ACTA OTORHINOLARYNGOLOGICA ITALICA 2021;41:371-376; doi: 10.14639/0392-100X-N1413

Otology

Pattern of cholesteatomas under a scanning electron microscope - a risk factor for bone resorption

Modello di colesteatomi al microscopio elettronico a scansione - un fattore di rischio per il riassorbimento osseo

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SUMMARY

Objective. The damaging effect of cholesteatomas presents mainly as bone resorption by osteoclasts located in the space between the bone and perimatrix. This process is initiated by the molecular cascade of osteoclast differentiation factors. The aim of the study is to analyse cholesteatoma microstructures via scanning electron microscope (SEM), and associate them with risk and grade of bone erosion.

Methods. Pathological middle ear tissue fragments with cholesteatoma visible under a microscope were collected from 58 patients operated on for chronic otitis media with features of bone defects in the middle ear walls. These fragments were examined under a scanning electron microscope.

Results. Analysis of the cholesteatomas' surface under a SEM revealed both regular and irregular structure of the matrix, most being the latter. Irregular matrix structures were observed in cases with a short disease history and in patients for whom this was the first surgical procedure. In our analysis, a cholesteatoma matrix with regular structures was associated with less bone destruction of the middle ear space.

Conclusions.The microstructure of cholesteatomas that showed regular layers under SEM coincides with reduced destruction of the middle ear bone walls. An irregular structure (pathognomonic for a process with a short medical history, and in patients operated on for the first time) is characterised by a tendency towards deeper destruction of bone tissue.

KEY WORDS: *chronic cholesteatoma otitis media, microstructure of cholesteatoma, scanning electron microscope, bone defects*

RIASSUNTO

Obiettivo. *L'effetto dannoso dei colesteatomi si presenta principalmente come riassorbimento osseo da parte degli osteoclasti situati nello spazio tra l'osso e la peri-matrice. Questo processo è avviato dalla cascata molecolare dei fattori di differenziazione degli osteoclasti. Lo scopo dello studio è analizzare le microstrutture del colesteatoma tramite microscopio elettronico a scansione (SEM) e associarle al rischio e al grado di erosione ossea.*

Metodi. *Sono stati raccolti frammenti patologici di tessuto dell'orecchio medio con colesteatoma visibile al microscopio da 58 pazienti operati per otite media cronica con difetti ossei delle pareti dell'orecchio medio. Questi frammenti sono stati esaminati al microscopio elettronico a scansione.*

Risultati. *L'analisi della superficie dei colesteatomi al microscopio elettronico a scansione ha rivelato una struttura irregolare della matrice nella maggioranza dei casi. Essa in particolare è stata osservata nei pazienti con una breve storia di malattia e nei pazienti sottoposti ad una prima procedura chirurgica. Nella nostra analisi, una matrice di colesteatoma con strutture regolari è stata associata a una minore distruzione ossea dello spazio dell'orecchio medio.*

Conclusioni. *1. La microstruttura dei colesteatomi che mostravano strati regolari al SEM coincide con una ridotta distruzione delle pareti ossee dell'orecchio medio. 2. Una struttura irregolare (patognomonica per un processo con una storia medica breve, e nei pazienti operati per la prima volta) è caratterizzata da una tendenza alla distruzione più profonda del tessuto osseo.*

PAROLE CHIAVE: *colesteatoma cronico otite media, microstruttura del colesteatoma, microscopio elettronico a scansione, difetti ossei*

Received: January 23, 2021
Accepted: February 27, 2021

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Funding
This research was supported by statutory funds of the Department of Otolaryngology of the Jagiellonian University, Krakow, Poland.

Conflict of interest
The Authors declare no conflict of interest.

How to cite this article: Wiatr A, Job K, Wiatr M. Pattern of cholesteatomas under a scanning electron microscope - a risk factor for bone resorption. Acta Otorhinolaryngol Ital 2021;41:371-376. https://doi.org/10.14639/0392-100X-N1413

Introduction

A joint opinion on the definition and classification of cholesteatoma was announced at the 10th International Conference on Cholesteatoma and Ear Surgery in June 2016 in Edinburgh [1]. Histologically, a cholesteatoma is made up of keratinised stratified squamous epithelium and keratin, resulting from the process of desquamation of keratinocytes without nuclei. These elements are parts of the cholesteatoma matrix and together with stromal cells form the cholesteatoma mass. About 3-6 in 100,000 people are diagnosed with cholesteatoma, and can be classified as either acquired or congenital.

