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ORIGINAL ARTICLE

Bacterial Infections and Osteoclastogenesis Regulators in Men and Women with Cholesteatoma

Wirginia Likus[1] · Krzysztof Siemianowicz[2] · Jarosław Markowski[3] · Jan Wiaderkiewicz[4,5] · Anna Kostrząb-Zdebel[6] · Edyta Jura-Szołtys[7] · Włodzimierz Dziubdziela[8] · Ryszard Wiaderkiewicz[9] · Marek J. Łos[10,11]

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Abstract One of the most distinct features of middle ear cholesteatoma is bone destruction. Aetiology of cholesteatoma is thought to be multifactorial. Endotoxins produced by bacteria are thought to initiate the inflammation process in the middle ear leading to cholesteatoma. There are physiological differences in bone metabolism between men and women. The aim of our study was the immunohistochemical evaluation of the contents of two key components of the OPG/RANK/RANKL triad—RANKL and OPG in cholesteatoma, to analyse if there are any differences between the sexes and to evaluate the bacteria species isolated from cholesteatoma just before surgical treatment and to evaluate their plausible influence on the expression of OPG and RANKL in cholesteatoma. Twenty-one adult patients with acquired cholesteatoma who underwent surgery were analysed. There were no statistically significant differences in the expression of both regulators of osteoclastogenesis between the sexes. In 38.1 % patients cholesteatoma was not infected, whereas in 61.9 % patients various bacterial infections or mycosis were found. The most frequently isolated species was *Pseudomonas aeruginosa* (14.29 % infections) followed by *Staphylococcus aureus* (9.52 % infections). There were no statistically significant differences in expression of both OPG and RANKL between uninfected and infected cholesteatomas.

Keywords Cholesteatoma · Osteoprotegerin · RANKL · Bacterial infection · Sexes

Introduction

Cholesteatoma is a non-neoplastic, progressive keratinizing cyst-like epithelial lesion of the temporal bone. It is lined by stratified squamous epithelium containing desquamated

✉ Wirginia Likus
wirginia.likus@gmail.com

✉ Marek J. Łos
mjelos@gmail.com

[1] Department of Human Anatomy, School of Medicine, Medical University of Silesia, 18 Medyków Str., Bldg. C-1, 40-752 Katowice, Poland

[2] Department of Biochemistry, School of Medicine, Medical University of Silesia, Katowice, Poland

[3] ENT Department, School of Medicine, Medical University of Silesia, Katowice, Poland

[4] Center for Experimental Medicine, Medical University of Silesia, Katowice, Poland

[5] Department of Pharmacology and Physiology, George Washington University, Washington, DC, USA

[6] Department of Pathomorphology, School of Medicine, Medical University of Silesia, Katowice, Poland

[7] Clinical Department of Internal Diseases, Allergology and Clinical Immunology, Medical University of Silesia, Katowice, Poland

[8] Outpatient Clinic for Treatment of Chronic Pain, Sosnowiec, Poland

[9] Department of Histology, School of Medicine, Medical University of Silesia, Katowice, Poland

[10] Department of Clinical and Experimental Medicine, Division of Cell Biology Integrative Regenerative Medicine Center (IGEN), Linköping University, Cell Biology Building, Level 10, 581 85 Linköping, Sweden

[11] Department of Pathology, Pomeranian Medical University, Szczecin, Poland

keratin. It can be either congenital or acquired. The main feature of cholesteatoma is the proliferation of epithelium with aberrant micro-architecture into the middle ear and mastoid. The uncontrolled progression of cholesteatoma leads to a destruction of the bony structures of the middle ear and sometimes the inner ear and/or mastoid (Bassiouny et al. 2012; Maniu et al. 2014). This disease leads to a conductive and sensoneurinal hearing loss, lateral semicircular canal fistula and facial palsy. If surgery is not applied at a proper time, cholesteatoma can lead even to intracranial complications. Surgery is the only available treatment of cholesteatoma. There are no available medicaments either to cure or to prevent disease or its recurrence (Welkoborsky 2011).

