker		Kruskal-Wallis	p value
Control group	**Children group**		
MMP-2 epithelium	MMP-2 matrix	1.005	0.945
MMP-2 connective tissue	MMP-2 perimatrix	0.370	> 0.999
MMP-9 epithelium	MMP-9 matrix	−1.761	0.235
MMP-9 connective tissue	MMP-9 perimatrix	−1.713	0.260
TIMP-2 epithelium	TIMP-2 matrix	−2.207	**0.082ʿ**
TIMP-2 connective tissue	TIMP-2 perimatrix	−1.779	0.226
TIMP-4 epithelium	TIMP-4 matrix	1.018	0.926
TIMP-4 connective tissue	TIMP-4 perimatrix	1.354	0.527

Abbreviations: MMP-2 – matrix metalloproteinase-2; MMP-9 – matrix metalloproteinase-9; TIMP-2 – tissue inhibitor of metalloproteinases-2; TIMP-4 – tissue inhibitor of metalloproteinases-4
Designations: **ʿ** – tendency to have a statistically significant difference

Table 2.3

Statistical differences in the numbers of remodelling factors between the control group and adult group

Tissue marker		*Kruskal-Wallis*	p value
Control group	**Adult group**		
MMP-2 epithelium	MMP-2 matrix	1.280	0.602
MMP-2 connective tissue	MMP-2 perimatrix	1.023	0.919
MMP-9 epithelium	MMP-9 matrix	−2.002	0.136
MMP-9 connective tissue	MMP-9 perimatrix	−0.860	> 0.999
TIMP-2 epithelium	TIMP-2 matrix	−1.826	0.203
TIMP-2 connective tissue	TIMP-2 perimatrix	−1.145	0.756
TIMP-4 epithelium	TIMP-4 matrix	0.883	> 0.999
TIMP-4 connective tissue	TIMP-4 perimatrix	1.460	0.433

Abbreviations: MMP-2 – matrix metalloproteinase-2; MMP-9 – matrix metalloproteinase-9; TIMP-2 – tissue inhibitor of metalloproteinases-2; TIMP-4 – tissue inhibitor of metalloproteinases-4

2.2.2 Proliferation marker Ki-67

In the **control group**, less than an occasional (0–0/+) Ki-67 positive cells were observed in both the epithelium and the connective tissue in two cases, while in the rest of the cases no Ki-67 positive cells were detected (0).

In general, an occasional (0/+) number of Ki-67 positive cells were observed in the **paediatric cholesteatoma** matrix, but less than an occasional (0–0/+) number of Ki-67 positive cells was observed in the perimatrix.

In the **adult cholesteatoma** matrix, in overall, an occasional (0/+) amount of Ki-67 positive cells were observed in the matrix, while the perimatrix generally showed less than occasional (0–0/+) Ki-67 positive cells (see Figure 2.7).

There were no statistically significant differences in the relative number of Ki-67 positive cells between the paediatric and adult cholesteatoma groups. **A statistically discernible increase** in Ki-67 positive cells was observed in the paediatric cholesteatoma matrix compared to the epithelium of the control group ($p = 0.006$), but no statistically significant differences were observed in the perimatrix compared to connective tissue, although the number of positive cells were greater in the perimatrix. A **statistically significant increase** in Ki-67 positive cells was observed in adult cholesteatoma matrix and perimatrix compared to control group epithelium and connective tissues ($p = 0.001$; $p = 0.030$) (see Table 2.4).

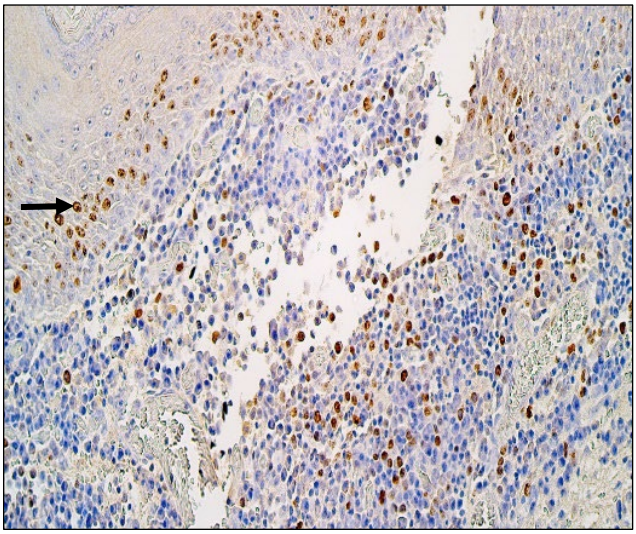

Figure 2.7 **A few to moderate (+/++) Ki-67 positive cells in the cholesteatoma matrix** (arrow**) and the perimatrix**

Adult cholesteatoma. Material obtained from the cholesteatoma of a 27-year-old woman. Ki-67 IHC × 200

Table 2.4

Statistical differences in the amount of proliferation marker Ki-67 between children, adult cholesteatomas, and control groups

Tissue marker		***Kruskal-Wallis***	**p value**
Children group	**Adult group**		
Ki-67 matrix	Ki-67 matrix	0.886	> 0.999
Ki-67 perimatrix	Ki-67 perimatrix	0.908	> 0.999
Control group	**Children group**	***Kruskal-Wallis***	**p value**
Ki-67 epithelium	Ki-67 matrix	3.110	**0.006***
Ki-67 connective tissue	Ki-67 perimatrix	1.977	0.144
Control group	**Adult group**	***Kruskal-Wallis***	**p value**
Ki-67 epithelium	Ki-67 matrix	3.697	**0.001***
Ki-67 connective tissue	Ki-67 perimatrix	2.577	**0.030***

Abbreviations: Ki-67 – *Kiel*-67
Designations: * – statistically significant difference

2.2.3 Nuclear factor kappa beta

In general, occasional (0/+) NF-κβ positive cells were observed in both the epithelial and the connective tissue of the **control group**.

In **paediatric cholesteatoma**, on average, a moderate (++) number of NF-κβ positive cells were observed in the matrix. And a few (+) NF-κβ positive cells were observed in the perimatrix (see figure 2.8).

In the **adult cholesteatoma** matrix a moderate (++) number of NF-κβ positive cells were observed in the visual field, while the median of the perimatrix was determined as an occasional (0/+) amount of NF-κβ positive cells.

When comparing the number of positive cells of NF-κβ in the children's cholesteatoma and adult cholesteatoma group, there were no statistically significant differences between the groups. A **statistically discernible increase** of NF-κβ positive cells was observed in the paediatric and adult cholesteatoma matrix compared to the epithelium of the control group epithelium ($p = 0.011$; $p = 0.013$). No statistically significant differences were observed when comparing the perimatrix of the cholesteatoma of children and adults with the connective tissue of the control group (see Table 2.5).

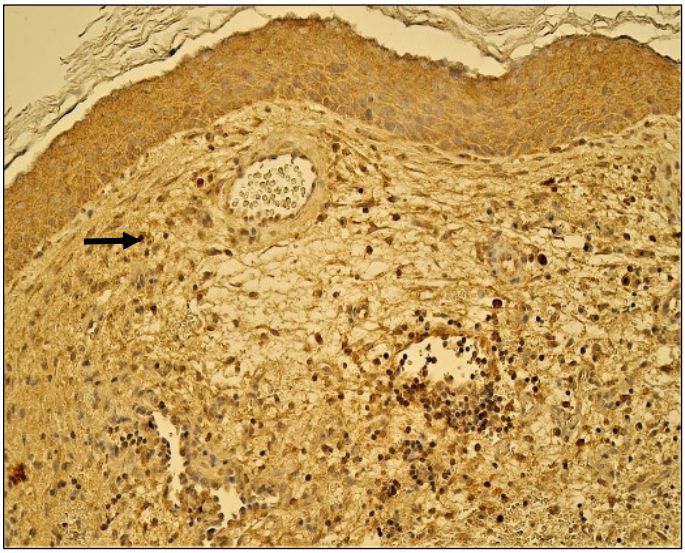

Figure 2.8 **Numerous to abundant (+++/++++) NF-κβ positive cells in the cholesteatoma matrix and moderate to numerous (++/+++) in the perimatrix** (arrow)

Child cholesteatoma. The material obtained from the cholesteatoma of a 14-year-old boy. NF-κβ IHC, × 250

Table 2.5

Statistical differences in the amount of transcription factor NF-κβ between children, adult cholesteatomas, and control groups

Tissue marker		***Kruskal-Wallis***	**p value**
Children group	**Adult group**		
NF-κβ matrix	NF-κβ matrix	−0.079	> 0.999
NF-κβ perimatrix	NF-κβ perimatrix	0.039	> 0.999
Control group	**Children group**	***Kruskal-Wallis***	**p value**
NF-κβ epithelium	NF-κβ matrix	2.915	**0.011***
NF-κβ connective tissue	NF-κβ perimatrix	1.779	0.226
Control group	**Adult group**	***Kruskal-Wallis***	**p value**
NF-κβ epithelium	NF-κβ matrix	2.864	**0.013***
NF-κβ connective tissue	NF-κβ perimatrix	1.805	0.213

Abbreviations: NF-κβ – nuclear factor kappa beta.
Designations: * – statistically significant difference.

2.2.4 Pro and anti-inflammatory cytokines

Interleukin-1 alfa

In the **control group**, an occasional (0/+) number of IL-1α positive cells were observed in both the epithelium and the connective tissue.

In the **paediatric group**, a few to moderate (+/++) IL-1α positive cells were observed in the cholesteatoma matrix, while an occasional (0/+) amount of IL-1α positive cells were observed in the perimatrix (see Figure 2.9).

A few to moderate (+/++) IL-1α positive cells, overall, in the matrix were observed in the **adult cholesteatoma** group. A few (+) IL-1α positive cells were observed in the perimatrix.

No statistically significant differences were observed between the relative amount of IL-1α in the cholesteatoma of children and adults. An increase in the number of IL-1α positive cells were observed in both cholesteatoma groups compared to the control group, but it did not reach statistical significance (see Table 2.6).

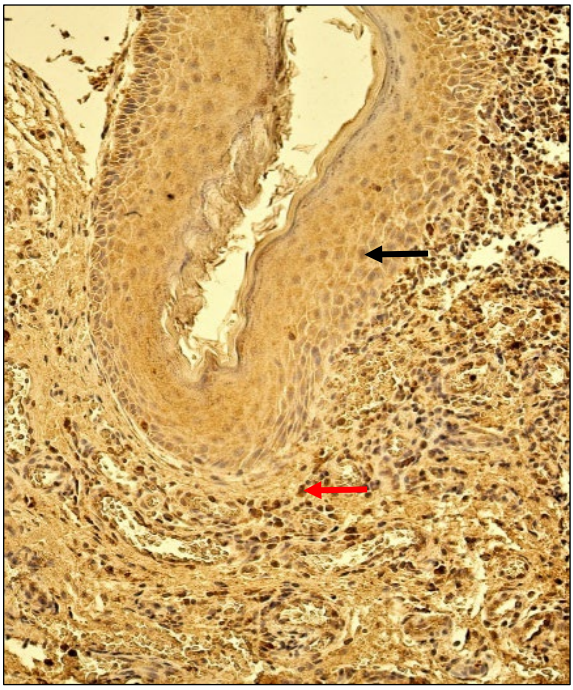

Figure 2.9 **Numerous (+++) IL-1α positive cells in the cholesteatoma matrix** (black arrow) **and in the perimatrix** (red arrow)

Child cholesteatoma. The material obtained from the cholesteatoma of a 13-year-old boy. IL-1α IHC, × 200.

Interleukin-10

In the **control group**, a moderate (++) number of IL-10 positive cells were observed in both epithelium and connective tissue.

A few to moderate (+/++) IL-10 positive cells were observed in **paediatric cholesteatoma** matrix, while a few (+) IL-10 positive cells were observed in the perimatrix (see Figure 2.10).

In the **adult cholesteatoma** group, a few to moderate (+/++) IL-10 positive cells were observed overall in the matrix and a few (+) IL-10 positive cells were observed in the perimatrix.

