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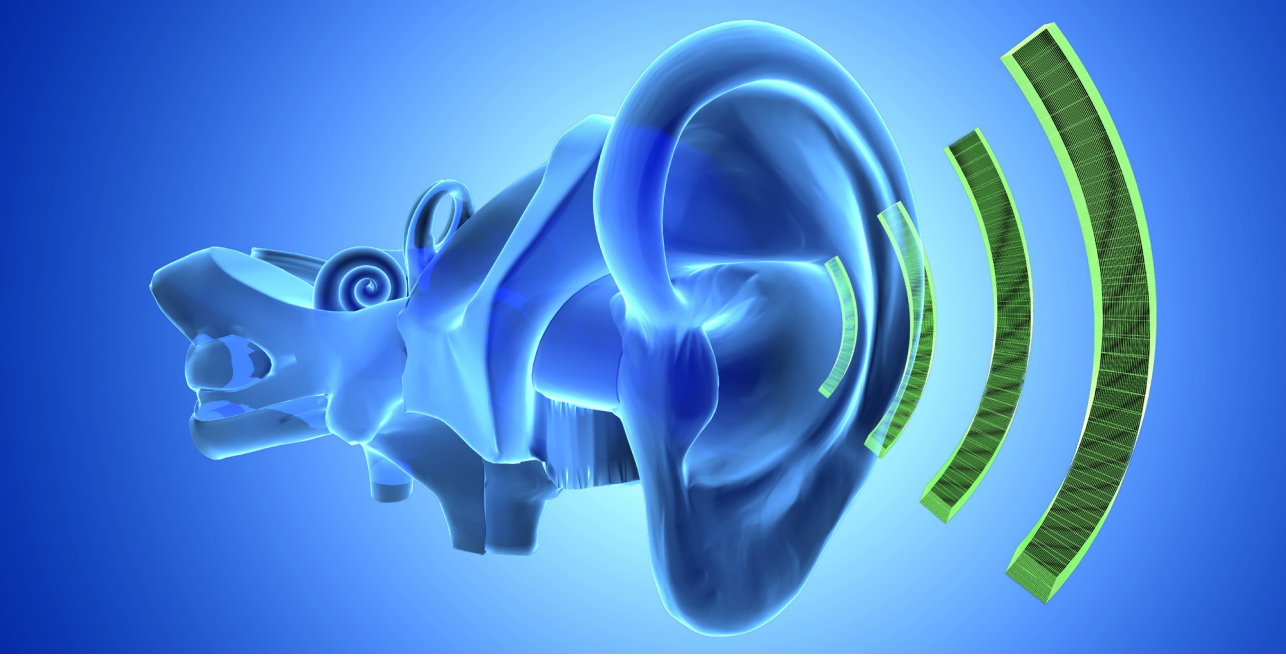

MIDDLE EAR PRESSURE REGULATION

COMPLIANT STRUCTURES
COUNTERBALANCING PRESSURE CHANGES

BY
SIMONA PADURARIU

DISSERTATION SUBMITTED 2018

AALBORG UNIVERSITY
DENMARK

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by

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PhD Dissertation

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CV

With a background in biomedical engineering and specialization within prosthetic technologies and biomaterials from Romania, I have followed my passion of helping people with hearing loss. I have started with learning practical aspects and challenges in an audiology center for three years, where I was left with unanswered questions regarding the middle ear physiology and pathology. This led me to an upgrade of my education with a research – oriented master, and my choice was Aalborg University. I graduated in 2012, with a Master dissertation within middle ear physiology in collaboration with Michael Gaihede, senior otosurgeon at the Department of Otolaryngology, Head and Neck Surgery at Aalborg University Hospital. Afterwards, I got a chance to delight in the middle ear research in the following 5 years as research assistant on the same department. The research work resulted in eight peer-reviewed articles, one proceeding abstract, and three poster presentations including the Best Clinical Poster prize at the international symposium MEMRO 2015.

ENGLISH SUMMARY

The middle ear is located deep within the temporal bone, and it is anatomically divided into two sections - *the tympanic cavity*, which lies behind the tympanic membrane, and the *mastoid* bone, which is a larger section divided into many small air-filled cavities (air cells). The middle ear is isolated from the surrounding atmosphere, mainly by hard bone walls, but also by the flexible tympanic membrane. Its function is to transfer sound pressure from the surroundings to the inner ear. This function requires frequent pressure compensation between the air entrapped in the middle ear and the surrounding atmosphere.

Middle ear pressure can be affected by several factors both in the atmosphere and due to the physiological processes, such as changes in body position, vertical movement in elevator, flight, diving, etc. In the middle ear there is a permanent exchange of gases between its air phase and the blood of the mucous membrane (mucosa) that covers the inside of the bone walls and the tympanic membrane cavity. The partial pressure of the gases usually causes a slight under-atmospheric pressure in the middle ear. Such minor or moderate pressure changes can cause small displacements of the tympanic membrane, while major changes cause openings of the Eustachian tube; the openings cause relief of underpressure by air supply from the nasopharynx. These two pressure-regulating mechanisms have been the focus of many studies over the past decades. However, recent clinical physiological tests have suggested that changes in the blood supply of the mucosa can lead to changes in its volume, thus changing the pressure in the middle ear. In view of the particular cellular structure of the mastoid with a large surface area relative to its volume, changes in the mucosa volume can be an effective factor in the pressure regulation of the middle ear.

Secretory otitis media and a number of later sequelae under the term of chronic otitis media are associated with under – pressure in the middle ear. That is, one or more factors contributing to the normal pressure regulation of the middle ear are affected. It is therefore important to have a good understanding of how and how much each single mechanism contributes to the physiology of the middle ear and pressure regulation.

This dissertation presents new knowledge about the roles that the Eustachian tube, the tympanic membrane and the mucosa have in the pressure regulation of the middle ear. The first two structures are characterized by clinical physiological measurements of pressure changes in healthy middle ears in short-term controlled experiments and in long-term monitoring over several hours. The third structure, which is not directly available in physiological tests, is characterized by histological methods. They consisted of both a systematic study of anonymous archive materials, including serial sections of the temporal bone in traditional H&E staining (hematoxylin & eosin) and new immunohistological preparations.

The results show that the Eustachian tube opens for approx. 0.34 s (SD 0.18), independent of the size of the pressure gradient or the resulting pressure equalization (Paper 1). This reminds of a biological reflex, which is in line with the literature, where possible mechanoreceptors in the tympanic membrane are discussed and presumed to be included in a neural reflex loop together with the Eustachian tube. Traditionally, openings of the Eustachian tube have been aligned with the swallowing reflex so that swallowing also triggers pressure equilibrium in the middle ear. Our results, however, show that the number of openings of the Eustachian tube is significantly lower than the number of swallowing events per day (Paper 2). This indicates that the Eustachian tube openings are an independent reflex mechanism.

Results associated with elevator trips have shown that the tympanic membrane can buffer about 23% of pressure changes in the ambient atmosphere. However, this is dependent on the volume of the middle ear, where the buffer effect can rise to more than 50% in middle ears with smaller volume (Paper 3).

The results of the histological studies have shown that the mucosa's morphology is not the same overall within the middle ear. Anteriorly and inferiorly in the tympanic cavity there is a higher epithelium with cilia, looser connective tissue and a thicker mucosa with blood vessels situated deeper under the epithelium. The other regions of the middle ear have a thinner mucosa with blood vessels closer to the surface, a flat epithelium without cilia and with closer connective tissue. However, the density of blood vessels is almost the same everywhere. These morphological variations indicate that the front and bottom parts of the middle ear are involved in clearance and local immune defense, while the other regions are adapted to gas exchange. In addition, a statistically significant difference was found in the mucosa thickness whether it contained filled blood vessels as opposed or its blood vessels were not apparent. This suggested the ability of the mucosa for volumetric changes, which may also affect the pressure of the middle ear (Paper 4).

A number of other observations on the archival materials illustrates stages of pathological changes of the mucosa and surrounding bone that seemed associated with under-pressure. There were described secretions in the airspace, invasive connective tissue or adipose tissue in the air cells and new bone formation that doubled the bone mass relative to healthy middle ear (Paper 5).

Preliminary results of immunohistological staining with endothelial – specific markers indicate that the mucosa has a greater number of blood vessels than identified by routine staining. The difference is probably due to the fact that some blood vessels are often closed or collapsed, or that they are cut peripherally. In addition, other special staining methods targeted nerve structures and smooth muscle in the mucosa. If these structures can be identified in future studies, they will underline the idea that volumetric changes in the mucosa can be neurally controlled and, therefore, included in an *active* regulation of the pressure of the middle ear along with openings of the Eustachian tube.

Our preliminary protein analyzes have demonstrated the applicability of mass spectrometry to formalin-fixed, decalcified and paraffin-embedded mucosal biopsies from the middle ear. These findings open up new perspectives for researching the middle ear structure and function in both normal and in pathologic cases.

DANSK RESUME

Mellemøret ligger dybt i tindingebenet, og det er anatomisk delt i to afsnit - *tympanum*, som ligger bagved trommehinden, og *mastoidet*, som ligger bagtil og, som er et større afsnit opdelt i mange små luftfyldte hulrum (*celler*). Mellemøret er isoleret fra den omgivende atmosfære væsentligst af hårde knoglevægge, men også af den fleksible trommehinde. Dets funktion er at overføre lyd tryk fra omgivelserne til det indre øre. Den funktion kræver hyppig trykudligning mellem luften i mellemøret og atmosfæren.

