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A Biochemical Analysis of the Stapes

Authors' Contribution:
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Data Collection B
Statistical Analysis C
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Background: Otosclerosis is a primary disease of the bony labyrinth. In the course of otosclerosis, abnormal resorption and recalcification of the endochondral layer of the temporal bone is observed. The otosclerotic process most commonly develops in the anterior part of the oval window.

Material/Methods: We analyzed stapes superstructures from 4 patients undergoing surgery for otosclerosis. The first step involved tissue assessment under a scanning electron microscope. The resulting images were analyzed in terms of morphological changes. The stapes superstructure was then divided into small "ossicles", including fragments from the closest vicinity of the stapes footplate and a fragment of the head of the stapes. This material was examined using a scanning electron microscope with a unit for chemical analysis in microareas.

Results: Chemical analysis confirms the appearance of considerable quantities of the following elements: carbon, oxygen, potassium, and calcium, and the appearance of small quantities of sodium and magnesium. Based on a detailed analysis of the chemical composition, these fragments could represent a calcium phosphate compound from the following system: CaO-P_2O_5-H_2O. Fragments of the superstructure from the region closest to the base of the stapes demonstrated a considerably larger presence of carbon, oxygen, and nitrogen, which most likely suggests an increased metabolic process in this region.

Conclusions: Our analysis revealed an increased metabolic activity in the closest vicinity of the otosclerotic focus, the fissula ante fenestram. The increased metabolism correlated with the bone tissue changes seen on scanning electron microscopy.

MeSH Keywords: Biochemical Processes • Microscopy, Electron, Scanning • Otosclerosis

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Background

Otosclerosis is a primary disease of the bony labyrinth. It is only seen in humans, affects the temporal bone, and is characterized by a progressive hearing loss and tinnitus [1–3]. According to the current knowledge, no osteoblast or osteoclast activity is observed in the otic capsule in healthy individuals. In the course of otosclerosis, abnormal resorption and recalcification of the endochondral layer of this part of the temporal bone is observed [5–7]. The endochondral layer is where small regions of immature cartilage are found. These are called globuli interossei and are populated by osteoblasts and osteoclasts. The histological activity of these sites, which are the earliest region of otosclerotic changes, has been divided according to the Schuknecht and Barber criteria in 2 phases: the active phase and the inactive phase:

- In the active phase (otospongiosis, otosclerosis), endochondral bone undergoes resorption by osteoclasts and new bone is formed by osteoblasts. During the process of resorption (otospongiosis), inflammatory cells such as the histiocytes, lymphocytes, and plasma cells, play an important role in addition to osteoclasts. The common result is the deposition of immune complexes. The next step involves an intensive mineralization and ossification of abnormal bone accompanied by atrophy of the spongy structure in favor of the mosaic structure.
- The inactive phase of the disease is its latent period during which no osteoblasts are observed. The bone formed in the final phase is very thick and highly cellular, contains many collagen fibers and little extracellular matrix compared to the normal bone [8–14].

The earliest manifestation of otosclerotic foci is the so-called blue mantle of Manasse, which is a basophilic area identified within the otic capsule on hematoxylin and eosin staining [15]. Histological studies have demonstrated that the otosclerotic process most commonly develops in the anterior part of the oval window, at a location called fissula ante fenestram. In addition to the aforementioned location, the pathological changes may also involve the round window, semicircular canals, the labyrinth, and endings of the nerve fibers of the superior ampullary and lateral branches of the vestibular nerve [14,16].

The etiology of otosclerosis is unclear. Otosclerosis is considered a heterogenous disease, which means that multiple factors and mechanisms (e.g., genetic, immune, viral, metabolic, hormonal) are necessary for its development.