The aetiology of cholesteatoma has not been elucidated yet. It is thought to be multifactorial (Maniu et al. 2014). The response of immune system to injury, irritation or infection in the middle ear is thought to play an important role in the pathogenesis of cholesteatoma. Endotoxins produced by bacteria are thought to initiate the inflammation process in the middle ear. The cells of inflammatory response, macrophages, produce pro-inflammatory cytokines, tumour necrosis factor (TNF)-α and interleukin (IL)-1β, which stimulate keratinocytes to produce other mediators of inflammation and augment the process. It leads to a formation of inflammatory granulation process, which starts the development of cholesteatoma. A transformation of keratinocytes into migratory and proliferative cells is an important step in a development of cholesteatoma. Inflammation and infection seem to be a universal setting for the development of acquired cholesteatoma (Maniu et al. 2014).

The destruction of bone structures is responsible for the most serious complications of cholesteatoma such as bone erosion of the ossicles, otic capsule, Fallopian canal, tegmen tympani and tegmen mastoideum (Maniu et al. 2014).

Osteoprotegerin (OPG), receptor activator of nuclear factor κB (RANK) and RANK ligand (RANKL) constitute a molecular triad associated with bone metabolism, vascular calcification and a development of the immune system through dendritic cells (Welkoborsky 2011).

Bone is a complex tissue undergoing simultaneously two processes: osteogenesis and osteolysis. The dynamic balance of these two processes enables remodelling of bones. The activity of both osteoclasts and osteoblasts is regulated by a complex network of hormones, cytokines, local growth factors and adhesion molecules. A set of cytokines, termed TNF superfamily, plays a pivotal role in regulating osteoclasts' cell functions. One system of this superfamily has been shown to play crucial role in controlling osteogenesis and pathophysiological bone remodelling. It is the triad: RANK, RANKL and OPG. RANKL is expressed as a membrane type molecule. However, it can undergo a proteolytic cleavage into soluble form retaining its biological activity. RANKL has been shown to stimulate osteoclast generation. OPG binds RANKL preventing in this way the association of RANKL with RANK. OPG plays a crucial role in regulating osteoclast formation. In this way OPG regulates osteolysis and prevents uncontrolled bone loss (Baud'huin et al. 2007; Boyce et al. 2008; Vitovski et al. 2007).

There are some differences of physiological bone metabolism between the sexes. Women are more prone to bone loss due to their hormonal conditions. It has not been evaluated yet if there are any differences between the sexes in the expression of OPG and RANKL in cholesteatoma.

The aim of our pilot study was the immunohistochemical evaluation of the contents of two key components of the OPG/RANK/RANKL triad—RANKL and OPG in cholesteatoma and to analyse if there are any differences between the sexes. The next goal of our study was to evaluate the bacteria species isolated from cholesteatoma just before surgical treatment and to evaluate their plausible influence on the expression of OPG and RANKL in cholesteatoma.

Materials and Methods

Twenty-one adult patients aged 41.72 ± 16.57 years (10 men aged 40.7 ± 16.0 years and 11 women aged 42.6 ± 17.7 years) with diagnosed acquired cholesteatoma were introduced into the study. There were no statistically significant differences of age between male and female patients. Only patients with previously histopathologically diagnosed cholesteatoma of middle ear were enrolled into the study. The duration of cholesteatoma was between 1 and 8 years. Patients were treated with topical antibiotics to obtain dry ear prior to surgery. In each case all efforts were made to create dry ear. This aim was obtained in 85.7 % patients. The surgical treatment usually was performed within 1–2 months after creating dry ear. In remaining 14.3 % subjects, despite intensive topical antibiotic drop treatment connected with careful ear clearing: daily vacuum pressure suck using a microscope and general antibiotic therapy (Amoxicillin with clavulanic acid twice 1.0 or Ciprofloxacin twice 0.5 or Cephuroxime twice 0.5) dry ear was not created. In these patients surgery was performed on wet ear. The swabs for bacterial cultures were collected from middle ear just before the surgical resection of cholesteatoma. Cholesteatoma tissues resected during surgery were sent to the department of pathomorphology for immunohistochemical analysis. Localization of cholesteatoma was as follows: attic (epitympanum)—16, atticoantrum—13, mesotympanum—9 and hypotympanum—6 cases. In some subjects more than one part of ear was affected.