Mellemøre trykket kan påvirkes af flere faktorer både i atmosfæren og pga. de fysiologiske processer, som for eksempel i forbindelse med ændringer i kropstilling, lodret bevægelse i elevator, flyvning, dykning, osv. I mellemøret findes en permanent udveksling af gasarter mellem dets luftfase og blodet i den slimhinde, som dækker knoglevæggene og trommehindens inderside. De partielle tryk af gasarterne forårsager normalt et let undertryk i mellemøret. Sådanne mindre eller moderate trykændringer kan medføre små forskydninger af trommehinden, mens større ændringer medfører åbninger af det Eustakiske rør; åbningerne bevirker udligning af undertryk ved tilførsel af luft fra svælget. Disse to trykregulerede mekanismer har været fokus i mange studier igennem de seneste årtier. Imidlertid har nyere klinisk fysiologiske forsøg peget på, at ændringer i slimhindens blodfylde kan medføre ændringer i dens volumen, og på den måde ændre trykket i mellemøret. I betragtning af særlig mastoidets cellulære struktur med et stort overflade areal i forhold til dets volumen, kan ændringer i slimhindens volumen være en effektiv faktor i mellemørets trykregulering.

Sekretorisk mellemørebetændelse og en lang række senfølger under betegnelsen kronisk mellemørebetændelse er forbundet med undertryk i mellemøret. Det vil sige, at en eller flere faktorer, som bidrager til mellemørets normale trykregulering, er påvirket. Det er derfor vigtigt at have en god forståelse af, hvordan og hvor meget hver enkel mekanisme bidrager til mellemørets fysiologi og trykregulering.

Denne afhandling præsenterer ny viden om de roller, som det Eustakiske rør, trommehinden og slimhinden har i mellemørets trykregulering. De første to strukturer bliver karakteriseret ved klinisk fysiologiske målinger af tryk ændringer i raske mellemører i korte veldefinerede forsøg og long-term monitorering over flere timer. Det tredje struktur, som ikke er direkte tilgængelig ved fysiologiske forsøg, bliver karakteriseret ved histologiske metoder. Der er inkluderet både et systematisk studie på anonymiseret arkiv materialer bestående af sekventielle snit fra tindingeben i traditionel hematoxylin & eosin farvning (H&E) og nye immunohistologiske præparater.

Resultaterne viser, at det Eustakiske rør åbner i ca. 0.34 s (SD 0.18), uafhængig af størrelsen af tryk gradienten eller den resulterende tryk udligning (Paper 1). Dette

minder om en biologisk refleks, hvilket er i overensstemmelse med litteraturen, hvor mulige mechano-receptorer i trommehinden diskuteres og formodes at indgå i et neuralt refleks loop sammen med det Eustakiske rør. Traditionelt har åbninger af det Eustakiske rør være sidestillet med synkerefleksen, således at synkning også udløser trykudligning i mellemøret. Vores resultater viser dog, at antallet af det Eustakiske rørs åbninger er signifikant lavere end antallet af synkninger over døgnet (Paper 2). Dette peger på, at det Eustakiske rørs åbninger er en selvstændig refleks mekanisme (Paper 2).

Resultater i forbindelse med elevator forsøg har vist, at trommehinden kan dæmpe eller buffer ca. 23 % af trykændringer i omgivende atmosfære. Dette er dog afhængig af mellemørets rumfang, hvor buffer effekten kan stige til over 50% i mellemører med mindre volumen (Paper 3).

Resultaterne af de histologiske studier har vist, at slimhindens morfologi er ikke ens overalt i mellemøret. Fortil og nedadtil i tympanum findes et højere epitel med cilier, løsere bindevæv samt en tykkere slimhinde med blodkar, der ligger dybere i epitelet. De øvrige regioner af mellemøret har en tyndere slimhinde med blodkar tættere ved overfladen, et fladt epitel uden cilier og med tættere bindevæv. Tætheden af blodkar er dog næsten den samme overalt. Disse morfologiske variationer peger på, at de forreste og nederste dele af mellemøret er involveret i bortskaffelse af sekret og mikroorganismer samt i et lokalt immunforsvar, mens de øvrige regioner er tilpasset gas-udveksling. Ydermere blev der fundet en statistisk signifikant forskel mellem slimhindens tykkelse dér, hvor den indeholdt fyldte blodkar i modsætning til, hvor blodkar ikke er tydelige. Dette er med til at understrege slimhindens mulighed for volumetriske ændringer, som også kan have betydning for regulering af mellemørets tryk (Paper 4).

En række andre observationer på arkivmaterialerne illustrerer stadier af patologiske forandringer af slimhinden og omgivende knogle, som syntes associeret med undertryk. Der fandtes sekret dannelser i luftrummet, invasion af bindevæv eller fedtvæv i luft celler og ny knogle dannelse, som sås at fordoble knoglemassen i forhold til raske mellemører (Paper 5).

Præliminære resultater af immuno-histologiske farvninger med endothel specifikke markører peger på, at slimhinden har et større antal blodkar end identificeret ved rutine farvninger. Forskellen skyldes formentligt, at en del blodkar ofte er lukkede eller kollapsede eller, at de er skåret perifert. Ydermere blev der arbejdet andre specielle farvninger målrettet nerve strukturer og glat muskulatur i slimhinden. Hvis disse strukturer kan identificeres i fremtidige studier, vil de underbygge ideen om, at volumetriske ændringerne i slimhinden kan være neuralt kontrollerede og dermed indgå i en aktiv regulering af mellemørets tryk sammen med åbninger af det Eustakiske rør.

Vores præliminære proteomanalyser har demonstreret anvendeligheden af mass-spektrometri til formalin-fikserede, afkalkede og paraffin-indstøbte slimhinde biopsier fra mellemøret. Disse fund åbner for nye perspektiver for forskning mellemørets struktur og funktion i både normale og i syge tilfælde.

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ABBREVIATIONS

ME = middle ear

ET = Eustachian tube

TC = tympanic cavity

TM = tympanic membrane

MACS = mastoid air cell system

OME = otitis media with effusion

HE = hematoxylin and eosin

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LIST OF PAPERS

The present thesis is based on the following publications:

1. Gaihede M, Padurariu S, De Greef D, Dirckx JJ. Eustachian tube pressure equilibration. Temporal analysis of pressure changes based on direct recordings with an intact tympanic membrane. Hearing Research 2013; 301:53-9. doi: 10.1016/j.heares.2013.01.003.

2. Nielsen, F.S., Larsen, M.S., Padurariu, S., Jacobsen, H., Joris, D.J., Østergaard, Lasse Riis Gaihede, M., 2018. Frequency of Eustachian tube openings based on long-term direct monitoring of mid-dle ear pressure. Hear. Res. MEMRO 2018 Special Issue. Manuscript.

3. Padurariu S, De Greef D, Jacobsen H, Kamavuako E, Dirckx JJ, Gaihede M. Pressure buffering by tympanic membrane. In vivo measurements of middle ear pressure fluctuations during elevator motion. Hear Res. 2015 Dec 15. pii: S0378-5955(15)30099-X. doi: 10.1016/j.heares.2015.12.004.

4. Padurariu, S., Röösli, C., Røge, R., Stensballe, A., Vyberg, M., Huber, A., Gaihede, M., 2018. On the functional compartmentation of the normal middle ear. Morpho-histological modelling parameters of its mucosa. Submitted to Hear Res, May, 31st 2018.

5. Padurariu, S., Röösli, C., Røge, R., Stensballe, A., Vyberg, M., Huber, A., Gaihede, M., 2018. On the mastoid pneumatization. Histological observations suggesting development of sclerotic changes. Manuscript.

OTHER PUBLICATIONS

Bennike TB, Kastaniegaard K, Padurariu S, Gaihede M, Birkelund S, Andersen V, Stensballe A. Proteome stability analysis of snap frozen, RNAlater preserved, and formalin-fixed paraffin-embedded human colon mucosal biopsies. Data in Brief 2016 Feb 6;6:942-7. doi: 10.1016/j.dib.2016.01.061. eCollection 2016 Mar.

Bennike TB, Kastaniegaard K, Padurariu S, Gaihede M, Birkelund S, Andersen V, Stensballe A. Comparing the proteome of snap frozen, RNAlater preserved, and formalin-fixed paraffin-embedded human tissue samples. EuPA Open Proteomics 2016, vol. 10, s. 9-18.

Gaihede M, Cros O, Padurariu S. The role of the mastoid in middle ear pressure regulation. Proceedings of the 9th International Conference on Cholesteatoma and Ear

Surgery, June 3-7th, 2012, Nagasaki, Japan. Ed. H. Takahashi, Kugler Amsterdam, 2013.

CONFERENCE PRESENTATIONS

Padurariu S, Gaihede M, Aernouts J, Dirckx JJ. Eustachian tube pressure equilibration. Quantitative analysis of the correlation between pressure gradient and pressure change rate. Forskningens Dag, Aalborg Sygehus, Aarhus Universitetshospital, 19. april, 2012.

Padurariu S. Greef D, Jacobsen H, Kamavuako EN, Dirckx JJ, Gaihede M. Pressure buffering by tympanic membrane. In vivo measurements of middle ear pressure fluctuations during elevator motion at The 6th International Symposium on Middle Ear Mechanics in Research and Otology, Aalborg, Danmark, 1-5. juli 2015.
Poster awarded with Best Clinical Poster prize.

Padurariu S, Röösli C, Røge R, Stensballe A, Vyberg M, Huber A, Gaihede M. On the functional compartmentation of the middle ear. A histological study of the middle ear mucosa. Presented at 100 Years Otorhinolaryngology, Zürich University Hospital, Aug. 30th – Sept. 2nd, 2017.