Material and Methods

Our analysis of stapes superstructures from 4 patients is a representative sample of the 10 patients included to the study. The study patients underwent surgery for otosclerosis at the Teaching Ward of Otolaryngology, University Hospital. The analyzed group contained only women aged between 25 to 45 years. The procedure was approved by the Jagiellonian University Bioethics Committee. The used fragments of stapes are normally removed and disposed of as medical waste during a stapedotomy. In 1 study patient, due to the very advanced otosclerotic process, the removal of the superstructure was accompanied by the removal of the base of the stapes (stapedectomy). Individual stapes were numbered from I to IV. The first step involved tissue assessment under a scanning electron microscope at the Scanning Electron Microscopy Laboratory, Teaching Ward of Otolaryngology, University Hospital. The resulting images were analyzed in terms of morphological changes of the surface by comparing changes in the anterior crus of the stapes (closest to the fissula ante fenestram) and the head of the stapes. The stapes superstructure was then divided into small "ossicles", including fragments from the closest vicinity of the stapes footplate, i.e., the inferior fragment of the anterior crus of the stapes, and a fragment of the head of the stapes. This material was examined using a scanning electron microscope, Nova Nanosem 200 (manufactured by FEJ Europe Company), with a unit for chemical analysis in microareas, EDS (Energy Dispersive X-Ray Spectroscopy, EDAX). In order to carry out the observations, the sample was affixed to the microscope stage using a conductive carbon tape. Measurements were carried out using a low vacuum detector (LVD) at an accelerating voltage of 15 kV and 10 kV and a spot size of 4 and 3.5. Observations were carried out in a secondary electron (SE) detection system. Scanning Electron Microscopy with EDS system enables analysis of surfaces of different materials, details of their morphology and chemical composition. During this process, the concentrated electron beam scans the analyzed surface. The electron beam penetrates the superficial layer of the material and elicits the signal from this layer. Stimulated secondary electron

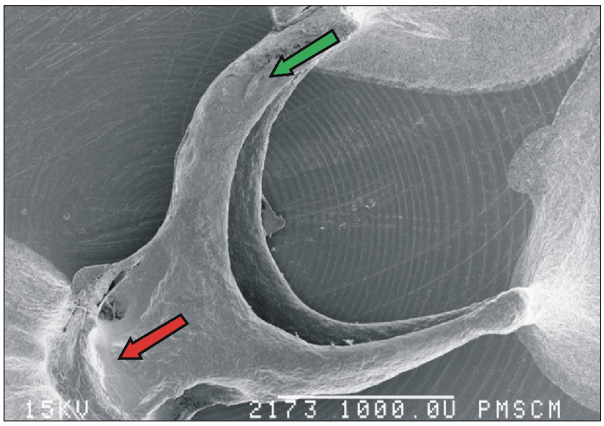

Figure 1. Stapes I (Scanning Electron Microscopy Laboratory, magnification 36×, BAR 1000, Jagiellonian University Medical College, K. Świeży).

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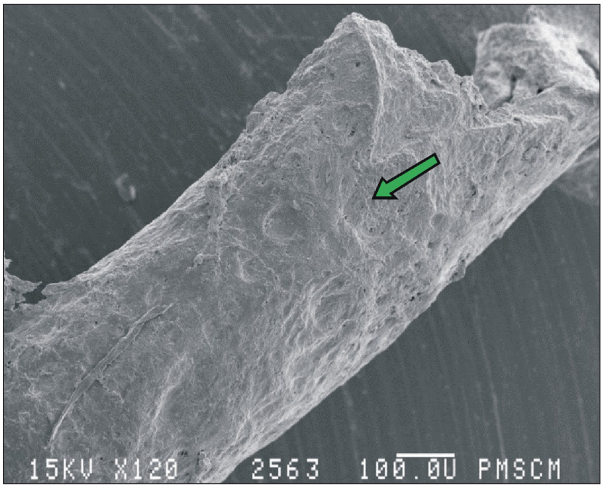

Figure 2. Anterior crus of stapes II (Scanning Electron Microscopy Laboratory, magnification 120×, BAR 100, Jagiellonian University Medical College, K. Świeży).

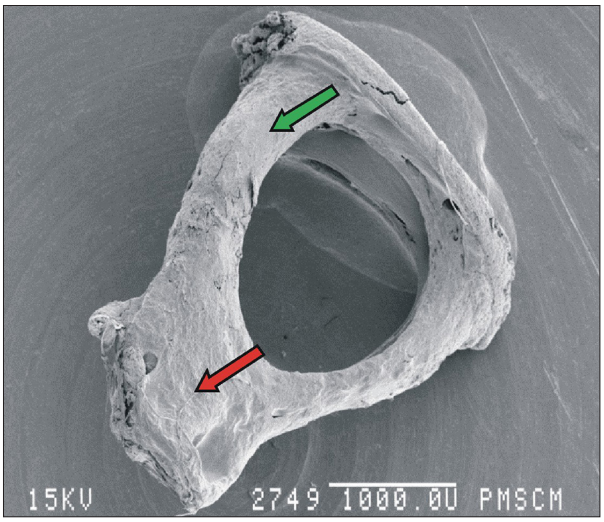

Figure 3. Stapes III (Scanning Electron Microscopy Laboratory, magnification 26×, BAR 1000, Jagiellonian University Medical College, K. Świeży).

signals allow for observation of the surface and performance of chemical analysis of the studied material.