Immunohistochemistry

Tissue fragments were fixed overnight in 10 % neutral-buffered formalin (phosphate buffer), subsequently passed through graded alcohol solutions, processed three times in xylene and finally embedded in paraffin blocks. Blocks were cut into 7 μm thick slices, placed on slides (Superfrost Ultra Plus, Thermo Scientific, Waltham, USA), deparaffinized and rehydrated. In order to uncover antigen binding sites, the slides were submerged in a buffered citric acid solution (Antigen Unmasking Solution, Vector Labs, Burlingame, USA) and microwaved for a total of 10 min in four equal sessions, left to cool to room temperature and washed with distilled water and PBS-T (phosphate buffered salt with 0.05 % Tween) subsequently for 5 min each. To prevent nonspecific binding of antigens, all slides were incubated with a 10 % rabbit serum for 90 min in room temperature. Next, all sections were incubated with primary anti OPG 1:200 (GTX62914, GeneTex, Irvine, USA) or anti RANK 1:500 (GTX108515, GeneTex, Irvine, USA) antibodies overnight in 4 °C. Slides were washed three times in PBS-T and blocked with 1.5 % (vol/vol) H_2O_2 for 10 min to quench endogenous peroxidase activity. Slides were again washed three times in PBS-T and then incubated with a biotinylated secondary antibody (Vectastain ABC Kit, Vector Laboratories) for 30 min. Slides were washed three times with PBS-T and incubated with the avidin–biotin complex peroxidase kit (Vectastain ABC Kit, Vector Laboratories) for another 30 min. After washing with water, freshly prepared DAB (Vectastain ABC Kit, Vector Laboratories) was added to the slides for 1 min. The slides were washed with water and passed through graded solutions of alcohol and xylene. The expression of OPG and RANK proteins was determined by assigning the measured mean grey value within the stratum basale of analysed tissue fragments to one of four levels: absent (−), low (+), medium (++) and high (+++) using the open source image analysis software Fiji (Prodanov and Verstreken 2012; Schindelin et al. 2012). The documentation of immunohistochemical reactions was performed with a DP-21 camera (Olympus) coupled with a Nikon Eclipse E400 optical microscope. HE and immunohistochemical stainings are presented in Figs. 1 and 2.

Statistical Analysis

The expression of OPG and RANK proteins was presented as mean and standard deviation of grey value within the stratum basale of analysed tissue fragments of cholesteatoma. In the evaluation of microbiological profile descriptive statistics was used. To compare OPG and RANKL expressions Cochran-Cox test was used. The statistical analysis was performed using the Statistica 10.0 PL software (StatSoft). A p value of ≤ 0.05 was statistically significant.

