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Role of the Mastoid in the Middle Ear Function

Histo-morphometric analysis of the mastoid mucosa versus middle ear mucosa in an animal model

Master Thesis

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Abstract

Secretory otitis media (SOM) is one of the causes of permanent hearing loss. The key factor in the pathogenesis of the SOM is a chronic under-atmospheric middle ear (ME) pressure, which is assigned mainly to an altered ME gas exchange in combination to an impaired Eustachian tube (ET) function. Recent research pointed to the importance of the mastoid in the ME pressure balance related to ambient, based on a much higher area-to-volume ratio compared to the ME cavity, which might be significant for pressure regulatory mechanisms like gas exchange and mucosal swallowing. Newer hypotheses require more studies. Ethical issues favour the use of animal models in studies for ME physiology and pathogenesis, often of rodents, which are readily available and easily maintained. They have a corresponding tympanic cavity, but not the mastoid. An extension of the tympanic cavity called bulla is considered to take over the role of the mastoid.

The present work raises the hypothesis that there is a significant difference in the diffusion and perfusion of the bulla versus the tympanic cavity mucosa in the rabbit. The data could be relevant in the interpretation of the studies on ME gas physiology.

The method consists of a manual measurement of the distances from the centres of the blood vessels to the surface of mucosa, of the thickness of mucosa layer, of the diameter and surface density of the vascular structures in 1 mm histological samples from tympanic cavity and bulla in 6 rabbits. Data from each region are used in comparative analysis.

The results show statistically significant shorter diffusion distances (30.7 vs. 14.8, $p < 0.001$) and thinner mucosa in the bulla (46.3 vs. 29.3, $p = 0.001$), but significantly more blood vessels in the tympanic cavity (307 vs. 284, $p = 0.002$). The vascular diameter did not differ significantly in the tympanic cavity vs. bulla (31.2 vs. 29.7, $p = 0.70$). Histologically, the mucosa of the tympanic cavity presents a more cubical epithelium and a looser connective tissue, whereas the bulla was lined by a flat surface epithelium. Generally, the results might suggest a higher specialization of the rabbit bulla for the trans-mucosal gas exchange vs. the tympanic cavity, but more efficient swallowing-congestion modifications of mucosa lining in the tympanic cavity.

Abbreviation list:

A/V ratio aria-to-volume ratio

EAC external auditory canal

ET Eustachian tube

H&E hematoxilin-eozyn staining

MACS mastoid air-cell system

ME middle ear

RBC red blood cells

SD standard deviation

SOM secretory otitis media

TM tympanic membrane

Table of contents

1. Introduction

Most of the hearing impairing conditions are caused by middle ear (ME) pathology, represented by secretory otitis media (SOM) and a range of serious sequelae. These pathological states are developed when a chronic disequilibrium occurs in the physiologic constants of the ME.

Because it is an isolated anatomical cavity (see *Appendix A*), supplemented with air only by intermittent openings of the Eustachian tube (ET), the ME is regarded as a semi-rigid gas pocket, normally characterized by an important pressure homeostasis. This is obviously related to its main function, which is the transmission of sound from the external auditory canal (EAC) to the inner ear, where the hearing receptors are located. The sound pressure coming from ambient is transmitted through the ME by vibrations of the tympanic membrane (TM) and the chain of ossicles (see *Appendix A*). Therefore, the optimal sound transmission is conditioned by an optimal mobility of the TM-ossicles system. This condition is only fulfilled when there is a pressure balance between the two sides of the TM, namely when the ME pressure equals the ambient pressure.

In pathological states, the ME chronically fails to keep this balance and the occurrence of this phenomenon is high (see *section 1.1*). The cause is not fully understood, even if several complementary mechanisms have been suggested to participate in the overall ME pressure regulation (see *section 1.2*).

Recent research has brought newer hypotheses into discussion, moving the focus from the ET, which was traditionally regarded as the only source of failure in ME pressure balance, to the role of the mastoid, which has long been regarded only as a passive pressure buffer, able to 'dilute' high pressure differences by its cellular or multi-chambered structure (see *section 1.3* and *Appendix A*).

More evidence has emerged that the mastoid might play an active role as well, based on its large surface covered with a well vascularised mucosa (see *sub-section 1.3.1*), which is involved in a continuous gas exchange between the air of the ME and the mucosal blood vessels (see *section 1.4)*.

The newer theories broaden the view on the complexity of the ME pressure regulation, but also bring newer unknowns in the overall equation. Therefore, more studies are required, but ethical issues favour the use of animal models in studies for ME physiology, with subsequent implications in humans.

Bulla is a term often assigned by authors to the animal ME (Daniel et al., 1982; Hanamure & Lim, 1987; Ar et al., 2007), and sometimes excluding the epitympanum (Uchimizu, 2007). The free medical dictionary defines the tympanic bulla as "a thin-walled bony capsule which houses an extension of the cavity of the middle ear, the tympanic cavity" (The Free Dictionary). The present study adopted the definition of The Free Dictionary for bulla, as distinctive from the ossicles-containing *tympanic cavity*.

The present work raises the question whether there is a morpho-functional partition in the rabbit ME corresponding to the one of the human ME and performs a histo-morphological comparison between the tympanic cavity and bulla mucosa (see *section 1.5*). The data can be useful in interpreting the studies on ME gas physiology and pathogenesis using rabbit models.

1.1 SOM: clinical background

By the age of 4 years, around 80% of children develop at least one episode of SOM (Zielhuis et al., 1990) and 28% further need to be treated with ventilation tubes inserted into the tympanic membrane (TM) until the age of 7 (Gaihede et al., 2007). This tubes restore the ME pressure and hearing function, and after a given time they are usually extruded with complete healing of the TM. Nevertheless, up to 24% of cases require longer or repeated use of ventilation tubes, resulting in permanent perforations and are candidates to surgical TM reconstruction (Strachan et al., 1996).

Moreover, repeated episodes of otitis media and long-term ventilation tube treatments often result in a structural degeneration of the TM (63% of cases) and can continue with a progressive retraction of the TM (Gaihede et al., 1997). In advanced stages of TM retraction, the bony structures in the ME start to be eroded, leading to a permanent hearing loss in 50%. Few of these cases get ultimately to the point of cholesteatoma formation (10/100,000 of inhabitants). (Nieland et al., 2012).

Whereas these conditions may not be overall frequent, they are still serious and their treatments require substantial resources along multiple out-patient control visits and longer surgeries with complicated reconstruction of the TM and ossicles. Often the hearing remains partially impaired despite optimal surgical reconstruction.

Among the patients admitted to the hospital for reconstructive otosurgery, 80% are related to *an under-atmospheric pressure in the ME,* hereafter called ***underpressure*** (Rasmussen et al., 2008). Despite the intense research on specific mechanisms generating and maintaining the ME pressure to negative values, there still are unknown parameters.

Traditionally, it was believed that the cause resided in an impaired function of the Eustachian tube, which is the only natural communication of the middle ear with ambient, though indirect, by the nasopharynx. But clinical experience as well as part of the experimental studies showed that the ET function in terms of capability to successfully perform the Valsalva manoeuvre did not correlate to the course of otosurgical patients. During the postoperative course of such patients; retraction pockets may develop independent of their ET function tested by the Valsalva manoeuvre, so that with a negative test their course may be uneventful without any problems related to formation of retractions, and vice versa (Sade & Ar, 1997). This means that other factors than the ET function determine whether retraction based on sustained underpressure will occur during the postoperative courses of these patients.

1.2 Pathogenesis of SOM: middle ear underpressure

SOM is generated by an accentuated underpressure created in the ME cleft, which leads to the formation of an inflammatory exudate in the tympanic cavity and to decresed hearing. Moreover, the sustained periods of underpressure are responsible for the formation of retraction pockets, atelectasis of the tympanic cavity and cholesteatoma (Sade & Ar, 1997) (see *Appendix B*).

Traditionally, the formation of chronic ME underpressure has been assumed to a long-term unbalance between two main pressure regulators: the ET openings and the gas diffusion through the mucosa layer of the tympanic cavity. This was usually explained by a constant net gas absorption from the ME air into the mucosal blood flow, insufficiently counterbalanced

by openings of the ET, which gets usually to impaired function in common cold or other upper respiratory tract infections (Sade & Ar, 1997; Doyle, 2000, Pau et al., 2009). Therefore, a huge amount of literature has been written on studies of the ET function related to SOM. Nevertheless, more recent studies revealed that the perturbation of the mucosal gas exchange is another cause for underpressure (Uchimizu, 2007).

In addition, other complementary causes have been correlated. Clinical practice has found that a diseased mastoid with sclerotic changes and decreased pneumatisation has been correlated with cases of chronic otitis media and sequelae related to ME underpressure. This finding suggests an important role of the mastoid in the overall regulation of the ME pressure. (Sade & Ar, 1997; Diamant, 1965; Tos & Stagerup, 1984).

Subsequently, more interest and basic work have emerged, and the mastoid has recently been underlined as an important field of future research (Dirckx & Sade, 2005).

1.3 ME pressure regulation and the role of the mastoid

The pressure balance between the ME and ambient can be easily disturbed by various physiological factors, such as changes in altitude (flying, elevator trip, diving), the sleep state (Tideholm et al., 1999) or changes in body position (Okubo & Watanabe, 1990), as well as by pathological factors, i.e. inflammations and infections. Moreover, the gases' law suggests that mere temperature changes already imply a change in the gas pressure (Magnuson, 2003). To face all these challenges, the ME is equipped with several complementary regulatory mechanisms.

One of them is the valve-function of the ET, which consists in openings of usually less than 1 second at times when a large or sudden pressure difference is created between the ME and the ambient, allowing that a small air bolus to be exchanged with the nasopharynx (Elner et al., 1971; McDonald et al., 2011).

Another regulatory mechanism is the movements of the flexible TM, which behaves as a passive buffer able to counterbalance small ME pressure deviations by bulging in or out (Elner, 1977; Cinamon & Sade, 2003). This action is obviously conditioned by the integrity of

the TM and limited by its own stiffness. Higher pressure differences would break it, if it was not for other complementary buffering mechanisms.

The *mastoid* part of the temporal bone (see Appendix A) plays a complementary buffering role, by its cellular structure, formed out by clusters of air-filled spaces or cells (also called *the mastoid air cell system*, MACS) separated by thin bony trabeculae, able to 'dilute' larger pressure differences. The tympanic cavity and MACS communicate in the postero-superior part of the tympanic cavity, and this region is called *aditus ad antrum.* Therefore, the two compartments have the same gas composition and pressure and form an entity called ***ME cleft***.

Moreover, the mucosa of the ME cleft is also involved in the permanent gas exchange between the air of the cavities and the gases dissolved in the mucosal blood supply. It is based on a passive diffusion of gases (O_2, CO_2. H_2O vapours, N_2 and Ar) between the two compartments, by virtue of the differences between the partial pressures of each gas (Yamamoto, 1999).