Results

Figures 1–7 show the images acquired using the scanning electron microscope. The chemical composition of the ossicles was then analyzed. Figure 8 (magnification 1000×) reveals the morphology of the sample surface, and is accompanied by the average chemical analysis revealing the change in the chemical composition of the examined fragment of the "ossicle". Chemical analysis confirmed the appearance of considerable quantities of the following elements: carbon, oxygen, potassium,

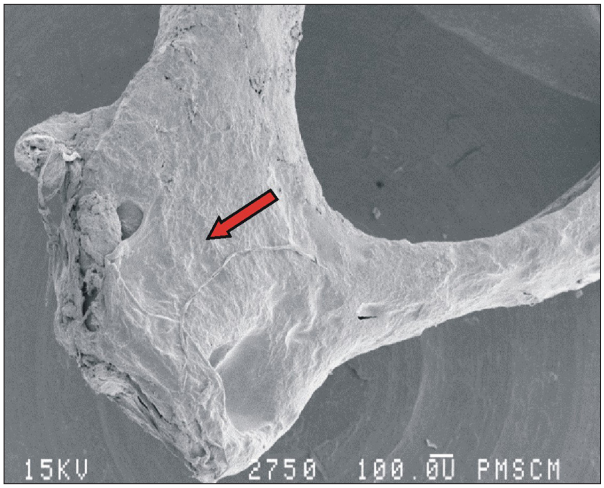

Figure 4. Head of stapes III (Scanning Electron Microscopy Laboratory, magnification 48×, BAR 100, Jagiellonian University Medical College, K. Świeży).

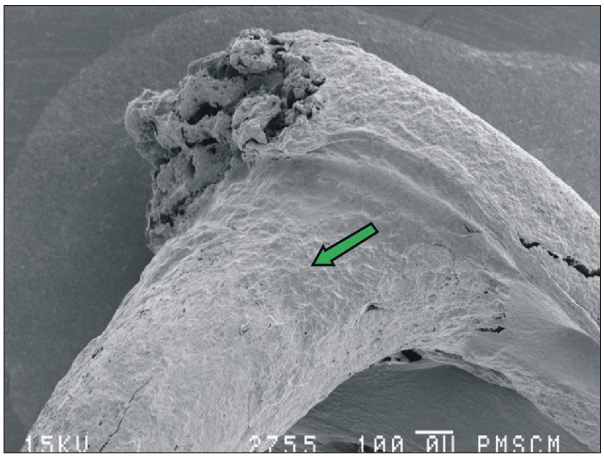

Figure 5. Anterior crus of stapes III (Scanning Electron Microscopy Laboratory, magnification 78x, BAR 100, Jagiellonian University Medical College, K. Świeży).

and calcium, and the appearance of small quantities of sodium and magnesium. Based on the quantitative analysis, the calcium-to-phosphorus molar ratio was 1.23 when all the elements were taken into account and 1.33 when the rest of the elements were omitted. See Figures 8 and 9.

For Figure 9 (a region of the anterior crus of stapes I, magnification 1000×), the sample morphology was similar, while the chemical composition also included nitrogen in addition to the elements already mentioned. As noted before, the calcium-to-phosphorus molar ratio was 1.19 (Case I) and 1.28 (Case II). See Figure 9.

For Figure 10 (a region of the head of stapes II, magnification 1000×), the sample morphology and chemical analysis were similar to those in the previous photograph and analysis.

