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Abstract

Background. Middle ear physiology includes both sound pressure transmission and homeostasis of its static air pressure. Pressure gradients are continuously created by gas exchange over the middle ear mucosa as well as by ambient pressure variations. Gas exchange models require actual values for regional mucosa thickness, blood vessel density, and diffusion distance. Such quantitative data have been scarce and limited to few histological samples from the tympanic cavity (TC) and the antrum. However, a detailed regional description of the morphological differences of the TC and mastoid air cell system (MACS) mucosa has not been available. The aim of the present study was to provide such parameters.

Methods. The study included sets of three histological H&E-slides from 15 archived healthy temporal bones. We performed a comparison of the mucosa morphology among the following regions: (1) anterior TC; (2) inferior TC; (3) posterior TC; (4) superior TC; (5) MACS antrum; (6) superior MACS; (7) central MACS; (8) inferior MACS.

Results. Regions (1) - (3), situated below the inter-attico-tympanic diaphragm, had the largest proportion of high respiratory epithelium, cilia and loose lamina propria within the mucosa, as well as the thickest mucosa and the largest diffusion distance. Regions (6) - (8), situated above the diaphragm, had the thinnest mucosa, the shortest distance to the blood vessels, together with the largest proportion of flat epithelium and very few cilia. Regions (4) - (5), still supradiaphragmatic, had intermediary values for these parameters, but generally closer to regions (6) - (8). The blood vessel density and the proportion of active mucosa were not significantly different among the regions.

Conclusion. Mucosa of regions (1), (2) and (3) represented a predominantly clearance-specific morphology, whereas in regions (4) - (8) it seemed adapted to gas exchange. However, the lack of statistically significant differences in blood vessel density and proportion of active mucosa indicated that all regions could be involved in gas exchange with the highest adaptation in the superior MACS. This pattern divides the middle ear functionally along the inter-attico-tympanic diaphragm rather than the anatomical division into TC and MACS.

1 **On the functional compartmentalization of the normal middle ear.**

2 **Morpho-histological modelling parameters of its mucosa.**

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25 1. Introduction

26 In the normal middle ear (ME) a pressure equilibrium with the ambient pressure must be maintained
27 in order to ensure an optimal sound transfer and normal hearing. This equilibrium is influenced by
28 several factors. One factor is the continuous bidirectional diffusion of gases between the ME cavity
29 and the mucosal blood vessels. This gas exchange normally leads to a net absorption of gas from the
30 ME cavity, which is counterbalanced by a gas supply from intermittent Eustachian tube openings
31 (Doyle, 2017; Gaihede et al., 2013; Sadé and Ar, 1997). Another factor is the displacement of the
32 tympanic membrane, which can counterbalance moderate pressure changes from either the ambient
33 atmosphere or from physiological effects with moderate inward and outward movements (Padurariu
34 et al., 2016; Sadé and Luntz, 1989). Finally, more studies have suggested that changes in the
35 volume of ME mucosa also can counterbalance changes in the ME pressure. Thus, small changes in
36 mucosal thickness over the large surface area of the mastoid air cell system (MACS) may have a
37 high impact on the ME pressure (Andréasson et al., 1976; Cros et al., 2016; Gaihede et al., 2010;
38 Magnuson, 2003).

39 Histo-morphological differences have been found between the mucosa of the tympanic cavity (TC)
40 and that of the MACS, which have pointed to functional differences between the two compartments
41 that are relevant for the understanding of the ME physiology including its overall pressure
42 regulation. Thus, compared to the TC, the mucosa of the MACS has been observed with a shorter
43 epithelium, which can be flat (Ars et al., 1997; Lim, 1979; Tos, 1984), cuboidal (Ars et al., 1997),
44 or a mixture of both (Hentzer, 1970). The mucosa of the MACS has also been found to have a
45 significantly shorter distance between the blood vessels and the epithelial basal membrane, as well
46 as a higher density of blood vessels compared to the antero-inferior part of the TC. Together with
47 the large surface area relative to the volume of the MACS, these features have been suggested to
48 represent an adaptation to an efficient gas exchange compared to the TC (Ars et al., 1997).
49 However, other authors have stated that the MACS only represents a passive buffer merely by
50 virtue of its larger volume compared to the TC (Alper et al., 2011; Sadé and Ar, 1997).

51 The TC mucosa has more types of epithelium, from stratified columnar with cilia to mono-layered
52 cuboidal and flat, but always with taller cells in the antero-inferior part, and shorter cells in the
53 postero-superior part (Ars et al., 1997; Hentzer, 1970; Palva et al., 1985; Sadé & Facs, 1966; Tos,
54 1984). Besides cilia, the TC mucosa may also contain secretory cells consistent with an immune

55 defense and clearance of effusion including cellular debris (Ars et al., 1997; Hentzer, 1984; Sadé
56 and Facs, 1966; Shimada and Lim, 1972).

57 Based on these histological differences, Ars et al. (1997) proposed a functional
58 compartmentalization of the ME cavity at the inter-attico-tympanic diaphragm, which is a plane
59 through the TC extending between the level of the tensor tympani tendon and the posterior incudal
60 ligament (Ars, 1998; Palva et al., 2001; Proctor, 1964). Thus, it has been suggested that the ME can
61 be divided into a postero-superior compartment consisting of the attic, the antrum, and the MACS,
62 which seems adapted to gas exchange, and the antero-inferior compartment of the TC, which may
63 contribute primarily to clearance function and immune defense (Ars et al., 1997).

64 The overall regulation of the ME pressure is of immense importance in clinical otology, where the
65 development of under-atmospheric pressure challenges the normal auditory function as well as the
66 surgical reconstruction of the ME; however, our basic understanding of these conditions is still
67 limited. Mathematical and experimental modelling have been employed to investigate the pressure
68 regulation, but they require anatomical and physiological input variables, which have not been
69 directly available. For instance, models of gas exchange have often used the traditional ME
70 compartments of TC and MACS, while assuming a uniform histo-morphology (Ar et al., 2007;
71 Doyle, 2017; Fink et al., 2003; Kania et al., 2004; Kanick et al., 2005; Swarts et al., 2010) and
72 approximating the ME diffusion distance to the thickness of the promontory mucosa (Ar et al.,
73 2007; Kanick et al., 2005; Yoon et al., 1990). In constructing such models, we need to know more
74 about regional variations in the blood vessel density, diffusion distance, mucosa thickness, density
75 of the lamina propria, and the surface area of active mucosa (Alper et al., 2017; Doyle, 2017;
76 Marcusohn et al., 2010; Swarts et al., 2010).

77 Based on the limited understanding of the functional properties of the ME mucosa and the requests
78 for detailed histo-morphometric parameters of the mucosa, we set out to investigate its regional
79 histological properties in archival histological sections from human temporal bones. Such structural
80 properties may be closely related to the ME physiology, and thus, the overall pressure regulation of
81 the ME.

82 2. Materials and methods

83 2.1 Material

84 The study material consisted of an anonymized archive of autopsy material from the Laboratory for
85 Temporal Bone Histology, Department of Otorhinolaryngology, Head and Neck Surgery, Zürich
86 University Hospital, Switzerland. It was represented by 15 horizontally sectioned normal temporal
87 bones of 4 female and 11 male cadavers, with a median age at death of 44 years (age range 21 to 89;
88 4 right and 11 left ears). The inclusion criteria were good tissue preservation and normal
89 pneumatization of the MACS. For each temporal bone a series of between 10 and 40 histological
90 slides were available. All these slides included areas from both the TC and the MACS along with
91 the inner ear. However, the MACS material was most variable among cases and always restricted to
92 sections through the lower level of the TC and above, and thus, the mastoid tip was not available for
93 analysis (Figure 1a).