The present work pays a special attention to the structures involved in the ME trans-mucosal gas exchange and therefore this mechanism is presented more detailed in a separate section (*section 1.4).*

1.3.1 Alternative hypotheses on the role of the mastoid

Whereas the tympanic cavity is relatively small (≈ 1 cm^3) with smoother walls, the mastoid bone is larger (≈6 cm^3), due to its cellular structure which greatly enhances its area relative to its volume, expressed as area-to-volume ratio (A/V ratio). By the advent of higher resolution in clinical CT scans more studies have determined the mastoid volume, whereas only relatively few have undertaken the more complicated task of determining also its surface area; this amounts to around 70 cm^2, resulting in an A/V ratio of 16 cm^{-1} (Park et al., 2000; Gaihede et al., 2012).

Considering the much higher A/V ratio of mastoid vs. the tympanic cavity, as well as the fact that all this surface is covered with a well vascularised mucosa, it results that the functions assigned to the whole cleft's mucosa might be much more efficient in the mastoid than in the

tymapnic cavity itself. This means that the contribution of the mastoid mucosa to the total ME trans-mucosal gas exchange might be more important than traditionally considered (Gaihede et al., 2010).

Moreover, the mucosa of the ME and of the mastoid is suggested to change the volume according to the degree of its congestion. Few decades ago, this phenomenon was described in the mucosa lining the ME as due to the compliance of the ME mucosa to pressure changes (Andreasson, 1976) and treated rather as a source of error in ME pressure studies (Elner et al., 1971).

Later on, these mucosal volume variations started to be seen as a potential mechanism to expand or compress the air content in the ME cleft, contributing in this way to ME pressure equilibration. Based on data on the mastoid volume and surface area (Park et al., 2000), it has been estimated that a change in thickness of the mucosa of 6 μm will change the pressure by 1 kPa (Magnuson, 2003). This mechanism can be supported by a relatively ample blood supply and high perfusion in order to cause significant changes in the congestion of the mucosa. Again, the high A/V ratio of the mastoid mucosa compared to the ME suggests a much higher efficiency in the mastoid.

1.3.2 Phylogeny: ME mechanisms in diving species

These alternative hypotheses have been corroborated with observations in diving species. Studies on sea lion (Odend'hal & Poulter, 1966), hooded seal (Stenfors et al., 2001) and penguin (Sade et al., 2008) described the ME mucosa having complex venous and sinusoid vascular structures embedded in a loose connective tissue, just like a corpum cavernosum, which allow important volumetric changes in response to its congestion. According to these studies, when these animals dive, their ME mucosa will be flooded with blood, and thus, the air content of the tympanic cavity will be compressed into a smaller air bubble with high pressure corresponding to the surrounding water pressure, as well as the air volume is replaced by a volume of an incompressible media (blood and/or tissue fluid) (see Figure 1). This enables the animals to dive to high depths without rupturing their TM's.

In both the sea lion and the hooded seal it has been described that multiple foramina perforate the bone from the outside into the tympanic cavity suggested to carry many blood vessels;

obviously an ample blood supply is needed for an effective and fast filling of the sinusoids or venous cavernous structures.

Figure 1. Drawing explaining the changes in ME mucosa of diving mammals; **A**. In atmospheric pressure, the venous sinuses are collapsed and mucosa is flat. **B**. During diving, the venous sinuses are enlarged; the mucosa is engorged with blood and the ME space is narrowed, increasing the pressure (Odend'hal & Poulter, 1966).

The swallowing of these structures may be passively controlled by the ME pressure itself, so that during submersion the generation of a ME underpressure will drive the filling, and vice versa (Odend'hal & Poulter, 1966). On the other hand, the idea of a possible active role by autonomous neural control has more recently been suggested (Stenfors et al., 2001; Nagaraj & Linthicum, 1998).

1.4 The ME pressure regulation by trans-mucosal gas exchange

As a biological gas pocket, the ME cleft has a steady-state condition, governed by the partial pressures of component gas species, which are carbon dioxide (CO_2), oxygen (O_2), nitrogen (N_2) and argon (Ar). Numerous studies tried to elucidate the kinetics of the ME gases, by monitoring the pressure variations after replacing the air in the ME cleft with gas mixtures of different composition. They used different animal models, e.g., rabbits (Hamada et al. 2002, Marcusohn et al., 2006, 2010), rats (Kania et al. 2004, 2006, Ar et al., 2007; Marcusohn et al., 2010), and monkeys (Doyle and Seroky 1994; Doyle et al. 1995, 1999), as well as humans (Aoki et al. 1998; Uchimizu et al. 2005). In addition, efforts have been made to describe the ME gas economy in mathematical models (Doyle and Alper, 1999; Fink et al. 2003).

The air of the ME cleft has a composition close to the venous blood, but different from the atmospheric air and also by the mixed air found in nasopharynx (Sade & Ar, 1997, Doyle, 2000). Thus gases passively diffuse from the compartment where they have a higher partial pressure to the one where they have lower partial pressure, until the equilibrium is reached. Whereas CO_2 and O_2 molecules have a high diffusion rate through tissues, re-equilibrating fast after a partial pressure disequilibrium, (Marcusohn et al., 2006, 2010), N_2 and Ar particles diffuse from the ME to the blood at a very low rate, causing a slow continue decrease of the total ME presure and governing the steady-state of the ME pressure (Pau et al., 2009; Kania 2006). The H_2O vapours have the same partial pressures and do not have any significant contribution in ME pressure balance (Marcusohn et al., 2010).

Generally, the CO_2 is the first diffusing molecule species from the mucosal blood flow to the air of the ME cleft until equilibrium is reached, producing a small increase in the total ME pressure. Immediately after, the O_2 molecules diffuse at a slower rate from the ME cleft to the blood flow and re-equilibrate very soon, as the partial pressure difference is usually very small. The inert gases (N_2 and Ar) diffuse from the ME cleft to the blood flow, producing a slow decrease of the total ME pressure.

While the pressure regulation assigned to the ET function is intermittent, very short and accounting only for a gas volume exchange of only 0.79 – 2.79 µL, the one assigned to gas exchange is continuous, on one hand because the ME gas content is often changed by ET openings, on the other hand due to the mucosal blood dynamics, and it has been described as an overall rate of gas gain and rate of gas loss, estimated at respectively 41µL/h and 34,8 µL/h in normal conditions (Mover-Lev et al., 1998).

1.4.1 The 'limiting' factors: diffusion and perfusion

The diffusion factor is represented by aspects such: the functional properties of the mucosal cells, the gas specific diffusion rate, permeability of blood vessels (Ars & Ars-Piret, 1994), as well as the tissue morphology, reflected by the distance from blood vessels to surface of epithelium and the density of the tissue (Ars et al., 1997).

The perfusion factor consists of the density of the vascular structures and the blood flow. If the vascular density can be estimated from measurements on histological sections, the blood

flow has been indirectly estimated by assuming proportionality between active mucosal surface and the body mass, based on its proportionality to the mass of the heart, which further correlates with the blood flow in the mucosa. By this means, a comparison was performed between rabbits and rats and the proportions were verified between these two species (Marcusohn et al., 2010).

1.4.2 Histological structure of the mucosal layer

The ME trans-mucosal gas exchange is assigned to the mucosa layer, which limits the diffusion by its histo-morphometric properties and by perfusion rate.

Mucosa is the innermost layer of hollow organs, thus covering all the bone surface of the ME cleft. It is further subdivided in an epithelial layer (*mucosal epithelium)*, standing on a *basal membrane* (also called basal lamina) and a connective tissue (CT) layer called *lamina propria*, adherent to the periosteum layer of the underlying bone (Telser et al., 2007) (see Figure 2).

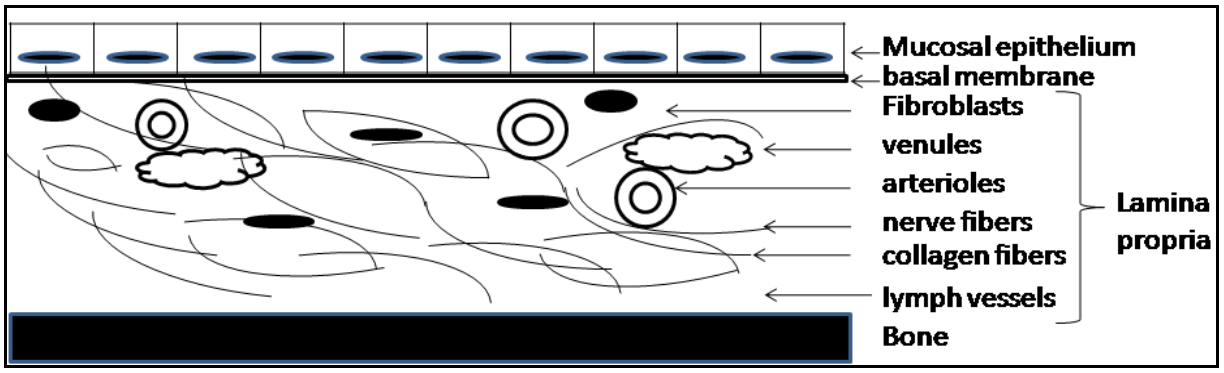

Figure 2. Drawing of the mucosa structure.

The *epithelium* of the antero-inferior human ME mucosa is respiratory, similarly to the mucosa from nasopharynx and ET, with 1-3 layers of columnar or cubical cells, often ciliated or with microvili, secretory and non-secretory, and intercalated with goblet cells (Sade et Ar, 1997). In the postero-superior part of tympanic cavity and in the mastoid, the epithelium is mono-layered, flat, alternating with very rare cuboidal cells (Hentzer, 1970).

The *lamina propria* is formed of loose CT, characterized by a less organized appearance and by being composed of relatively few cells (mezenchymal cells, basal cells, fibroblasts,

lymphocytes, macrophages, etc) in a large volume of extracellular matrix. The major matrix components are collagen and elastic fibres.

Generally, the loose CT contains many blood vessels, nerve endings, lymphatic vessels and interestingly, misses a basal membrane, making it easier to be crossed by macromolecules and cells (Telser et al., 2007). The postero-superior and antrum mucosa seems to be more abundant in superficial blood vessels compared to tympanic cavity (Ars & Ars-Piret, 1994).

1.4.3 Two morpho-functionally distinct regions of ME cleft mucosa

Whereas the mucosa lining the tympanic cavity has been systematically described, the histological properties of the mastoid mucosa have only been sporadically reported. In the only 2 systematic papers emerging from the same institute, the structure of the human ME mucosa has been compared with the mastoid represented by specimens taken from *the antrum* part of the mastoid (Fig. 1) (Ars & Ars-Piret, 1997; Matanda et al., 2006).