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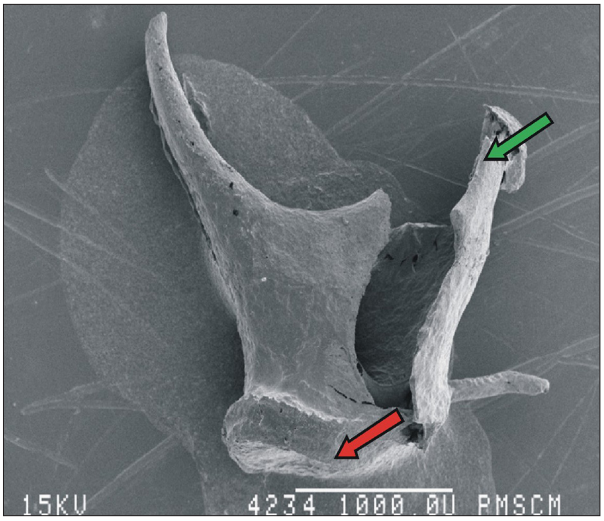

Figure 6. Stapes IV (Scanning Electron Microscopy Laboratory, magnification 78×, BAR 100, Jagiellonian University Medical College, K. Świeży).

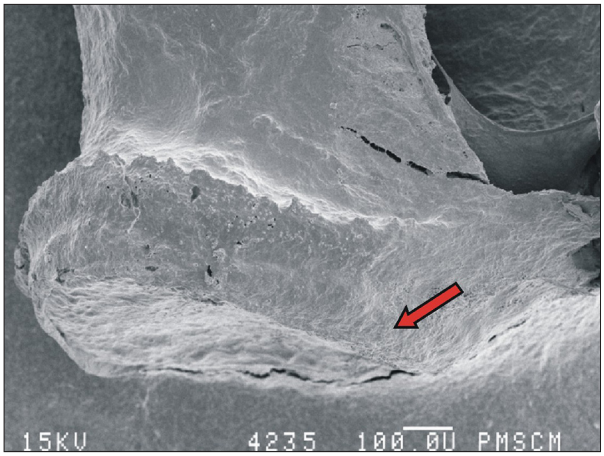

Figure 7. Head of stapes IV (Scanning Electron Microscopy Laboratory, magnification 100×, BAR 100, Jagiellonian University Medical College, K. Świeży).

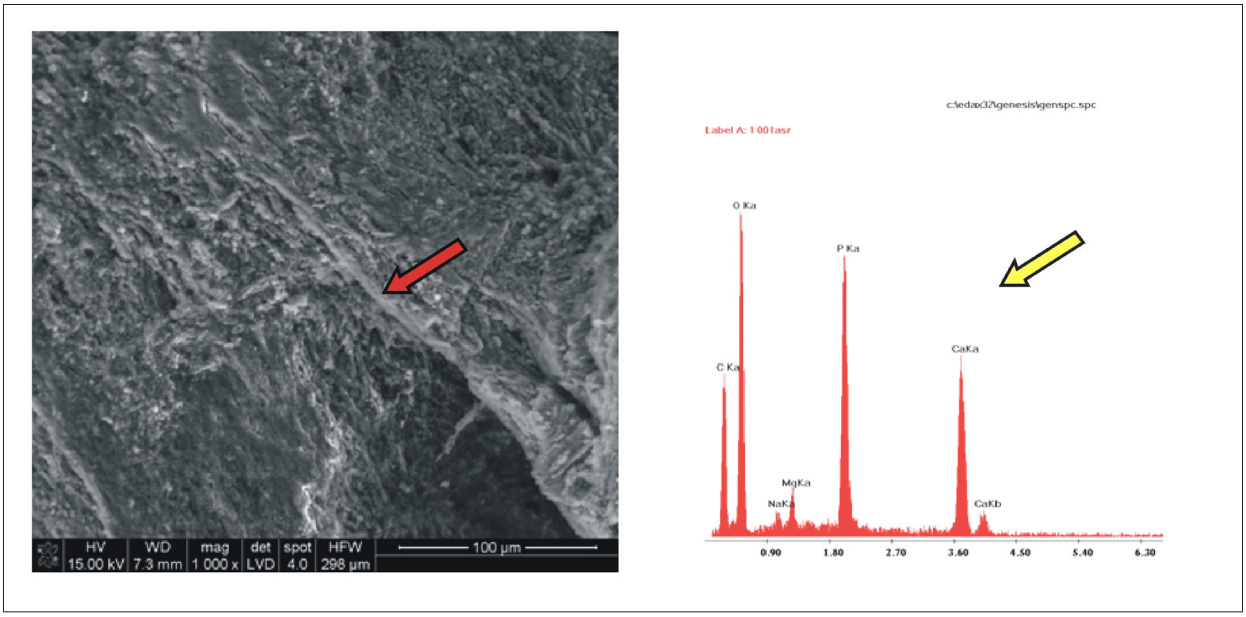

Figure 8. A fragment from the close vicinity of the head of stapes I, magnification 1000×; courtesy of Faculty Laboratory of Scanning Electron Microscopy, AGH University of Science and Technology; M. Ziąbka).