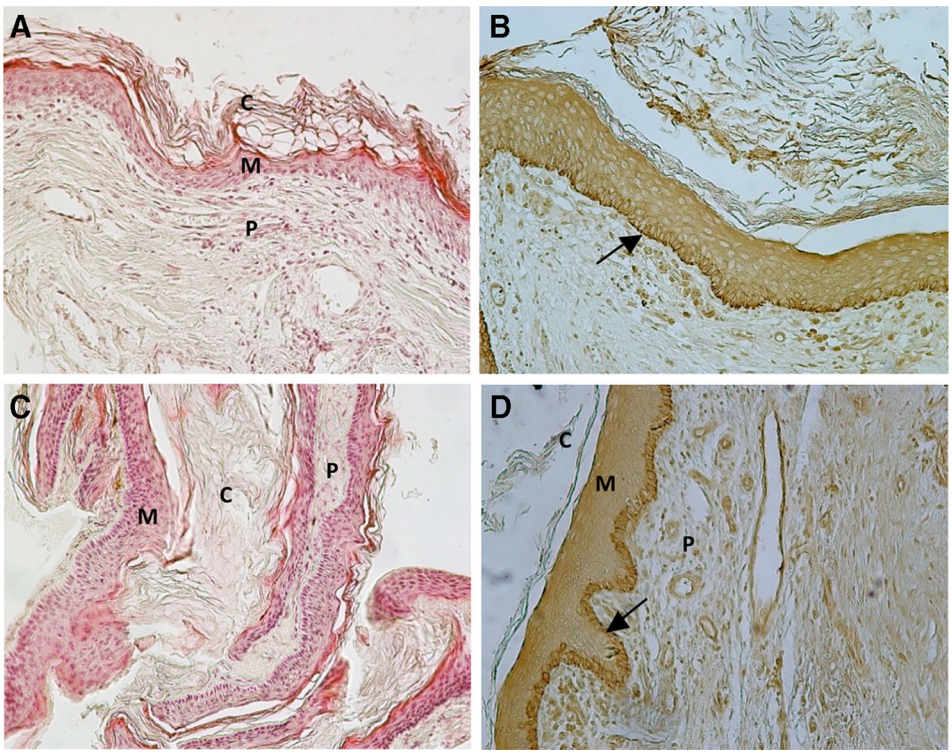

Fig. 1 Cholesteatoma tissue from the patients with confirmed (**a**, **b**) or not confirmed (**c**, **d**) infection (magnifition: ×400). *M* matrix (stratified squamous epithelium), *P* perimatrix (subepithelial connective tissue), *C* cystic content (keratin). Staining—HE (**a**, **c**); anti-RANKL antibody (**b**, **d**). *Arrows* point to strong reaction in basal layer of epithelium. Note: The bone was not included in the fragments of cholesteatoma tissue as to stain bones the tissue fragments have to be treated with strong acids (14 % HCl) what destroys the epitops required for immunostaining

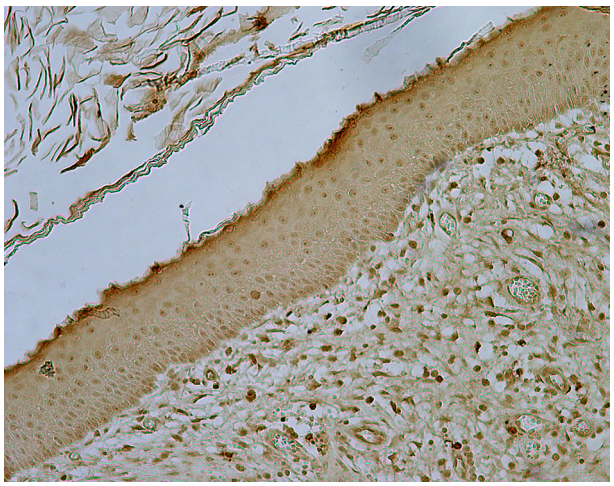

Fig. 2 Cholesteatoma tissue stained with the use of anti-OPG antibody (magnifition: ×200). Expression visible mostly in individual connective tissue cells. Level of expression differs (as shown in Table 1)

Results

The distribution of levels of OPG and RANKL expression among studied patients is presented in Fig. 3.

Sexes

Statistical analysis did not show any significant differences in the expression of both regulators of osteoclastogenesis between the sexes (Table 1).

In men a number of cells both OPG-negative and RANKL negative in cholesteatoma tissue was twice more frequent than in women (Table 1). In men a percentage of OPG-negative cells in cholesteatoma was three times more frequent than in women (30 and 9.1 %, respectively; Table 1). However, these differences did not reach the statistical significance.