94 The slides had been prepared according to routine pathology procedures, which consisted of
95 formalin fixation, decalcification in nitric acid (HNO₃), celloidine embedding, serial sectioning at
96 20 µm thickness, and finally staining with haematoxylin and eosin (H&E) of every 10th or 20th
97 section (Merchant, 2010).

98 2.2 Sampling of mucosa

99 The best preserved slides from each of the 15 cases were scanned by a NanoZoomer robotic
100 scanning microscope (Hamamatsu, software version 2.5.88) with a source lens of 20 times and a
101 further digital zoom of 2 times (resolution 0,227 µm/pixel). Whole slides were digitally archived as
102 '.ndpi' files equivalent to a JPEG compression (Figure 1b). All samples were analyzed in
103 Nanozoomer Viewer version 2.5.88 (Hamamatsu Photonics K.K.) at 40 times mode. On each slide
104 the TC and MACS regions were identified; the length of each varied up to respectively 5 and 15
105 mm. In order to standardize the different samples, it was decided to include four sampling regions
106 from each TC and MACS. These regions consisted of respectively (1) anterior, (2) inferior, (3)
107 posterior, and (4) superior TC, (5) antrum, as well as (6) superior, (7) central and (8) inferior
108 MACS. They could be harvested on respectively (1) the most superior available slide through the
109 TC containing the incudo-malleolar joint and antrum (including regions 6, 5 and 4); (2) the first
110 available slide through the TC under the inter-attico-tympanic diaphragm (including regions 7, 3,

111 and 1), and (3) the most inferior available slide through the TC (including regions 8 and 2) (Figure
112 1a). Thus, a total of 120 regions, 8 from each of 15 temporal bones, were selected.

113 In each of the above mentioned regions, a cross-sectional mucosa sample completely attached to the
114 underlying bone was selected corresponding to a 1920 x 1016 pixels image (435 μ m sample length)
115 (Figure 1c). Further, each sample was selected such that the tissue was intact without local
116 inflammatory changes. The clarity of the digital image at the sampling site because of the lens focus
117 at scanning was an additional selection factor narrowing down the sample eligibility within the
118 regions.

119

120 *Please insert **Figure 1** around here.*

121

122 2.3 Histo-morphometric investigations

123 A preliminary assessment of the mucosa morphology included observations of the type of the
124 superficial epithelium and the presence or absence of cilia (Figure 2, zones 2 and 8 vs. the others).
125 The columnar and the cuboidal types were noted as high epithelium, whereas the flat (squamous)
126 epithelium was noted as low. Moreover, the evaluation referred to the underlying lamina propria,
127 which contain the blood vessels as well as connective tissue fibers and cells (fibrocytes). This
128 included the degree of its organization, classified as either tight or loose; thus, it was tight when the
129 connective tissue fibers and fibrocytes nuclei had a parallel orientation without spacing in between,
130 and when the staining intensity was relatively close to that of the underlying bone (Figure 2, zones
131 4, 5). By contrast, the loose mucosa was characterized by less organized or irregular connective
132 tissue fibers, an aerated appearance of lamina propria and a lighter staining (Figure 2, zones 1-3, 6-
133 8). The occurrence of these three features was expressed as samples counts out of total number per
134 region. As the archival slides often presented differences in staining intensity and sectioning, all the
135 analyses were performed dynamically under different digital magnification lenses in order to
136 minimize interpretation errors.

137

138 A quantitative analysis was carried out by using digital image analysis. The H&E mucosa sample
139 and the contained blood vessels were manually segmented to overcome the challenges of automatic
140 segmentation due to differences in staining and sectioning. The blood vessels were defined by their

141 endothelial cells and the presence of erythrocytes. Afterwards, the following morphological
142 measurements were performed (Figure 1c):

- 143 1) the **mucosa thickness**, for which there were made minimum eight measurements per sample,
144 both through the centers of the blood vessel sections and in between the blood vessel sections
145 (μm); the two types of measurements were annotated as two different categories and further
146 compared;
- 147 2) the **blood vessel density**, which was quantified by the density of the blood vessel sections
148 within the mucosa cross-section; this was determined by the ratio of the summed area of the
149 blood vessel sections related to the total mucosa cross-sectional area (%);
- 150 3) the **diffusion distance**, which was measured by the shortest distance between the surface
151 epithelial cells and the center of the major axis of the blood vessel sections (μm);
- 152 4) the ratio of **active mucosa**, representing the proportion of surface mucosa crossed by underlying
153 blood vessels, was calculated by the sum of the horizontal projections of the blood vessel
154 sections, normalized to the sample length of 435 μm ;
- 155 5) the **diffusion distance-to-thickness ratio**, was calculated to investigate whether there was any
156 region with a preferentially superficial expression of the blood vessels relative to their thickness.

157 All the measurements were performed by the same observer (SP).

158 2.4 Statistical analysis

159 Measurements were exported from Nanozoomer Viewer as comma-separated-values (.csv) files for
160 analysis. The data of the five variables were first checked for normal distribution by Shapiro-Wilk
161 test. Two variables failed to prove normal distribution (mucosa thickness and diffusion distance);
162 however, their distributions (negatively skewed) were similar in shape. Variable transformations
163 such as log-transformation were avoided due to difficulties in data interpretation. The five variables
164 were tested for the assumption for homogeneity of variances by Leneve's test and analyzed by one-
165 way ANOVA. A series of inter - regional comparisons was performed by post-hoc tests as follows:
166 Tukey HSD test was applied to the variables meeting the assumption of equality of variance by
167 Levene's test (blood vessel density and length of active mucosa), whereas Games-Howell test was
168 used for the remaining variables failing to prove equality of variances.

169 A paired *t*-test was also performed to compare the mucosa thickness across sections with underlying
170 blood vessel versus sections with no blood vessels.

171 Linear correlation analyses by Pearson test were applied to investigate correlations between any
172 variable and age, between thickness subgroups, and between thickness and diffusion distance
173 respectively.

174 Intra-observer reliability was calculated based on repeated measurements of 11 samples included in
175 the study belonging to 5 different cases and including 323 repeated measurements by the same
176 observer and over several months. The analysis was performed by a Chronbach's Alpha intraclass
177 correlation with a two-way mixed model and a consistency definition.

178 All statistical analyses were performed in IBM SPSS Statistics 24.

179 3. Results

180 There was a large variation in the histo-morphological appearance of the mucosa sampled from the
181 different regions of the ME with respect to its thickness and vascularization pattern, type of
182 epithelium and the density of the lamina propria as illustrated in Figure 2.

183 The epithelial layer of the mucosa varied from high i.e. columnar and cuboidal, to flat, as well as
184 from pseudo- or multi-layered to simple. High epithelium was encountered in 11 – 12 out of 15
185 samples in each of the TC regions 1 and 2, in 3 – 6 out of 15 in the TC regions 3 and 4 and in
186 MACS region 5, as well as in 1 – 3 samples out of 15 in each of the MACS regions 6, 7 and 8
187 (Figure 3a). In all the other samples, the epithelium was simple flat.