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CHAPTER 1. INTRODUCTION

Hearing is an important factor for the language acquisition and social integration, in which the middle ear (ME) accomplishes the mechanical conversion, amplification and further transmission of the soundwaves from the ambient atmosphere to the inner ear.

ME pathology may often cause conductive hearing loss, and it affects particularly children. Most episodes of common cold associate with ME inflammation or otitis media, which may result in creation of under-atmospheric pressure (i.e. *underpressure*) and accumulation of effusion in the ME air space. In many cases, this episode resolves within days or weeks. However, the chronic cases, where the effusion lasts longer, are typically treated with insertion of ventilation tubes into the tympanic membrane (TM) for the re-equilibration of ME pressure and aeration of ME space. Nevertheless, this procedure may sometimes result in a weak scar tissue within the TM, which can degenerate in case of recurrence of underpressure later on, after the removal of the ventilation tubes. (Alper et al., 2017; Sadé and Ar, 1997). The recurrence of underpressure, indicates that the actual understanding and approach of the ME pathology still has some limitations, and require more of basic research on the factors involved in the overall ME pressure regulation.

The studies included in this thesis answer some of the unknowns in the basic science otology, formulated as research goals at the international panel meetings of the American Academy of Otolaryngology – Head and Neck Surgery (Alper et al., 2017). The scope of this thesis is to bring evidence about the mechanisms of ME pressure regulation from in vivo physiological measurements of the ME pressure as well as from the human ME histology.

The overall understanding of ME pressure regulation is of immense importance in otology for the improvement of otosurgical treatment strategies. For instance, the formation of cholesteatoma with bony destruction of ME structures as well as the high recurrence rates of cholesteatoma surgeries depend highly on sustained ME underpressure; and, while obliteration of the mastoid may decrease recurrence rates significantly, we still do not know the exact events behind (Alper et al., 2017; Takahashi, 2017). Bringing new and basic evidence together can clarify such mechanism and point at rational treatment strategies.

The following paragraphs offer an overview over the ME anatomy, physiology and pathology, highlighting uncovered knowledge areas, to which the current thesis is trying to respond. We will highlight that the ME has a mainly rigid bony structure, closed up laterally by a thin and flexible tympanic membrane (TM), and anteriorly by the muscular and usually closed Eustachian tube (ET). It has a large air space, in which pressure homeostasis is essential for the optimal auditory function. The function of pressure homeostasis is mostly fulfilled by the combined action of the soft structures,

which are the mucosal lining the bony structures (i.e. TC, MACS, ET), the muscles surrounding the ET and the TM.

1.1. MIDDLE EAR – A GAS POCKET OF THE TEMPORAL BONE

ME is a cluster of communicating bony cavities embedded in the temporal bone, and divided into two anatomical compartments, i.e. the tympanic cavity (TC), including the bony part of the ET, and the mastoid air cell system (MACS) (Ars and Dirckx, 2016;

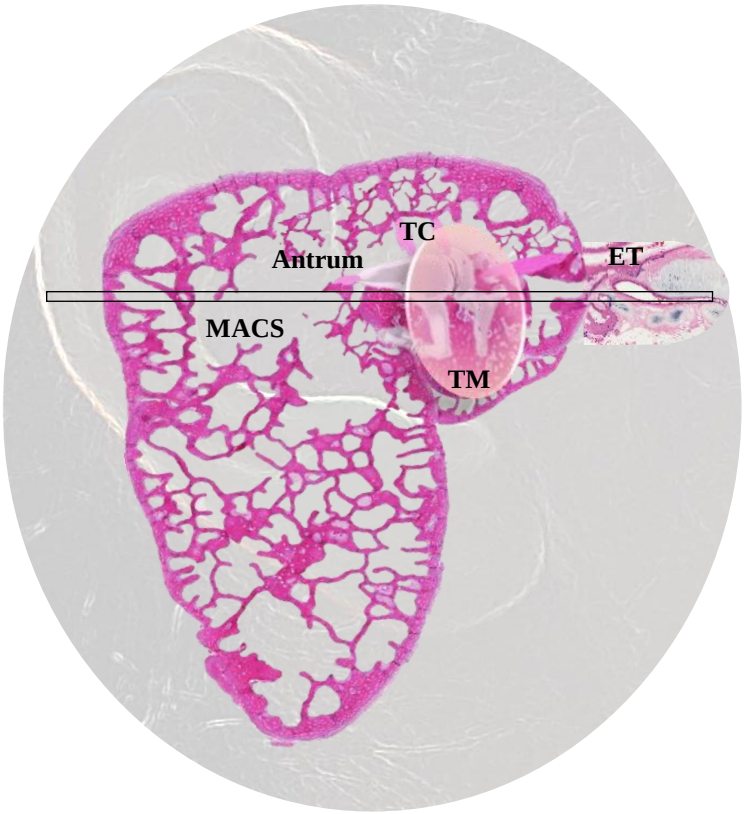

Figure 1-1. Lateral view of the middle ear. MACS = mastoid air cell system; TC = tympanic cavity, communicating with MACS by the antrum; ET = Eustachian tube, TM = tympanic membrane.

Bhutta, 2015; Tos, 1984). The TC is a larger air cell of up to 1 cm^3, containing the auditory transmission chain, and separated from the ambient atmosphere by the TM. The ET is a one third bony – two thirds cartilaginous tube connecting the TC towards the nasopharynx, having the lumen closed most of the time (Sadé and Ar, 1997). The TC is connected posteriorly and superiorly to the MACS, consisting of a cluster of air-filled intercommunicating small cavities within the bone behind the ear canal. MACS is

connected to the TC in the postero-superior part by a quite large opening called mastoid antrum (Figure 1-1 and 1-2).

The physiology of the ME is closely related to pressure homeostasis, consisting of the continuous maintenance of a near-ambient pressure, which ensures the optimal sound conduction.

Considering the predominantly hard walls of the ME cavities, it becomes evident that the soft or compliant structures may have an important role in counterbalancing any pressure changes. They will be presented in the next section.

1.2. COMPLIANT STRUCTURES OF THE MIDDLE EAR

Soft tissues are generally characterized by compliance, which is the ability to distend or compress at the application of a transmural strain (Carter et al., 2001; Kim et al., 2008; Richardson et al., 1972). The compliance is a function of the structure (Gaihede, 1996; Koike et al., 2004), thus we start with a description of the ME compliant components.

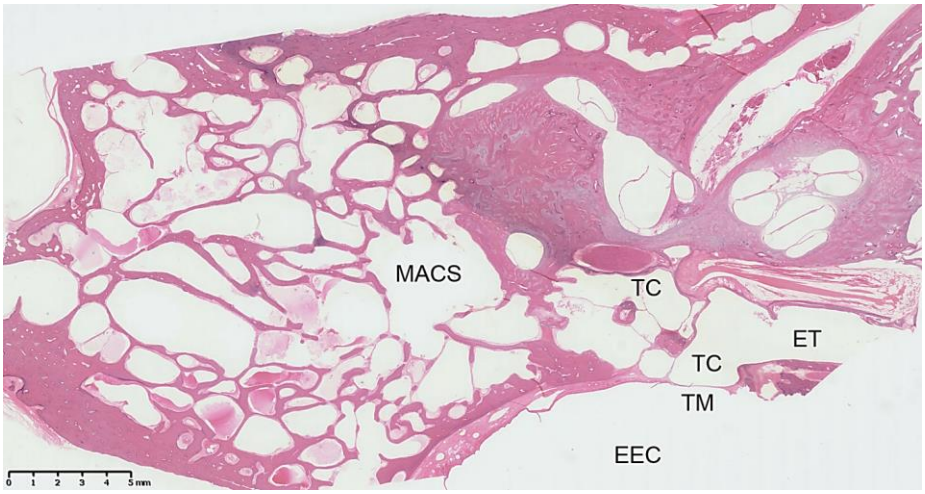

Figure 1-2. Horizontal overview of the middle ear structures at the level marked on Figure 1-1. EEC external ear canal.

1.2.1. EUSTACHIAN TUBE

The ET is the connection between the TC and the nasopharynx, and at the same time the only direct source of a gas supply. It is a distensible tube, which is closed most of the time, in order to prevent the ME from regurgitation of saliva, bacteria invasion or noise from the nasopharynx (Takahashi, 2017) It is surrounded by hard walls, cartilaginous in the anterior 2/3 towards the nasopharynx and osseous in the posterior

1/3, proximal to the TC (Figure 1-3). The junction between the two portions describes a narrow part called isthmus.

The ET lumen is sheathed with mucosa containing loose connective tissue, blood vessels and a high ciliated epithelium. The ET mucosa presents with extensions of folds, which might act as valves at the transition between the osseous and cartilaginous parts (Sadé and Ar, 1997)(Figure 1-3).

The ET has been investigated by direct approaches, where its muscular compliance could be calculated as the inverse of the resistance to different rates of air flow forced by the nasopharynx (Leuwer et al., 2002; Takahashi et al., 1987).