When comparing the children and adult cholesteatoma groups, it did not show any statistically significant differences in the relative amount of IL-10 positive cells between these two groups. Comparison of the control group with both groups of patients, showed that the number of IL-10 positive cells in the control group material were increased compared to both patient groups, but this did not reach a statistically significant difference (see Table 2.6).

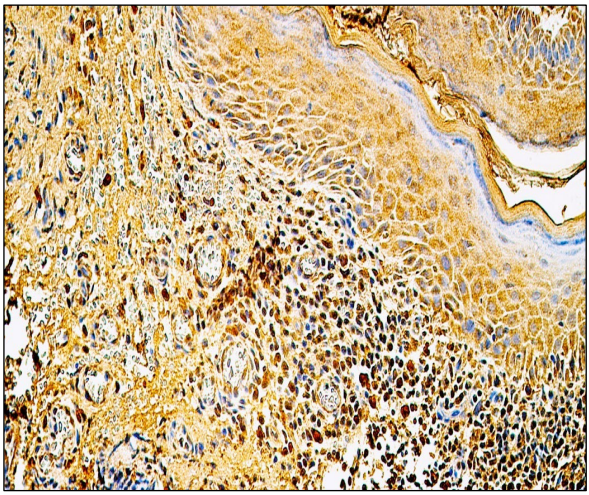

Figure 2.10 **Numerous (+++) IL-10 positive cells in the cholesteatoma matrix and moderate to numerous (++/+++) in the perimatrix**

Child cholesteatoma. The material obtained from the cholesteatoma of a 13-year-old boy.IL-10 IHC × 250

Table 2.6

Statistical differences in the amount of pro and anti-inflammatory cytokines between children, adult cholesteatomas, and control groups

Tissue marker		***Kruskal-Wallis***	**P – value**
Children group	**Adult group**		
IL-1α matrix	IL-1α matrix	−0.039	> 0.999
IL-1α perimatrix	IL-1α perimatrix	0.717	> 0.999
IL-10 matrix	IL-10 matrix	0.043	> 0.999
IL-10 perimatrix	IL-10 perimatrix	0.868	> 0.999
Control group	**Children group**	***Kruskal-Wallis***	**P – value**
IL-1α epithelium	IL-1α matrix	1.773	0.229
IL-1α connective tissue	IL-1α perimatrix	0.526	> 0.999
IL-10 epithelium	IL-10 matrix	−0.456	> 0.999
IL-10 connective tissue	IL-10 perimatrix	−1.780	0.225
Control group	**Adult group**	***Kruskal-Wallis***	**P – value**
IL-1α epithelium	IL-1α matrix	1.747	0.242
IL-1α connective tissue	IL-1α perimatrix	1.000	0.952
IL-10 epithelium	IL-10 matrix	−0.427	> 0.999
IL-10 connective tissue	IL-10 perimatrix	−1.205	0.684

Abbreviations: IL-1α – interleukin-1 alfa; IL-10 – interleukin-10

2.2.5 Human beta defensins

Human beta defensin-2

In general, a few (+) HβD-2 positive cells were observed in the skin epithelium of the **control group** and less than occasional (0–0/+) HβD-2 positive cells were observed in the connective tissues. A few to moderate (+/++) relative number of HβD-2 positive cells, in overall, were observed in the **paediatric cholesteatoma** matrix, while occasional (0/+) number of HβD-2 positive cells were observed in perimatrix (see Figure 2.11). In general, a few to moderate (+/++) HβD-2 positive cells were observed in **adult cholesteatoma** matrix. The perimatrix showed a few (+) HβD-2 positive cells. There were no statistically significant differences in the number of HβD-2 positive cells between the adult and child cholesteatoma groups. When comparing both groups

of patients with the control group, it showed a **statistically discernible increase** of HβD-2 positive cells in the perimatrix of the cholesteatoma (children and adults) compared to the connective tissue of the skin ($p = 0.038$ and $p = 0.015$). The amount of HβD-2 in the cholesteatoma matrix was also higher than in the skin epithelium but did not reach statistical significance (see Table 2.7).

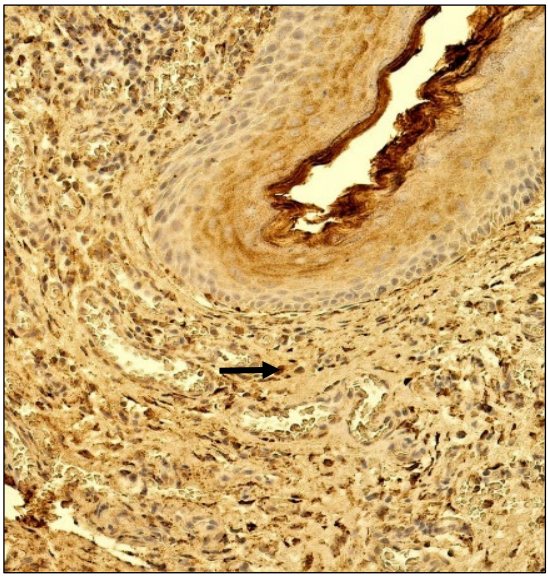

Figure 2.11 **Moderate (++) HβD-2 positive cells in the cholesteatoma matrix and moderate (++) in the perimatrix** (arrow)

Child cholesteatoma. The material obtained from the cholesteatoma of a 9-year-old girl. HβD-2 IHC × 200

Human beta defensin-4

Overall, a few (+) HβD-4 positive cells were observed in the **control group** epithelium and occasional (0/+) HβD-4 positive cells were observed in connective tissue.

In the **paediatric cholesteatoma** matrix less than occasional (0-0/+) HβD-4 positive cells were seen in the visual field. An occasional (0/+) HβD-4 positive cells were observed in perimatrix.

An occasional (0/+) relative number of HβD-4 positive cells were observed in **adult cholesteatoma** matrix and perimatrix (see Figure 2.12).

No statistically discernible differences in the amount of HβD-4 positive cells between children and adult cholesteatoma groups were found. Comparing the paediatric cholesteatoma group with the control group, it showed **a tendency** for a **statistically significant increase** in the number of HβD-4 positive cells in the epithelium of the control group compared to the cholesteatoma matrix ($p = 0.099$). Comparing the adult cholesteatoma group with the control group, it showed an increased amount of HβD-4 positive cells in the control group tissue, but it was not statistically significantly increased (see Table 2.7).

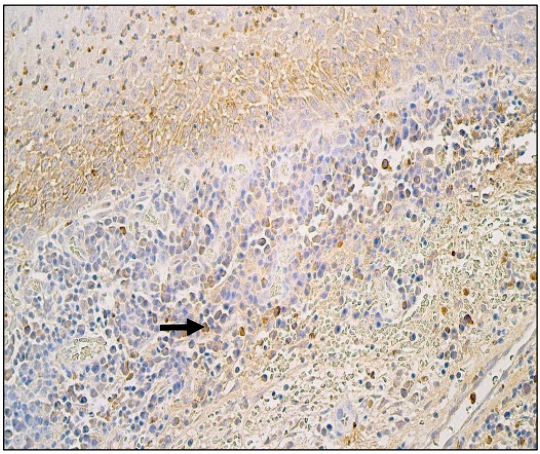

Figure 2.12 **Moderate to numerous (++/+++) HβD-4 positive cells in the cholesteatoma matrix and a few (+) in the perimatrix** (arrow)

Adult cholesteatoma. Material obtained from the cholesteatoma of a 27-year-old woman. HβD-4 IHC × 250

Table 2.7

Statistical differences in the amount of human beta defensins between children, adult cholesteatomas, and control groups

Tissue marker		***Kruskal-Wallis***	**P – value**
Children group	**Adult group**		
HβD-2 matrix	HβD-2 matrix	0.587	> 0.999
HβD-2 perimatrix	HβD-2 perimatrix	0.480	> 0.999
HβD-4 matrix	HβD-4 matrix	0.868	> 0.999
HβD-4 perimatrix	HβD-4 perimatrix	−0.719	> 0.999
Control group	**Children group**	***Kruskal-Wallis***	**P – value**
HβD-2 epithelium	HβD-2 matrix	1.339	0.542
HβD-2 connective tissue	HβD-2 perimatrix	2.498	**0.038***
HβD-4 epithelium	HβD-4 matrix	−2.132	**0.099 t**
HβD-4 connective tissue	HβD-4 perimatrix	−0.627	> 0.999
Control group	**Adult group**	***Kruskal-Wallis***	**P – value**
HβD-2 epithelium	HβD-2 matrix	1.727	0.253
HβD-2 connective tissue	HβD-2 perimatrix	2.815	**0.015***
HβD-4 epithelium	HβD-4 matrix	−1.558	0.358
HβD-4 connective tissue	HβD-4 perimatrix	−1.103	0.811

Abbreviations: HβD-2 – Human beta defensin-2; HβD-4 – Human beta defensin-4
Designations: * – statistically significant difference; t – tendency for statistically significant difference

2.2.6 Vascular endothelial growth factor

The relative number of VEGF positive cells in the **control group** epithelium was, on average, moderate to numerous (++/+++). A few (+) VEGF positive endotheliocytes were observed in the blood vessels of the connective tissue.

On average, a moderate (++) number of VEGF positive cells were observed in the **paediatric cholesteatoma** matrix, while an occasional (0/+) amount of VEGF positive endotheliocytes were observed in perimatrix (see Figure 2.13).

In the **adult cholesteatoma** group, a few to moderate (+/++) VEGF positive cells were observed in the matrix. An occasional (0/+) VEGF positive endotheliocytes were observed in perimatrix.

Comparing the children's cholesteatoma and adult cholesteatoma groups, it showed no statistically plausible differences between the two groups. There were also no statistically significant differences when comparing a group of paediatric patients with a control group. Comparing the adult cholesteatoma group with the control group, it showed **a tendency for statistically significant increase** in the number of VEGF positive cells in the control group epithelium compared to the cholesteatoma matrix ($p = 0.096$), but this tendency was not observed when comparing the connective tissue of the control group and the perimatrix of the cholesteatoma (see Table 2.8).

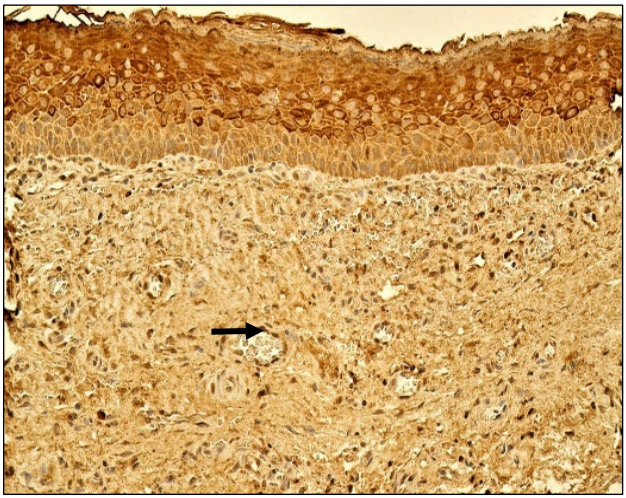

Figure 2.13 **Numerous to abundant (+++/++++) VEGF positive cells in the cholesteatoma matrix and, moderate to numerous (++/+++) in the perimatrix, both endoteliocytes** (arrow) **and inflammatory cells**

Child cholesteatoma. The material obtained from the cholesteatoma of a 16-year-old girl. VEGF IHC × 250

Table 2.8

Statistical differences in the amount of VEGF between children, adult cholesteatomas, and control groups

Tissue marker		***Kruskal-Wallis***	**p value**
Children group	**Adult group**		
VEGF matrix	VEGF matrix	−0.694	> 0.999
VEGF perimatrix	VEGF perimatrix	0.486	> 0.999
Control group	**Children group**	***Kruskal-Wallis***	**p value**
VEGF epitheliums	VEGF matrix	−1.687	0.275
VEGF connective tissue	VEGF perimatrix	−0.973	0.991
Control group	**Adult group**	***Kruskal-Wallis***	**p value**
VEGF epithelium	VEGF matrix	−2.146	**0.096** [t]
VEGF connective tissue	VEGF perimatrix	−0.652	> 0.999

Abbreviations: VEGF – vascular endothelial growth factor
Designations: [t] – tendency for statistically significant difference

2.2.7 *Sonic hedgehog* gene protein

In general, a few to moderate (+/++) SHH positive cells were observed in the epithelium of the **control group** tissue. And a few (+) SHH positive cells were observed in the connective tissue of the skin.