Based on different structure between the human mastoid mucosa and tympanic cavity mucosa, it has been suggested that the antero-inferior part of the tympanic cavity is more specialized in clearance, by serous and mucus secretion and cilia movements serving as conveyor-belt for the secretions and pathogens towards the ET and thus to the nasopharynx (Sade & Ar, 1997). By contrast, the properties of the mastoid facilitate an efficient gas exchange by significantly shorter diffusion distance and higher perfusion compared with the tympanic cavity (Holmquist, 1978; Ars & Ars-Piret, 1994).

Moreover, the aforementioned differences between the tympanic cavity and mastoid mucosa in histological structures are illustrated in images posted on the home page of the Massachusetts Eye and Ear Infirmary Temporal Bone Consortium, from which we exemplify in Appendix C. They have displayed a series of histological sections of the ME from one temporal bone, which by chance also present only the adjacent parts of the mastoid (Massachusetts, 2011).

In summary, the two compartments of the ME cleft seem to have different histological properties indicating different functions (Ars et al., 1997; Matanda et al., 2006; Massachusetts, 2011). However, these findings are limited by the facts that in one study only *the antrum* part of the mastoid was investigated quantitatively (Ars et al., 1997; Matanda et

al., 2006), and in the other source only mastoid cells close to the tympanic cavity are visible Massachusetts, 2011).

1.5 Problem formulation

Deregulation of ME pressure is a very frequent pathogenetic factor involved in SOM and related sequelae resulting in permanently decreased hearing. The perturbation of the trans-mucosal gas exchange is one of the main causes responsible for the development of an under-atmospheric ME pressure.
More evidence has emerged that the mastoid plays an important role in the gas exchange, favoured firstly by a high A/V ratio involved, and secondly by the histological structure of its mucosal lining.

This includes clinical evidence, structural studies of mastoid anatomy and histology, as well as phylogenetic evidence in diving mammals. Further, new clinical experiments indicate that the mastoid mucosa might be an active component in regulation of the ME pressure (Gaihede et al., 2010), by its congestion-swelling volumetric changes. Altogether these findings point to the importance of the mastoid, and warrant further research in this direction (Dirckx & Sade, 2005).

Studies on mastoid and ME mucosa are very difficult to be performed in humans and can arise ethical issues. The main reason is that if mucosa contains nerve structures responsible for a reflex of pressure regulation, harvesting human samples might affect them, producing a permanent disequilibrium in the donor's ME pressure regulation.

Primates have a ME very similar to human, but their use as model for ME gas exchange studies are very expensive and difficult, and also lead to ethical issues.

Rodents have been the elective model for many physiological ME studies, because they are cheap, easy to handle and with a ME relatively similar to human. They have a corresponding tympanic cavity separated from EAC by a tympanic membrane and hosting 2 or 3 ossicles, whereas the MACS is replaced by a postero-inferior prolongation of the tympanic cavity called ***bulla*** (Ar et al., 2007).

Bulla is a term often assigned by authors to the whole ME and bulla cavity, including the upper ossicles part as well as the inferior prolongation (Daniel et al., 1982; Hanamure & Lim, 1987; Ar et al., 2007), and sometimes as the whole cavity containing the ossicles together with the broadened inferior part, as distinct part of the epitympanum (Uchimizu, 2007). The free medical dictionary defines the tympanic bulla as "a thin-walled bony capsule which houses an extension of the cavity of the middle ear, the tympanic cavity" (The Free Dictionary). The present study adopted the definition of The Free Dictionary for bulla and tympanic cavity.

The human ME mucosa proved to have 2 distinct morphological compartments related to the gas exchange, i.e. the tympanic cavity and the mastoid antrum, and this implies differences at functional level as well (Ars et al., 1997). Thus, it is expected that the animal models often used for studies on ME gas physiology and pathology, for instance the rabbit, will also present two corresponding partitions in their ME cleft, characterized by different morphology, suggesting functional differences.

Consequently, the present study *hypothesizes* that there is a significant difference between the morphological properties of the mucosa of the tympanic cavity, compared to those of the bulla mucosa in rabbit. These properties refer to the basic histological properties of the mucosa, i.e. the epithelium type, the thickness of the connective tissue, the abundance of blood vessels, as well as to some parameters important for the tissue resistance to the diffusion: the distances from the blood stream to the mucosal surface and the size of the blood vessels.

The purpose of investigating this hypothesis is to form evidence for an important role of the mastoid in ME pressure regulation, and to explain clinical and experimental observations of changes in ME pressure, where ETO's are not involved.

2. Methods and Materials

2.1 Experimental design

The method consisted of a qualitative and quantitative histo-morphometrical comparison between samples harvested from ME and bulla mucosa respectively in rabbits.

Bulla is a term often assigned by authors to the whole ME and bulla cavity, including the upper ossicles part as well as the inferior prolongation (Daniel et al., 1982; Hanamure & Lim, 1987; Ar et al., 2007), and sometimes as the whole cavity containing the ossicles together with the broadened inferior part, as distinct part of the epitympanum (Uchimizu, 2007). The free medical dictionary defines the tympanic bulla as "a thin-walled bony capsule which houses an extension of the cavity of the middle ear, the tympanic cavity" (The Free Dictionary). The present study adopted the definition of The Free Dictionary for bulla, in contrast to the ossicles-containing part of the ME cleft, called tympanic cavity.

2.1.1 ME specimens

The heads of six healthy New Zeeland White rabbits were provided by the Animal Laboratory of Aalborg Hospital. All animals were about 8 months old and had a mean weight of 3850g. They were donated after being used in experiments for nervous electrical stimulation. Their MEs were harvested within 2 - 4 hours after the animals were sacrificed, by means of an otomicroscope and micro-surgical tools in a manner illustrated in Figure 3.

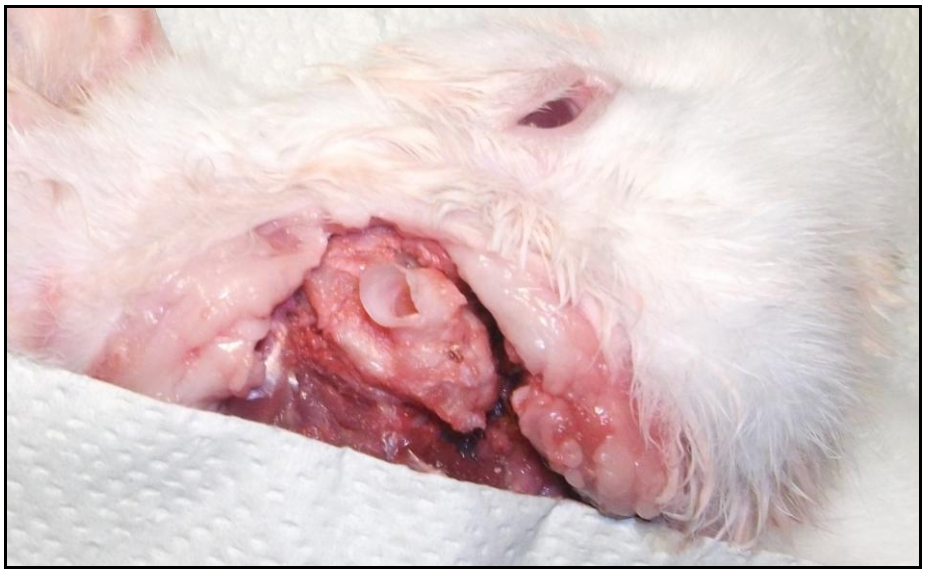

Figure 3. Harvesting approach of the rabbit ear.

The specimens were set up for fixation in 4% neutral buffered formalin immediately after harvesting, for periods from two days to 2 weeks, until they could start decalcification procedure in the assigned laboratory. The fixation inside the cavities was facilitated by 1-2 small holes created at the harvesting in the bony wall with a thin needle, superiorly and inferiorly.

2.2 Histological preparations and slicing

All histological procedures, as well as the subsequent steps until the image acquisition and storage were carried out by the Pathologic Institute of Aalborg Hospital according to standardized protocols.

After 2 to 14 days of formalin fixation at room temperature, the specimens underwent decalcification in 85% formic acid for periods varying between 3-6 weeks, until the samples could be cut with a knifeblade (Feather Trimming Blade, no.130).

Each specimen underwent 2 parallel coronal dissections, in order to include the TM on the slides (see Figure 4). They were first half-cut through the middle of the EAC, in order to include the TM on each slide. Afterwards, one half was half-cut again at approximately 0.5 cm. The purpose was to get a sample from the central region and one more from another location. Cut 2 could be either from anterior or posterior part.

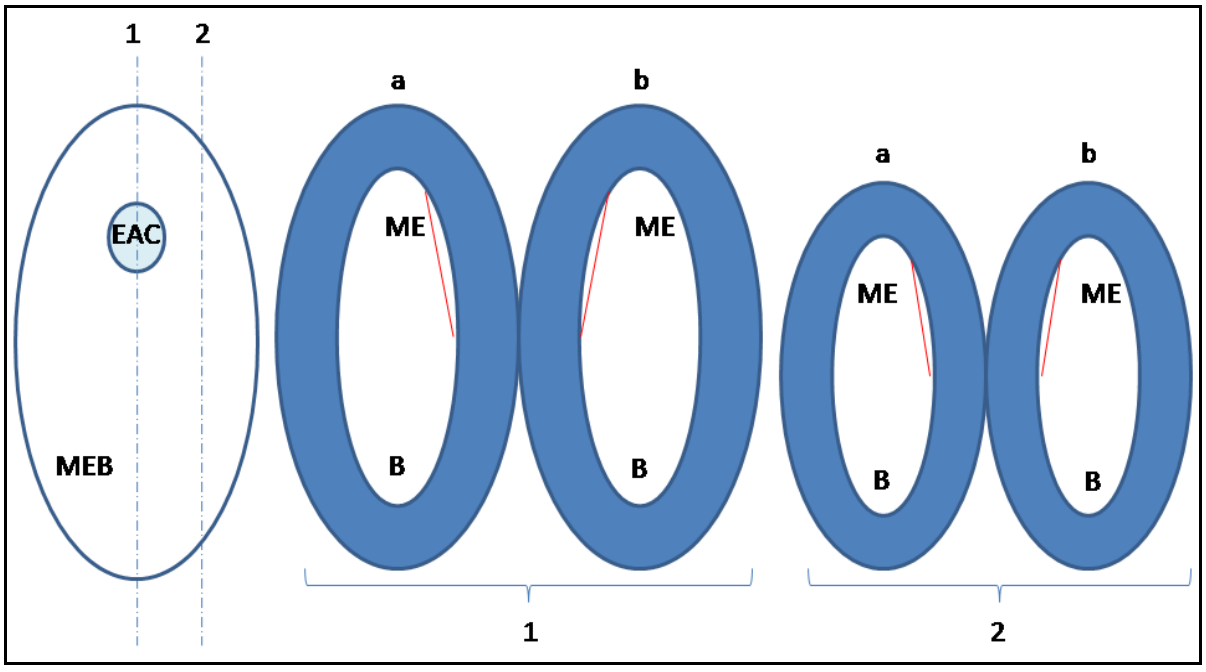

Figure 4. The cutting procedure. **EAC** external auditory canal; **MEB** middle ear and bulla common cavity; **ME** middle ear cavity; **B** bulla. The tympanic membrane is represented with the red line.