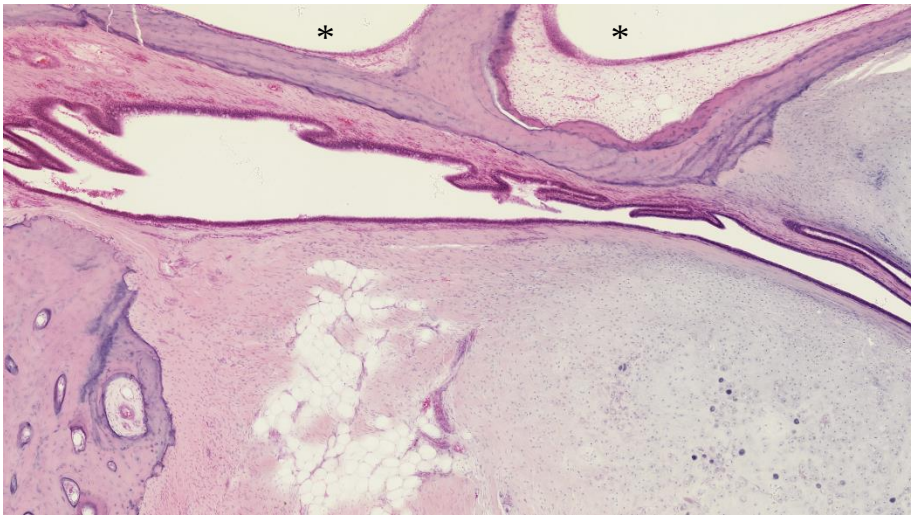

*Figure 1-3. Eustachian tube on a horizontal section at a level of the inferior TC. To the left, the osseous part is surrounded by pneumatized bone (the peri-tubal air cells, *). In this part the ET lumen is larger (1.3 mm). To the right, the cartilaginous part (blue) surrounds a narrower ET lumen (0.1 mm). The narrowing between the two parts is called the isthmus and presents with mucosal folds (magnification 5x).*

1.2.2. TYMPANIC MEMBRANE

The TM is an approximately 0.1 mm thick membrane with an about 10 mm diameter separating the ME from the external ear canal and the ambient atmosphere (Luers and Hüttenbrink, 2016). It is anchored to the medial end of external ear canal by a horseshoe-shaped fibrous annulus and has a conical shape with the tip oriented inwards (Kassem et al., 2010). The structure of the TM is three - layered and comprises a thicker lamina propria covered by epithelia on each side, flat mucosal on the inner side, and keratinized stratified on the outer side (Hentzer, 1969; Lim, 1970). The structure of the lamina propria divides the TM into a larger well-organized fibrous region called pars

tensa, and a limited elastic amorphous region, called pars flaccida (Hentzer, 1969; Lim, 1970). The mechanical properties of the pars tensa are attributed to its collagen fibers organized radially, concentrically and tangentially, as well as to the fibrous annular ligament (Lim, 1970) (Figure 1-4).

The TM is the only membrane in the body with air on both sides, and its particular structure enables an optimal balance between stiffness and compliance, so that both transmission of acoustic pressures are facilitated and strength against high static pressures as well as compliance buffering low static pressures (Ars et al., 1989; Von Unge and Dircks, 2009).

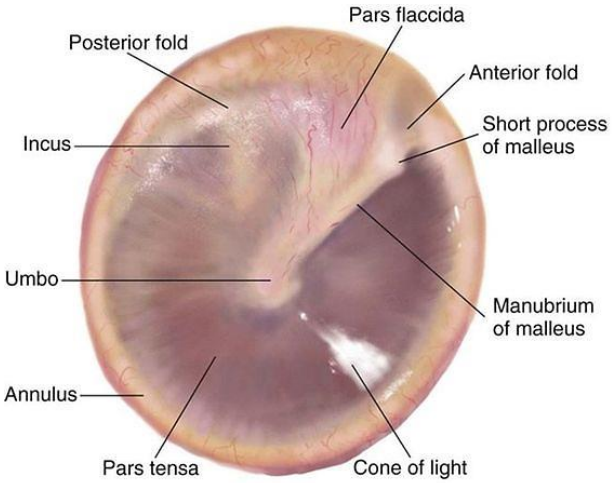

Figure 1-4. Tympanic membrane (used with permission from www.pinterest.com)

The high content of less organized elastic fibers combined with fewer collagen fibers in pars flaccida makes the TM in the region around the malleus insertion onto it more compliant and more vulnerable to long-lasting pressure loads than pars tensa (Sadé, 1997).

1.2.3. MIDDLE EAR MUCOSA

The ME mucosa is built up of loose connective tissue paved with luminal epithelium and interspersed with blood vessels presenting a variable content of blood (Figure 9 in Results) (Hentzer, 1970; Marcusohn et al., 2010). The epithelium can vary within the ME. It has been described as higher (cuboidal or cylindrical) and ciliated in the anterior and inferior parts of the TC, suggesting involvement in the clearance of the accumulations, microorganisms, etc. by the ET (Hentzer, 1970; Lim, 1979; Sadé and Facs, 1966). In the MACS and superior TC, the epithelium was descried as cuboidal or flat and normally without cilia (Hentzer, 1970; Lim, 1979; Sadé and Ar, 1997). The

diffusion distance was also found shorter in the superior TC and MACS, suggesting a possible specialization in gas exchange (Ars et al., 1997).

1.2.4. MUSCLES AND LIGAMENTS

The ME muscles and ligaments are also compliant structures (Gentil et al., 2011; Kwacz et al., 2015). As they are organically linked to the ossicular chain, which in turns leans on the TM, any muscular contraction will pull the TM inwards, causing a slight ME overpressure (Ingelstedt and Jonson, 1966). However, their contribution in the ME pressure regulation is minor, and will not be discussed in the present work.

1.3. MIDDLE EAR PRESSURE REGULATION

There is a continuous and bidirectional gas exchange between the ME gas and the gas content of the blood vessels of the ME mucosa, resulting in a net absorption from the ME and thus a slight underpressure (Doyle et al., 2011; Sadé and Ar, 1997). The larger pressure gradients between the ME and ambient atmosphere can be quickly equilibrated by intermittent Eustachian tube openings, supplying the ME cavity with gas from the nasopharynx, having a different gas composition from the venous blood within mucosa.

Despite its appearance of a closed gas pocket, the ME demonstrates dynamic pressure changes. The pressure balance between the ME and ambient is permanently challenged by any change in temperature, body position, altitude, physical effort, etc. (Doyle, 2017). A recording of the pressure fluctuations occurring in the ME in daily conditions reveals permanent deviations from the baseline around the ambient pressure, and is illustrated in Figure 1-5.

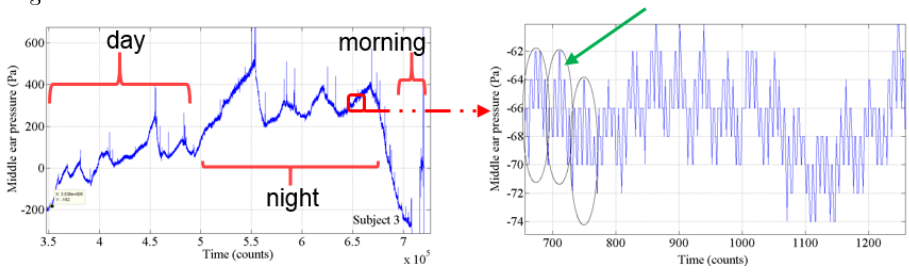

Figure 1-5. Pressure fluctuations over 21 hours in a healthy human middle ear displayed in counts = 1/10 seconds. Left panel: recording of the day-night fluctuations of the ME pressure; right panel: a 1-minute detail of the recording; the groups of high-frequency oscillations as encircled might reflect the respiratory rate, whereas the individual peaks might be related to the heart rat.

A closer look will reveal 4 levels of oscillation frequencies:

1) Low-frequency day-night oscillations, with a general trend of high overpressure during the night, a steep fall in the morning, and lower pressure close to baseline during the day. This is in accordance with other studies, and is related to body position affecting the hydrostatic pressure (Brattmo et al., 2003).

2) Moderate-frequency of irregular oscillations around baseline especially during the day, with a frequency varying largely between 3 and over 30 and very different amplitudes;
3) Respiratory-related oscillations of 15 - 20/min frequency (Figure 1-5, right panel);
4) Heart rate-related oscillations of 60-80/min frequency (Fig. 1-5, right panel).

In summary, the ME pressure is characterized by the summation of different factors contributing to the ME pressure changes. Some examples are pressure increase in erect position (Brattmo et al., 2003), pressure decrease in supine position (Tideholm et al., 1996), increase followed by decrease after experimental inflation inducing an ET opening (Doyle, 1997) etc. However, interpretation of the frequency patterns on the ME pressure monitoring over 20-hour period is very challenging, due to the overlap of the different factors.

According to the current knowledge, these fluctuations reflect mainly the combined participation of the following **mechanisms** (Doyle, 2000)**:**

1) The permanent **gas exchange** with the blood vessels crossing the mucosa;
2) The fast and intermittent **ET openings**;
3) Slow pressure changes due to momentary **displacements of the TM**;
4) Pressure variations due to **volumetric changes of mucosa**.

However, the unknowns come when the signal should be decomposed into the different mechanisms. The current thesis has focused on the extraction of the patterns of the ET opening and the buffering of the TM.

1.3.1. EUSTACHIAN TUBE OPENING

The ET opens momentarily several times a day allowing quick gas exchanges between the TC and the nasopharynx at pressure gradients beyond 165-545 daPa (Takahashi, 2017). The opening occurs often in association with swallowing or yawning, and therefore considered to be controlled by the swallowing reflex (Brattmo et al., 2003; Cinamon et al., 2017; Flisberg et al., 1963; Mover-Lev et al., 1998). However, mechanical stimulation of the TM generated brain action potentials in the brain area responsible for the muscles surrounding the ET. This suggested that the ET opening might be controlled by a neural reflex loop, which in turn might be fed by the mechanoreceptors described in the TM (Nagai and and Tono, 1989; Sami et al., 2009; Songu et al., 2009).