On average, moderate to numerous (++/+++) SHH positive cells were observed in **paediatric cholesteatoma** matrix, while the perimatrix showed a few to moderate (+/++) SHH positive cells.

In the **adult cholesteatoma** matrix, on average, moderate to numerous (++/+++) SHH positive cells were observed, and a moderate (++) number of SHH positive cells were observed in the perimatrix (see Figure 2.14).

Comparing the cholesteatoma groups of children and adults, we found no statistically significant differences between them. When comparing the paediatric and adult cholesteatoma groups with the control group, it showed **a statistically significant increase** in SHH positive cells in the cholesteatoma perimatrix compared to the control skin connective tissue ($p = 0.011$ and $p = 0.005$). When comparing the cholesteatoma matrix of the two groups with

the control skin epithelium, it did not show statistically significant differences between the groups, but SHH positive cells were increased in the cholesteatoma groups (see Table 2.9).

Figure 2.14 **Numerous (+++) SHH positive cells in the cholesteatoma matrix and a few to moderate (+/++) in the perimatrix** (arrow)

Adult cholesteatoma. Material obtained from the cholesteatoma of a 27-year-old woman. SHH IHC, × 250

Table 2.9

Statistical differences in the amount of SHH gene protein between children, adult cholesteatomas, and control groups

Tissue marker		***Kruskal-Wallis***	**p value**
Children group	**Adult group**		
SHH matrix	SHH matrix	0.482	> 0.999
SHH perimatrix	SHH perimatrix	0.363	> 0.999
Control group	**Children group**	***Kruskal-Wallis***	**p value**
SHH epithelium	SHH matrix	0.999	0.954
SHH connective tissue	SHH perimatrix	2.906	**0.011***

Table 2.9 continued

Tissue marker		*Kruskal-Wallis*	p value
Control group	**Adult group**		
SHH epithelium	SHH matrix	1.318	0.563
SHH connective tissue	SHH perimatrix	3.146	**0.005***

Abbreviations: SHH – *Sonic hedgehog* gene protein
Designations: * - Statistically significant difference

2.3 Statistical correlations of data

2.3.1 Statistical correlation of data in control group

29 statistically significant correlations were observed in the control group.

Of these, 20 statistically significant correlations were a very strong positive (rs = 0.8–1.0). Two correlations were very strong negative (rs = −1.0–−0.8) and seven statistically significant correlations were strong positive correlations (rs = 0.6–0.8).

The **strongest statistically significant correlations** in the control group are summarised in Table 2.10.

Table 2.10

Statistically significant correlations between different tissue factors in the control group

Correlation tightness	Tissue markers between which correlation has been established	*Spearmann* correlation coefficient	p value
A very strong positive correlation (0.8–1.0)	VEGF epithelium and HβD-2 epithelium	**0.971**	<0.001
	TIMP-2 epithelium and NF-κβ epithelium	**0.925**	0.003
	MMP-9 epithelium and SHH epithelium	**0.924**	0.003
	TIMP-2 connective tissue and HβD-2 epithelium	**0.921**	0.003
	VEGF epithelium and VEGF connective tissue	**0.911**	0.004
	MMP-9 epithelium and SHH connective tissue	**0.896**	0.006
	IL-10 epithelium and IL-10 connective tissue	**0.891**	0.007

Table 2.10 continued

Correlation tightness	Tissue markers between which correlation has been established	*Spearmann* correlation coefficient	p value
A very strong positive correlation (0.8–1.0)	TIMP-2 connective tissue and VEGF epithelium	**0.885**	0.008
	TIMP-4 connective tissue and SHH epithelium	**0.882**	0.009
	HβD-4 epithelium and HβD-4 connective tissue	**0.870**	0.011
	TIMP-4 epithelium and TIMP-4 connective tissue	**0.868**	0.011
	VEGF connective tissue and HβD-2 epithelium	**0.856**	0.014
	SHH epithelium and NF-κβ epithelium	**0.851**	0.015
	TIMP-4 connective tissue and NF-κβ connective tissue	**0.845**	0.017
	TIMP-2 connective tissue and VEGF connective tissue	**0.845**	0.017
	MMP-2 epithelium and TIMP-2 epithelium	**0.833**	0.020
	NF-κβ epithelium and NF-κβ connective tissue	**0.827**	0.022
	TIMP-2 epithelium and SHH epithelium	**0.824**	0.023
	SHH epithelium and NF-κβ connective tissue	**0.819**	0.024
	TIMP-4 epithelium and SHH epithelium	**0.811**	0.027
A very strong negative correlation (−1.0 to −0.8)	IL-1α epithelium and IL-10 connective tissue	**−0.829**	0.021
	MMP-2 epithelium and IL-1α connective tissue	**−0.808**	0.028

Abbreviations: MMP-2 – matrix metalloproteinase-2; MMP-9 – matrix metalloproteinase-9; TIMP-2 – tissue inhibitor of metalloproteinases-2; TIMP-4 – tissue inhibitor of metalloproteinases-4; NF-κβ – Nuclear factor kappa beta; IL-1α – Interleukin-1 alfa; IL-10 – Interleukin-10; HβD-2 – Human beta defensin-2; HβD-4 – Human beta defensin-4; VEGF – Vascular endothelial growth factor; SHH – Sonic hedgehog gene protein.

2.3.2 Statistical correlation of data in paediatric patient group

A total of 174 statistically significant very strong, strong, a moderately strong and weak positive correlations were observed in the children's cholesteatoma group.

Of all statistically significant correlations, six were very strong positive correlations (rs = 0.8 – 1.0), 62 were strong positive correlations (rs = 0.6 – 0.8). Moderately strong positive correlations (rs = 0.4 – 0.6) were 104 and statistically significant weak positive correlations (rs = 0.2 – 0.4) were two.

The **strongest statistically significant correlations** in the children's cholesteatoma group are summarised in Table 2.11.

Table 2.11

Statistically significant correlations between different tissue factors in the children cholesteatoma group

Correlation tightness	Tissue markers between which correlation has been established	*Spearmann* correlation coefficient	p value
A very strong positive correlation (0.8–1.0)	TIMP-4 matrix and TIMP-4 perimatrix	**0.841**	<0.001
	IL-10 matrix and HβD-2 matrix	**0.828**	<0.001
	IL-1α matrix and NF-κβ matrix	**0.827**	<0.001
	SHH matrix and SHH perimatrix	**0.813**	<0.001
	SHH matrix and Ki-67 matrix	**0.803**	<0.001
	MMP-2 matrix and MMP-2 perimatrix	**0.803**	<0.001
A strong positive correlation (0.6–0.8)	MMP-2 matrix and SHH matrix	0.786	<0.001
	MMP-2 perimatrix and SHH matrix	0.786	<0.001
	Ki-67 matrix and Ki-67 perimatrix	0.780	<0.001
	SHH matrix and NF-κβ matrix	0.753	<0.001
	NF-κβ matrix and HβD-2 matrix	0.750	<0.001
	HβD-2 matrix and HβD-2 perimatrix	0.748	<0.001
	SHH perimatrix and Ki-67 matrix	0.746	<0.001
	VEGF matrix and VEGF perimatrix	0.745	<0.001
	TIMP-4 matrix and NF-κβ matrix	0.738	<0.001
	NF-κβ matrix u and n Ki-67 matrix	0.726	<0.001
	TIMP-4 perimatrix and IL-10 perimatrix	0.721	<0.001
	IL-1α matrix and IL-10 perimatrix	0.720	<0.001
	IL-1α matrix and IL-1α perimatrix	0.716	<0.001
	MMP-9 perimatrix and IL-1α perimatrix	0.714	<0.001
A strong positive correlation (0.6–0.8)	IL-1α perimatrix and VEGF perimatrix	0.714	<0.001
	MMP-9 matrix and MMP-9 perimatrix	0.710	<0.001
	IL-1α matrix and IL-10 matrix	0.709	<0.001
	SHH matrix and NF-κβ perimatrix	0.702	<0.001
	IL-1α matrix and HβD-2 matrix	0.700	<0.001

Abbreviations: MMP-2 – matrix metalloproteinase-2; MMP-9 – matrix metalloproteinase-9; TIMP-4 – tissue inhibitor of metalloproteinases-4; NF-κβ – Nuclear factor kappa beta; IL-1α – Interleukin-1 alfa; IL-10 – Interleukin-10; HβD-2 – Human beta defensin-2; VEGF – Vascular endothelial growth factor; SHH – *Sonic hedgehog* gene protein.

2.3.3 Statistical correlation of data in adult patient group

A total of 76 statistically significant correlations were observed in the adult cholesteatoma group.

Of these, four were very strong positive correlations (rs = 0.8–1.0). In 24 cases, strong positive correlations were observed (rs = 0.6–0.8). Moderately strong positive correlations (rs = 0.4–0.6) were 46 and statistically significant weak positive correlations (rs = 0.2–0.4) were two.

The **strongest statistically significant correlations** in the adult cholesteatoma group are summarised in Table 2.12.

Table 2.12

Statistically significant correlations between different tissue factors in the adult cholesteatoma group

Correlation tightness	Tissue markers between which correlation has been established	*Spearmann* correlation coefficient	p value
A very strong positive correlation (0.8–1.0)	IL-10 matrix and HβD-2 matrix	**0.841**	<0.001
	IL-1α matrix and HβD-2 matrix	**0.827**	<0.001
	IL-1α matrix and IL-10 matrix	**0.813**	<0.001
	IL-10 matrix and IL-10 perimatrix	**0.801**	<0.001
A very strong positive correlation (0.6–0.8)	MMP-9 perimatrix and NF-κβ perimatrix	0.790	<0.001
	IL-1α matrix and IL-10 perimatrix	0.762	<0.001
	IL-10 perimatrix and NF-κβ perimatrix	0.751	<0.001
	TIMP-4 perimatrix and NF-κβ perimatrix	0.749	<0.001
	NF-κβ perimatrix and HβD-2 perimatrix	0.748	<0.001
A very strong positive correlation (0.6–0.8)	MMP-2 matrix and SHH matrix	0.719	<0.001

Abbreviations: MMP-2 – matrix metalloproteinase-2; MMP-9 – matrix metalloproteinase-9; TIMP-4 – tissue inhibitor of metalloproteinases-4; NF-κβ – Nuclear factor kappa beta; IL-1α – Interleukin-1 alfa; IL-10 – Interleukin-10; HβD-2 – Human beta defensin-2; SHH – *Sonic hedgehog* gene protein

3 Discussion

The scientific literature finds a relatively broad range of studies related to acquired human cholesteatoma. However, studies are predominantly based on clinical and/or radiological evaluation, while morphology is less studied. There are even fewer comparative studies that evaluate acquired adult and paediatric cholesteatoma. Of these comparative studies, few compare these two groups in morphological terms. Consequently, different opinions exist as to whether and how the children and adult cholesteatoma differs.

The pathogenesis of cholesteatoma has not been fully explained; however, it is known that cholesteatoma is related to bone tissue remodulation, inflammation, and distinct cell proliferation and growth. Our study evaluated MMP-2, MMP-9, TIMP-2, TIMP-4, Ki-67, NF-κβ, IL-1 α, IL-10, HβD-2, HβD-4, VEGF and SHH in children, adult acquired cholesteatoma tissue, and control tissue, which is the skin of the outer ear passage. Selected tissue markers represent the clinical signs of the cholesteatoma. It should be emphasised that this is one of the most extensive studies by number of tissue markers, reflecting the different morphofunctional processes characterising cholesteatoma, and this makes our study one of the most detailed available in the scientific literature when comparing groups of children and adults. The study also looked at several tissue markers that have not previously been studied in cholesteatoma tissues, such as TIMP-4, HβD-4 and SHH, making it impossible in these cases to compare the results with other researchers. However, this made it necessary to investigate studies of other pathologies so that cautious conclusions could be drawn on the results obtained. Finally, by studying the different tissue factors, we showed complex cholesteatoma pathogenesis by evaluating, explaining, and substantiating the different correlations between tissue factors in the cholesteatoma tissue of both patient groups.