The slices 1b and 2b from figure 4 were chosen for further processing and analysis from each ME bulla. They were paraffin-embedded, and consecutively cut in 2 sections of 4 µm thickenss at 300 µm distance. Finally, they were stained with hematoxilin-eozyn (HE).

2.3 Image acquisition

Full slide images were acquired by scanning of the stained slides by Nanozoomer HT 1.0 (Hamamatsu) with the 20X "source lens". The digital full slide images were stored in a database accessed using the Nanozoomer HT Viewer 1.0.

2.4 Defining the regions of interest

The tympanic cavity was defined according to the presence of the TM and the ossicles. In samples where the TM was missing due to processing procedures, the shape and dimensions of the two cavities were compared to the corresponding samples where the TM was present.

One mucosa sample of 1mm length was picked from the endpoints of the longest diameter of each ME and bulla cavity (see figure 5). Basically, from each image, one sample was picked up from epitympanum or superior tympanic cavity, and the other sample from the middle part of the floor of the cavity, making sure that in was harvested from the bulla region.

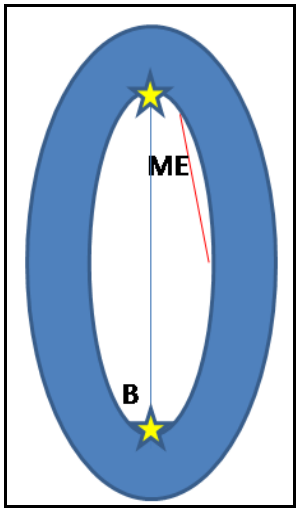

Figure 5. The sampling procedure. **EAC** external auditory canal; **ME** tympanic cavity; **B** bulla. The tympanic membrane is represented as red line and the yellow stars represent the sampling places.

The reason for choosing the furthest regions possible is that no literature to define the separation between ME and bulla was available.

The mucosal blood vessels were defined as structures delimited by endothelial cells and containing red blood cells (RBCs), with diameter ranging from 5 to more than 100 μm and with a structure of the walls as described by histology textbooks (Tesler et al., 2007). The spare RBCs were seen outside or within the epithelial lining, without having a net contour of endothelial cells were not counted for blood vessels. At each cutting step, one image was used for analysis and the other one as control to support the identification of the blood vessels on the analyzed images. All the identifiable blood vessels on the selected samples were included in the analysis.

2.5 Quantitative examination

The images could be accessed from the database by Nanozoomer Viewer 2.0 HT (Hamamatsu). Using the annotating tools of this viewer, linear measurements could be performed. A zooming scale ranging up to x100 allowed an annotation accuracy of approximately 1μm. Because curvilinear lengths were not possible to be performed, whereas the cavities have both linear and curvilinear surfaces, the 1mm sample lengths were chosen to be as flat as possible, in the predefined regions. The lengths were measured as parallel to the bone.

The following parameters were measured:
- the longest diameter of the identified blood vessels;
- the distance from the midpoint of the longest diameters to the surface of the epithelium and all the underlying vascular structures found in the selected area;
- mucosa thickness in 5 sites of the picked samples.

It was possible to annotate the longest diameter or the shortest distance quite accurately due the property of the annotation tool of the Nanozoomer Viewer to permanently display the current distance as soon as the first position was already chosen. Moreover, it was possible to change the magnification during the annotation process, increasing the chance of correctly positioning the second point. In this way, the cursor could ‘slide’ along the surface and the operator could fix the end point when the displayed distance had the minimal value. Similarly, after choosing a point on the margin of a blood vessel, the pointer could ‘slide’ along the contour, until the maximal value of the diameter could be reached and marked.

The density of the blood vessels was estimated by dividing the number of blood vessels identified in 1 mm sample length to the extrapolated area of the sample, which was calculated as the product between the sample length and its mean thickness.

Data extracted from the two regions of each ear were analyzed in SPSS version 19, using independent-samples *t*-test and one-sample *t*-test at 95% confidence interval.

3. Results

From the 6 rabbits 10 ears could be analyzed, resulting in 21 images. One ear was excluded from quantitative analysis due to major defects in the structure resulted from cutting procedures (right ear of rabbit 2), and another one because it presented signs of inflammation (left ear of rabbit 6). Each image gave a sample for both ME and bulla, resulting in a total of 42 samples for analysis.

3.1 Gross anatomy of the ME cleft in the rabbit

The ME cleft of the rabbit is a air-filled cavity of around 11 mm high, which is much larger inferiorly, where the diameter can get to around 7-8 mm. The narrower superior part contains the ossicles and is incompletely separated by the lower region by a thin bony plate (see Figures 6 and 7).

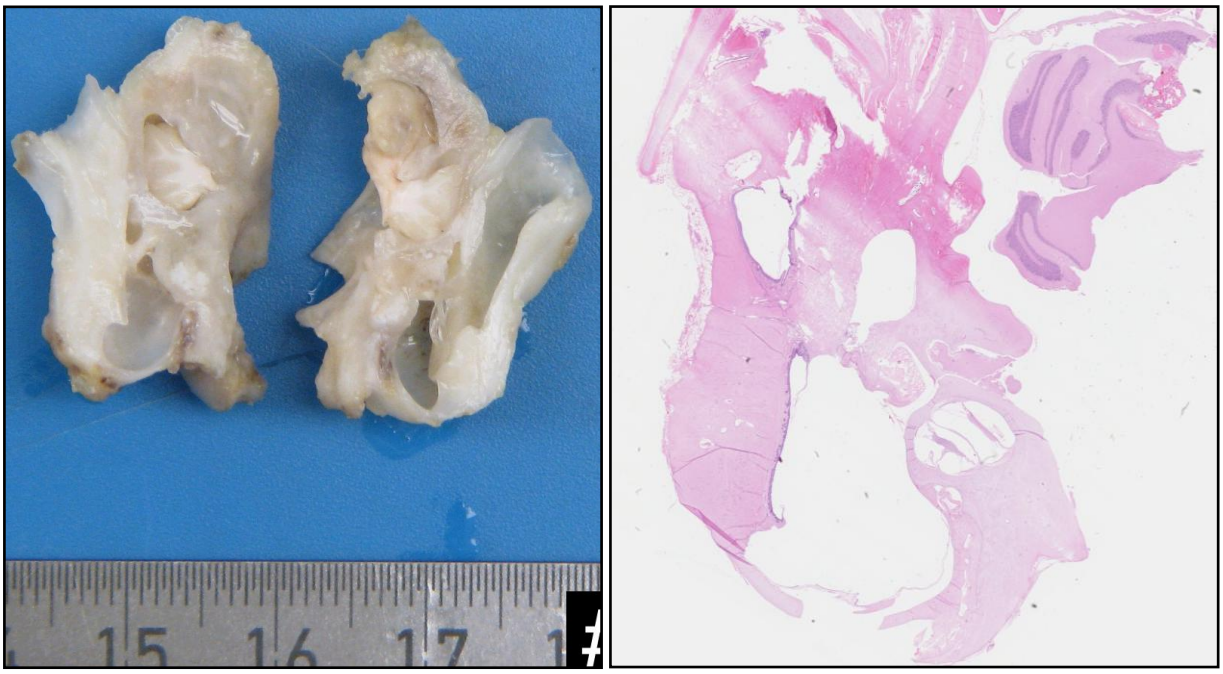

Figure 6. Coronal section through the rabbit ME cleft. *Left panel*: coronal section through a right ME cleft *Right panel*: Inverted histological image of the left sample from the left panel, (rabbit 4, right ear; ×0.45)

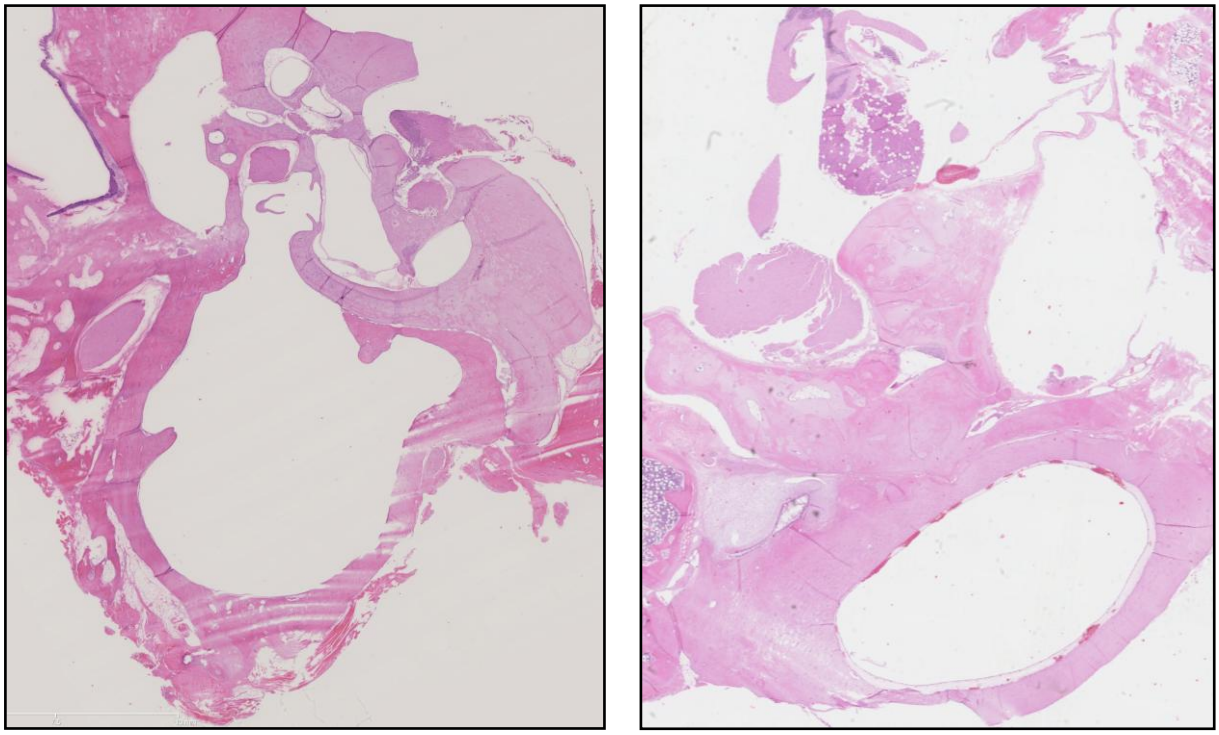

Figure 7. Delimitation between the tympanic cavity and bulla *Left panel*: section close to the central plane of the cavity. *Right panel:* more marginal section through the ME cleft (left panel: rabbit 5, left ear: right panel: rabbit 6, right ear, ×0.48).