The calcium-to-phosphorus molar ratio was 1.17 (case I) and 1.26 (case II). See Figure 10.

A detailed morphological analysis of the calcium phosphates along with the chemical assessment of the anterior crus of stapes II revealed much higher quantities of carbon, oxygen and nitrogen (Figure 11). Figure 12 shows the anterior crus of stapes III, magnification 5000×.

The chemical analysis result for stapes IV was almost identical, as was the chemical composition of the samples collected from stapes I (see Table 1).

The results are summarized in Table 1. We tried to find out whether the region in the closest vicinity of the fissula ante fenestram demonstrated differences in chemical composition compared to the region of the stapes located the furthest to this region (surface of the head of the stapes).

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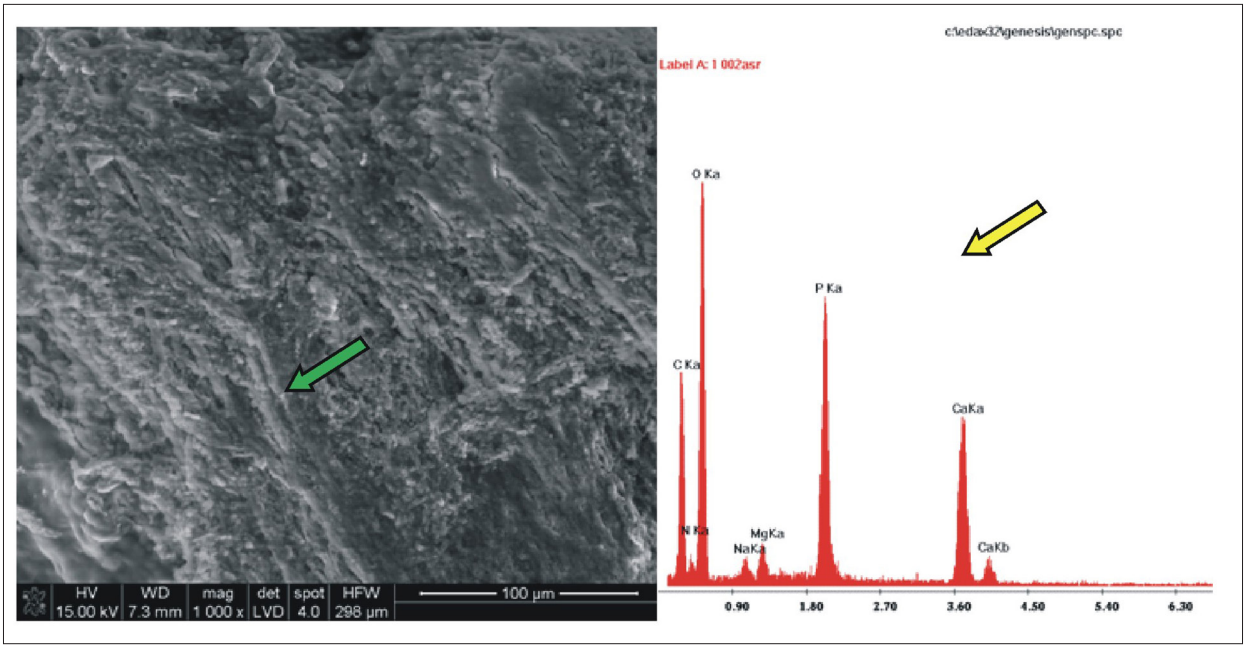

Figure 9. A region of the anterior crus of stapes I, magnification 1000×; courtesy of Faculty Laboratory of Scanning Electron Microscopy, AGH University of Science and Technology; M. Ziąbka.