In routine microscopy samples, hyperproliferation of the epithelial layer, or matrix, was observed in both groups of cholesteatoma compared to the unchanged skin epithelium of the outer ear canal of the control group. The cholesteatoma matrix was similar to the epithelium of unchanged skin, with the same layers (*basale, spinosum, granulosum* and *lucidum*), yet they were hyper-proliferated in the case of cholesteatoma. These observations also coincide with the data described by other authors (Lim and Saunders, 1972). There were no visible differences between the two groups of patients. This can be explained by the fact, that the causes and mechanism of the occurrence of cholesteatoma are similar in patients of all ages (Louw, 2010).

No marked differences were observed between the groups of paediatric and adult patients when evaluating subepithelial connective tissues, or the perimatrix. However, there were individual differences in each group of patients. In particular, in some patients, the perimatrix was thicker and significantly more infiltrated with inflammatory cells, while in others, the perimatrix was thinner and the infiltration of inflammatory cells was lower. Such a picture was seen in both groups of children and adults. Other authors published similar observations (Dornelles et al., 2006). Comparing the perimatrix of the cholesteatoma with the subepithelial connective tissues of the control skin showed that the connective tissue in the cholesteatoma group was looser and much more infiltrated with inflammatory cells, as well as significantly higher amounts of small blood vessels were observed. These data show a common tumour picture, dominated by inflammation and neo angiogenesis, which contribute to tumour formation, but these are nonspecific changes.

3.1 Tissue remodulation

In the case of human acquired cholesteatoma, the action of remodelling factors initiates the destruction of surrounding bone tissue (Morales et al., 2007; Juhász et al., 2009). When evaluating the relative amount of **MMP-2**, no

statistically significant differences were observed between the groups of cholesteatomas of children and adults. The findings differ from the only other study comparing MMP-2 expression in groups of children and adult patients. Dornelles et al. in their study show that MMP-2 in children's cholesteatoma tissues was statistically significant increased compared to adults (Dornelles et al., 2009). The difference between studies is that in the Dornelles et al. study, the semiquantitative counting system included only four values (0-3), which can lead to potentially higher odds of error in statistical calculations. However, the number of comparative studies of such two groups is so small, that objective comparisons of data between studies are difficult.

When comparing the expression of MMP-2 in tissue of both groups of cholesteatomas with the tissue of the control group, the relative amount of MMP-2 was higher in the patient group than in the control group, but no statistically significant differences were observed. Banerjee et al. also observed similar results in their study (Banerjee et al., 1998). These results are supported by Morales et al., who in their study shows that clinically aggressive cholesteatomas have higher expression of MMP-2, while the relative amount of MMP-2 in less aggressive cholesteatoma will be like any other pathological-free tissue (Morales et al., 2007). The author of this study did not correlate the clinical data of the patients with biomarkers; however, when evaluating each patient individually, it could be seen that there were patients with higher and lower expression of MMP-2 in the tissue, and if evaluated clinically, there could be correlations between MMP-2 expression and the clinical manifestation of the tumour. However, this is the direction of the prospective study.

The relative amount of **MMP-9** in children's and adults' cholesteatoma tissues was similar and no statistically plausible differences were observed between these groups. Palkó et al. also obtained similar data and found no statistically significant differences between the groups of children and adults in

their study (Palkó et al., 2018), but distinguishing the study groups further, it showed higher rates of MMP-9 in adults with recurrent cholesteatoma. The opposite results were obtained by Dornelles et al., who shows in their study that the relative amount of MMP-9 was higher in children's cholesteatoma than in adult cholesteatoma (Dornelles et al., 2009). It should be stressed that the different results obtained may be influenced by the selection of patients: the higher the inflammation during tissue analysis, the higher the expression of MMP-9 in the tissues. In addition, Juhász et al. showed in their study that antibacterial therapy can reduce the amount of MMP-9 in tissues (Juhász et al., 2009). As we know, in the children's population, the incidence of middle ear inflammation is significantly higher than in the adult population (Al-Shehri et al., 2021), which may help explain why the studies of some authors in the children's group show higher expression of MMP-9 in cholesteatoma tissue compared to adult cholesteatoma.

Comparing the cholesteatoma tissue of both groups and the skin of the control group and the relative amount of MMP-9 in them, we found no significant differences between the groups. However, in the control group, the relative amount of MMP-9 was slightly higher than in both groups of cholesteatoma. Banerjee et al. obtained similar data, which found no statistically significant differences between cholesteatoma and control skin in their study but showed slightly higher factor rates in the cholesteatoma group (Banerjee et al., 1998). Also, Rezende et al. in their study did not detect MMP-9 signal in cholesteatoma tissue using PCR (Rezende et al., 2012). The data obtained by Rezende et al. can be attributed in part to the results we obtained, as MMP-9 was very poorly expressed or could not be found at all in many micro-preparations. On the contrary, the opposite results, where MMP-9 is statistically significantly more expressed in cholesteatoma tissue compared to the control group, have been obtained by several authors (Olszewska et al., 2016; Juhász et al., 2009;

Suchozebrska-Jesionek et al., 2008; Kaya et al., 2020). These authors also correlate the amount of MMP-9 with clinical aggressiveness of the cholesteatoma. But there are studies showing that decreased amount of MMP-9 exacerbate angiogenesis in tumour tissue (Pozzi et al., 2002). Although this study was conducted in the animal model and does not bind to the cholesteatoma, it may explain that not only an increased level of MMP-9, but also a decrease in it, may induce angiogenesis in tumour tissue, possibly including the cholesteatoma.

The relative amount of **TIMP-2** in cholesteatoma tissue in children and adults was similar and no statistically significant differences were observed between these groups. In the scientific literature available to us, we could not find another comparative study evaluating the expression of TIMP-2 in cholesteatoma of children and adults. When comparing the cholesteatoma of children and adults with the control group, there was a tendency to have a statistically significant lower expression of TIMP-2 in the group of children than in the control tissue. The relative number of TIMP-2 positive cells was also fewer in the adult group than in the control group, however, the difference was not as pronounced as in children. Kaya et al. obtained similar data, although in a study with a different design where the number of genes was studied, showing that TIMP-2 was reduced in cholesteatoma tissue compared to control skin tissue (Kaya et al., 2020).

The relative amount of **TIMP-4** in children and adults cholesteatoma was similar and no statistically significant differences were observed between the groups. There were also no statistically significant differences when comparing the groups of both patients with the control group. However, the expression of TIMP-4 was higher in the cholesteatoma tissue of both groups than in the control skin tissue. No studies, on the relationship between TIMP-4 and cholesteatoma, can be found in the available scientific literature. Since

TIMP-4 is significantly more expressed in cholesteatoma tissue than TIMP-2, we concluded that, regardless of the patient's age, it plays an important role in the pathogenesis of cholesteatoma.

When evaluating our findings on remodelling factors, we observed that the expression of these factors in cholesteatoma tissues was variable (MMP-2 expression tended to increase, MMP-9 tended to decrease, and TIMP-2 tended to decrease, TIMP-4 tended to increase) compared to the control group. This means that an important balance between MMPs and TIMPs in cholesteatoma tissues has been altered. This change in this balance results in tissue remodelling in the middle ear in patients with cholesteatoma. This is also supported by other researchers, who explain that the imbalance between MMPs and TIMPs complexes in cholesteatoma tissues suggests ECM proteolysis, which causes bone tissue deformation in patients with cholesteatoma (Schönermark et al., 1996). These changes are observable and similar regardless of patient age, as demonstrated by similar relative amounts of remodelling factors and similar correlations between these tissue markers in the two cholesteatoma patient groups. In this study, we observed strong positive correlations between all remodelling factors in both children and adult cholesteatoma groups, indicating that there is a regulation between them.

3.2 Proliferation of cholesteatoma tissue

Increased cell proliferation is one of the main characteristics of cholesteatoma (Yeşilova et al., 2017). We used the proliferation marker **Ki-67**, one of the most reliable markers that show cell division, as well as being used in oncology to determine tumour aggressiveness (Petrelli et al., 2015). When comparing the expression of Ki-67 in tissues of cholesteatoma of children and adults, it showed no statistically significant differences between the groups. Research can be found in the scientific literature that the relative amount of Ki-67 is similar in both children's and adults' cholesteatoma tissues (Sikka et al.,

2012; Mallet et al., 2003). However, there are different opinions, such as Bujía et al., in their study, showing that the proliferation index of children's cholesteatoma cells is higher than that of adults (Bujía et al., 1996). Differences between the study results can, of course, also be searched in the study designs, however, in our view, a more important aspect has been mentioned by other authors. Respectively, not all cholesteatomas will be "active." In their study, Mallet et al. divided cholesteatoma into two groups, active and inactive. In the active cholesteatomas, Ki-67 positive cells will be seen throughout the basal layer, whereas in nonactive tumours, Ki-67 positive cells were scattered intermittently across the basal layer (Mallet et al., 2003). Inactive cholesteatomas show a lower proliferation index. This applies to both groups – children and adults. Our study also had these "active" and "inactive" cholesteatomas in both groups of patients. This is consistent with what Bujía et al. mention in their study, that the tissue of one individual's cholesteatomas expresses Ki-67 unequally across all fields of vision.

The results we obtained show a statistically significant increase in the relative amount of Ki-67 positive cells in the tissues of the cholesteatoma of children and adults compared to the skin of the control's outer ear canal. Other researchers (Akdogan et al., 2013; Chung et al., 2015) have similar results, notably, no studies showing different results were found.

By evaluating our findings, we can conclude that the proliferation of cholesteatoma cells does not depend on the patient's age. In our study, both groups of cholesteatoma were homogeneous and both groups showed similar individual changes between patients. Additionally, Ki-67 is a highly reliable proliferation marker for assessing the activity of cholesteatoma cell division and shows differences between comparable tissues. Ki-67 is potentially significant in clinical medicine, where this marker could be used to determine how each patient's cholesteatoma tissue is capable of proliferating, i.e. to evaluate

the proliferation index of these tissues. That would allow prediction of the potential rate of recurrence development, and, in patients with a potentially higher cholesteatoma proliferation index, control MRI scan or reoperation would be needed earlier than in patients with a cholesteatoma with a low proliferation index.

3.3 Transcription factor

NF-κβ is a significant factor in cell regulation. It mostly regulates inflammatory processes in the body, but has also been associated to cell proliferation and apoptosis processes (Schürmann et al., 2022; Giuliani et al., 2018). When comparing children's cholesteatoma with adult cholesteatoma tissues and NF-κβ expression in them, it did not show statistically discernible differences between these groups. From the results obtained, we can conclude that the processes regulated by NF-κβ do not depend on the age of the patient. Jesic et al. also found no differences between the adult and paediatric groups in the relative amount of NF-κβ in tissues acquired from the middle ear in the case of a chronic inflammation (including cholesteatoma) in their study (Jesic et al., 2014).

Statistically significant differences were observed when comparing NF-κβ expression in cholesteatoma tissues of children and adults with control skin. NF-κβ was statistically more likely to be expressed in both children's cholesteatomas and adult cholesteatomas matrix compared to the control skin epithelium. Similar results, where increased expression of NF-κβ is observed in the epithelium of the cholesteatoma compared to the skin of the ear region, are also demonstrated by other authors (Byun et al., 2010; Xu et al., 2009; du et al., 2016; Liu et al., 2014). Byun et al. also speculate that activated and increased excretion of NF-κβ limits cell apoptosis resulting in hyperproliferation of epithelial cells, which is significant in the pathogenesis of cholesteatoma (Byun et al., 2010). Liu et al. also conclude in their study that the increased NF-κβ, along with other cellular factors, induce hyperproliferation of cholesteatoma cells through the signal pathway (Liu et al., 2014). Our study observed close and

moderate correlations in the cholesteatoma groups of children and adults between NF-κβ and Ki-67. These results are similar to the conclusions of the two authors mentioned above on the involvement of NF-κβ in cell proliferation in cholesteatoma.