188 Cilia were encountered in all regions except 7; there were 4 to 5 samples out of 15 in each of TC
189 regions 1 and 2, 2 to 3 samples out of 15 in TC regions 3 and 4, and up to 1 sample out of 15 per
190 MACS region (Figure 3a). Goblet cells were only seen occasionally.

191 The lamina propria of the mucosa was loose in 8 samples out of 15 in each of the TC regions 1 - 3,
192 in 4 to 5 samples out of 15 in each of TC region 4 and MACS regions 5 and 6, and only in 2 to 3
193 samples out of 15 in the remaining MACS regions (Figure 3a).

194

195 *Please insert Figure 2 around here.*

196 The means and standard deviations of the raw anatomical measurements are listed in Table 1.

197 **Mucosa thickness** of all samples varied generally between 5 and 212 μm (detailed values in Table
198 1 and Figure 3b) having decreasing values from region 1 in TC through regions 6 – 8 in MACS,

199 though with the lowest peak in region 6. Regions below the inter-attico-tympanic diaphragm i.e.
200 regions 1, 2 and 3 presented a significantly thicker mucosa compared to all the other regions ($p \leq$
201 0.033 in all paired comparisons), except region 3, which had values very close to regions 4 and 5.
202 However, regions 3 – 5 still had significantly thicker mucosa compared to MACS regions 6 – 8 ($p \leq$
203 0.035), which had values close to each other.

204

205 *Please insert Table 1 around here.*

206

207 The thickness varied also for individual samples with the presence or absence of blood vessels. The
208 means of the mucosa thickness over blood vessel sections was higher than the means of the mucosa
209 thickness measured in the places not crossing over blood vessel sections with an average difference
210 of 4 μm (SD 10) (paired t -test, $N = 107$, $p < 0.001$). There was though a strong correlation between
211 the two types of thickness measurements (Pearson $\rho = 0.94$, $p < 0.001$).

212

213 The **blood vessel density** generally ranged between 2 and 44 %, but failed to show any statistically
214 significant difference by paired comparisons by regions ($p > 0.05$) (Table 1 and Figure 3c).

215

216

217 *Please insert Figure 3 around here.*

218

219 The **diffusion distance** showed a large variability on the range from 1 to 188 μm . The regions
220 below the inter-attico-tympanic diaphragm presented significantly longer diffusion distances (with p
221 ≤ 0.013) than the above-regions, except that region 3 did not differ from regions 4, 5 and 8. In fact,
222 MACS regions 5 – 8 had very close value to the TC region 4. Moreover, there was a significant
223 correlation between the diffusion distance and the thickness of the mucosa layer (Pearson's $\rho =$
224 0.789, $p < 0.001$) (Table 1 and Figure 3d).

225

226 The proportion of the **active mucosa** ranged between 56 to 100 % without any statistically
227 significant differences between regions.

228

229 The **thickness-relative diffusion distance** varied between 8 and 97 % across all regions, and there
230 was a tendency of region 6 to express blood vessels closer to the mucosa top compared to the other

231 regions, although the differences were significant only between region 6 and respectively regions 2
232 and 8 ($p \leq 0.038$).

233 The intra-observer reliability of measurements was of 0.992 for single measures and of 0.996 for
234 average measures ($p < 0.001$).

235 Correlation analysis between ages and any of the measured parameters yielded no statistically
236 significant results ($p > 0.05$ for any parameter).

237 4. Discussion

238 The current study compared morphometric parameters of the ME mucosa in eight different regions
239 of the ME, and found few statistically significant differences between the regions above and below
240 the inter-attico-tympanic diaphragm related to the mucosa thickness and the diffusion distance.

241 Thus, our more extensive sampling of the MACS region provided results consistent with those of
242 Ars et al. (1997). However, the means of the diffusion distance with values between 12 and 48 μm
243 in any of the 8 sampled regions including the epithelium were generally lower than the averages of
244 40 μm and 71 μm for respectively postero-superior and antero-inferior compartments excluding the
245 epithelium reported by Ars et al (1997). This difference may primarily reside in the fact that mucosa
246 investigated in the present study was anchored to the bone that prevented it from curling and
247 becoming thicker. Another possible factor may be a different degree of tissue shrinkage due to
248 longer time of histological processing of the full mount archive materials used in the present study.

249 In a histo-morphological study on 100- μm length promontory mucosa from normal ME's, Yoon et
250 al. (1990) found an average thickness of 37.5 μm excluding the epithelial layer. This is in good
251 agreement with the mean of 55 (SD 34) μm including the epithelial layer for region 2, which may
252 be the closest sampled regions in the present study. Moreover, they reported an average blood
253 vessel density of 12.8 %, which was also in quite good agreement with the mean of 15 % for the
254 same region in the current study.

255 Overall, the current results showing that the mucosa of regions above the inter-attico-tympanic
256 diaphragm, having typically a one-layered flat epithelium and normally lacking cilia, seems to
257 correspond to the neural crest origin described by Thompson & Tucker (2013) in the mammalian
258 attic. Together with a shorter diffusion distance, this part of the ME seems specialized in facilitating
259 the gas exchange. By contrast, the parts below the inter-attico-tympanic diaphragm, described as of

260 endodermal origin, is characterized by a better clearance and defense functionality (Thompson and
261 Tucker, 2013; Tucker et al., 2018).

262 **4.1 Trans-mucosal gas exchange**

263 The MACS regions presented a remarkably thinner mucosa and shorter diffusion distance compared
264 with the remaining regions. The ratio between the two parameters also indicated that the blood
265 vessel sections were situated most superficially in region 6. Together with the mostly flat
266 epithelium and a relatively loose lamina propria, this region looked like the ideal site for gas
267 exchange.

268 There was no evident correlation between the diffusion distance and the blood vessel density. The
269 latter suggested the highest blood supply in the central MACS (region 7), where the lamina propria
270 was predominantly dense. Moreover, it was noticed that the looser appearance of the lamina propria
271 associated negatively with the blood vessel density (Pearson's $\rho = -0.71$; $p = 0.05$), so that the
272 looser the appearance of the lamina propria, the lower the density of the blood vessels.

273 Generally, the regions situated under the inter-attico-tympanic diaphragm (regions 1, 2, and 3)
274 presented a looser lamina propria, as well as a thicker mucosa, and a higher ciliated epithelium,
275 compared with the regions above the diaphragm, in agreement with the previous studies (Ars et al.,
276 1997; Hentzer, 1984; Sadé and Facs, 1966; Shimada and Lim, 1972). Moreover, despite the thicker
277 mucosa and deeper blood supply in the sub-diaphragmatic compartment, the ratio between the
278 diffusion distance and the respective mucosa thickness as well as the proportion of active mucosa
279 and the cross-sectional density of the blood vessels are comparable among all the ME regions.
280 Thus, the sub-diaphragmatic compartment altogether appears also to be adapted to an efficient
281 trans-mucosal gas exchange. However, the muco-ciliary function in this region also involves
282 secretion of mucus; this forms a mucous blanket on the top of the epithelium, which may constrain
283 the gas exchange by acting as a relative barrier for the gas molecules.