Microbiological Isolates

In 38.1 % patients cholesteatoma was not infected, whereas in 61.9 % patients various bacterial infections or mycosis were found. Microbiological profile of species isolated from cholesteatoma is presented in Table 2. The most frequently isolated species was *Pseudomonas aeruginosa* (14.29 % infections) followed by *Staphylococcus aureus* (9.52 % infections; Table 2). Bacteriological culture and antibiogram showed that there were no antibiotic resistant bacteria species either in discharge or flake masses.

Our research also revealed that more infections were caused by more than one species, i.e. *S. aureus* and *Haemophilus influenzae*, *Coagulase negative staphylococcus* and *Aspergillus spp*, *Proteus penneri* and *Corynebacterium spp*, *Candidia albicans* and *Proteus mirabilis* or *Morganella morganii* and *Escherichia coli*.

The percentage of cholesteatomas uninfected and infected was similar in men (36.36 and 63.64 %, respectively) and women (40 and 60 %, respectively; Table 3). In men cholesteatoma was most often infected with *Coagulase negative staphylococcus* (20 % patients) while in women the most often isolated pathogen was *P. aeruginosa* (18.18 % patients). In women more frequently the infection was caused by more than one species (Table 3).

There were no statistically significant differences in expression of both OPG and RANKL between uninfected and infected cholesteatomas.

Discussion

Acquired middle ear cholesteatomas are characterized by intense inflammatory reaction leading to soft tissue and bone destruction. Many scientists have focused their research on mechanisms regulating bone resorption. OPG/

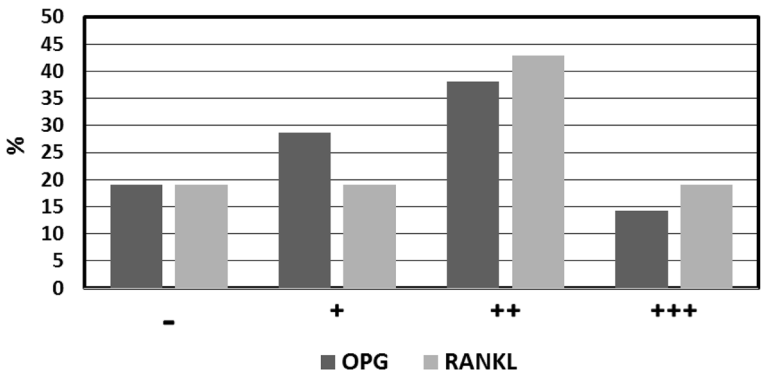

Fig. 3 Percentage of patients with various levels of OPG and RANKL protein expression ("–": absent, "+": low expression, "++": medium expression, "+++": high expression of examined protein in cholesteatoma tissue)

Table 1 Level of expression of OPG and RANKL in both sexes ("−": absent, "+": low expression, "++": medium expression, "+++": high expression of examined protein in cholesteatoma tissue)

Level expression of OPG and RANKL	OPG		RANKL	
	% Male	% Female	% Male	% Female
−	30.00	9.10	20.00	18.18
+	30.00	27.27	20.00	18.18
++	30.00	45.45	40.00	45.45
+++	10.00	18.18	20.00	18.18

Table 2 Microbiological profile of isolates obtained from swabs taken from middle ear ($n = 21$)

Infection	% Patients
Without bacterial infection	38.10
Staphylococcus aureus	9.52
Staphylococcus aureus and *Haemophilus influenza*	4.76
Serratia marcescens	4.76
Pseudomonas aeruginosa	14.29
Coagulase negative staphylococcus	9.52
Coagulase negative staphylococcus and *Aspergillus spp.*	4.76
Proteus penneri and *Corynebacterium spp*	4.76
Candidia albicans and *Proteus mirabilis*	4.76
Morganella morganii and *Escherichia coli*	4.76

Table 3 Microbiological profile of isolates obtained from swabs taken from middle ear in men and women