The cholesteatoma perimatrix is made of connective tissue (collagen and reticulin fibres) and inflammatory cells (neutrophils, histiocytes and lymphocytes). Operated patients who were diagnosed with chronic cholesteatomatous otitis media tend to suffer from bone tissue erosion. The damaging effect of cholesteatoma presents mainly in the destruction of the ossicular chain, caused by bone resorption by osteoclasts located in the space between the bone and the cholesteatoma perimatrix. The process of bone resorption in cholesteatoma is initiated by a molecular cascade of osteoclast differentiation factors, which include osteoprotegerin (OPG), the receptor activator of nuclear factor κB (RANK), located on osteoclasts and the receptor activator of nuclear factor κB ligand (RANKL), located on the superficial layer of osteoblasts. This system is the most important cascade regulating bone erosion. The long process of the incus is usually the fastest to erode. Bone defects may occur within the prominence of the lateral semicircular canal and the facial canal. The inflammatory process may spread to the labyrinth, sigmoid sinus and into the cranial cavities [2-4].

The purpose of the study is to analyse cholesteatoma microstructures via scanning electron microscope (SEM) and associate them with risk and degree of bone erosion.

The aim of the analysis is to assess the relationship between the microstructure of cholesteatoma and degree of damage to adjacent bone structures.

The specific goal is to identify variants of the cholesteatoma characterised by a tendency towards aggressive osteolysis of bone tissue and consequently, causing damage to the elements of the auditory ossicle chain as well as the bones of the middle ear, which opens the way for the development of ear-related complications

Materials and methods

Pathological middle ear tissue fragments with cholesteatoma visible under SEM were collected from 58 patients operated on due to chronic otitis media with middle ear wall damage. The group included 27 women and 31 men. The youngest patient was 27 years old, and the oldest was 70.

The Local Bioethics Committee issued approval no. 112.6120.206.2016 for this study on 27 June 2016. The procedures were performed from 2016 to 2019. All methods were performed in accordance with relevant guidelines and regulations. Informed consent was obtained from all subjects or their parents and/or legal guardian if subjects were under 18 years old. Afterwards, the histopathological characteristics were observed.

The extracted material was prepared by fixing it in 4% formalin, and then dehydrated in a series of ethyl alcohols at increasing concentrations: 35%, 50%, 75%, 96% and 99.9%. Once prepared, the samples were subjected to air-drying and subsequently attached onto slides using glue (Electrodag 915 Silver Paint by TAAB). The samples were then sprayed with a thin layer of gold using a JEOL JEE-4x vacuum evaporator. An analysis of the prepared samples was performed with the use of the JEOL JSM35CF scanning microscope at the Laboratory of Scanning Microscopy.

SEM analysis took into account the following parameters and data:

1. Analysis of images at 30x, 100x, 200x, 1000x, 2000x magnification.
   a. The structure of the cholesteatoma: matrix and perimatrix (m/p).
   b. Matrix structure (regular and irregular).
   c. Blood vessels present in cholesteatoma (matrix and perimatrix).
   d. Surgery I – first operation, II – subsequent operation.

The statistical analysis was performed using Statistica software. A Chi-square test with cross tabulation was used to verify non-parametric hypotheses. This test was used for qualitative variables. The test was then confirmed by the Chi-square goodness of fit test. Statistically significant results were considered for $p < 0.05$ [5].

Results

Between 2016 and 2019, 440 operations were performed on patients due to chronic otitis media. Cholesteatoma was found in 130 cases. Destruction of the bone walls of the middle ear was present in 58 patients with cholesteatoma, which constitutes about 10% of patients operated on for chronic otitis media. These patients were further analysed.

Analysis of the cholesteatoma specimens under SEM in all discussed cases showed structures consisting of a matrix and perimatrix.

In 35% of cases (20 of 58 patients), the matrix structure was regular, with separate layers. In the remaining 65% of

Table I. The differences between regular and chaotic matrix structure of cholesteatoma.

	Regular (20 patients)	Irregular (38 patients)
Facial nerve canal defect	18 (90%)	22 (70%)
Lateral semicircular canal defect	1 (5%)	4 (16%)
Base of middle cranial fossa defect	1 (5%)	12 (16%)
Vascularisation of cholesteatoma	No	No
Perimatrix evaluation	Very high cell density	Very high cell density
First surgery	4 patients	29 patients
Reoperation	16 patients	9 patients

cases (38 of 58 patients), the structure was chaotic and irregular.