In our study, we found strong positive correlations between MMP-2, MMP-9 and NF-κβ in cholesteatoma tissue of both ages. These results can be explained by research from other authors, where NF-κβ has been shown to be one of the regulators of MMP-2 and MMP-9 (Schürmann et al., 2022) in cholesteatoma and other tissues, and stimulates tumour growth, especially angiogenesis by activating MMP activity (Shuman Moss et al., 2012). We also found strong and moderate correlations between NF-κβ and VEGF in both groups of patients, suggesting that NF-κβ may affect angiogenesis in cholesteatoma tissue. This claim is supported by Fukudome et al., who demonstrate in their study that NF-κβ initiates VEGF activation (Fukudome et al., 2013). VEGF, MMP-2 and MMP-9 are known to be related to each other, and both promote and inhibit angiogenesis in tissues of different tumours (Zhu et al., 2019), and the correlations between these factors shown in our study suggest that the mechanisms of angiogenesis and tissue remodelling are similar in cholesteatoma tissues as well, and that one of the regulators for these mechanisms is NF-κβ. Importantly, the results of our study are similar in both cholesteatoma groups.

NF-κβ is mentioned mainly in relation to inflammation regulation. IL-1 is known to activate NF-κβ in different tissues. Activated NF-κβ further promotes the regulation of inflammatory genes and the binding of inflammatory cells (Guo et al., 2024). IL-10, in turn, weakens NF-κβ activation through signal pathways by limiting inflammatory development (Li et al., 2023). In an experimental study, mice, inhibited by IL-10 activity, were shown to increase NF-κβ by producing inflammation (Saadane et al., 2005). Although no

such studies have been conducted in tissues with cholesteatoma, we also found inflammatory superiority in our study and observed decreased relative levels of IL-10 compared to control tissues. As a result, we may cautiously conclude that decreased expression of IL-10 in cholesteatoma tissue affects that the relative number of NF-κβ is increased and enhances inflammatory processes. This theory is also reinforced by the close correlations between IL-1, IL-10 and NF-κβ in both children's and adults' cholesteatoma tissues.

NF-κβ is involved in many different processes in the human body and disease pathogenesis (Giuliani et al., 2018), which we also observe in our study. Specifically, we discovered the involvement of this factor in inflammation, cell proliferation, angiogenesis and tissue remodelling processes, explaining the correlations with all cell factors examined in this study. Thus, we emphasise the complex and important function of NF-κβ also in the pathogenesis of cholesteatoma.

3.4 Pro and anti-inflammatory cytokines

No statistically significant differences were observed in the relative amounts of either IL-1α or IL-10 in cholesteatoma tissues between the children and adult groups. The findings suggest that the action of pro- and anti-inflammatory cytokines and the regulation of the inflammatory process is similar in both groups of patients. It is supported by the strong and very strong correlations between IL-1α and IL-10 in both groups. However, the available literature lacks comparative research between groups of children and adults.

There were also no statistically discernible differences in the relative amounts of the two cytokines when comparing the groups of children and adults with the control group. The relative number of **IL-1α** positive cells in cholesteatoma tissues was higher than in the control group but was not statistically significant. In studies by other authors, the amount of IL-1α in cholesteatoma tissues was statistically significantly increased compared to

the control group (Bujía et al., 1996; Yetiser et al., 2002; Kuczkowski et al., 2011; Çandar et al., 2022). However, these studies have been conducted with quantitative IL-1α detection methods such as *Western blot* and ELISA. Our results are similar to those of other authors because we found a large infiltration of inflammatory cells in the perimatrix of the cholesteatoma, although only a fraction of these inflammatory cells emitted IL-1α. Its absolute amount was higher than in the skin, suggesting an involvement of this cytokine in inflammation process.

It has been described that IL-1α induce the growth of the cholesteatoma and the deformation of bone tissue in the middle ear in patients with cholesteatoma (Bujía et al., 1996; Lee et al., 2010). IL-1α initiates osteoclasts and increases bone matrix degradation (Artono et al., 2020). MMP-2 and MMP-9 are also known to be directly related to damage to the bone matrix in the case of cholesteatoma (Morales et al., 2007; Juhász et al., 2009). The role of IL-1α as a trigger for MMP-2 and MMP-9 and the subsequent initiation of osteoclasts has previously been demonstrated (Kusano et al., 1998; Jia et al., 2021). These studies also explain the data we obtained. In our study, we observed correlations between IL-1α and MMP-2 and MMP-9. We assume that in cholesteatoma tissue, IL-1α, is a significant inflammatory aggravating factor that can affect bone tissue deformation in patients with cholesteatoma regardless of age, as the resulting correlations were observed in both the children and adult groups.

When evaluating the relative amount of **IL-10** in both patient groups and control groups, it was found that it was decreased in both patient groups compared to the control group, but no statistically reliable differences were found between the groups. Kuczkowski et al. also found no statistically significant differences between the cholesteatoma group and the control group in their study (Kuczkowski et al., 2011). In other studies, the authors mention that a decreased amount of IL-10 can cause chronic inflammation of the middle ear and that

the factors that promote and suppress inflammation must be in balance (Smirnova et al., 2004; Juhn et al., 2008). Interestingly, our study found strong positive correlations between IL-1α and IL-10 in both groups of patients, but very strong and negative correlations in the control group. These differences could be explained by the fact that dysregulation develops in cholesteatoma tissue between pro- and anti-inflammatory cytokines, resulting in chronic inflammation, leading to further changes in tissues and the function of tissue factors, for example bone tissue remodulation. This assumption was also made by Kuczkowski et al. (Kuczkowski et al., 2011).

IL-10 has been shown to inhibit MMP-9 as an inflammation suppressant cytokine, but it activates TIMP-2 (Mertz et al., 1994; Lee and Kim, 2014;). However, since studies with IL-10 and its role in the pathogenesis of the cholesteatoma are scarce, explanations should be sought elsewhere. In our study, we found close positive correlations between IL-10 and MMP-9, TIMP-2, TIMP-4 in cholesteatoma tissue of children and adults. These results could indicate that IL-10 is involved in the regulation of MMPs and TIMPs, also in the case of cholesteatoma. Since IL-1α is known to regulate the functioning of MMPs, we assume that the already proven dysregulation between IL-1α and IL-10 may also affect the balance between MMP and TIMP complexes.

3.5 Human beta defensins

The acquired human cholesteatoma is characterised by chronic bacterial inflammation. One of the most common bacteria in chronic inflammation of the middle ear is P. aeruginosa, against which HβD-2 and HβD-4 produce strong local antibacterial effects (Ricciardiello et al., 2009). When comparing the relative amounts of these two defensins, it did not show statistically significant differences between the groups of children and adults. Comparable comparative studies could not be found in the available scientific literature. From

our findings, we can conclude that the local antimicrobial function of tissue factors in patients with cholesteatoma does not depend on the age of the patient.

Statistically significantly increased **HβD-2** expression in the cholesteatoma perimatrix were observed when comparing the relative amount of HβD-2 in both groups of cholesteatoma patients and control skin. There was also an increase in the amount of HβD-2 in the epithelial layer for the cholesteatoma groups, but it did not reach statistical significance. The data of our study coincide with the results of other authors, where HβD-2 is more expressed in cholesteatoma compared to control tissue (Park et al., 2003; Ren et al., 2005; Song et al., 2007). These results are supported by studies where increased expression of HβD-2 in tissues is initiated by cytokines and bacteria (Meyer et al., 2000; harder et al., 2000). Our study also finds similar very strong and strong correlations in the case of cholesteatoma in children and adults between HβD-2 and IL-1α, as well as between HβD-2 and NF-κβ, and between NF-κβ and IL-1α. This relationship between the factors listed above is not accidental and has also been shown in other studies where IL-1α has been identified as a trigger for HβD-2, while NF-κβ is a regulator of this process (Moon et al., 2002; Wehkamp et al., 2006). When evaluating anti-inflammatory activity in the cholesteatoma, we found similar correlations in the children and adult groups between HβD-2 and IL-10 in the perimatrix of the cholesteatoma, where there are many white blood cells, which could mean that in anti-inflammatory activity, both factors help each other. The results we have obtained, help explain Kanda et al., who demonstrate that HβD-2 stimulates IL-10 activity in T cells (Kanda et al., 2011). From these results, we can conclude that HβD-2 is a significant antimicrobial peptide involved in the regulation of inflammatory processes and is similar in function to patients with cholesteatoma of all ages.

We obtained interesting results by comparing the amount of **HβD-4** in both patient and control groups. Although the tendency to decrease in HβD-4 is observed only in the paediatric cholesteatoma matrix compared to the epithelium of the control group, the relative amount of HβD-4 was also lower in the adult group than in the control group. According to the information available to us, our study is the only one where HβD-4 cholesteatoma tissues have been evaluated and comparisons have been made between groups of patients of different ages and control tissues. However, a study was found in which, in chronic periodontitis, the amount of HβD-4 in the mucous membranes was not different from that of healthy tissues (Li et al., 2016). Further studies analysed that cell factors such as IL-1, IL-6, INF-α, INF-γ do not increase the expression of HβD-4 in the respiratory epithelium compared to control tissues, as well as in inflammation of the skin caused by lipopolysaccharides, the level of HβD-4 do not increase and is similar to control tissue (Poiraud et al., 2012; García et al. 2001). Based on these data, the theory was presented that HβD-4 does not participate in the cell-initiated immune response (Li et al., 2016). The results of our study support this theory because there were no correlations between HβD-4 and, for example, IL-1, IL-10, NF-κβ or remodelling factors in the tissue of cholesteatoma of both children and adults and, overall, there were a very few correlations between HβD-4 and the other tissue factors (compared to HβD-2).

Although there is very little research on the function of human beta defensins in cholesteatoma tissues, a review of all studies suggests that of the human beta defensins studied, it is HβD-2 that has the most active expression in cholesteatoma tissues. For example, Song et al. shows HβD-2 more activity in cholesteatoma tissue than it is in HβD-3, and Park et al. show that HβD-2 is more active than HβD-1 (Song et al., 2007; Park et al., 2003). Finally, the results of our study strongly show that HβD-2 is more expressed in cholesteatoma tissue when compared to HβD-4 in both patient groups We conclude that HβD-2 is

a more significant antimicrobial peptide in cholesteatoma than HβD-4 and is supported by significantly higher correlations between HβD-2 and other tissue factors in both children and adult cholesteatoma.

3.6 Vascular endothelial growth factor

Angiogenesis is important for the cholesteatoma to grow and spread to the middle ear (Hamed et al., 2019). No statistically significant differences in **VEGF** expression were observed when comparing the group of children with the adult group. Therefore, we can conclude that angiogenesis in children and adults' cholesteatoma tissue is similar and not age dependent.

There were no statistically significant differences when comparing the control group with the two groups of patients. However, a tendency was observed, that the relative amount of VEGF in the skin epithelium of the outer ear passage was more expressed than in the matrix of adult patients with cholesteatoma. There was no such tendency in children, but, still, there were fewer VEGF positive cells than in the control group. These results differ from those of other authors, such as Zang et al., who in their study show that VEGF in cholesteatoma was more present in the matrix than in skin epithelium (Zang et al., 2019). The difference in results could be determined by the fact that Zang et al. used the Western blot method to detect VEGF in their study, which shows different results from the factor concentration we obtained. It should be mentioned, that our findings could be explained by studies of other tumours. This should include basal cell carcinoma, where VEGF is reduced in the epithelial layer, and VEGF in various other epithelial tumours is secreted in an extracellular space. VEGF is also known to be secreted paracrine in the case of cholesteatoma, where keratinocytes release VEGF that enters the perimatrix and induces angiogenesis (Fukudome et al., 2013). This could explain why in our study the relative number of VEGF-positive cells in the matrix was less than in the control skin epithelium. This is also confirmed by the close correlations found

in our study between VEGF in the matrix and VEGF in perimatrix in the cholesteatoma tissue of patients of both ages.