3.2 Microscopic observations

The mucosa presented a high inhomogeneity concerning the thickness, the density of the mucosal tissue, the number, diameter and distribution of the blood vessels. The epithelium lining the ME cleft was usually mono- or pseudo-stratified, with either flat or cubical cells. In epitympanum, mucosa presented more often cubical epithelium, and a more expanded lamina propria, whereas the bulla was characterized by a thinner mucosa with flat epithelium and denser lamina propria. Figures 8 and 9 exemplify the tympanic cavity and bulla mucosa in the same ear.

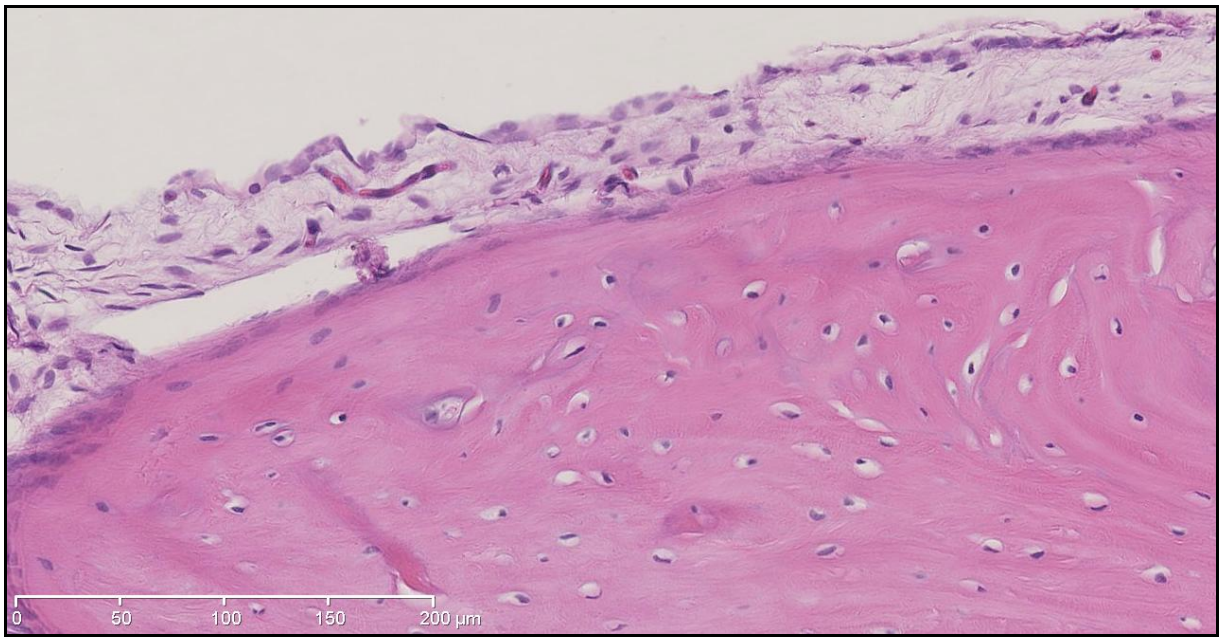

Figure 8. ME mucosa. The epithelium is cubical mono or pseudo-stratified. A capillary oriented to the mucosal surface is sectioned longitudinally (left ear of rabbit 6, ×20)

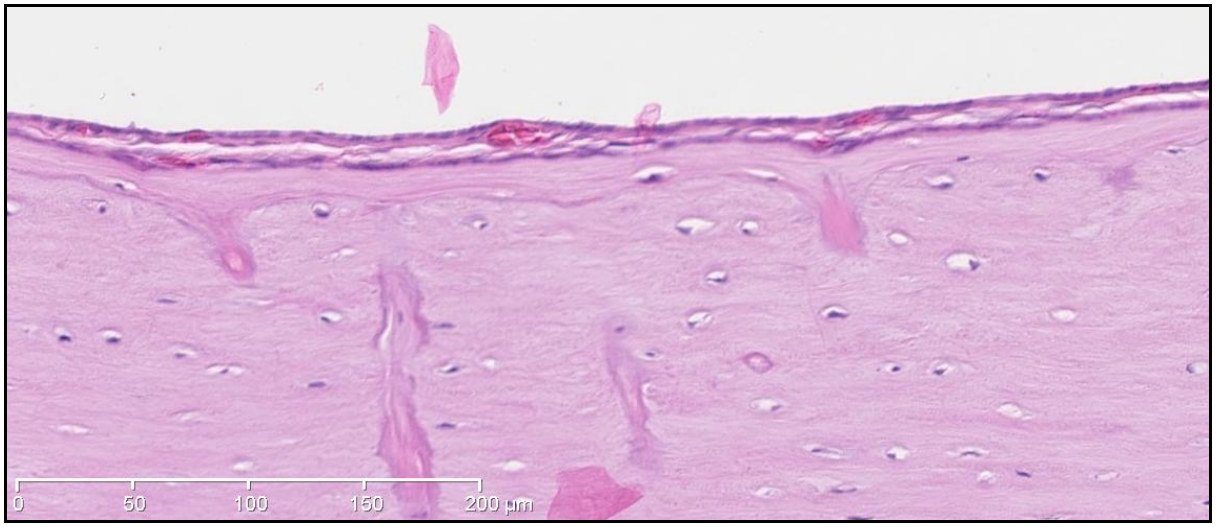

Figure 9. Bulla mucosa. The epithelium is flat and mono-layered. To be noted the presence of several superficial blood vessels, just underlying the mucosal epithelium (rabbit 6, left ear; ×20).

Closer to the ET orifice, as well as towards the TM, portions of cubical or columnar cells, sometimes organized in 2-3 layers could be seen (see Figures 10 and 11).

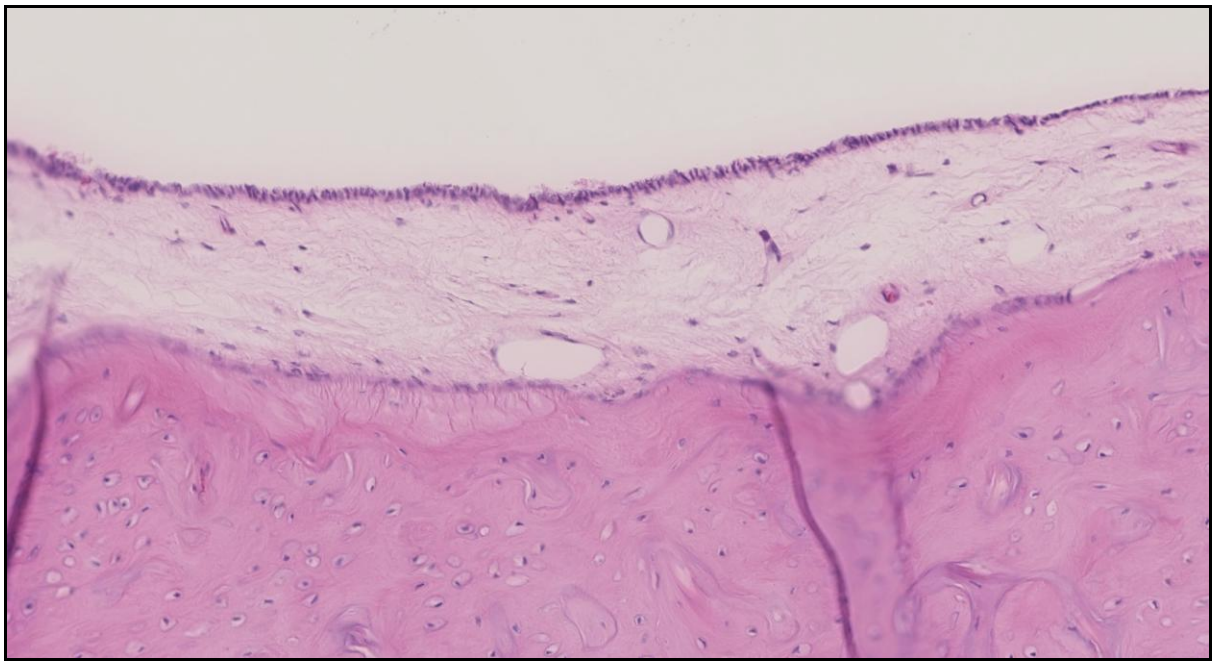

Figure 10. Portion of columnar epithelium in the tympanic cavity in vicinity of the TM. Note the transition from flat to columnar epithelium in the right part of the image and few ciliated cells in the centrum (rabbit 5, right ear; ×10).

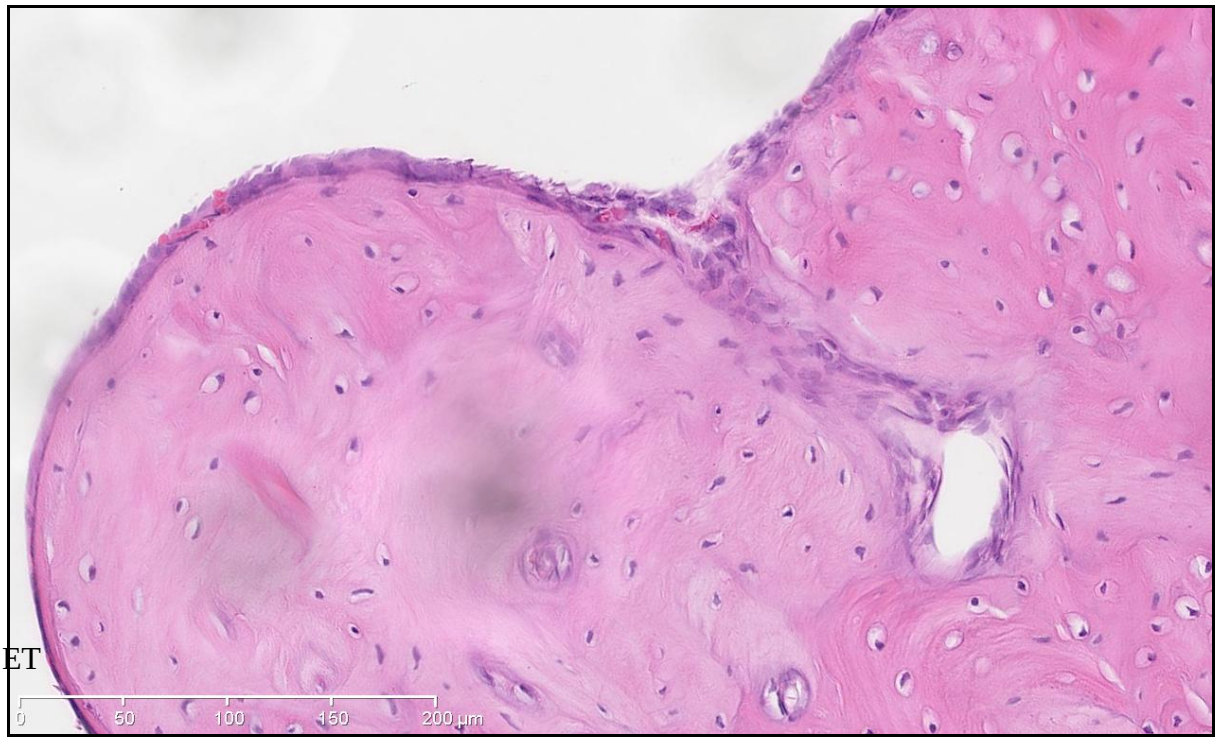

Figure 11. Pseudo-stratified epithelium in vicinity of the ET. Remark the mucosa folding into the bone (rabbit 5, left ear, ×20).