In otitis media, the ability of the ET to open is reduced. This is probably explained by mucosal inflammation and blockage of the ET lumen (Alper et al., 2011; Takahashi et al., 1987), most probably at the level of the pharyngeal orifice (Takahashi et al., 1996).

This leads to a decreased gas supply, which cannot counterbalance the net absorption of the gas molecules occurring over the mucosal blood vessels.

A recent study was performed in unconscious humans under rehabilitation, less able of ET openings due to paralysis of their pharyngeal muscles including inhibition of swallowing. It was found that only half of the subjects developed ME underpressure, whereas it could be expected that all should have done it. This study suggested that the role of the ET in the overall pressure regulation has probably been overestimated (Cinamon et al., 2017).

1.3.2. TYMPANIC MEMBRANE BUFFERING

Based on its structure presented in Section 1.2.2., the TM is able to buffer smaller pressure changes by inwards and outwards displacements. It has been estimated that for pressure gradients up to 160 daPa, the TM is able to displace up to 0.4 mm representing up to 25% of the pressure gradient as measured under experimental conditions (Dirckx and Decraemer, 1991). Beyond this pressure, the TM behaves like a rigid wall.

TM been the most accessible for study, and its compliance estimation by tympanometry has been serving as a diagnostic parameter in otology and audiology for decades (Cheng et al., 2007; Gaihede, 1996).

The role which the TM plays in the ME pressure regulation by buffering was set as research goal in 2011, and it has been studied in various experimental conditions so far (Alper et al., 2017). However, physiological experiments have not been available.

1.3.3. GAS EXCHANGE OVER THE MIDDLE EAR MUCOSA

There is a permanent bidirectional gas exchange between the air enclosed within the ME and the blood perfusion of the ME mucosa, which is predominantly venous (Figure 1-6). The exchange of CO_2 and O_2 molecules occurs within few minutes after each aeration by the ET due to the small difference in partial pressures. However, the exchange of N_2 is a much slower process, probably due to both its reduced tissue solubility and its higher partial pressures gradient of approximately 6 kPa (Kania et al., 2006; Pau et al., 2008) (Figure 1-6). The overall result is a net gas absorption from the ME leading to underpressure.

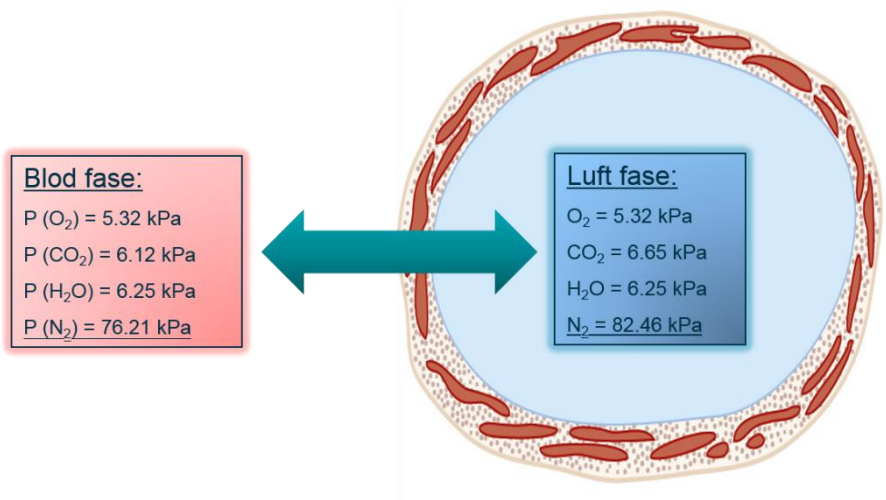

Figure 1-6. Schematic representation of the gas exchange between the blood perfusing the ME mucosa and the air filling the ME cavities by the virtue of the partial pressures (P) of the involved gases species (Doyle, 2000). In normal MEs the differences in partial pressures between the air space and the venous blood results in a net absorption of gas (by courtesy of Dr. Michael Gaihede).

1.3.4. THICKNESS CHANGES OF THE MUCOSA

By engorging more blood, the mucosa may expand, as well as by emptying its blood and constricting blood vessels it may shrink. Due to its large surface-to-volume ratio, it has been estimated that a mere thickness change of 6 μm in of a 10-mL ME, the mucosa may be able to change the ME pressure with 1 kPa (Magnuson, 2003). This hypothetical role of the mucosa in ME pressure change seems to agree with physiological studies measuring indirectly the contribution of mucosa thickness changes to ME pressure (Andréasson et al., 1976; Gaihede et al., 2010), but morpho-histological evidence for its abilities has been lacking so far (Alper et al., 2017).

1.4. MIDDLE EAR PRESSURE DYSREGULATION

The ME pressure dysregulation consists typically of the formation of *underpressure* or under-ambient pressure, which reduces very much the mobility of the auditory chain and thus induces conductive hearing loss. It occurs in relation with any type of otitis media, starting already in the acute episodes associated with the common cold (Bhutta et al., 2017).

Otitis media includes inflammatory changes of the ME and ET mucosa, as well as an apparently enriched vascularization, which results in an intensified gas exchange (Matanda et al., 2006). The inflamed ET loses its ability to counterbalance the intensified gas absorption over the mucosa, combined with an increased oxygen

consumption by the inflammation and eventually infectious agents, as well as with the negative pressure generated by the effusion clearance by the ET (Takahashi, 2017). This accentuates the formation of underpressure, which may cause a retraction of the TM inwards until it adheres to the ossicles and the opposite wall of the ME, limiting its buffer capacity, as well as its mobility. In the longer course, the TM becomes increasingly flaccid and adheres at some point to the auditory ossicles and the promontory (atelectasis) (Sadé and Luntz, 1989) (Figure 1-7).

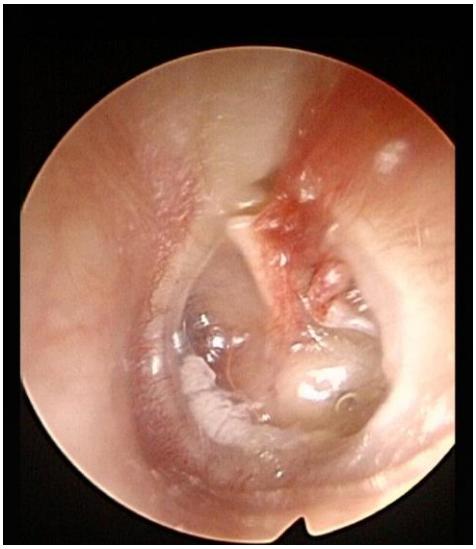

Figure 1-7. Atelectatic middle ear, with retracted tympanic membrane, adhering to the ossicles and inner wall of the ME. The ME contain fluid with small air bubbles (By courtesy of Dr. Michael Gaihede).

Accentuated underpressure is the most important etiological factor of secretory otitis media (Merchant and Nadol, 2010; Takahashi, 2017). It is characterized by the accumulation of fluid in the air space of ME. This becomes a great impediment for the gas exchange causing the underpressure to become a permanent situation. A long – lasting underpressure can complicate with retraction of the TM, atelectasis of the ME, and cholesteatoma formation, which may erode the ME bony structures and expand within the ME and its adjacent regions (Ars et al., 1989; Tos, 1983). These conditions may cause permanent conductive hearing loss and has also a quite high recurrence rate (Puria et al., 2013; Takahashi, 2001).

Another effect of prolonged underpressure may be sclerotization of the mastoid, consisting of filling of the air space with thickened mucosa and bone tissue. This can be seen as an opacification of the mastoid on CT scanning of the temporal bones, and often noticed during mastoid surgery (Takahashi, 2017) (Figure 1-8).

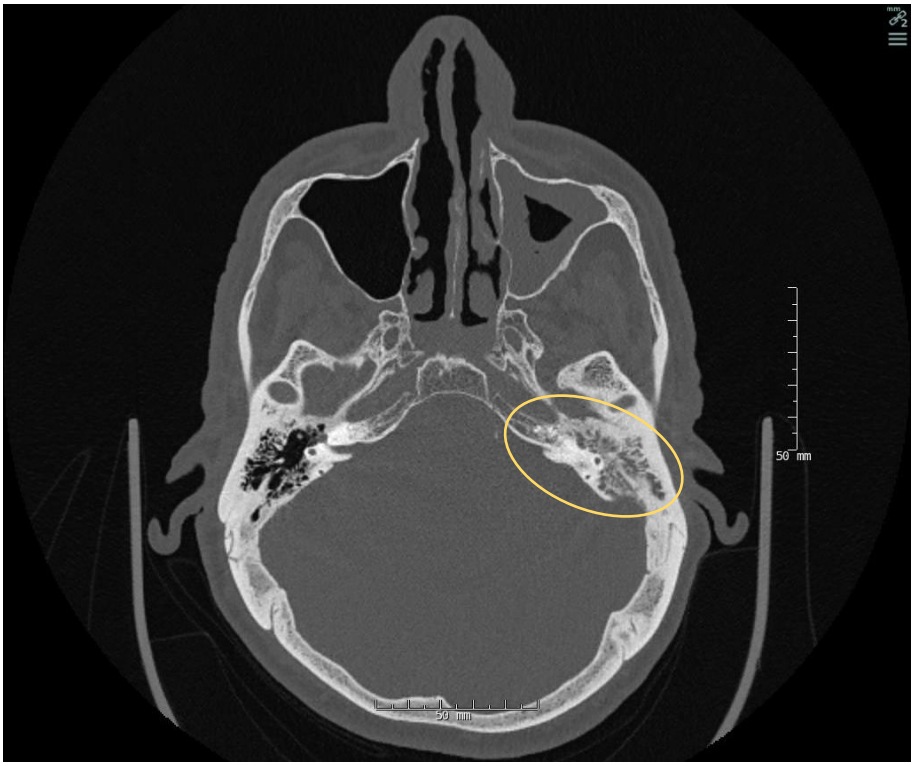

Figure 1-8. CT-scanning of a patient, presenting normal pneumatization of the right ear (situated at the left of the image), and sclerotic mastoid on the opposite ear (encircled) (by courtesy of Dr. Michael Gaihede).