When comparing the relative amount of VEGF in the cholesteatoma perimatrix and control subcutaneous connective tissue, we also did not see statistically significant differences between the groups here. However, in other studies, the results show an increase in the VEGF distribution compared to the control group. For example, Fukudome et al. show in its study the increased amount of VEGF in the perimatrix compared to control tissues (Fukudome et al., 2013). In this case, the number of patients was small, which could affect the results. Sudhoff et al. show in their study that the number of small blood vessels and VEGF expression are higher in cholesteatoma than in control skin (Sudhoff et al., 2000). We can explain these differences between studies by the fact that in our study we only looked at endotheliocytes in the perimatrix. The amount of blood vessels compared to the subcutaneous portion of the control group was significantly higher for cholesteatoma; however, not all endotheliocytes in these vessels expressed VEGF and their relative amount was not higher than the relative amount of subcutaneous positive endotheliocytes. Studying the absolute amount of blood vessels and VEGF positive cells would probably produce similar results with data from other authors. However, in our study, we stuck to one evaluation system for all markers. Importantly, neoangiogenesis is significantly more pronounced in cholesteatoma tissue compared to control skin (Olszewska et al., 2004).

Correlations between VEGF and MMP-2, MMP-9 and NF-κβ indicate the importance of this cell factor in various cholesteatoma processes as tissue remodulation and angiogenesis (Fukudome et al., 2013; Zhu et al., 2019).

3.7 *Sonic hedgehog* gene protein

The relative amount of the **SHH gene protein** in adult and children cholesteatoma tissue was similar and did not achieve statistically significant differences. This was the first study to evaluate the amount from protein of this gene in cholesteatoma tissue, and a comparison of the factor finding was performed for the first time between different age groups.

When comparing the SHH gene protein in both cholesteatoma groups and in the skin of the control group, we obtained statistically significant increased relative amounts of SHH in the cholesteatoma perimatrix of both groups. In the cholesteatoma matrix, the relative amount of SHH compared to the skin epithelium did not achieve statistically significant differences.

The SHH gene is also associated with ear development, such as the loss of SHH affects the development of the outer and middle ears (Rees and Gillis, 2022; Grevellec et al., 2011). An increased amount of SHH has been shown to be present in both malignancies and precancerous conditions in the oral cavity (Gonzalez et al., 2016; Takabatake et al., 2019). In other organ systems, the amount of SHH in tumour cells has also been increased compared to control tissue, as well as shown to be associated with tumour growth (Jing et al., 2023). Perimatrix in the case of cholesteatoma is known to be important for the aggressiveness and growth of the cholesteatoma. It contains many inflammatory cells and is a layer of cholesteatoma in direct contact with the surrounding healthy tissues (Dornelles et al., 2006). Dornelles et al. correlated the thickness of the perimatrix with the aggressiveness of the cholesteatoma in their study: the thicker the perimatrix, the greater the infiltration of inflammatory cells and the greater the aggressiveness of the tumour in surrounding tissues.

Observations on the role of perimatrix in the aggressiveness of cholesteatoma, as well as studies on the association with tumour growth, and the fact that SHH has increased in both malignant and benign aggressive

formations, such as precancerous conditions in the oral cavity and also in the case of cholesteatoma, indicate that SHH plays a significant role in the pathogenesis of the cholesteatoma. We assume that SHH affects the growth of the perimatrix, thus also amplifying the growth of the cholesteatoma itself and its destructive effects on the surrounding tissue of the middle ear. Importantly, these processes are similar in both children's and adults' cholesteatoma tissues.

By summarising the results we have obtained, we can conclude that the hypothesis put forward by this work will not be confirmed. No differences were found between tissue remodelling, proliferation, pro and anti-inflammatory activity, as well as its regulation, local defence of the tissue, angiogenesis, and expression of the *Sonic hedgehog* gene protein in cholesteatoma tissue in **patients of different ages**. **The morphological findings in the tissues of the children and adult cholesteatoma tissues were similar and the distribution of different tissue factors was more similar than different**. This is demonstrated by similar relative quantities of tissue markers, similar correlations between tissue factors, as well as their similar variations in cholesteatoma tissue in both age groups. Other researchers have also obtained the findings that children and adult cholesteatoma are not morphologically different (Sikka et al., 2012; Welkoborsky et al., 2007; Dornelles et al., 2006). However, there are also different opinions when studies have found differences between groups of children and adults in different tissue markers, concluding that children's cholesteatoma is more aggressive than adults (Bujía et al., 1996; Dornelles et al., 2009). There is no denying that the disease is progressing more aggressively in children and that there is a more frequent recurrence, also shown by numerous clinical studies and radiological studies (Jackson et al., 2018; Kalia et al., 2022; Lima et al., 2020; Lynrah et al., 2013) (compared to the small number of morphological studies). However, we did not find morphological differences between these groups. Differences such as more frequent upper respiratory

diseases and inflammation of the middle ear in children or differences in the pneumatization of the mastoid bone, mastoid cell development, anatomical changes of the Eustachian tube, and variations in hormonal activity (Preciado et al., 2012) may affect the clinical course of disease between groups of children and adults.

When comparing the two groups of patients, we are aware that children (especially the oldest) are physiologically similar to the adults (especially the youngest) and, of course, a more correct group distribution would be after skin maturation, which becomes so at about age 7, but we were guided by the nationally accepted age distribution scale.

The study also has limitations. Although the IHC and semi-quantitative tissue evaluation method are accurate and widely applied in research, these methods cannot establish marker concentrations in investigational tissues that allow for a more accurate comparison of groups, in particular investigational groups, with the control group. Methods such as ELISA and Western blot would be useful for detecting even more accurate results. Another limitation is the relatively small number of patients in the control group, as well as the fact that the tissue was taken from the bodies of deceased people, but ethical conditions dictate precisely the choice of material in that group.

Conclusions

1 Hyperproliferation of the cholesteatoma matrix, variable perimatrix, and neoangiogenesis with infiltration of inflammatory cells are non-age-related nonspecific tissue changes of the cholesteatoma.

2 Variable expression of remodelling factors (tendency in increase of MMP-2 and, in particular, TIMP-4 and decrease of MMP-9 and TIMP-2) in cholesteatoma tissue indicates non-age related disbalance of remodelling factors with specific, mainly compensatory, involvement of TIMP-4.

3 The statistically significant increase in the number of Ki-67 positive cholesteatoma cells indicates the aggressiveness of the tumour, which is variable even within a single formation and does not correlate with age.

4 The pronounced finding of NF-κβ in the epithelium of the cholesteatoma and the close correlations with Ki-67, IL-1α, IL-10, MMPs, and VEGF indicate inflammatory modelled epithelial proliferation, maintained balance between pro- and anti-inflammatory cytokines, and active remodelling in conditions of ischemia-stimulated neoangiogenesis in longitudinal aspect.

5 The increase in the number of IL-1α positive structures and the decrease in the number of positive IL-10 cells in the cholesteatoma tissue, indicate a small, tumour-specific pro- and anti-inflammatory cytokine imbalance.

6 The statistically significant increase in HβD-2 positive cells in the perimatrix of the cholesteatoma and its increased tendency in the epithelium, along with a close correlation with IL-10, indicate a common age-independent intensification of local tissue defence factors.

7 The statistically significant increase in the number of SHH positive cells in the perimatrix of the cholesteatoma, indicates the intensification of this gene protein in tumour growth, although with a vague morphopatogenetic path of action.

8 In children and adults' cholesteatoma, without minor individual variations, there is virtually **no difference in tissue remodelling, cell proliferation,**

pro and anti-inflammatory cytokine and antimicrobial peptide secretion, angiogenetic processes, and SHH gene protein stimulation.

Proposals

A human acquired cholesteatoma clinically is relatively aggressive. More studies would be needed to assess the correlation between the **clinical presentation of the disease and the individual anatomy of the temporal bone with different tissue markers**. Such studies could help better define tissue markers that could be used in daily practice, help better understand and predict disease progression in a particular patient, and provide better control of the disease even after surgery.

Gene research is now rapidly evolving, opening opportunities to **evaluate the involvement of different genes** in the development of cholesteatoma.

Finally, the only treatment option for cholesteatoma is surgical excision, but more **studies in the development of a variety of topical or systemic medications** that could reduce the recurrence of cholesteatoma.

List of publications, reports and patents on the topic of the Thesis

Publications (Scopus, Web of Science, ERIH PLUS):

1 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2024. Comparison of Tissue Factors in the Ontogenetic Aspects of Human Cholesteatoma. *Diagnostics (Basel, Switzerland)*, *14*(6), 662. doi:10.3390/diagnostics14060662

2 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2023. Morphopathogenesis of Adult Acquired Cholesteatoma. *Medicina (Kaunas, Lithuania)*, *59*(2), 306. doi:10.3390/medicina59020306

3 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2022. Remodeling Factors, Transcription Factors and Angiogenetic Factors in Cholesteatoma in Ontogenetic Aspect. *Iranian journal of otorhinolaryngology*, *34*(121), 71–81. doi:10.22038/IJORL.2021.53716.2842

4 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2021. Complex Evaluation of Tissue Factors in Pediatric Cholesteatoma. *Children (Basel, Switzerland)*, *8*(10), 926. doi:10.3390/children8100926

Reports and theses at international congresses and conferences:

1 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2024. Morphological comparison of paediatric and adult acquired cholesteatoma. 11th Baltic Morphology Meeting. Riga, Latvia Abstracts, 13. – 15.11.2024, 25 (Oral presentation)

2 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2024. Comparison of Adult and Children Acquired Cholesteatoma. 7th Congress of European ORL-HNS. Dublin, Ireland. 15. – 19.06.2024 (Oral presentation)

3 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2023. Morphological differences between an adult and pediatric acquired cholesteatoma. 8th Baltic ENT congress. Vilnius, Lithuania. 09. – 10.06.2023 (Oral presentation)

4 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2023. Comparison of proliferation and remodelation markers in adult and pediatric cholesteatoma. 16th Congress of the European Society of Pediatric Otorhinolaryngology. Liverpool, United Kingdom. 20. – 23.05.2023 (Oral presentation)

5 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2023. Morphological Characterisation of Paediatric and Adult Acquired Cholesteatoma. Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice" 29.–31.03.2023 (Oral presentation)

6 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2023. Immunohistochemical comparison of adult and children cholesteatoma. International Practical Ophthalmology. Medical and Environmental Problems of our Days: Collection of Works International Scientific and Practical Interdisciplinary Conference. State Institution "National

Research Center for Radiation medicine of the National Academy of Medical Sciences of Ukraine". Abstracts 23.–24.02.2023, 31-33 (Oral presentation)

7 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2021. Transcription and remodelling factors in the development of cholesteatoma from an ontogenetic aspect compared to deep external ear skin controls. Rīga Stradiņš University International Research Conference on Medical and Health Care Sciences "Knowledge for Use in Practice". Abstracts, 24.–26.03.2021, 430 (Poster presentation)

8 **Dambergs, K.,** Sumeraga, G., Pilmane, M. 2021. Proliferation markers, remodelling factors and antimicrobial peptides in cholesteatoma. The 15th Congress of the European Society of Pediatric Otorhinolaryngology, Marseille, France. 13. – 16.02.2021 (Poster presentation)

References

1 Akdogan, V., Yilmaz, I., Canpolat, T. and Ozluoglu, L. N. 2013. Role of Langerhans cells, Ki-67 protein and apoptosis in acquired cholesteatoma: prospective clinical study. *The Journal of laryngology and otology*, *127*(3), 252–259.

2 Al-Shehri, A. M., Sr, Al-Zomia, A. S., Alayash, A. F., M Al Hunaif, A., A Mansour, A., Alqahtani, M., A Asiri, O. and A Alserhan, S. 2021. Association Between Acute Otitis Media and Inner Ear Disorders Among Adults in Aseer Region. *Cureus*, *13*(11), e19556.

3 Artono, Surarto, B., Purnami, N., Hutahaen, F. and Mahardhika, M. R. 2020. The Association of IL-1 Alpha Level and TNF Alpha Expressions on Bone Destruction in Chronic Suppurative Otitis Media and Cholesteatoma. *Indian journal of otolaryngology and head and neck surgery : official publication of the Association of Otolaryngologists of India*, *72*(1), 1–7.