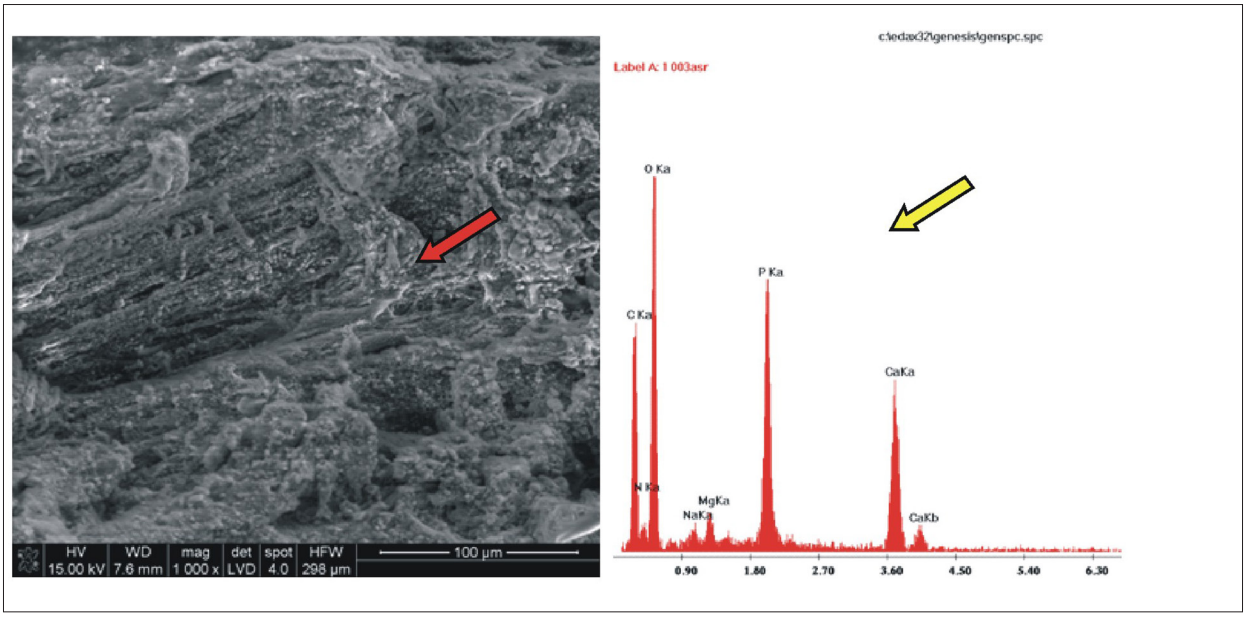

Figure 10. A region of the head of stapes II, magnification 1000×; courtesy of Faculty Laboratory of Scanning Electron Microscopy, AGH University of Science and Technology; M. Ziąbka.

We analyzed the calcium-to-phosphorus ratio to identify the chemical compound that formed the stapes. The calcium-to-phosphorus ratios in individual samples ranged from 1.17 to 1.33. Based on a detailed analysis of the chemical composition, these fragments could represent a calcium phosphate compound from the following system: $CaO\text{-}P_2O_5\text{-}H_2O$ (Table 2).

Based on our analysis, we observed increased quantities of oxygen and nitrogen in the closest vicinity of the fissula ante fenestram compared to the head of the stapes [17–20].

We found no correlation between duration of the otosclerotic process and the degree of intensification of metabolic activity in stapes. On the other hand, increased metabolism was observed in the patients who had a significant perceptive