284 Overall, the mucosa had a moderate vascularization. However, it was interspersed with segments of
285 more intense vascularization, where the blood vessels were more congested, the mucosa was
286 thicker, and the epithelium was higher with cilia. This has been noticed in both the TC and the
287 MACS, and it suggested either a localized defense reaction and/or sequelae of earlier episodes of
288 inflammation.

289 **4.2 Mucosal congestion**

290 The same structural properties of the mucosa that may enhance the physiological gas exchange –
291 vascularization, connective tissue that might change between loose and dense, together with a large
292 mucosal surface area of the MACS – may also point to another role of the mastoid mucosa in the
293 overall ME pressure regulation. Thus, it has been suggested that physiological changes in the
294 mucosal volume or thickness may influence or counter-balance changes in ME pressure effected by
295 changes in its congestion (Gaihede et al., 2010; Magnuson, 2003). This is almost similar to the
296 mechanism found in the nasal mucosa that controls the airflow through the nose by changes in the
297 mucosal congestion, which is efficiently managed by specialized venules or sinusoids
298 (Widdicombe, 1997). Such specialized venules have not been demonstrated in the ME, but
299 increasing its vascular congestion may still be likely to increase the mucosal thickness, and
300 ultimately the ME pressure (Figure 4). It follows that this mechanism would work in either
301 direction, so that increasing or decreasing the mucosal congestion would result in increasing or
302 decreasing the ME pressure.

303

304 *Please insert **Figure 4** around here.*

305 It has been estimated that for normal sized ME's, a change in mucosal thickness of only 6 μm is
306 enough to induce a pressure change of 1 kPa (Magnuson, 2003). In our samples, we found a mean
307 difference of 4 μm (SD 10) between paired mucosa measurements (N = 107 pairs) in the presence
308 versus absence of blood vessel sections. Since venules often are found collapsed in tissue samples,
309 the difference between the presence and absence of venules may more likely represent the
310 difference, whether the venules are blood-filled and visible, or collapsed and invisible. Thus, the
311 difference in mucosal thickness of 4 μm found here may simply reflect changes in congestion,
312 which are in the same order of magnitude (6 μm) as suggested for physiological pressure changes
313 by Magnuson (2003).

314 In diseased ME mucosa, which is relatively thicker, an apparently new blood vessel formation has
315 been observed (Ar et al., 2007; Matanda et al., 2006). In addition, the lamina propria seems to
316 become less organized with a looser appearance when the blood vessels become more prominent or
317 congested. Figure 4 illustrates such a situation with rich blood filled venules and an expanded
318 mucosa, where the looser appearance of the lamina propria may result from the expansion of the
319 connective tissue. Thus, these changes may result from a response to counter-balance under-
320 atmospheric pressure related to inflammatory conditions, and it may also be attributed merely to the

321 fact that venules largely collapsed in normal tissue preparations become expanded. This becomes
322 evident at immunostaining of the mucosal blood vessels with CD31 staining, where a very high
323 density of mucosa blood vessels are visible including many collapsed vessels (Figure 5).

324

325 *Please insert Figure 5 around here.*

326

327 **4.3. Cilia and metaplasia**

328 The cilia distribution in the present samples generally agreed with earlier systematic studies, since
329 they were noticed to be most numerous in the inferior and anterior TC, and less frequent in superior
330 and posterior TC (Sadé and Facs, 1966; Shimada & Lim, 1972). However, in one case numerous
331 cilia were found in the antrum and MACS (Figure 6), which is in agreement with few of the
332 previous studies (Hentzer, 1970; Shimada & Lim, 1972). The presence of numerous cilia in the
333 lateral MACS in one of the best-preserved cases suggested that metaplasia might have occurred as
334 result of earlier ME pathology (Shimada & Lim, 1972).

335

336 *Please insert Figure 6 around here.*

337 The currently used material underwent prolonged fixation and decalcification, which could
338 disintegrate cilia, thus, their frequency might be underestimated (Sadé & Facs, 1966). However,
339 cilia were also occasionally found in peri-antral MACS of more subjects of the present material
340 outside the samples used in our analysis. This may also suggest that antrum and the peri-antral
341 MACS can be a transition site between the clearance and gas exchange functions. Altogether, cilia
342 distribution and clearance may be dynamic and include the MACS probably in response to local
343 inflammatory factors (Sadé & Facs, 1966).

344 **4.4 Strengths and limitations of the study**

345 There are unique advantages of using this archival material such as the larger availability of whole
346 samples and serial sections; moreover the mucosa is much better protected against shrinkage and
347 curling due to its firm attachment onto the bone compared to separate mucosa pieces harvested
348 during the surgery. However, due to the anonymity of the material, we had no information about

349 specific ME disorders, and the judgement of normality was subjective and only based on a normal
350 appearance of the mucosa and the MACS pneumatization.

351 One specific aspect of archive materials is that they are usually only available in H&E staining and
352 embedded often in celloidine. While the latter offers a very good morphological preservation, the
353 H&E staining gives a good overview on the tissue composition. However, it makes the blood vessel
354 identification more challenging, especially if they are collapsed and emptied of blood. A special
355 marker for endothelial cells would highlight them and this would be an advantage for an automatic
356 segmentation of the digital images (Figure 5), whereas a safe quantitative analysis of the H&E
357 samples requires a time-consuming analysis slide-by-slide by a pathologist.

358 Another limitation is that the inferior-most sections of the mastoid are missing in this analysis,
359 providing an incomplete image. This may become the object of further studies, where the whole
360 mastoid will be harvested and prepared histologically.

361 The study is also limited by the manual method, which was not favorable to a quantitative
362 measurement of the parameters in all the mucosa available, but rather to a sample-based design. A
363 systematic study on the effect of changing samples was not performed. However, it could
364 occasionally be noticed that by replacing a sample within a region did not affect the levels of
365 significance. This might be assumed to the relatively low rate of statistically significant differences
366 of the measured parameters among the regions.

367 A known issue of morphological analyses is the possible bias induced by preparation-related
368 shrinkage. The current study was performed in a comparative manner, so an eventual shrinkage bias
369 should be relatively the same in all the sample groups. However, if the results should be used in a
370 mathematical model, correction would be necessary considering that the underlying bone might
371 shrink about 6 %, and the mucosa may also follow this phenomenon (Buytaert et al., 2014).

372 The present study has been limited by the planimetric design of the sampling, which may
373 correspond to screenshots through the mastoid mucosa. In vivo, mucosa is subject to dynamic
374 behavior regulated by chemical mediators with effects on blood flow and blood vessel permeability,
375 which may allow for large adaptive variations. Moreover, the longitudinal blood vessel sections and
376 the diffusion distances cannot be considered absolute values, but rather relative values dependent on
377 the angle of sectioning at its time. The blood vessel sections may represent a cut through the most
378 central section or just through the endothelial wall. Thus, when the blood vessels are just identified

379 by their endothelial cells, they may represent only the wall of a blood-filled vessel or a collapsed
380 blood vessel. A clear judgment was not possible due to the large cutting steps to the next available
381 section, which was often 200 μm or more, clearly larger than the capillary or venule cross-diameter.