When the persisting underpressure occurs in younger ages, the mastoid development is inhibited (Aoki et al., 1990). This seems to be a risk factor as well as an unfavorable prognostic factor for ME disease both in childhood and as adult (Sadé and Fuchs, 1997, 1996).

"…For what we know is incomplete…;
but when what is complete comes,
then what is incomplete will be done away with."

The Bible, International Standard Version,
1st Corinthians 13:9 – 10.

CHAPTER 2. AIMS

2.1. STUDY I: CONTRIBUTION OF THE EUSTACHIAN TUBE

PAPER 1 aimed at characterizing physiological ET openings by measuring their opening time, pressure gradient and rate of pressure change in normal human MEs. This included also investigations of possible correlations between these physiological variables.

PAPER 2 aimed at assessing the number of ET openings in normal humans from long-term monitoring of the ME pressure. This included the development of an algorithm, which could automatically detect ET opening patterns and determine their number over time. Thus, the number of daily ET openings could be assessed and compared with the number of swallow events reported in the literature.

2.2. STUDY II: CONTRIBUTION OF THE TYMPANIC MEMBRANE

PAPER 3 aimed at a description of the ME pressure changes in response to the atmospheric pressure variation during elevator trips, and to determine the buffering capacity of the TM at such low pressure changes. In addition, the ME volume was determined and its correlation to the TM buffering capacity was investigated.

2.3. STUDY III: EVIDENCE FOR THE CONTRIBUTION OF THE MUCOSA

PAPER 4 aimed to compare the mucosa in different regions of the normal human ME in order to evaluate its properties relevant for an efficient pressure regulation. This includes a histo-morphological description of the mucosa as well as the determination of a series of histo-morphometric variables regarding the blood vessel density and distribution, mucosa thickness and diffusion density in different ME regions.

PAPER 5 aimed at the description of certain histological changes that were incidentally found in PAPER 4. These changes included both – inflammatory morphological findings and quantitative investigation of the relative ratios among bone, soft tissue and air determined by image analysis. These findings seem relevant in the development of decreased pneumatization and sclerotic changes of the mastoid.

In addition, a preliminary immuno-histochemical study aimed at testing the use of immuno-histochemical biomarkers for the identification of blood vessels, muscular and neural structures within the ME mucosa.

Vision without action is a daydream. Action without vision is a nightmare.

-Japanese proverb

CHAPTER 3. METHODOLOGY

3.1. PHYSIOLOGICAL APPROACH (STUDIES I AND II)

This method served the ET and TM studies and was rooted in an earlier series of physiological investigations of the ME pressure in normal MEs conducted by Gaihede et al. (2010). They consisted of a long-term continuous monitoring of the pressure fluctuations within healthy MEs as described below.

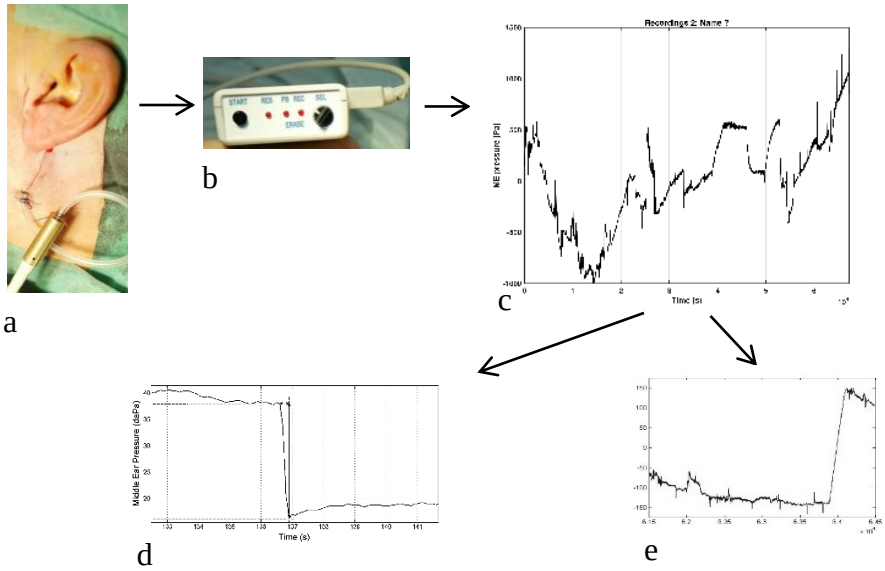

Figure 3-1. Method for physiological measurements of the ME pressure. (a) Catheter inserted into the mastoid tip of a subject and connected to pressure transducer; (b) pressure logger recording the pressure fluctuations within the middle ear; (c) exemple of full recording; (d) the pattern of the Eustachian tube opening, (e) a sequence of slow pressure fluctuations in normal daily conditions ending up with a steep rise corresponding to an elevator ascend.

A catheter was inserted into the lateral mastoid air cells and connected to a pressure transducer (Figure 3-1, a). The measured pressure fluctuations were recorded over a time interval in the range of 13 to 22 hours by a pressure logger (Figure 3-1, b), which also allowed online monitoring by connection to a PC (Figure 3-1, c). The full recordings had a sample frequency of 10 Hz and a pressure resolution of 1 Pa. The material was afterwards divided in smaller studies for the extraction and analysis of the ET openings (Figure 3-1, d) and elevator trips (Figure 3-1, e). The recordings were

available as Matlab files, in which the extraction and analysis of the patterns was performed. SPSS was also employed for parts of the statistical analysis.

3.1.1. CONTRIBUTION OF THE EUSTACHIAN TUBE

The ET opening features consisting of opening time and pressure change were extracted respectively manually as described in PAPER 1 (Gaihede et al., 2013), and automatically in PAPER 2 (Nielsen et al., 2018), the latter based on an algorithm including rule-based decision, continuous wavelet transformation and Pearson's correlation for 1-D template matching.

3.1.2. CONTRIBUTION OF THE TYMPANIC MEMBRANE

The TM buffer capacity was calculated by the ratio between the pressure changes during the elevator trips and the ambient pressure. This was further correlated with the volumes of the MEs, calculated by the application of Boyle's ideal gas law from on the ME pressure changes consecutive inflation-deflation tests with different gas volumes (Gaihede et al., 2013). Further, the ratio between the ME pressure change, assumed to be proportional with the TM displacement, and the difference in pressure change within ME and ambient during the trips gave a TM compliance estimate (Padurariu et al., 2016).

3.2. HISTOLOGICAL APPROACH (STUDY III)

The ME mucosa was investigated by a histological approach consisting of both systematic morphologic observations and measurements, as well as immuno-histochemical staining.

3.2.1. HISTO-MORPHOLOGY AND MORPHOMETRY OF THE MUCOSA

The systematic morphological investigations were made possible by the Temporal Bone Histology Lab, Otolaryngology, Zürich University Hospital, who provided access to 21 sets of archive slides of temporal bones stained in hematoxylin – eosin (HE).

For PAPER 4, cases with normal pneumatization were selected with the simultaneous availability of both TC and MACS, which counted to a total of 15 cases. Only sections crossing through the TC were available in between the planes 1 - 3 represented on the Figure 3-2, upper panel.

The sampling and the measurements were performed using hand free tools in Nanozoomer Viewer v. 2.8.8 (Hamamatsu, Japan). Full-screen mucosa samples (435 μm, magnification of 40x) from 8 sites from both TC and MACS (Figure 3-2, upper panel) were used for morphological observations as well as measurements.