In cases with a regular matrix structure, bone destruction was visible under SEM at 100x-200x magnification and greater. In cases of irregular matrix structure, the damage in some patients was already visualised at 30x magnification. Most cases of regular, layered structure in cholesteatomas were found in patients who were reoperated on, with a long disease duration. An irregular structure of cholesteatoma was observed in 65% of patients, mostly operated on for the first time due to chronic otitis media. In these patients, the features of chronic otitis media were observed no later than 2 years prior to surgical treatment, indicating a short medical history.

The differences between patients with regular and irregular cholesteatoma matrix structures are presented in Table I.

In all patients, damage to the elements of the ossicular chain was observed, most often involving 2 or 3 ossicles. Cholesteatoma with regular, layered structures were characterised by a significantly lower comorbidity of bone wall defects in the middle ear ($p < 0.05$). In this group, facial nerve fistulas were often observed.

In cases of irregularly structured cholesteatoma, defects in the bone walls with the potential for ear-related complications were statistically significant ($p < 0.05$) compared to cholesteatomas with regular structures. These complications can be both intratemporal (defects of the facial nerve canal, or defects in the horizontal semicircular canal) and intracranial (defect of the skull in the medial fossa).

A defect in the base of the middle cranial fossa was observed in 12 patients. In 4 patients, a fistula on the horizontal semicircular canal was also present. In these patients, the most common form of bone defect was damage to the facial nerve canal, found in over 60% of cases with an irregular matrix structure.

The following results were obtained from the SEM assessment of cholesteatoma (Figs. 1-4).

No vessels were found in any of the preparations analysed, regardless of the structure of the cholesteatoma. In each

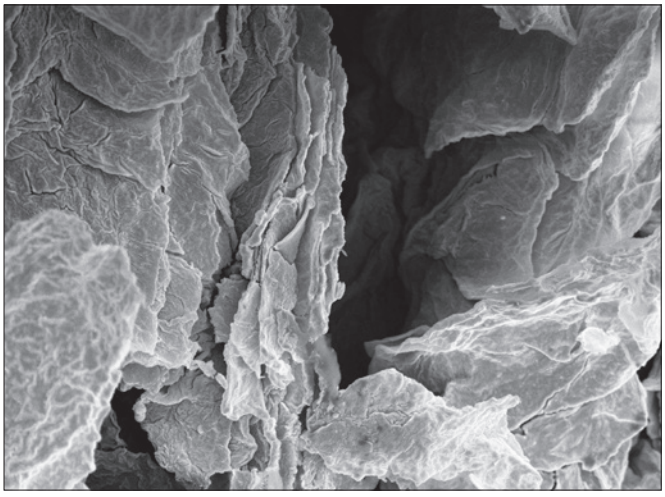

Figure 1. Matrix, x2000. (Focal corneocyte desquamation in the matrix. Squamous keratinocytes of the matrix, showing unordered stacking pattern and irregular microplicae on the surface. Workshop of Scanning Microscopy).

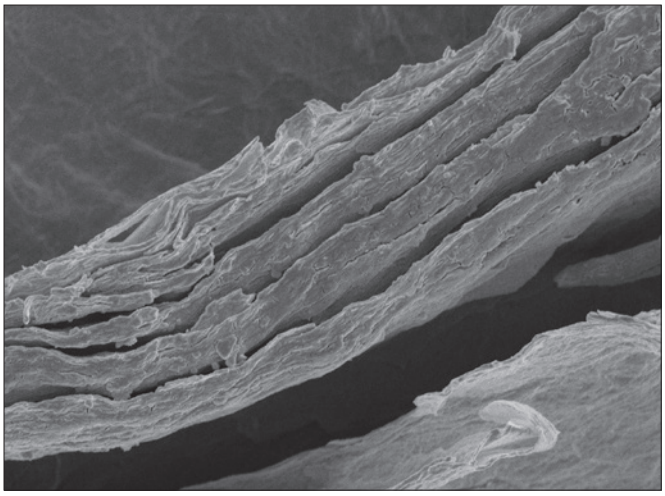

Figure 2. Matrix, x1000. (Squamous keratinocytes of the matrix, regular microplicae on the surface. Workshop of Scanning Microscopy).