4 Banerjee, A. R., James, R. and Narula, A. A. 1998. Matrix metalloproteinase-2 and matrix metalloproteinase-9 in cholesteatoma and deep meatal skin. *Clinical otolaryngology and allied sciences*, *23*(4), 345–347.

5 Barton, B. and Peat, J. K. 2014. *Medical statistics: a guide to SPSS, data analysis and critical appraisal / Belinda Barton, Jennifer Peat*. Chester, West Sussex; Hoboken, NJ: Wiley Blackwell/ BMJ Books. From: via GoogleBooks [viewed: 15.03.2024]

6 Bhutta, M. F., Williamson, I. G. and Sudhoff, H. H. 2011. Cholesteatoma. *BMJ (Clinical research ed.)*, *342*, d1088.

7 Byun, J. Y., Yune, T. Y., Lee, J. Y., Yeo, S. G. and Park, M. S. 2010. Expression of CYLD and NF-kappaB in human cholesteatoma epithelium. *Mediators of inflammation*, *2010*, 796315.

8 Britze, A., Møller, M. L. and Ovesen, T. 2017. Incidence, 10-year recidivism rate and prognostic factors for cholesteatoma. *The Journal of laryngology and otology*, *131*(4), 319–328.

9 Bujía, J., Holly, A., Antolí-Candela, F., Tapia, M. G. and Kastenbauer, E. 1996. Immunobiological peculiarities of cholesteatoma in children: quantification of epithelial proliferation by MIB1. *The Laryngoscope*, *106*(7), 865–868.

10 Bujía, J., Kim, C., Ostos, P., Sudhoff, H., Kastenbauer, E. and Hültner, L. 1996. Interleukin 1 (IL-1) and IL-1-receptor antagonist (IL-1-RA) in middle ear cholesteatoma: an analysis of protein production and biological activity. *European archives of oto-rhino-laryngology*, *253*(4-5), 252–255.

11 Çandar, T., Baklaci, D., Bilgin, E., Erdem, D. and Dalgıç, M. 2022. Investigation of EGF, IL1-α and IL-6 levels and selected hematological parameters (NLR, MPV) in patients with the chronic cholesteatomatous otitis media. *Acta biochimica Polonica*, *69*(3), 633–637.

12 Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. 1996. Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. *Nature*, *383*(6599), 407–413.

13 Chung, J. H., Lee, S. H., Park, C. W., Kim, K. R., Tae, K., Kang, S. H., Oh, Y. H. and Pyo, J. Y. 2015. Expression of Apoptotic vs Antiapoptotic Proteins in Middle Ear Cholesteatoma. *Otolaryngology--head and neck surgery*, *153*(6), 1024–1030.

14 Dornelles, C., Meurer, L., Selaimen da Costa, S. and Schweiger, C. 2006. Histologic description of acquired cholesteatomas: comparison between children and adults. *Brazilian journal of otorhinolaryngology*, *72*(5), 641–648.

15 Dornelles, C.deC., da Costa, S. S., Meurer, L., Rosito, L. P., da Silva, A. R. and Alves, S. L. 2009. Comparison of acquired cholesteatoma between pediatric and adult patients. *European archives of oto-rhino-laryngology*, *266*(10), 1553–1561.

16 Du, L., Xie, Y. D. and Liu, M. 2016. Expression and significance of PDK1 and NF-κB in the middle ear cholesteatoma. *Journal of clinical otorhinolaryngology, head, and neck surgery*, *30*(10), 770–773.

17 Fischer, A. H., Jacobson, K. A., Rose, J. and Zeller, R. 2008. Hematoxylin and eosin staining of tissue and cell sections. *CSH protocols*, *2008*, pdb.prot4986.

18 Fontes Lima, A., Carvalho Moreira, F., Sousa Menezes, A., Esteves Costa, I., Azevedo, C., Sá Breda, M. and Dias, L. 2020. Is pediatric cholesteatoma more aggressive in children than in adults? A comparative study using the EAONO/JOS classification. *International journal of pediatric otorhinolaryngology*, *138*, 110170.

19 Forthofer, R., Lee, E. S. and Hernandez, M. (Eds.). 2007. Descriptive methods. In: Forthofer, R., Lee, E. S. and Hernandez, M., ed. *Biostatistics (Second Edition).* Academic Press, 21–69. From: doi.org/10.1016/B978-0-12-369492-8.50008-X. [viewed 15.03.2024]

20 Fukudome, S., Wang, C., Hamajima, Y., Ye, S., Zheng, Y., Narita, N., Sunaga, H., Fujieda, S., Hu, X., Feng, L. and Lin, J. 2013. Regulation of the angiogenesis of acquired middle ear cholesteatomas by inhibitor of DNA binding transcription factor. *JAMA otolaryngology-- head & neck surgery*, *139*(3), 273–278.

21 García, J. R., Krause, A., Schulz, S., Rodríguez-Jiménez, F. J., Klüver, E., Adermann, K., Forssmann, U., Frimpong-Boateng, A., Bals, R. and Forssmann, W. G. 2001. Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. *FASEB journal*, *15*(10), 1819–1821.

22 Giuliani, C., Bucci, I. and Napolitano, G. 2018. The Role of the Transcription Factor Nuclear Factor-kappa B in Thyroid Autoimmunity and Cancer. *Frontiers in endocrinology*, *9*, 471.

23 Givvimani, S., Tyagi, N., Sen, U., Mishra, P. K., Qipshidze, N., Munjal, C., Vacek, J. C., Abe, O. A. and Tyagi, S. C. 2010. MMP-2/TIMP-2/TIMP-4 versus MMP-9/TIMP-3 in transition from compensatory hypertrophy and angiogenesis to decompensatory heart failure. *Archives of physiology and biochemistry*, *116*(2), 63–72.

24 Gonzalez, A. C., Ferreira, M., Ariel, T., Reis, S. R., Andrade, Z. and Peixoto Medrado, A. 2016. Immunohistochemical evaluation of hedgehog signalling in epithelial/mesenchymal interactions in squamous cell carcinoma transformation: a pilot study. *Journal of oral pathology & medicine*, *45*(3), 173–179.

25 Grevellec, A., Graham, A. and Tucker, A. S. 2011. Shh signalling restricts the expression of Gcm2 and controls the position of the developing parathyroids. *Developmental biology*, *353*(2), 194–205.

26 Guo, Q., Jin, Y., Chen, X., Ye, X., Shen, X., Lin, M., Zeng, C., Zhou, T. and Zhang, J. 2024. NF-κB in biology and targeted therapy: new insights and translational implications. *Signal transduction and targeted therapy*, *9*(1), 53.

27 Hamed, M. A., Sayed, R. H., Shiogama, K., Eltaher, M. A., Suzuki, K. and Nakata, S. 2019. Localisation of basic fibroblast growth factor in cholesteatoma matrix: an immunochemical study. *The Journal of laryngology and otology*, *133*(3), 183–186.

28 Harder, J., Bartels, J., Christophers, E. and Schröder, J. M. 1997. A peptide antibiotic from human skin. *Nature*, *387*(6636), 861.

29 Harder, J., Meyer-Hoffert, U., Teran, L. M., Schwichtenberg, L., Bartels, J., Maune, S. and Schröder, J. M. 2000. Mucoid Pseudomonas aeruginosa, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia. *American journal of respiratory cell and molecular biology*, *22*(6), 714–721.

30 Jackson, R., Addison, A. B. and Prinsley, P. R. 2018. Cholesteatoma in children and adults: are there really any differences? *The Journal of laryngology and otology*, *132*(7), 575–578.

31 Jesic, S., Jotic, A., Tomanovic, N. and Zivkovic, M. 2014. Expression of toll-like receptors 2, 4 and nuclear factor kappa B in mucosal lesions of human otitis: pattern and relationship in a clinical immunohistochemical study. *The Annals of otology, rhinology, and laryngology*, *123*(6), 434–441.

32 Jia, Y., Chen, X. and Sun, J. 2021. Apremilast ameliorates IL-1α-induced dysfunction in epidermal stem cells. *Aging*, *13*(15), 19293–19305.

33 Jing, J., Wu, Z., Wang, J., Luo, G., Lin, H., Fan, Y. and Zhou, C. 2023. Hedgehog signaling in tissue homeostasis, cancers, and targeted therapies. *Signal transduction and targeted therapy*, *8*(1), 315.

34 Juhász, A., Sziklai, I., Rákosy, Z., Ecsedi, S., Adány, R. and Balázs, M. 2009. Elevated level of tenascin and matrix metalloproteinase 9 correlates with the bone destruction capacity of cholesteatomas. *Otology & neurotology*, *30*(4), 559–565.

35 Juhn, S. K., Jung, M. K., Hoffman, M. D., Drew, B. R., Preciado, D. A., Sausen, N. J., Jung, T. T., Kim, B. H., Park, S. Y., Lin, J., Ondrey, F. G., Mains, D. R. and Huang, T. 2008. The role of inflammatory mediators in the pathogenesis of otitis media and sequelae. *Clinical and experimental otorhinolaryngology*, *1*(3), 117–138.

36 Kaya, İ., Avcı, Ç. B., Şahin, F. F., Özateş, N. P., Sezgin, B., Kurt, C. Ç., Bilgen, C. and Kirazlı, T. 2020. Evaluation of significant gene expression changes in congenital and acquired cholesteatoma. *Molecular biology reports*, *47*(8), 6127–6133.

37 Kalia, M., Dass, A., Singhal, S. K. and Gupta, N. 2022. Comparative study of cholesteatoma in paediatric and adult patients. *The Journal of laryngology and otology*, *136*(8), 765–768.

38 Kanda, N., Kamata, M., Tada, Y., Ishikawa, T., Sato, S. and Watanabe, S. 2011. Human β-defensin-2 enhances IFN-γ and IL-10 production and suppresses IL-17 production in T cells. *Journal of leukocyte biology*, *89*(6), 935–944.

39 Kuczkowski, J., Sakowicz-Burkiewicz, M., Iżycka-Świeszewska, E., Mikaszewski, B. and Pawełczyk, T. 2011. Expression of tumor necrosis factor-α, interleukin-1α, interleukin-6 and interleukin-10 in chronic otitis media with bone osteolysis. *ORL; journal for oto-rhino-laryngology and its related specialties*, *73*(2), 93–99.

40 Kuo, C. L., Shiao, A. S., Yung, M., Sakagami, M., Sudhoff, H., Wang, C. H., Hsu, C. H. and Lien, C. F. 2015. Updates and knowledge gaps in cholesteatoma research. *BioMed research international*, *2015*, 854024.

41 Kusano, K., Miyaura, C., Inada, M., Tamura, T., Ito, A., Nagase, H., Kamoi, K. and Suda, T. 1998. Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. *Endocrinology*, *139*(3), 1338–1345.

42 Lee, E. J. and Kim, H. S. 2014. The anti-inflammatory role of tissue inhibitor of metalloproteinase-2 in lipopolysaccharide-stimulated microglia. *Journal of neuroinflammation*, *11*, 116.

43 Lee, Y. M., Fujikado, N., Manaka, H., Yasuda, H. and Iwakura, Y. 2010. IL-1 plays an important role in the bone metabolism under physiological conditions. *International immunology*, *22*(10), 805–816.

44 Li, K., Li, J., Wei, X., Wang, J., Geng, M., Ai, K., Liang, W., Zhang, J., Li, K., Gao, H. and Yang, J. 2023. IL-10 Negatively Controls the Primary T Cell Response of Tilapia by Triggering the JAK1/STAT3/SOCS3 Axis That Suppresses NF-κB and MAPK/ERK Signaling. *Journal of immunology (Baltimore, Md.: 1950)*, *210*(3), 229–244.

45 Li, X., Duan, D., Yang, J., Wang, P., Han, B., Zhao, L., Jepsen, S., Dommisch, H., Winter, J. and Xu, Y. 2016. The expression of human β-defensins (hBD-1, hBD-2, hBD-3, hBD-4) in gingival epithelia. *Archives of oral biology*, *66*, 15–21.