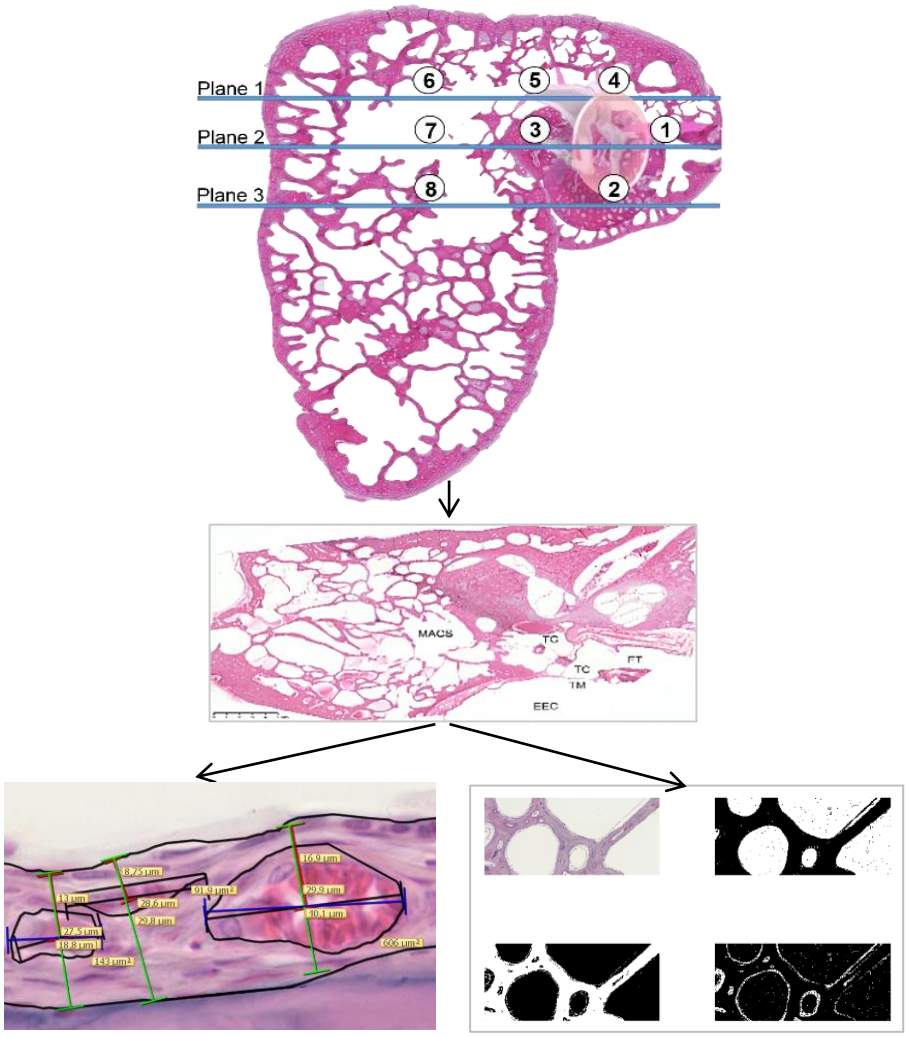

Figure 3-2. Method for histo-morphological measurements of ME structure. The eight sampling regions represented on the upper panel are: (1) anterior TC; (2) inferior TC; (3) posterior TC; (4) superior TC; (5) mastoid antrum; (6) superior MACS; (7) central MACS; (8) inferior MACS. The middle panel is an example of a slide, where the mastoid air cell system (MACS), the tympanic cavity (TC), the tympanic membrane TM, the Eustachian tube (ET) and the external ear canal (EEC) are represented. The lower panel to left is a mucosa sample (PAPER 4); the green lines are thickness measurements the black contours represent surface area measurements of the blood vessel sections, and the red lines represent diffusion distances. The lower panel to right is a sample of the morphological measurements of the mastoid pneumatization (PAPER 5); (up to left) original image; (up to right) air space segmentation; (down to left) bone segmentation; (down to right) mucosa segmentation.

The morphological observations consisted of a classification and counting of the samples regarding the epithelial type, the presence of cilia, and looseness of the lamina propria.

The morphological measurements (morphometry) consisted of the mucosa thickness; blood vessels density; the distance from the center of the blood vessel sections to the top of the mucosa (diffusion distance), the length of mucosa including blood vessel sections (active mucosa).

The proportion calculations for PAPER *5* were performed in Matlab 2017b on mastoid samples at the 5x magnification mode using a GMModels algorithm. This consisted in clustering the image components into three phases (air, bone, soft tissue) in the normally pneumatized mastoid samples, and respectively in two in the sclerotic mastoid samples (Figure 3-2).

3.2.2. IMMUNOHISTOCHEMISTRY OF THE MUCOSA (PRELIMINARY TESTS)

Fresh mastoid bone samples from cochlear implant donors were fixated in 10% neutral buffered formalin for 24-96 hours, depending on their size, decalcified in 10% EDTA for 5-20 months, and embedded in paraffin. Three-µm-thick sections were cut and stained with Haematoxylin-Eosin (HE) for morphologic evaluation and Alcian-PicroSirius (APS) for assessing the connective tissue and acidic polysaccharides. For immunohistochemistry the sections were mounted on positively charged slides (SuperFrost + , Menzel Gläser, Germany), dried at room temperature and baked for 1 h at 60 °C, and stained for biomarkers as listed in Table 3-1, using staining protocols optimized for the purpose. In short, deparaffinization, rehydration, and heat-induced epitope retrieval were performed on the Ventana Benchmark Ultra. Epitope retrieval was performed in Cell Conditioning 1 (cat. 950-124; Ventana, USA), pH 8.5 at 99 °C for 48 min. Endogenous peroxidase was blocked with 3 % hydrogen peroxide (ultraView DAB, cat. 760–500; Ventana). Dilutions of primary antibodies were applied on serial slides and incubated 20 min at 36 °C. Diaminobenzidine (ultraView DAB, cat. 760–500; Ventana) staining was developed using a multimer-based visualization system (ultraView DAB, cat. 760-500; Ventana). Finally, the slides were counterstained with hematoxylin and cover-slipped. The antibodies which were tested is presented in Table 3-1.

Tabel 3-1. Immunohistochemical staining biomarkers tested preliminarily on paraffin-embedded mastoid samples

Staining	Biomarker for	Purpose
CD31	Endothelial cells	Identification of blood vessels
ERG-PCK	Endothelial cells and Epithelial cells	Distinguish between endothelial cells and epithelial inclusions in mucosa expansions
ERG-D240	Endothelial cells and Endothelial cells of the lymphatic vessels	Distinguish lymphatic endothelial cells from the endothelial cells of blood vessels
DES (Desmosin)	Smooth muscle cells Striated muscle cells	Identify muscle fibers within lamina propria of the mucosa
ASMA (Alpha – smooth muscle actine)	Smooth muscle cells Perictytes	Identify more specifically smooth muscle fibers within the mastoid mucosa
SOX10	Schwann cells of the peripheral nerves Melanocytes	Identify nerves within mucosa, and neural crest differentiation
NF (Neurofilament)	Neurons	Identify nerves within the mucosa
SYP (Synaptophysine)	Neurons Neuroendocrine cells	Identify nerves within the mucosa
S100	Nerves Schwann cells	Identify nerve and neuroendocrine cells
LAM (Laminine)	Basement membranes	Visualize the continuity of the basement membrane of the mucosal blood vessels
CD3	T-lymphocytes	Identification of inflammation
CD20	B-lymphocytes	Identification of inflammation
CD68	Monocytes; Histiocytes; Macrophages; Osteoclasts	Identification of inflammation

"Research is an organized form for curiosity."

- Anonym

CHAPTER 4. RESULTS

4.1. STUDY I. CONTRIBUTION OF THE EUSTACHIAN TUBE

4.1.1. DESCRIPTION OF THE EUSTACHIAN TUBE OPENING (PAPER 1)

This first part of the study focused on the characterization of the ET openings in response to supervised experimental gas volume changes. The pressure gradients ranged between 325 and 390 daPa following controlled "inflations" and "deflations" with ±50, ±100, ±200, ±300 µL. This study revealed that only 4 out of 9 subjects (44%) responded to gas "inflations" and "deflations" predominantly by ET openings (≥10), illustrated on recordings by step-wise pressure changes towards baseline (Fig. 4-1). Thus only N = 4 subjects were included in the further analysis.

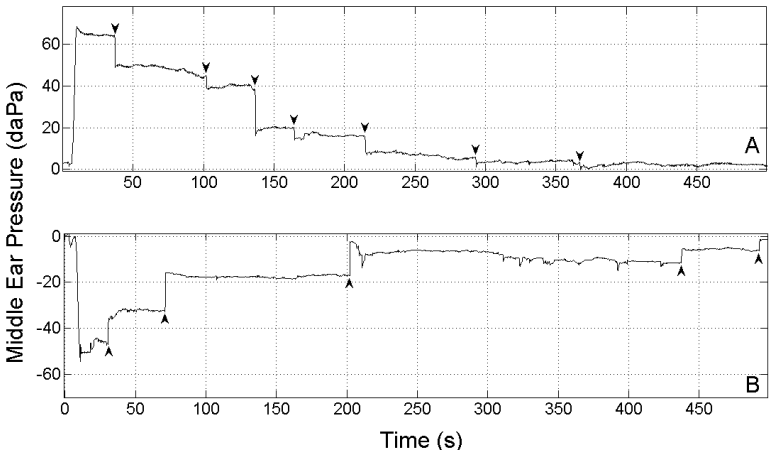

Figure 4-1. Recording of ME pressure regulation after experimental inflation (upper panel) and deflation (lower panel) of the ME with 100 µL air in a subject who responded predominantly by ET openings, which are marked by arrow tips in the direction of the counterbalance.

Results and interpretation

Mean ET opening time was 0.34 s (SD 0.18), ranging from 0.1 to 0.9 s. The ET opening time showed a narrow variation, tending to behave relatively constant reminding of a predefined reflex time.

Mean pressure change by ET opening was 30 daPa (SD 51), ranging from -213 to 312 daPa. There was, however, no linear correlation between the ET opening time and the

pressure change ($r^2 = 0.01$; $p = 0.34$). In other words, **a longer ET opening time did not necessarily produce a higher pressure change.**

Mean rate of pressure change was 97 daPa/s (SD 124), ranging from -533 to 568 daPa. However, there was no linear correlation between the ET opening time and the rate of pressure change ($r^2 = 0.00$; $p = 0.57$). This means that **a longer ET opening time did not equilibrate for instance at a lower rate.**

There was no linear correlation between the ET opening time and the pressure gradient ($r^2 = 0.03$; $p = 0.16$). A **larger pressure gradient did not increase the ET opening time**, but would produce successive ET openings. The mean number of ET openings per subject over the 10 min. in this experiment was 19.

However, there was a good linear correlation between the pressure gradient and the rate of pressure change by ET openings ($r^2 = 0.75$; $p < 0.001$), suggesting that the pressure gradient dictates the rate of pressure change or, in other words, the efficiency of the ET openings.