Pathogen	% Male	Pathogen	% Female
Without bacterial infection	40.00	Without bacterial infection	36.36
Staphylococcus aureus	10.00	*Staphylococcus aureus*	9.09
Pseudomonas aeruginosa	10.00	*Staphylococcus aureus* and *Haemophilus influenzae*	9.09
Coagulase negative staphylococcus	20.00	*Serratia marcescens*	9.09
Coagulase negative staphylococcus and *Aspergillus spp.*	10.00	*Pseudomonas aeruginosa*	18.18
Morganella morganii and *Escherichia coli*	10.00	*Proteus penneri* and *Corynebacterium spp*	9.09
		Candidia albicans and *Proteus mirabilis*	9.09

RANK/RANKL system has been widely studied in cholesteatomas. Researchers evaluated the expression of this protein either in various parts of cholesteatoma or compared it with a normal skin obtained from external auditory meatal. Kuczkowski et al. (2010a, b) found a higher expression of OPG and RANKL in cholesteatoma. This team observed that RANKL-positive cells were detected mainly in stroma, whereas OPG-positive cells were present in cholesteatoma epithelium. That study also revealed a higher expression of TNF-α in cholesteatoma. However, the level of the studied proteins did not correlate with a stage of bone destruction (Kuczkowski 2010b). Xia et al. (2007) evaluated the expression of all elements of this triad finding that RANK and RANKL were overexpressed in infiltrated lymphocytes in cholesteatoma whereas OPG was significantly higher in cholesteatoma tissue compared with normal skin. Interesting results were obtained by Jeong et al. (2006) who observed the contradictory results of OPG and RANKL in cholesteatoma. The count and rate of RANKL-positive cells were significantly higher in cholesteatoma, but the count and rate of OPG-positive cells were significantly higher in normal skin comparing to cholesteatoma. An increased expression of RANKL in cholesteatoma was also detected by Ma and Ye (2008). However, this research team did not observe any statistically significant differences in OPG expression between cholesteatoma and normal skin. They used a similar methodology as in our study, the analysis of optical density (Ma and Ye 2008).

Some researchers pointed out that there were some differences in the histopathological and immunohistochemical characterization of cholesteatoma in children and adults. Acquired cholesteatoma in children had a higher cellular inflammatory infiltrates while in adults the degree of fibrosis was significantly higher. They concluded that paediatric cholesteatoma was more aggressive and invasive (Bassiouny et al. 2012). These findings are confirmed by our results. HE staining showed a very modest inflammatory component.

We are the first who compared the adult acquired cholesteatoma in men and women and found no significant differences in the expression of both OPG and RANKL.

Recently, more and more attention is paid to the role of cytokine-mediated inflammation in the pathogenesis of cholesteatoma. Haruyama et al. (2010) observed that expression of IL-17 was correlated with RANKL and the degree of bone destruction. Bacterial infections have been

thought to be important complications of cholesteatoma. However, recent years have put new light on their role in the pathogenesis of this pathology. Bacterial biofilms seem to play an important role in this process. This term describes bacterial communities enclosed in matrix excreted by them which is adherent to the surface of mucosa or implant. Bacteria creating biofilm are resistant to antibiotics leading to a chronicity of the infection. Many species are capable to create a bacterial biofilm, e.g. *P. aeruginosa, Haemophilus influenzae, Streptococcus pneumoniae* and *S. aureus* (Lampikoski et al. 2012; Maniu et al. 2014). Some researchers have postulated that bacterial biofilms may be responsible for the aggressiveness of cholesteatoma. In our research we did not observe any statistically significant difference in the expression of OPG and RANKL between patients with and without bacterial infections.