382 Future studies with systematic application of immuno-histochemical staining would offer a more
383 detailed investigation of the mucosa samples including the vascular density by CD31 as specific
384 marker of the endothelial cells (Figure 5); however, this demands paraffin-embedded tissue
385 specimens. We have attempted this in a series of cases, but a longer decalcification process also
386 lead to problems with the quality of the subsequent staining of the tissue samples. Improved
387 techniques are needed, where for instance smooth muscle fibers within the lamina propria of the
388 blood vessels as well as neural fiber components may be detected by immuno-staining. This may
389 further elucidate the functional properties of the mucosa with regard to the possibility of a neural
390 control of changes in its perfusion and congestion. Such findings may point to an overall active role
391 of the mucosa in the ME physiology and pressure regulation, and should be aimed for in future
392 studies.

393 **Conclusion**

394 The histomorphometric variables provided useful measures for detailed ME modeling including the
395 mucosa thickness, the diffusion distance, and the active mucosal surface area. Since the assessment
396 of the mucosal perfusion is impossible to obtain with current techniques, the density of the blood
397 vessels may serve as an indirect measure of the mucosal blood supply.

398 Regions of antero-inferior TC presented significantly thicker mucosa and longer diffusion distances
399 from blood vessels to surface than the than the regions of the remaining TC and MACS, whose
400 shorter diffusion distances and much larger mucosa surface area should facilitate gas exchange.

401 However, the relatively uniform blood vessel density and proportion of active mucosa suggest that
402 all ME regions may be involved in gas exchange. Moreover, a potential role in pressure regulation
403 by changes in mucosa congestion is also suggested based on a significant difference in mucosa
404 thickness depending on the presence or absence of underlying blood vessels. Thus, the size of the
405 MACS surface area contributes to its efficacy in gas exchange and pressure regulation via changes
406 in congestion and mucosal thickness.

407 In many respects, the TC and MACS compartments might be treated as a unity in normal
408 conditions. However, in inflammatory conditions with changes in mucosa thickness, blood vessel

409 density, and blockage of the inter-attico-tympanic diaphragm including dysfunction of the
410 Eustachian tube, the two compartments may become totally isolated gas pockets, which may reach
411 neither a balance with each other, nor with the ambient pressure, and thus, throwing the ME in the
412 vicious circle of underpressure.

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420

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509 continuum of otitis media.

510

511 **Figure captions:**

512 **Figure 1.** (a) Sagittal representation of the middle ear, where the sampling planes and regions are
513 represented. TC = tympanic cavity; MACS = mastoid air cells system. The sampled regions are: (1)
514 anterior TC; (2) inferior TC; (3) posterior TC; (4) superior TC; (5) MACS antrum; (6) superior
515 MACS; (7) central MACS; (8) inferior MACS; (b) Example of a horizontal slide including three
516 regions of mucosal sampling (slide 2; digital lens 0.36; scale bar = 5 mm). (c) Example of histo-
517 morphometric measurements in a mucosa (M) sample, oriented upwards, whereas the bone (B) is at
518 the bottom of the image. The green lines are thickness measurements, the black line represents the
519 diffusion distance, and the dark blue ellipse represents a blood vessel section. Sample region 5;
520 H&E, magnification lens 40x; scale bare = 25 μm .

521 **Figure 2.** Samples of mucosa from respectively each of the eight regions defined in Figure 1. All
522 samples belong to the same ear (case 5, H&E) and at the same magnification (digital lens 80x).
523 Mucosa is oriented with the air interface upwards, and attached to the underneath bone (B). Notice
524 much thicker mucosa in zones 1 and 2 compared to the others regions. Mucosa elements referred in
525 the study are emphasized as follows: the epithelium of each sample is marked with black arrows,
526 the lamina propria (lp) is marked with braces, the blood vessels are marked with stars, and cilia μm
527 are marked with blue arrows. In the illustrated case, the epithelium of regions 1, 4, 6, and 8 was
528 considered low, whereas in the other regions it was considered high; mucosa of regions 4, 5 and 8
529 was considered tight, whereas in the other regions it was considered loose. Scale bars = 25.

530 **Figure 3.** Summary of main morphological and morphometric analyses of the mucosa in 8 regions
531 of 15 normal ME's. Panel (a) represents the proportion of samples per region presenting each
532 mentioned feature. The three boxplot panels represent the middle 50% of the morphological
533 measurements. The horizontal lines within are the medians, and the whiskers represent the 95%
534 confidence intervals. The small circles represent the outliers within 1.5 interquartile range, whereas
535 the stars indicate the outliers beyond this limit.

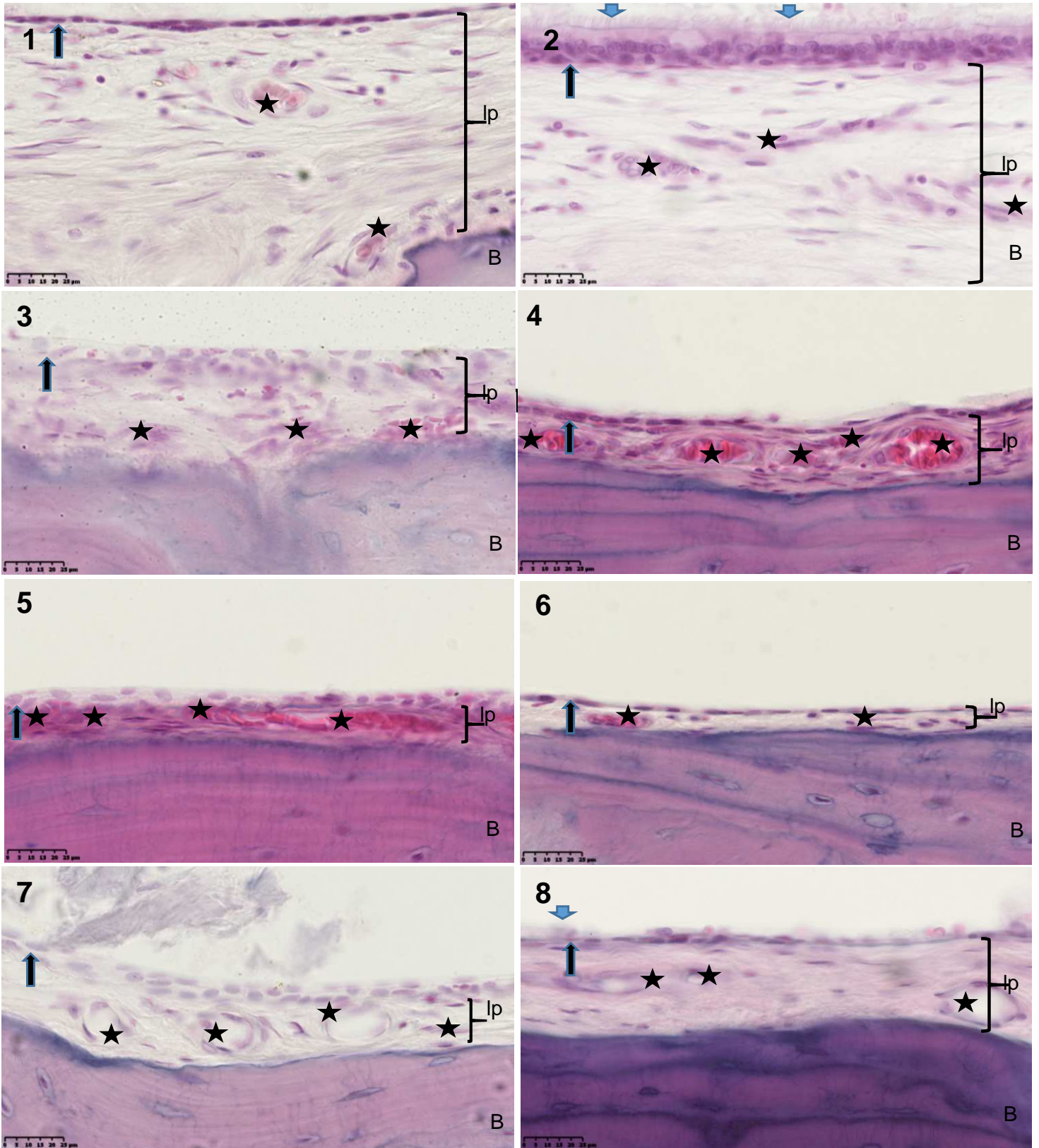
536 **Figure 4.** Mastoid air cell with expanded/looser mucosa and many distended venules. The sampling
537 site is marked within a black box on the slide map to the right (case 6, magnification lens 5x; scale
538 bar = 500 μm).