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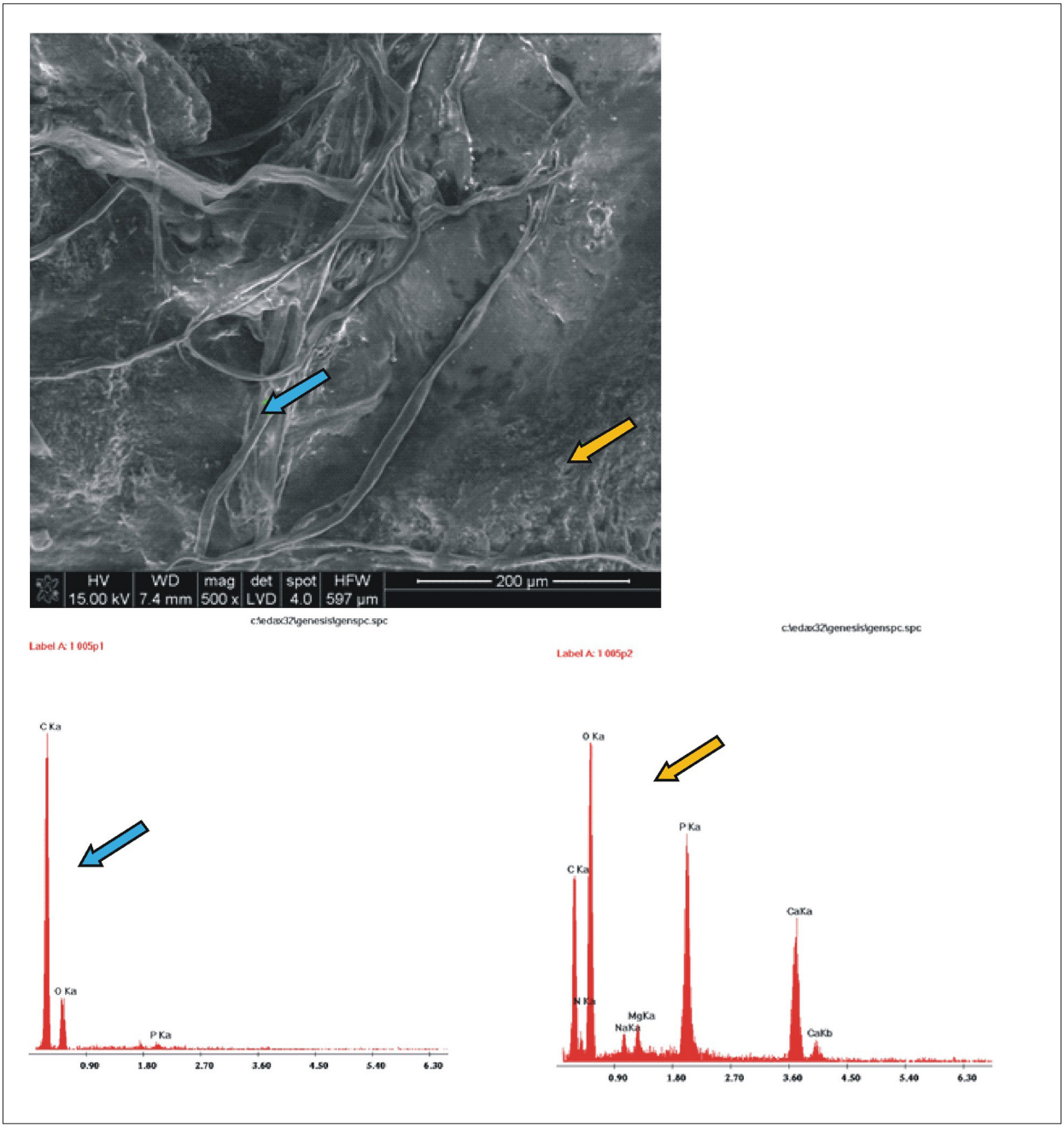

Figure 11. Anterior crus of stapes II, magnification 500×; courtesy of Faculty Laboratory of Scanning Electron Microscopy, AGH University of Science and Technology; M. Ziąbka and chemical analyses at 2 points: point 1 (carbon, oxygen, small quantity of nitrogen); point 2 (calcium phosphate and increased quantities of oxygen and nitrogen).

component of hearing loss. These observations might be a reflection of the negative influence of metabolites freed from the otosclerotic bone on inner ear function.

Discussion

Bone growth and mineralization depend on the activity of 2 types of cells: osteoclasts and osteoblasts, although chondrocytes and vascular cells are also involved in the critical aspects of these processes. In developmental terms, bone formation occurs through 2 mechanisms: endochondral ossification and intramembranous ossification, with the former being responsible