A study of Madana et al. (2011) showed that *P. aeruginosa* was the most often isolated bacteria in children with chronic suppurative otitis media. In our study comprising adult patients this bacteria was detected in 10 % male and 18 % female patients. Si et al. (2013) used *P. aeruginosa* in his mouse experimental model of cholesteatoma. When autologous meatal skin graft had been implanted into the middle ear and infected with *P. aeruginosa*, cholesteatoma developed in 92 % experimental animals. In another animal model, gerbils with induced cholesteatomas, a concomitant *P. aeruginosa* infection caused an increase of cholesteatoma size and invasiveness comparing to uninfected animals. This infection also increased morbidity among studied gerbils (Jung et al. 2011).

In our study we detected one case of *Escherichia coli* infection concomitant with *Morganella morgani*. This infection may be potentially dangerous. Mirza et al. (2014) reported a case of *E. coli* positive infratentorial subdural empyema secondary to mastoiditis and underlying cholesteatoma.

In our study only one female had *Candida albicans* infection. This mycosis was accompanied by *Proteus mirabilis* infection. Our microbiological research was based on isolates obtained from swabs. Effat and Madany (2014) used keratin samples obtained during surgery. In his study fungal infections were present in 89 % samples. The differences in percentages of bacterial infections detected by various researchers may be caused by various techniques of obtaining material for microbiological cultures. Bacterial biofilms may be nearly impossible to detect with standard techniques (Kaya et al. 2013). In our study we did not collect bacterial swabs from external auditory tube, what can be done in out-patient department settings. The swabs were collected from middle ear just before the surgical resection of cholesteatoma. The very fact of reaching a "dry ear" condition in the course of pre-surgery procedure, revealed during physical examination, as a local condition of the middle ear being ready for surgery (qualifying it for surgical treatment), does not give the evidence of the fact that no single colony of bacteria is present in deeper layers of cholesteatoma shells and at the border with bones. It is not possible to reach a completely sterile ear condition using conservative therapy only. Microbial biofilms can be often met in patients with cholesteatoma. Kaya et al. (2013) observed bacterial biofilms by 61.5 % patients suffering from cholesteatoma. Chole and Faddis (2002) point out that existence of bacterial biofilms may explain clinical characteristics of cholesteatoma, that is, persistence and recurrence of infection with surgical treatment being the only effective method of therapy. The nature of recurrence may explain the seeming discrepancy between the findings of clinical examination suggesting the eradication of bacterial infection and findings of microbiological examination on swabs taken from middle ear just before the excision of cholesteatoma. Thus, the achievement of "dry ear" condition on pre-surgery examination in case of topical and general application of antibiotics in 85.7 % of the cases is not in discrepancy with the fact that only in 38.1 % of microbiological examinations (swabs taken on the operating table) the cultures were sterile.

A conclusion from our research could be drawn, that the "dry ear" condition is not synonymous with its actual sterile condition. Only surgical procedures really allow to free the ear from pathological bacteria. It is not possible to have a sterile middle ear by means of conservative treatment, in the course of inflammations with cholesteatoma. Thus, the old principle followed in laryngology has been confirmed again, that each ear with cholesteatoma inflammation must be operated on, not only due to possible complications, but also because of chronic inflammatory condition. Our results demonstrate that neither the presence of bacterial infections nor the sex influence the expression of two most important components of OPG/RANK/RANKL system. As our research is a pilot study and it has some limitations a continuation of this research on a larger group of patients is required.

No standard treatment is available to either cure or to prevent the recurrence of cholesteatoma (Welkoborsky 2011). Thus, it is important to examine the disease from a different angle. Perhaps the pathophysiology of the disease involves local deregulation of apoptosis, necroptosis, or autophagy (Chaabane et al. 2013; Jain et al. 2013). Alternatively, the underlying mechanism causing cholesteatoma may involve local deregulation of stem cell growth and differentiation. Recently identified novel drugs, like, for example, salinomycin that preferentially targets cancer-stem cells (Jangamreddy et al. 2013; Wasik et al. 2014), and apoptins that preferentially target cells that exhibit

uncontrolled growth (Chaabane et al. 2014; Rollano Peñaloza et al. 2014), may need to be tested as novel experimental treatments for cholesteatoma.

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