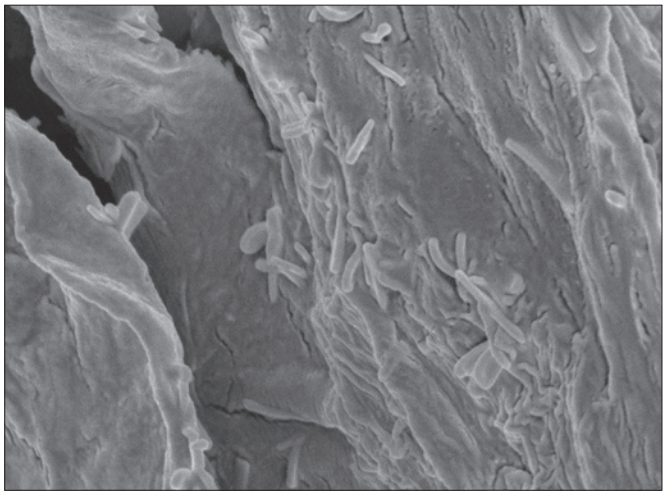

Figure 3. Perimatrix/Matrix, x6000. (Single settlements of bacteria on the border perimatrix/matrix, erythrocyte. Workshop of Scanning Microscopy, magnification x 6000 to improve analysis in this case).

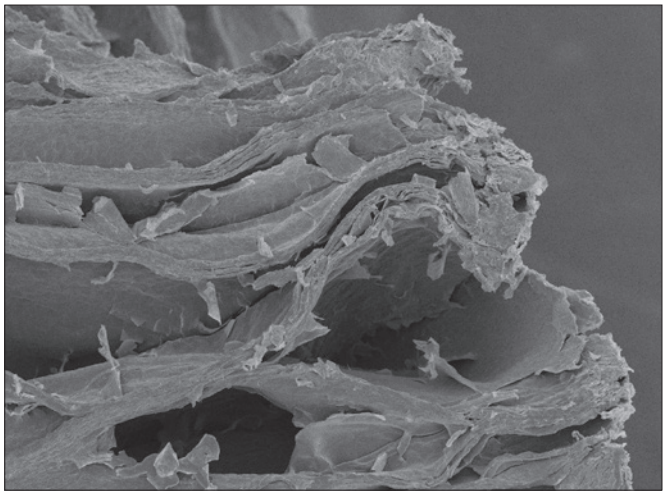

Figure 4. Matrix x100. (Squamous keratinocytes of the matrix, irregular microplicae on the surface. Workshop of Scanning Microscopy).

case, the perimatrix was characterised by a very high cell density. Parallel histopathological examination confirmed the diagnosis of acquired cholesteatoma wherever relevant. Microscopic analysis of defects and degree of damage to the surface of the ossicular chain revealed changes in the form of bone loss, fissured cracks and irregular surfaces (Fig. 5).

Discussion

Advanced histological and immunohistochemical studies have allowed for more detailed discoveries regarding the complex process of cholesteatoma expansion and development, and are the basis for further analyses aimed at developing an effective method for combating this disease. The analysis carried out in the presented work complements numerous publications on this topic.

We conducted a SEM analysis of the surface of the cholesteatomas, which revealed both regular, and in most cases, irregular matrix structures. An irregular matrix structure was observed in cases with a short disease history and in patients who underwent their first surgical procedure. Observations of this type of structure are in line with the research by Miodoński et al. [6]. Similar to previous findings, this suggests that the proliferation of keratinocytes in cholesteatomas is much less coordinated than in normal epidermis, where the pattern is regular. Such surface features suggest abnormal keratinisation and a predominance of cells in the formation of cholesteatoma.

The regular arrangement of layers in cholesteatoma was observed using SEM during the investigation by Youngs and Rowels [7]. Based on our observations, this type of matrix system was present in patients with many years of medical history, who underwent multiple surgeries due to recurrence of cholesteatoma. In our analysis, the regular structure of the cholesteatoma matrix was associated with less bone destruction of the middle ear space. This implies that a regular matrix structure and its associated tight intercellular connections between keratinocytes reduces molecular permeability, while limiting the destructive activity of the cholesteatoma on the bone structures, compared to a chaotic and irregular matrix structure. It is worth considering whether or not the regularity of the matrix structure of the cholesteatoma contributes to its overall permeability to agents involved in the complex mechanism of bone resorp-