Cillia could be rarely noticed in small patches distributed in various parts of both, the tympanic cavity and bulla, but being apparently higher in the former than in the latter (see Figures 12 and 13).

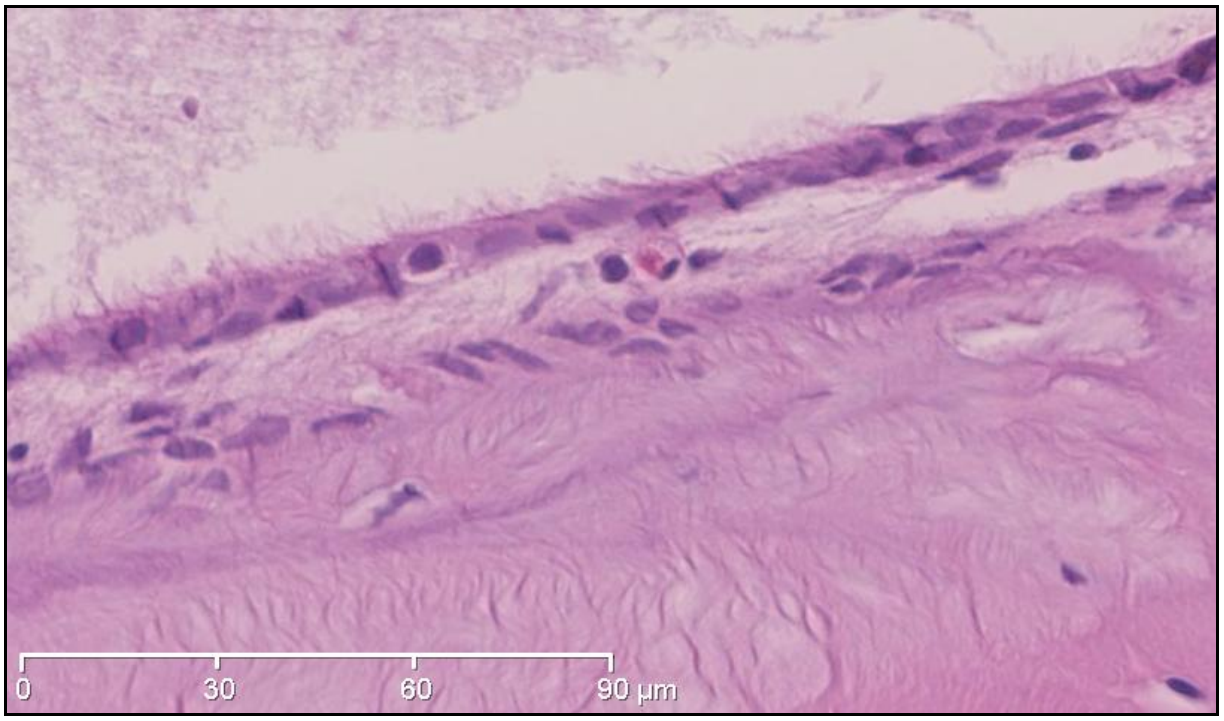

Figure 12. Ciliated pseudo-stratified epithelium in the upper tympanic cavity (rabbit 3, left ear, ×63).

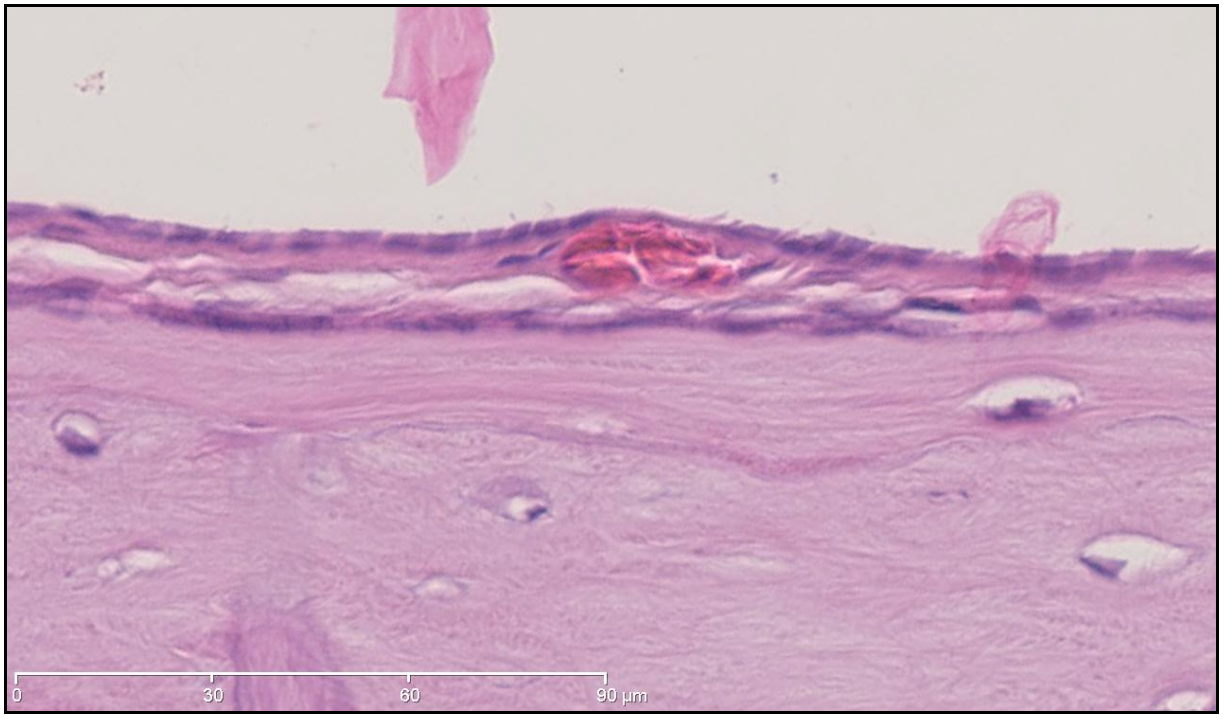

Figure 13. Ciliated epithelium in the bulla, at the transition to the tympanic cavity. (rabbit 6, left ear, ×63).

3.3 Quantitative analysis of mucosa morphology in the tympanic cavity and bulla

A comparative description of the morphological parameters between the mucosa lining the upper and lower segments of the rabbit ME cleft is presented in Tables 1-4.

The mean distance between blood vessels and mucosal surface was significantly higher ($p < 0.001$) in the upper tympanic cavity (mean 30.7 µm), as compared to the bulla region (mean 14.8 µm) (see Table 1). If rabbit 1 is excluded, the means become 16.4 µm vs. 10.9 µm respectively, with a $p = 0.007$.

The diameters of mucosal blood vessels was not significantly different between the two regions ($p = 0.7$), but their average was slightly larger in the tympanic cavity (31.2 µm) vs. bulla (29.7 µm) (see Table 2).

The thickness of the mucosa gave a significantly larger mean in the tympanic cavity (46,3 µm) as compared to the bulla (14.8 µm), with a p-value < 0.001 (see Table 3). Also, there is a significant difference between the distances to the blood vessels and the thickness ($p < 0.001$). If rabbit 1 was excluded, the overall mean thickness became 26.2 µm vs. 25.4 µm respectively, which did not differ significantly ($p = 0.8$).

Table 1. The distances between blood vessels and mucosal surface (mean ± SD) in tympanic cavity vs. bulla

Rabbit no.	Ear	Tympanic cavity		Bulla		P-value
		N	Mean ± SD (µm)	N	Mean ± SD (µm)	
1	R	43	68.9 ± 42.9	26	28.4 ± 11.3	<0.001
	L	7	68.7 ± 26.0	5	32.0 ± 08.4	0.009
2	L	5	13.5 ± 12.1	7	7.4 ± 07.4	0.35
3	R	26	11.6 ± 6.9	13	19.0 ± 25.2	0.31
	L	20	14.3 ± 07.0	27	9.1 ± 05.3	0.01
4	R	23	32.0 ± 29.5	18	10.8 ± 10.9	0.003
	L	9	13.8 ± 13.2	12	21.9 ± 30.7	0.42
5	R	18	7.6 ± 8.4	7	5.1 ± 1.7	0.24
	L	12	14.5 ± 11.2	5	7.2 ± 3.9	0.06
6	L	20	17.2 ± 12.8	24	6.3 ± 4.5	0.001

Overall		183	30.7 ± 34.6	144	14.8 ± 15.9	<0.001

Table 2. The diameters (mean ± SD) of blood vessels in tympanic cavity vs. bulla

Rabbit no.	Ear	tympanic cavity		Bulla		P-value
		N	Mean ± SD (μm)	N	Mean ± SD (μm)	
1	R	43	35.2 ± 34.0	26	46.2 ± 40.2	0.25
	L	7	65.6 ± 91.3	5	48.3 ± 17.1	0.63
2	L	5	61.7 ± 52.3	7	35.7 ± 27.0	0.35
3	R	26	14,3 ± 15.5	13	34.7 ± 34.2	0.06
	L	20	28.4 ± 32.6	27	26.0 ± 32.3	0.80
4	R	23	40.2 ± 34.2	18	22.8 ± 16.6	0.04
	L	9	27.0 ± 28.4	12	35.4 ± 50.5	0.63
5	R	18	19.3 ± 14.0	7	27.2 ± 27.1	0.48
	L	12	52.0 ± 71.3	5	18.7 ± 17.9	0.15
6	L	20	17.4 ± 15.0	24	13.2 ± 7.82	0.26
Overall		183	31.2 ± 38.2	144	29.7 ± 31.9	0.70

Table 3. The thickness of mucosa (mean ± SD) in tympanic cavity vs. bulla

Rabbit no.	Ear	Tympanic cavity		Bulla		P-value
		N	Mean ± SD (μm)	N	Mean ± SD (μm)	
1	R	18	118.0 ± 32,5	19	44.0 ± 9.4	<0.001
	L	5	109.7 ± 22.4	4	37.3 ± 10.3	0.001
2	L	5	21.6 ± 9.3	5	18.7 ± 7.6	0.60
3	R	10	24.0 ± 9.6	10	31.6 ± 22.4	0.34
	L	15	25.4 ± 9.0	15	27.9 ± 8.3	0.43
4	R	10	41.8 ± 62.2	9	28.0 ± 15.3	0.51
	L	10	17.6 ± 11.8	10	36.0 ± 26.2	0.06
5	R	10	18.5 ± 12.2	10	18.3 ± 7.8	0.97
	L	10	23.6 ± 12.0	10	23.5 ± 16.9	0.98
6	L	10	35.4 ± 11.5	10	14.9 ± 5.0	<0.001
Overall		103	46.3 ± 45.5	102	29.3 ± 16.7	0.001

The estimated density of blood vessels in a sample of 1 mm^2 mucosa section was significantly higher ($p = 0.002$) in the tympanic cavity (307 mm^{-2}) compared to bulla (284 mm^{-2}) (see Table 4).