46 Lillie, R. D., Pizzolato, P. and Donaldson, P. T. 1976. Hematoxylin substitutes: a survey of mordant dyes tested and consideration of the relation of their structure to performance as nuclear stains. *Stain technology*, *51*(1), 25–41.

47 Lim, D. J. and Saunders, W. H. 1972. Acquired cholesteatoma: light and electron microscopic observations. *The Annals of otology, rhinology, and laryngology*, *81*(1), 1–11.

48 Liu, W., Yin, T., Ren, J., Li, L., Xiao, Z., Chen, X. and Xie, D. 2014. Activation of the EGFR/Akt/NF-κB/cyclinD1 survival signaling pathway in human cholesteatoma epithelium. *European archives of oto-rhino-laryngology*, *271*(2), 265–273.

49 Lynrah, Z. A., Bakshi, J., Panda, N. K. and Khandelwal, N. K. 2013. Aggressiveness of pediatric cholesteatoma. Do we have an evidence? *Indian journal of otolaryngology and head and neck surgery, 65*(3), 264–268.

50 Louw L. 2010. Acquired cholesteatoma pathogenesis: stepwise explanations. *The Journal of laryngology and otology*, *124*(6), 587–593.

51 Mallet, Y., Nouwen, J., Lecomte-Houcke, M. and Desaulty, A. 2003. Aggressiveness and quantification of epithelial proliferation of middle ear cholesteatoma by MIB1. *The Laryngoscope*, *113*(2), 328–331.

52 Meyer, J. E., Harder, J., Görögh, T., Schröder, J. M. and Maune, S. 2000. hBD-2-Genexpression in nasaler Schleimhaut [hBD-2 gene expression in nasal mucosa]. *Laryngo- rhino- otologie*, *79*(7), 400–403.

53 Mertz, P. M., DeWitt, D. L., Stetler-Stevenson, W. G. and Wahl, L. M. 1994. Interleukin 10 suppression of monocyte prostaglandin H synthase-2. Mechanism of inhibition of prostaglandin-dependent matrix metalloproteinase production. *The Journal of biological chemistry*, *269*(33), 21322–21329.

54 Moon, S. K., Lee, H. Y., Li, J. D., Nagura, M., Kang, S. H., Chun, Y. M., Linthicum, F. H., Ganz, T., Andalibi, A. and Lim, D. J. 2002. Activation of a Src-dependent Raf-MEK1/2-ERK signaling pathway is required for IL-1alpha-induced upregulation of beta-defensin 2 in human middle ear epithelial cells. *Biochimica et biophysica acta*, *1590*(1-3), 41–51.

55 Morales, D. S., Penido, N.deO., da Silva, I. D., Stávale, J. N., Guilherme, A. and Fukuda, Y. 2007. matrix metalloproteinase 2: an important genetic marker for cholesteatomas. *Brazilian journal of otorhinolaryngology*, *73*(1), 51–57.

56 Mosser, D. M. and Zhang, X. 2008. Interleukin-10: new perspectives on an old cytokine. *Immunological reviews*, *226*, 205–218.

57 Olszewska, E., Chodynicki, S. and Chyczewski, L. 2004. Znaczenie angiogenezy w patogenezie perlaka ucha środkowego u dorosłych [Role of angiogenesis in the pathogenesis of cholesteatoma in adults]. *Otolaryngologia polska = The Polish otolaryngology*, *58*(3), 559–563.

58 Olszewska, E., Matulka, M., Mroczko, B., Pryczynicz, A., Kemona, A., Szmitkowski, M., Mierzwinski, J. and Pietrewicz, T. 2016. Diagnostic value of matrix metalloproteinase 9 and tissue inhibitor of matrix metalloproteinases 1 in cholesteatoma. *Histology and histopathology*, *31*(3), 307–315.

59 Ozola, L. and Pilmane, M. 2023. Local Defense Factors in Cleft-Affected Palate in Children before and during Milk Dentition Age: A Pilot Study. *Journal of personalised medicine*, *14*(1), 27.

60 Palkó, E., Póliska, S., Sziklai, I. and Penyige, A. 2018. Analysis of KRT1, KRT10, KRT19, TP53 and MMP9 expression in pediatric and adult cholesteatoma. *PloS one*, *13*(7), e0200840.

61 Park, K., Moon, S. K., Choung, Y. H. and Choi, H. S. 2003. Expression of beta-defensins in human middle ear cholesteatoma. *Acta oto-laryngologica*, *123*(2), 236–240.

62 Petrelli, F., Viale, G., Cabiddu, M. and Barni, S. 2015. Prognostic value of different cut-off levels of Ki-67 in breast cancer: a systematic review and meta-analysis of 64,196 patients. *Breast cancer research and treatment*, *153*(3), 477–491.

63 Pilmane, M., Jain, N. and Vitenberga-Verza, Z. 2021. Expression Analysis of *FGF/FGFR* and *FOX* Family Proteins in Mucosal Tissue Obtained from Orofacial Cleft-Affected Children. *Biology*, *10*(5), 423.

64 Poiraud, C., Quereux, G., Knol, A. C., Zuliani, T., Allix, R., Khammari, A. and Dreno, B. 2012. Human β-defensin-2 and psoriasin, two new innate immunity targets of zinc gluconate. *European journal of dermatology: EJD*, *22*(5), 634–639.

65 Pozzi, A., LeVine, W. F. and Gardner, H. A. 2002. Low plasma levels of matrix metalloproteinase 9 permit increased tumor angiogenesis. *Oncogene*, *21*(2), 272–281.

66 Preciado D. A. 2012. Biology of cholesteatoma: special considerations in pediatric patients. *International journal of pediatric otorhinolaryngology*, *76*(3), 319–321.

67 Rees, J. M. and Gillis, J. A. 2022. Distinct proliferative and middle ear skeletal-patterning functions for SHH-expressing epithelia in the chick hyoid arch. *Developmental biology*, *489*, 98–108.

68 Ren, X., Cui, P., Chen, W. and Xu, M. 2005. The expression and significance of human beta-defensin 2 in middle ear cholesteatoma epithelium. *Journal of clinical otorhinolaryngology*, *19*(7), 313–314.

69 Rezende, C. E., Souto, R. P., Rapoport, P. B., Campos, L.d, and Generato, M. B. 2012. Cholesteatoma gene expression of matrix metalloproteinases and their inhibitors by RT-PCR. *Brazilian journal of otorhinolaryngology*, *78*(3), 116–121.

70 Ricciardiello, F., Cavaliere, M., Mesolella, M. and Iengo, M. 2009. Notes on the microbiology of cholesteatoma: clinical findings and treatment. *Acta otorhinolaryngologica Italica: organo ufficiale della Societa italiana di otorinolaringologia e chirurgia cervico-facciale*, *29*(4), 197–202.

71 Saadane, A., Soltys, J. and Berger, M. 2005. Role of IL-10 deficiency in excessive nuclear factor-kappaB activation and lung inflammation in cystic fibrosis transmembrane conductance regulator knockout mice. *The Journal of allergy and clinical immunology*, *115*(2), 405–411.

72 Schönermark, M., Mester, B., Kempf, H. G., Bläser, J., Tschesche, H. and Lenarz, T. 1996. Expression of matrix-metalloproteinases and their inhibitors in human cholesteatomas. *Acta oto-laryngologica*, *116*(3), 451–456.

73 Schürmann, M., Goon, P. and Sudhoff, H. 2022. Review of potential medical treatments for middle ear cholesteatoma. *Cell communication and signaling: CCS*, *20*(1), 148.

74 Shuman Moss, L. A., Jensen-Taubman, S. and Stetler-Stevenson, W. G. 2012. matrix metalloproteinases: changing roles in tumor progression and metastasis. *The American journal of pathology*, *181*(6), 1895–1899.

75 Sikka, K., Sharma, S. C., Thakar, A. and Dattagupta, S. 2012. Evaluation of epithelial proliferation in paediatric and adult cholesteatomas using the Ki-67 proliferation marker. *The Journal of laryngology and otology*, *126*(5), 460–463.

76 Smirnova, M. G., Birchall, J. P. and Pearson, J. P. 2004. The immunoregulatory and allergy-associated cytokines in the aetiology of the otitis media with effusion. *Mediators of inflammation*, *13*(2), 75–88.

77 Song, J. J., Chae, S. W., Woo, J. S., Lee, H. M., Jung, H. H. and Hwang, S. J. 2007. Differential expression of human beta defensin 2 and human beta defensin 3 in human middle ear cholesteatoma. *The Annals of otology, rhinology, and laryngology*, *116*(3), 235–240.

78 Stamatas, G. N., Roux, P. F., Boireau-Adamezyk, E., Lboukili, I. and Oddos, T. 2023. Skin maturation from birth to 10 years of age: Structure, function, composition and microbiome. *Experimental dermatology*, *32*(9), 1420–1429.

79 Stefanini, M., De Martino, C. and Zamboni, L. 1967. Fixation of ejaculated spermatozoa for electron microscopy. *Nature*, *216*(5111), 173–174.

80 Suchozebrska-Jesionek, D., Szymański, M., Kurzepa, J., Gołabek, W. and Stryjecka-Zimmer, M. 2008. Gelatinolytic activity of matrix metalloproteinases 2 and 9 in middle ear cholesteatoma. *Journal of otolaryngology - head & neck surgery*, *37*(5), 628–632.

81 Sudhoff, H., Dazert, S., Gonzales, A. M., Borkowski, G., Park, S. Y., Baird, A., Hildmann, H. and Ryan, A. F. 2000. Angiogenesis and angiogenic growth factors in middle ear cholesteatoma. *The American journal of otology*, *21*(6), 793–798.

82 Takabatake, K., Shimo, T., Murakami, J., Anqi, C., Kawai, H., Yoshida, S., Wathone Oo, M., Haruka, O., Sukegawa, S., Tsujigiwa, H., Nakano, K. and Nagatsuka, H. 2019. The Role of *Sonic hedgehog* Signaling in the Tumor Microenvironment of Oral Squamous Cell Carcinoma. *International journal of molecular sciences*, *20*(22), 5779.

83 Teibe, U. 2007. Bioloģiskā statistika. LU

84 Wehkamp, K., Schwichtenberg, L., Schröder, J. M. and Harder, J. 2006. Pseudomonas aeruginosa- and IL-1beta-mediated induction of human beta-defensin-2 in keratinocytes is controlled by NF-kappaB and AP-1. *The Journal of investigative dermatology*, *126*(1), 121–127.

85 Welkoborsky, H. J., Jacob, R. S. and Hinni, M. L. 2007. Comparative analysis of the epithelium stroma interaction of acquired middle ear cholesteatoma in children and adults. *European archives of oto-rhino-laryngology*, *264*(8), 841–848.

86 Xu, Y., Tao, Z. Z., Hua, Q. Q., Wang, X. C., Xiao, B. K. and Chen, S. M. 2009. Activation of nuclear factor-kappaB and aberrant gene expression of interleukin 6 in middle ear cholesteatoma. *Chinese journal of otorhinolaryngology head and neck surgery*, *44*(3), 192–196.

87 Yeşilova, M., Görür, K., Ismi, O., Özcan, C. and Büyükafşar, K. 2017. The Role of Rho/Rho-Kinase Pathway in the Pathogenesis of Cholesteatoma. *Otology & neurotology*, *38*(4), 516–520.

88 Yetiser, S., Satar, B. and Aydin, N. 2002. Expression of epidermal growth factor, tumor necrosis factor-alpha, and interleukin-1alpha in chronic otitis media with or without cholesteatoma. *Otology & neurotology*, *23*(5), 647–652.

89 Zang, J., Yang, B., Feng, S. and Jiang, X. 2019. Low expression of microRNA-125b enhances the expression of STAT3 and contributes to cholesteatoma growth. *Archives of medical science: AMS*, *18*(6), 1596–1606.

90 Zhu, J., Zhang, X., Ai, L., Yuan, R. and Ye, J. 2019. Clinicohistopathological implications of MMP/VEGF expression in retinoblastoma: a combined meta-analysis and bioinformatics analysis. *Journal of translational medicine*, *17*(1), 226.

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