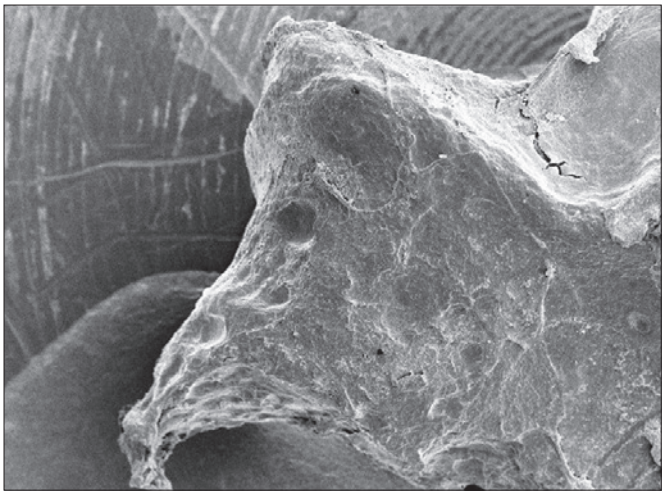

Figure 5. Incus, x30, (Defect of the ossicle's surface). Workshop of Scanning Microscopy).

tion in cholesteatoma. This could be similar to how healthy skin tissue acts as a barrier to disease, but irregular, less healthy skin is more permeable to foreign agents. Moreover, it is worth considering whether or not the lower expression of transient receptor potential channels (TRPVs), observed in cholesteatomas in the studies by Ba Hung Do et al. is important in the formation of a regular or irregular structure [8]. Studies conducted by Koizumi et al. [9] compared the increased permeability of the cholesteatomas' epithelium with skin tissue. The difference in the permeability of molecules between cholesteatoma epithelium and normal skin may be a clue to understanding the mechanism of bone resorption in cholesteatoma by acid lysis. Answers to this question require further research. However, it seems likely that a better understanding of this mechanism may lead to the development of a more effective treatment for cholesteatoma.

Herein, particular attention was paid to the occurrence and degree of erosion of bone tissue due to the destructive effect of cholesteatoma. Based on the literature, destruction of the middle ear bone wall in the skull base was observed in approximately 5-10% of surgical operations, and intracranial complications were found in 1% of patients operated on as a result of diseases of the middle ear [10]. The destruction of bone elements leads to damage to the ossicular chain, and consequently to frequently worse outcomes in terms of hearing improvement. The occurrence of bone structure defects within the temporal bone is an introduction to the development of intratemporal and intracranial complications. According to the literature, the frequency of the defect in the semicircular canal found during surgery for chronic cholesteatoma in otitis media varies between 2.7% and 12.5%, and in most cases affects the lateral semicircular canal. Grewal et al. showed this in 96% of cases and Faramarzi et al. in 95.8% of all fistulas described, which is consistent with our observations [11-13]. It should be noted that the analysis and statistical comparison of the results of bone defects of the middle ear structures in the present work was difficult due to the small number of patients, and is only an additional aspect analysed in the context of the conclusions from the assessment of cholesteatomas using a scanning microscope. The analysis of a larger group of patients by the authors of this work in another study indicates that defects in the middle cranial fossa occur almost five times more often than in the posterior one and at the same rate in the middle and posterior cranial fossa [14].

A chronic cholesteatomatous inflammatory process involving the ossicles destroys the individual elements of the ossicular chain. Bone resorption in the ear cholesteatoma is controlled by the OPG/RANKL/RANK system. In cholesteatoma, there is an increase in the expression of RANKL, which is the main bone resorption factor, often excluding their use as reconstruction materials [15]. In our observation, the process of destruction of the ossicular chain elements was at varying degrees of advancement, and in most cases prevented the use of these elements for the reconstruction stage.

The conducted study indicated the microstructure of cholesteatoma as a prognostic factor of the destruction of bone elements. On the one hand, this can lead to hearing impairment, but on the other it can also open the way for the development of complications of chronic otitis media. The most aggressive course of cholesteatoma with the development of ear-related complications was correlated with the presence of an irregular, chaotic structure combined with a short medical history. The above observation is very important in clinical practice because it justifies the earliest possible initiation of surgical treatment after diagnosis of chronic cholesteatoma otitis media.

The long-term course of cholesteatoma was reflected in the evolution of its microstructure observed under SEM to a regular pattern in the majority of patients, with less aggressiveness towards bone tissue. The main limitation is the necessity to prepare the collected material in vitro according to the SEM sample evaluation protocol, which limits the simultaneous evaluation of biochemical pro-inflammatory factors responsible for the bone destruction process in the preparations analysed.

Conclusions

The microstructure of cholesteatoma assessed using SEM in the form of regular layers coincides with reduced destruction of the middle ear bone walls. An irregular matrix structure (pathognomonic for a process with a short medical history, patients operated on for the first time) is characterised by a tendency for deeper destruction of the bone tissue.

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