Table 4. The mean number of observed blood vessels per 1 mm sample length in tympanic cavity and bulla and the mean density of blood vessels in a sample of 1 mm^2 mucosa section in tympanic cavity and bulla

Rabbit no.	Ear	Mean number of blood vessels		Mean density of blood vessels in 1 mm^2	
		Tympanic cavity	Bulla	Tympanic cavity	Bulla
1	R	11	11	93	159
	L	7	7	64	135
2	L	5	5	263	368
3	R	13	13	406	219
	L	7	7	250	321
4	R	12	12	429	321
	L	5	5	139	167
5	R	9	9	500	222
	L	6	6	261	125
6	L	10	10	667	800
Overall		9 ± 3	7 ± 3	307 ± 192	284 ± 200

4. Discussion

The role of ME trans-mucosal gas exchange in ME pathology is an important topic in medical research. Efforts have been put in quantifying the parameters involved and to formulate mathematical models of the trans-mucosal gas exchange mechanism that would help in explaining for instance the simultaneous decrease in ME pressure and increased mucosal thickness. In this respect, Ar and co-workers proposed a model to estimate "the effective mucosal blood flow rate" as related to inflammatory conditions, based on partly empirical, partly measured data. Thus he used the mucosa thickness as an estimate of the diffusion distance (Ar et al., 2007). Incorporaion of measured morphometric parameters into the model would better reflect the histologic contributions to the predicted data.

Even though the exact diffusion path of the gases might not be a straight line, the current study started with the assumption that the gas economy would choose the shortest distance to the blood stream, which could be represented by the actual distance from the blood vessels to the surface and might be quite different from the thickness of the mucosa. Moreover, the blood vessels can be placed superficially in the mucosa, so as the gas might not need to diffuse in the whole thickness, but up to the closest blood vessels. The net difference between the values of the mean thickness and the distances between blood vessels and surface might make a difference in the result obtained by Ar's model, which could be significant in ears with so small volume like the rabbit's.

The present work aimed at comparing these distances between two regions of the ME cleft in the rabbit model in the light of a similar study in humans, which found a functional partition of the human ME cleft, in a region corresponding the most part of the tympanic cavity, and a region corresponding to the MACS and its antrum, based on the distances between blood vessels and mucosal epithelium (Ars et al., 1997).

The current study similarly found a significantly lower diffusion distance, a thinner mucosa and a more flat epithelium in the bulla, suggesting that the gas exchange is more favoured in this region compared to the upper tympanic cavity. On the other hand, the significantly thicker mucosa, combined with the higher mucosal blood vessel density and slightly larger vascular diameters in the tympanic cavity might suggest a more efficient swallowing-congestion changes at this level compared to bulla.

Moreover, there could be seen regions in mucosa of both the tympanic cavity and the bulla presenting folds or invaginations in the underlying bone (see Figure11), apparently tending to enhance the surface to volume ratio, fact important for the gas exchange as well as for the ME gas compression by mucosal swallowing.

The small patches of cilia noticed to be better represented in tympanic cavity than in the bulla suggest the clearance function of the ME epithelium, which has been more detailed described in different rodents elsewhere (Daniel et al., 1982; Hanamure & Lim, 1987; Chole & Chiu,

1985). Basically, in these studies cilia has been described to be represented on few linear tracts from different portions of tympanic cavity or bulla towards the ET orifice, which is usually situated antero-medially in rodents, at the junction between the two ME regions.

The results of the current work might be influenced by the fact that the first rabbit presented a much thicker mucosa compared to the others, which could be due to either edema or autolysis of the tissue. At the preliminary otoscopic examination, this rabbit did not present signs of otitis media, and no infiltration tissue was noticed on the histological sections, but the time lag between death and starting of the fixation procedure might have been longer than 3 hours, when the normal processes of autolysis start (Hentzer, 1970). However, the exclusion of this specimen from analysis would less affect the difference in the distances from vessels to surface, but the difference in thickness between the 2 regions would no longer be significant.

The extrapolation of the section area based on the mean thickness might be forced, due to the irregular profile of the sample, which this time was approximated to a rectangle. A better estimate of the vascular density could result based on the total vascular area in a sample of measured area, which could not be measured in Nanozoomer Viewer.

Although the rabbit is one of the animals often used in ME experiments (Marcusohn et al., 2006, 2010; Uchimizu, 2007), to our knowledge no histo-morphometrical comparison between tympanic cavity and bulla mucosa is available. It is generally accepted that the rabbit ear, likewise in other rodents, misses the MACS described in human, but the bulla, situated inferiorly, appears much broader than the superior part where the structures involved in sound transmission are located. Thus, this portion enhances the surface of the ME cleft and this might influence the trans-mucosal gas exchange as well as changes of the ME pressure by mucosal congestion or swallowing, properties assigned also to the human MACS.

The thickness of mucosal layer can be relevant for estimating the possible changes in ME pressure by swelling and congestion of mucosa, and can be explained by the ability of engorging much blood, but a limitation could be the possible shrinkage due to decalcification agent (Neves et al., 2011).

The current method had a very similar principle to the one used by Ars and colleagues (Ars et al., 1997), i.e. the measurement of the linear distance between the surface epithelium and the centre of blood vessels. Whereas this is the simpler approach suggested by the basic stereolgy textbook (Weibel, 1979), but still reliable, Kanick et colleagues considered the alternative method mentioned in the same textbook, i.e. the harmonic mean of all simulated pathway lengths from mucosal surface to the capillary wall for different mucosal geometries (Kanick, et al., 2005). The latter compared the two methods with a detailed, two-dimensional finite element analysis applied for the oxygen diffusing capacity and concluded that the predictive accuracy was improved by including the harmonic mean of all contributing pathway lengths, based on capturing information about the capillary shape and distribution.

Despite the limitations in mathematical accuracy, the current study presents the advantage of an easy and cheap preliminary set of experiments to organize the methods to be used in more detailed human studies. On one hand, the animals were readily available and could be used without asking for ethical approval.

On the other hand, some difficulties were encountered in choosing the staining method, as there was no standardized protocol for the use of immunohistochemical methods more specific to the vascular structures in animals, and there were not enough time and material resources for "trial and error" procedures. Therefore, the general H&E staining was used.
The staining quality was good, but the close nuances of pink and violet of the histological elements as well as the biological shape heterogeneity prevented an appropriate segmentation of the blood vessels, thus limited the use of image analysis tools. Moreover, the exporting of the images from the database in order to be used in image analysis programs encountered technical problems related to resolutions higher than ×20.

This limitation, on the background of a serious time constraint from the availability of the microscopic images, led to the use of the presented manual method, which increases the likelihood of intra-individual variability in the measurements. Even though semi-automated software packages are available on the marked, they can be expensive and still require a definition of the regions of interest by a trained operator. More advanced studies make use of several 'blinded' operators who define the regions of interest on the same image in order to decrease the intra-individual variability (Brügmann et al., 2012).

In further studies, the use of CD31 staining would increase very much the specificity to blood vessels, which gives them a dark brown staining on a blue background, facilitating the set up of a semi-automated method. It would be thus possible to set up an algorithm of an automated calculation of the shortest distance from a manually pointed region of interest to the automatically smoothed epithelial surface.

Even if the methods can be much improved by automation, a persistent limitation can be foreseen even for future semi-automated algorithms, related to the accuracy of the identified blood vessel count. Many capillaries might be congested at the time of sectioning and this prevents them to be identifiable in common light microscopic methods. A serial step-cutting of a tissue sample is the common solution that gives a spatial view in the tissue, also bringing the big advantage of supplementing studies on temporal bone using other imagistic methods with model histological parameters.

A good example is the Figure 9 of the present work, which is a nice illustration of the blood supply crossing the bone towards the mucosa, which might confirm the interpretation of the micro-channels described by micro-CT scanning (Gaihede et al., unpublished data). Thus, corroborating data about the mastoid and bulla morphology from various study angles, i.e. CT methods, light and even electron microscopy, clinical experiments on ME pressure fluctuations with the clinical experience of SOM, a better understanding of the underlying mechanisms is obtained.

The diffusion and perfusion parameters are especially interesting in inflammatory conditions. If the increase in the mucosal thickness during inflammation would be expected to limit the gas diffusion, experimental studies have revealed that actually the gas exchange is more intense in this case (Ar et al., 2007). An explanation comes from histo-morphological quantification of mucosa-related parameters: a looser connective tissue, a significantly increased in blood flow and an increased permeability, factors which accelerate the gas diffusion and can correlate with the impairment of the ET ventilation, based on the general inflammatory background (Uchimiz, 2007).

All these factors together are able to lead to very low values of ME pressure. The blood flow might be much increased and the diffusion distances much decreased by the possible opening of more superficial capillaries, that can located immediately under the epithelial layer. At very low underpressures, based on large hydrostatic pressure, a transsudate can be forced to effuse from the blood vessels in the ME cavity. Basically, this means that the impermeable basal membrane of the blood vessels gets to allow fluid effusion. This would be easily explained using electron microscopy to explore the continuity of the basal membrane, as well as the junctions between the endothelial cells that line the mucosal blood vessels.

Finally, an interesting remark is that the functional partition of the ME cleft based on the diffusion distances estimated by the same method as Ars et coworkers (Ars et al., 1997) as well as the current study, has been also described in in inflammation (Matanda et al., 2006). All in all, the present study opened the perspective of performing similar histo-morphological measurements in normal and diseased human mastoids, by improving of the technique with a combination of more specific staining methods, stereological sampling and semi-automated methods for image analysis that allow a degree of flexibility in defining of the regions of interest.

5. Acknowledgements

The author is deeply grateful to Mogens Vyberg, Giedrius Lelkaitis and Kent Amond, who supported the progress of the current work despite their very tight schedule, as well as to the Pathological Institute of Aalborg Hospital for supporting all the expenses of the experiments. Special thanks to supervisor Michael Gaihede for the large availability in supervising the project, besides many clinical and research responsibilities.