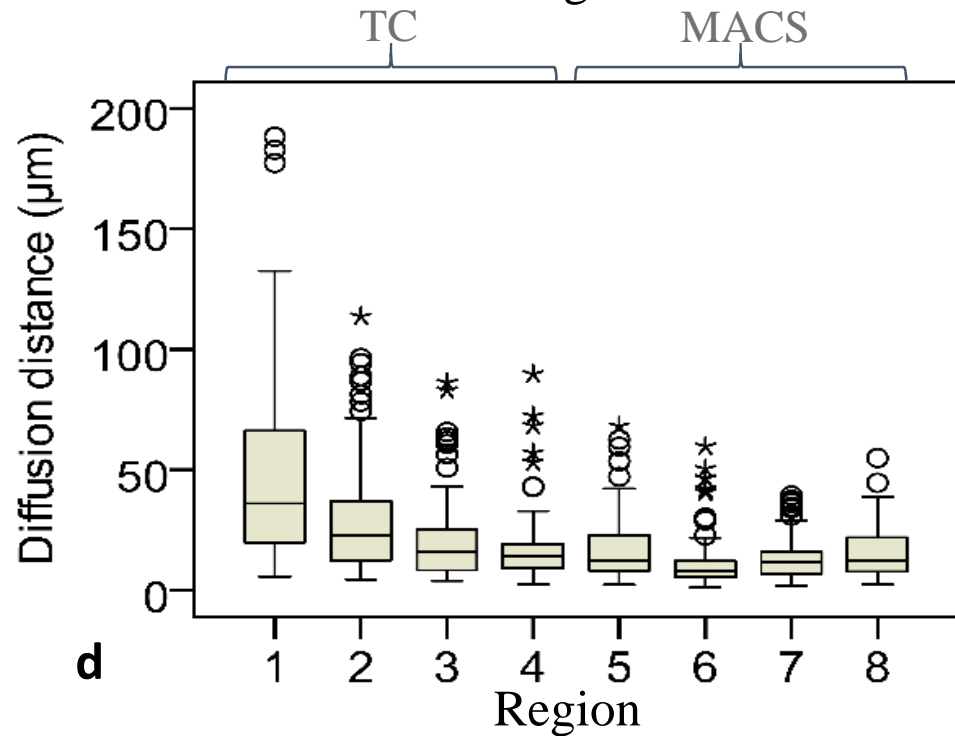
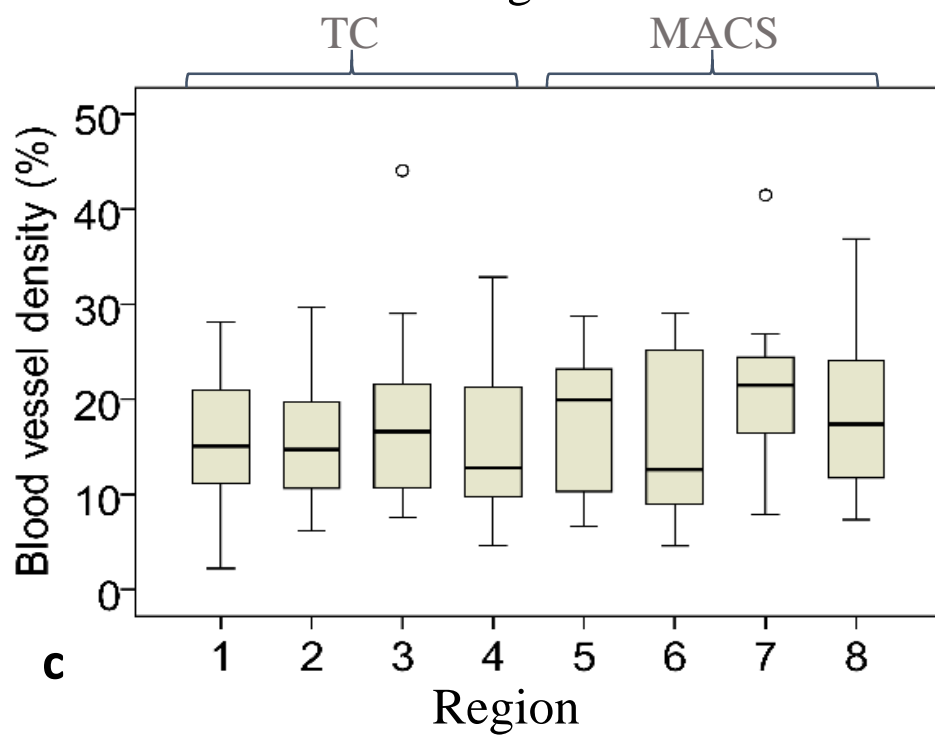
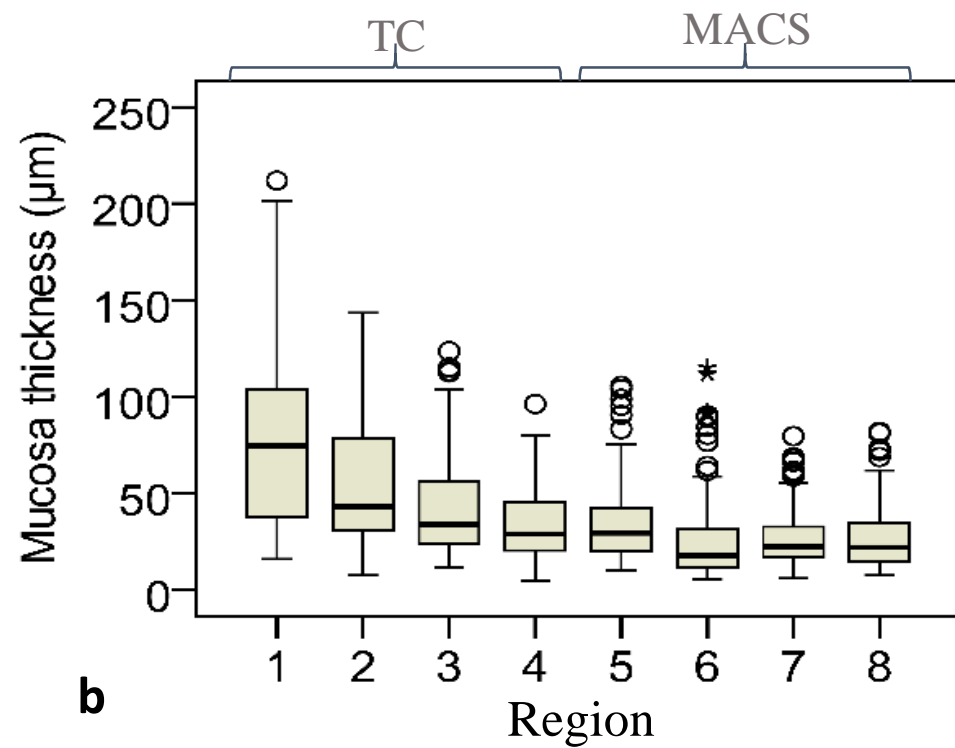
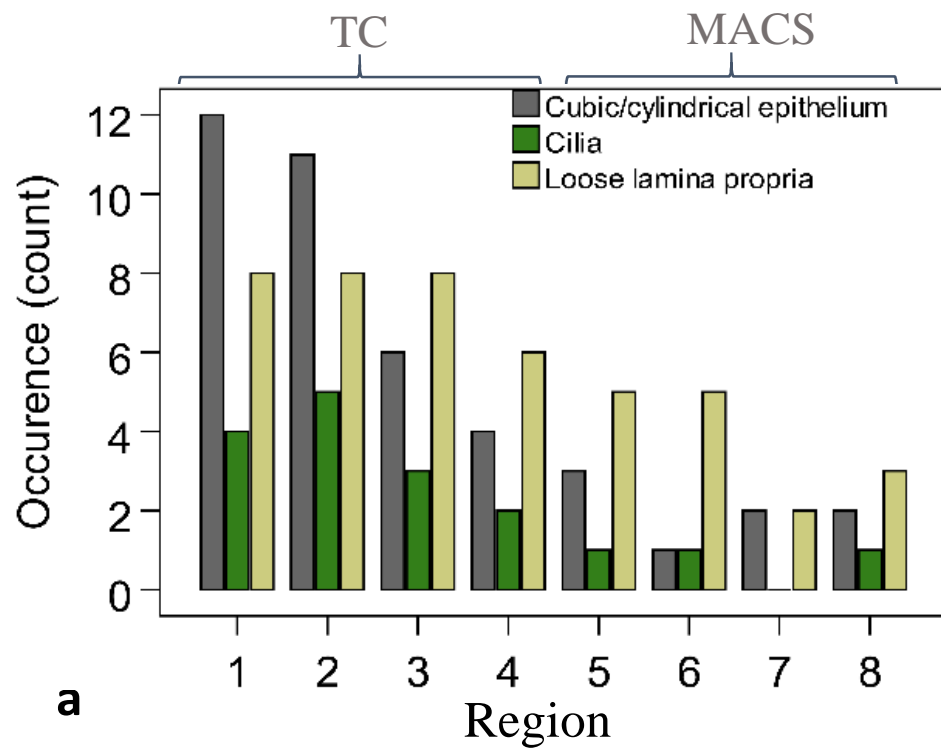
539 **Figure 5.** MACS mucosa stained with CD31 marking endothelial cells of blood vessels in brown.
540 Notice the contour of several blood vessels with the lumen almost collapsed. These blood vessels
541 may be concealed in H&E stained preparations (magnification lens 20x; scale bar = 100 μm).