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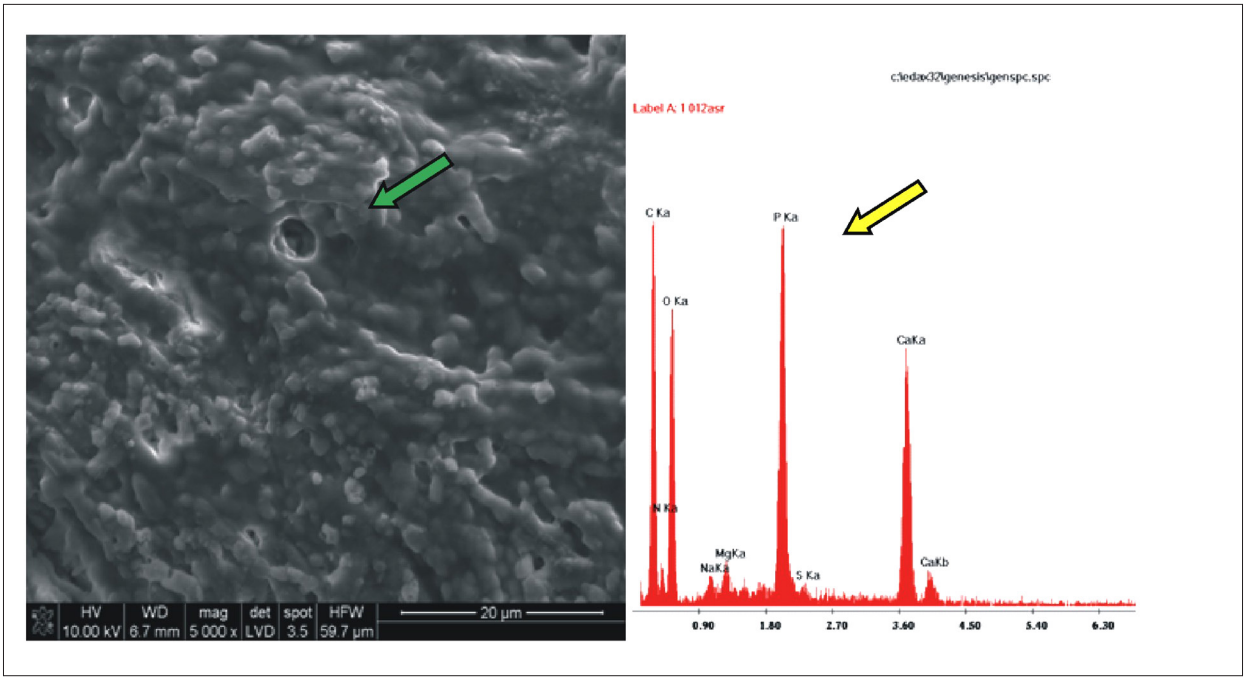

Figure 12. Anterior crus of stapes III, magnification 5000x; courtesy of Faculty Laboratory of Scanning Electron Microscopy, AGH University of Science and Technology; M. Ziąbka). Morphology of calcium phosphates.

Table 1. The chemical analysis of stapes.

Stapes (No.)	Head of stapes Chemical composition	Anterior crus of stapes Chemical composition
I	C, O, K, Ca, Na, Mg, P	C, O, K, Ca, Na, Mg, P, N
II	C, O, K, Ca, Na, Mg, P, N	C, O, K, Ca, Na, Mg, P, N considerable increase of O and N
III	C, O, K, Ca, Na, Mg, P	C, O, K, Ca, Na, Mg, P, N considerable increase of O and N
IV	C, O, K, Ca, Na, Mg, P	C, O, K, Ca, Na, Mg, P, N

Table 2. Calcium phosphate compounds from the following system: $CaO\text{-}P_2O_5\text{-}H_2O$.

Ca/P (calcium-to-phosphorus) ratio	Compound name	Chemical formula
1.0	Calcium pyrophosphate(V)	$Ca_2P_2O_7$
1.333	Hexa calcium dihydro hexa orthophosphate(V) 5-hydrate	$Ca_8H_2(PO_4)_6\ 2H_2O$
1.0	Calcium hydrogen orthophosphate(V)	$CaHPO_4$

for the formation of long bones, and the latter for the formation of topologically flat bones, such as the bones of the skull. Endochondral ossification requires a sequential formation and degradation of chondral structures on growth plates, which serve as a matrix for the formation of osteoblasts, osteoclasts, vessels and subsequent mineralization.

During intramembranous ossification, bone is formed directly within the connective tissues. Both processes require osteoblast infiltration and subsequent deposition of the matrix. Based on a detailed analysis of the chemical composition, these fragments could represent a calcium phosphate compound from the following system: $CaO\text{-}P_2O_5\text{-}H_2O$. Fragments of the superstructure from the region closest to the base of the stapes, i.e., the otosclerotic foci, demonstrated a considerably larger presence of carbon, oxygen, and nitrogen, which most likely suggests an increased metabolic process in this region. Given the complexity of the mechanism that underlies otosclerosis and

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the impossibility, for ethical reasons, to compare it with the healthy bone of the stapes, the study requires further observations and comparisons with factors proven to affect the process of otosclerosis development.

Conclusions

Our analysis revealed an increased metabolic activity in the closest vicinity of the otosclerotic focus, the fissula ante fenestram. The increased metabolism correlated with the bone tissue changes seen on scanning electron microscopy.

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