Appendix A. ME anatomy

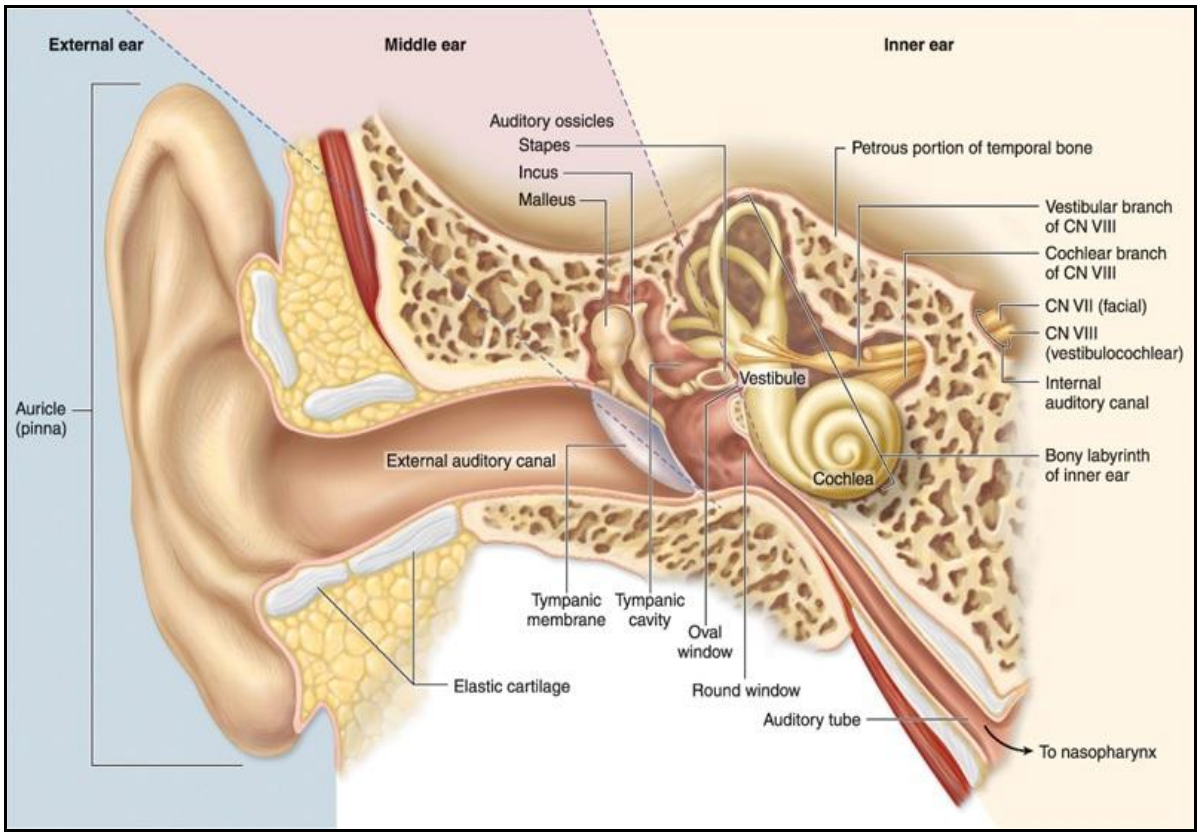

Figure A. The human middle ear

Source: http://academic.kellogg.edu/herbrandsonc/bio201_mckinley/Nervous%20System.htm

Appendix B: ME pathology

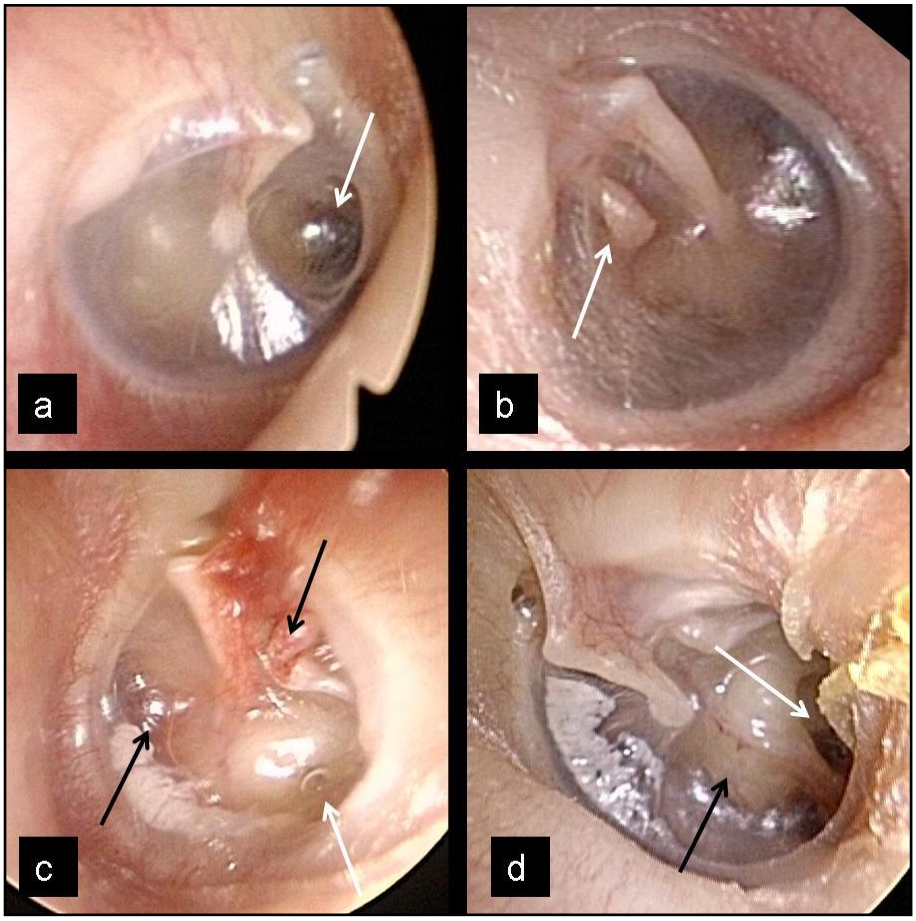

Figure B.1 Otomicroscopy illustrating various degrees of TM atrophy, retractions, and atelectasis (by courtesy of dr. Michael Gaihede)

a. Normally appearing TM, but with a smaller atrophic scar area anteriorly with retraction corresponding to the position of a previous ventilation tube (white arrow; right ear)

b. TM with diffuse atrophy and retraction, where the TM adheres to the incudo-stapedial joint in the upper posterior quadrant; the long process of the incus appears not to be eroded here, and the joint is intact (white arrow; right ear)

c. TM with pronounced atrophy and retraction, where the TM in the upper posterior quadrant surrounds the incudo-stapedial joint as well as the stapedial tendon and minor erosion appears on the lateral aspect of the long process of the incus (upper black arrow). In the lower part, the TM adheres to the medial wall of the ME cavity (promontory) and a small air bubble is seen (white arrow), as well as fluid level is seen anteriorly as signs of middle ear effusion (lower black arrow; left ear)

d. TM with pronounced atrophy and atelectasis of the posterior parts – note the steeper position of the manubrium of the malleus. The TM is adherent to the promontory (black arrow) and with a deep retraction down into the tympanic sinus postero-inferiorly (white arrow); the bottom cannot be seen, but the yellow crusts appearing on the ear canal wall from the retraction may indicate an incipient cholesteatoma formation (left ear).

Appendix C: MEEI Temporal bone consortium histology of the temporal bone

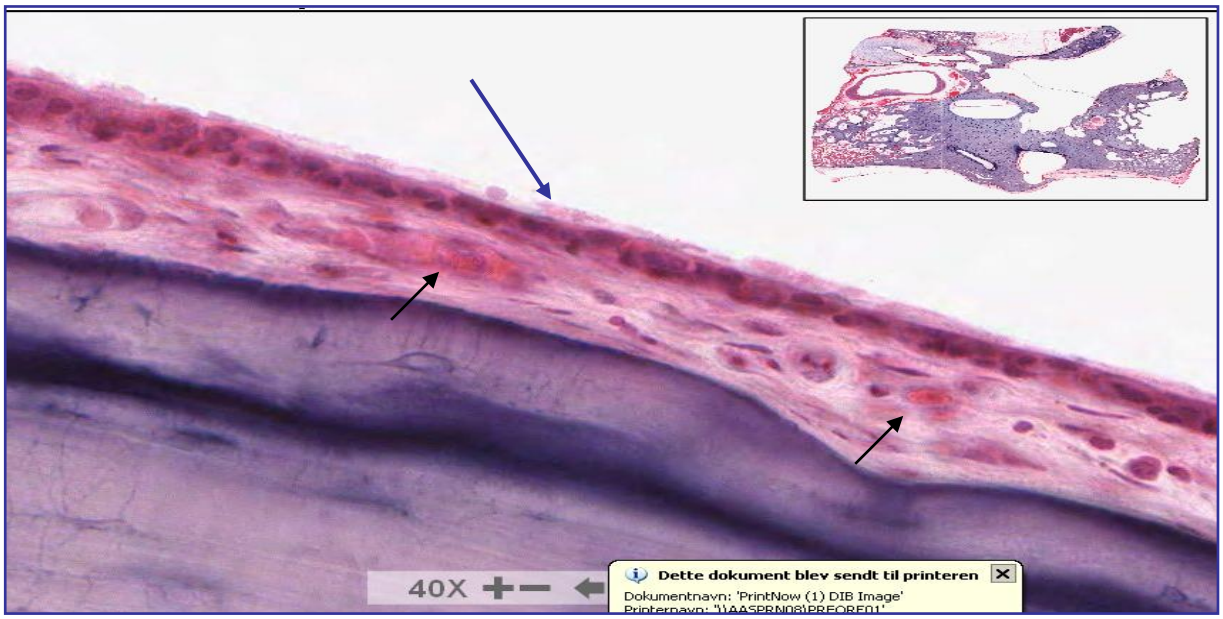

Fig. C.1. Microphotography of ME mucosa (HE staining; 40×). Pseudostratified epithelium with ciliated cells and superficial mucus layer (blue arrow). More vessels can be seen (black arrows). From Massachushetts Eye and Ear Infirmary, Temporal bone consortium, Boston – at http://temporalboneconsortium.org/educational-resources/atlas/image-library/

Fig.C.2. Microphotography of mastoid mucosa (HE staining; 40×). Flat monolayer epithelium (blue arrow) with underlying subepithelial loose connective tissue (SE) and abundant vessels (black arrows). From Massachusetts Eye and Ear Infirmary, Temporal bone consortium, Boston – at http://temporalboneconsortium.org/educational-resources/atlas/image-library/

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