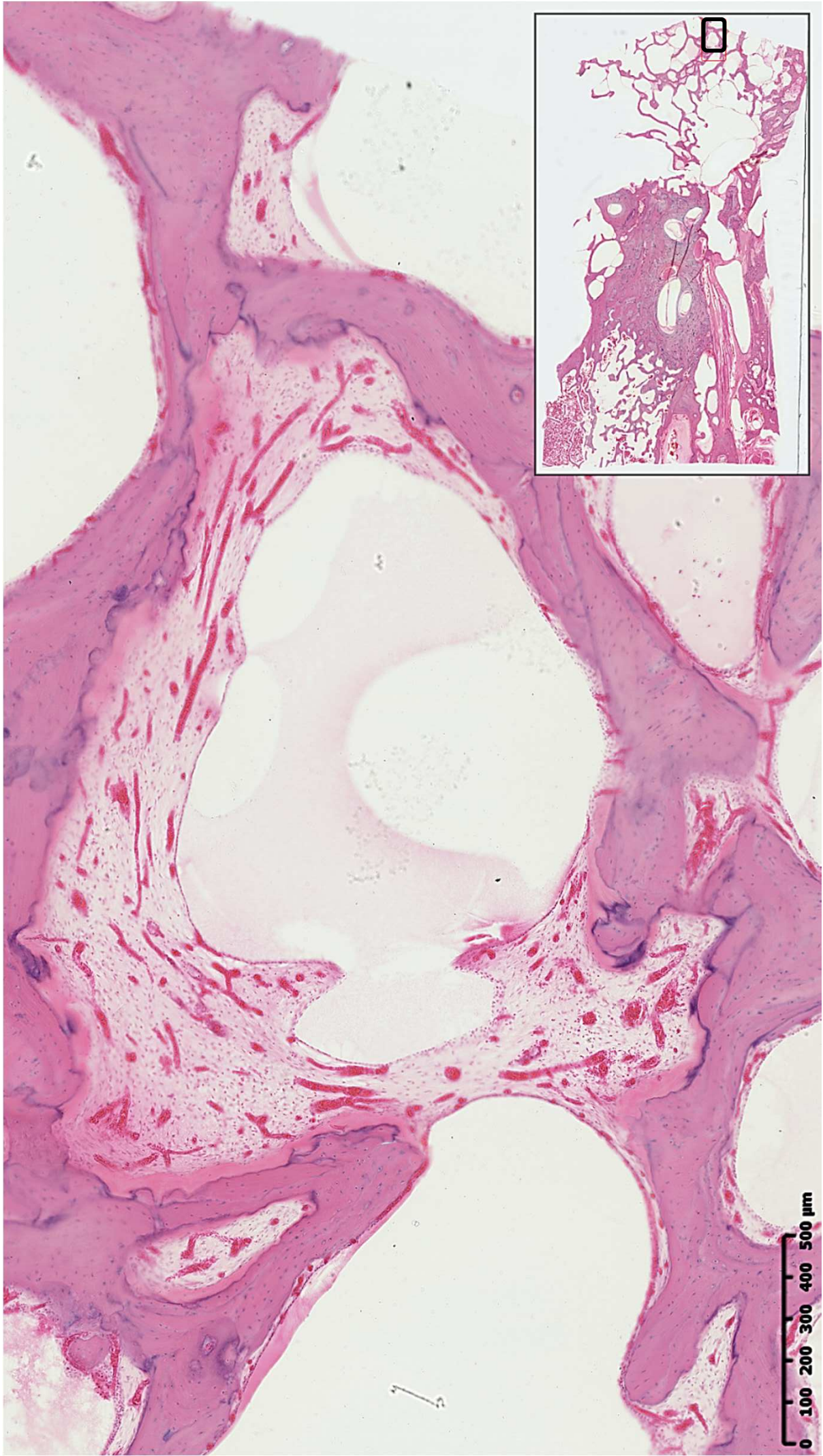
542 **Figure 6.** Cilia within lateral MACS represented with arrows (Case 8, magnification lens 80x). The
543 sampling site is marked within a black box on the overview map to the right. Scale bare = 25 μm .

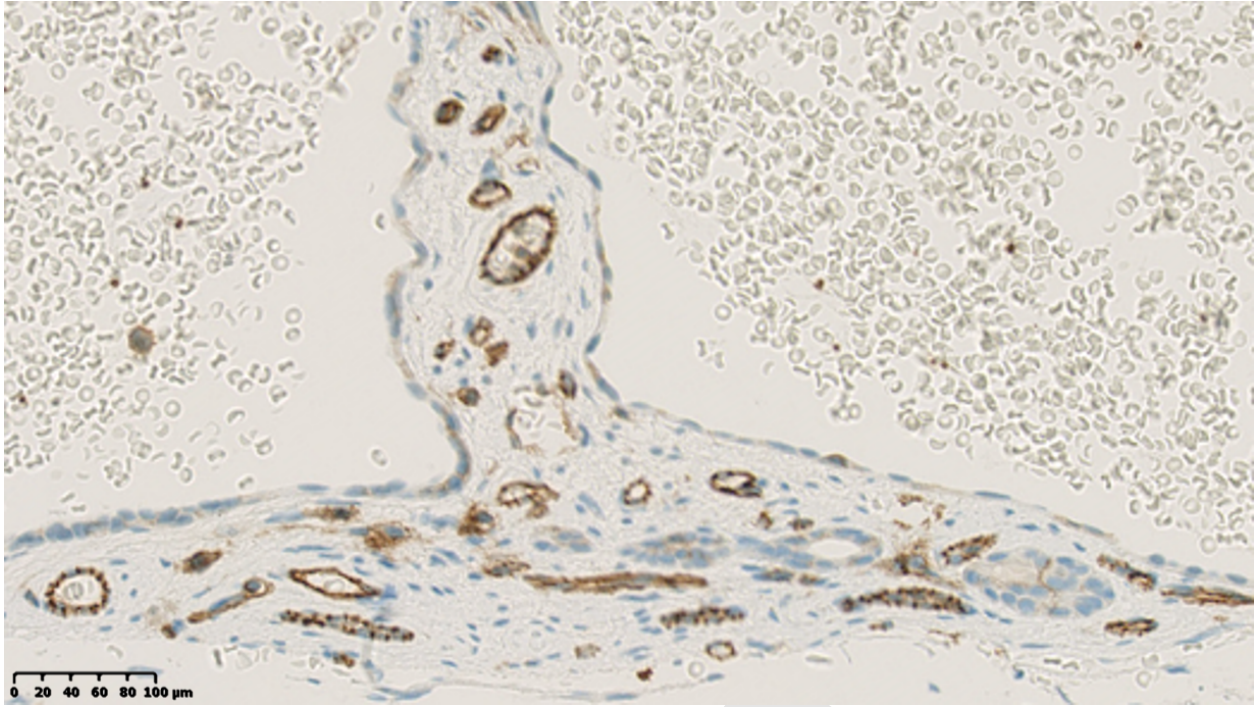
Table 1. Distributions of raw morphometric parameters in eight sampled regions of the middle ear mucosa (N = 15). Results are expressed as rounded mean (standard deviation). The last column presents the level of significance for oneway ANOVA test for differences among regions with respect to each parameter

	Middle ear region								ANOVA
	1	2	3	4	5	6	7	8	p-value
Mucosa thickness (μm)	84 (55)	55 (34)	44 (28)	35 (29)	36 (21)	26 (23)	27 (15)	27 (16)	< 0.001
Blood vessel density (%)	15 (8)	15 (7)	18 (10)	16 (9)	18 (8)	16 (9)	21 (8)	18 (8)	0.518
Diffusion distance (μm)	48 (40)	29 (23)	20 (17)	17 (15)	18 (13)	12 (12)	14 (8)	15 (10)	< 0.001
Active mucosa (%)	66 (26)	59 (19)	61 (23)	55 (19)	61 (24)	49 (25)	58 (19)	53 (22)	0.481
Diffusion distance/ thickness (%)	50 (23)	51 (20)	44 (20)	48 (18)	48 (20)	43 (16)	49 (14)	51 (16)	0.036

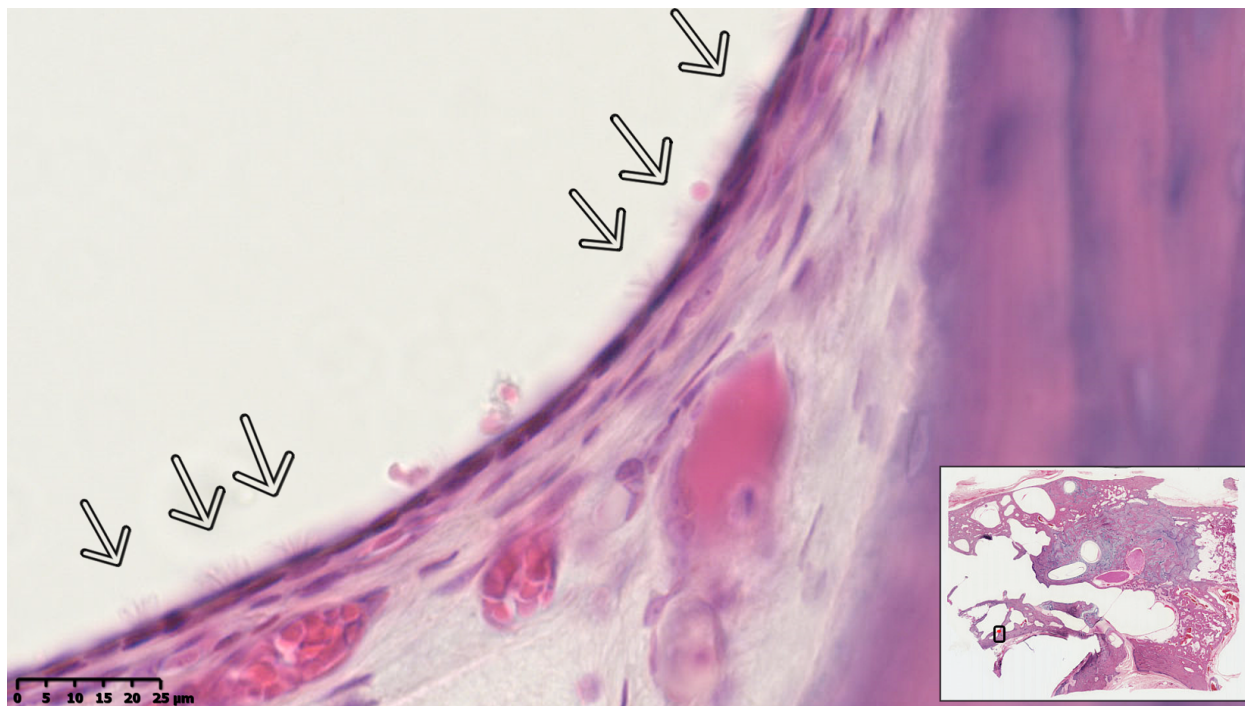








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Highlights

- Mucosa morphology differs between antero-inferior and postero-superior middle ear
- Mucosa morphology is divided by the inter-attico-tympanic diaphragm
- The postero-superior mucosa is thinner with shorter diffusion distances for gases
- The blood vessel density is approximately uniform across the middle ear regions
- Mucosa structure of the main middle ear regions seems efficient for gas diffusion