There was also good linear correlation between the pressure change by ET openings and the pressure gradient between the ME and ambient ($r^2 = 0.77$; $p < 0.001$. This means that a larger pressure gradient results in a larger pressure change. Moreover, the slope of the regression line between the pressure gradient and the pressure change may be a measure of the individual **compliance of the ET**.

4.1.2. FREQUENCY OF THE EUSTACHIAN TUBE OPENING (PAPER 2)

The second part of this study focused on the ET openings naturally occurring over long-term monitoring. Due to numerous sources of noise, only N = 4 subjects were included in the further analysis.

Results and interpretation

The mean number of ET openings was 84/day (SD 60), significantly lower than the average number of swallowing events reported in literature, calculated at a mean of 1228/day. This points to a distinct and separate reflex, which may be part of a neural feedback control arc for the overall control of ME pressure.

4.2. STUDY II. CONTRIBUTION OF THE TYMPANIC MEMBRANE

4.2.1. PRESSURE BUFFERING DURING ELEVATOR TRIPS (PAPER 3)

This study described the physiological pressure changes in normal MEs in response to low atmospheric pressure changes induced by elevator trips (Figure 4-2), extracted from the same long-term monitoring as in PAPER 2. The TM buffering capacity was determined from the ratio between the pressure changes in the ME versus ambient.

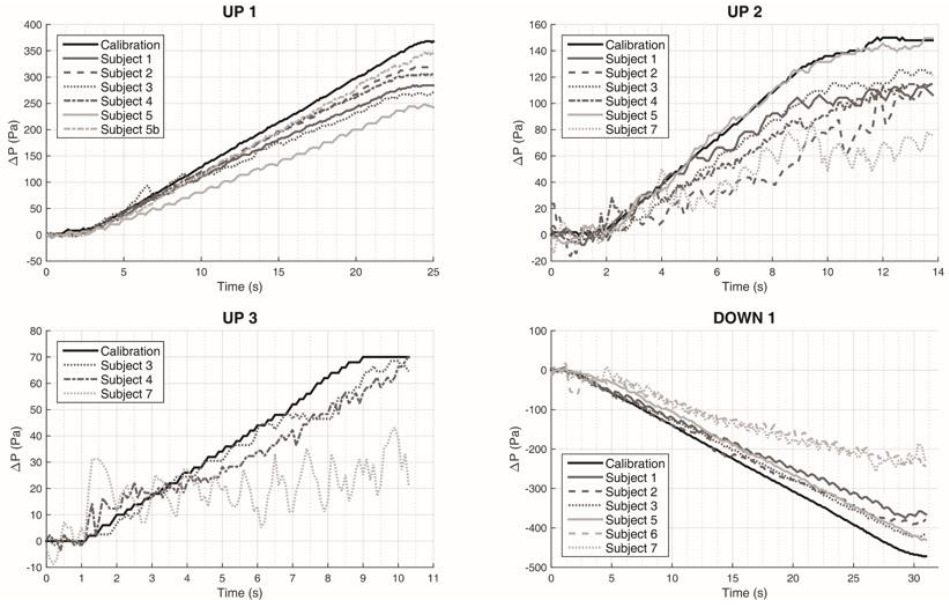

Figure 4-2. Continuous recordings of the ME pressure fluctuations in healthy human MEs during elevator trips. UP 1 = from basement to the 7th floor; UP 2 = from the 7th to the 10th floor; UP 3 = from the basement to the 1st floor; DOWN 1 = from the 10th floor to the basement. Note: the building has basement (underground) and ground floor (floor 0). Calibration = recordings of changes in ambient pressure during the same elevator trips.

Results and interpretation

The pressure in the ambient during the elevator trips changed within the interval -47 to 37 daPa at a rate of approximately 2 daPa/s, whereas the pressure in the MEs changed within the interval -42 to 35 daPa. The elevator trips produced a smooth pressure change proportional with the height change (Figure 8). Thus, no ET openings were elicited at low pressure changes (in the order of few hundred daPa) and a low pressure rate.

The overall mean buffer capacity of the TM was 23.3%; it ranged between 55% in a smaller ME (5 mL) and down to 5% in the largest MEs (11 mL).

The individual ME volumes could be calculated based on Boyle's gas law from the "inflation-deflation" experiments described in PAPER 1, averaging 9.7 mL. The individual ME volumes presented a strong negative linear correlation with the individual TM buffer capacity ($r^2 = 0.92$ for N = 5 subjects; Kendall's rank correlation: $p = 0.005$). The TM buffer capacity was highest in the smallest MEs and decreased with the increase in ME volume.

The mean TM compliance was 32.7 x 10^{-3} µL/Pa for upwards elevator trips, and 25.8 10^{-3} µL/Pa for downward trips, showing asymmetry between inwards and outwards TM displacements. The volume displacement by TM buffering ranged between 0.8 and 15.4 µL.

4.3. STUDY III. EVIDENCE FOR CONTRIBUTION OF THE MUCOSA

4.3.1. MUCOSA MORPHOLOGY AND MORPHOMETRY (PAPER 4)

Results and interpretation

The general morphology of the ME mucosa presented a large variation in the different sampling regions as illustrated in Figures 4-3 and 4-4, a.

The thickness of the MACS mucosa sampled from 3 different sites averaged 20.63 µm was significantly lower than mucosa of anterior, inferior and posterior TC, averaging 50.66 µm ($p = 0.000$ by independent-samples *t*-test). The mucosa of superior TC and antrum averaged 29.22 µm, intermediating the two compartments. Moreover, the thickness increased from the superior plane through both MACS and TC towards the inferior plane.

The blood vessel density averaged 15 % for the TC (17 % without superior TC) and 18 % in the MACS ± antrum, presenting no significant variation. General values ranged between 2 to 44 %. The mucosa of the MACS and the TC did not differ in vascularization, but presented a broad range for the blood vessel density within ME mucosa. The mucosa of the MACS and the TC did not differ in vascularization, but presented a broad range for the blood vessel density within ME mucosa, suggesting a high potential of volumetric contribution of the blood vessels in the ME mucosa morphology.

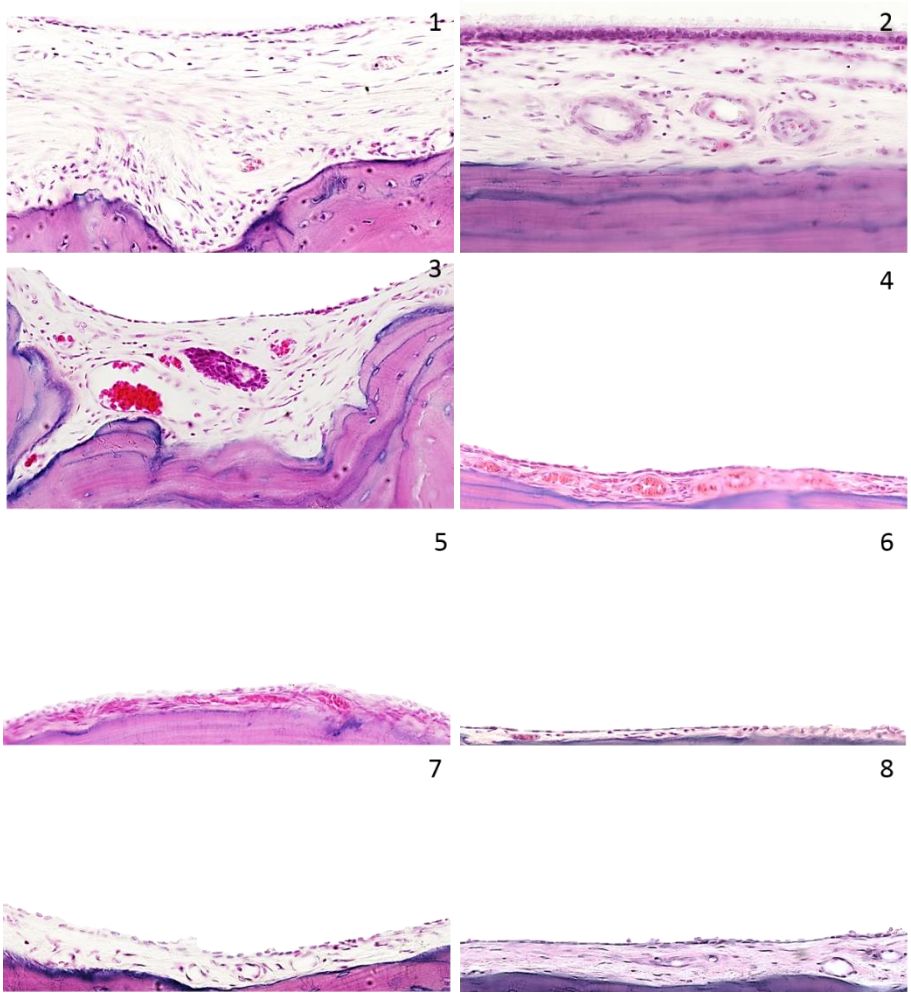

*Figure 4-3. Mucosa samples from 8 different regions within TC and MACS of the same ear. **(1)** anterior TC; **(2)** inferior TC; **(3)** posterior TC; **(4)** superiorTC; **(5)** aditus ad antrum and MACS antrum; **(6)** superior MACS; **(7)** central MACS; **(8)** inferior MACS, corresponding to the inferior TC (all images at a magnification of 40x).*

The mucosa thickness measured across blood vessels was in average 4 ± 10 µm higher than in between blood vessels within the individual samples ($p = 0.000$ by paired t-test). An increased perfusion with blood seems to cause a significant change in mucosa thickness.

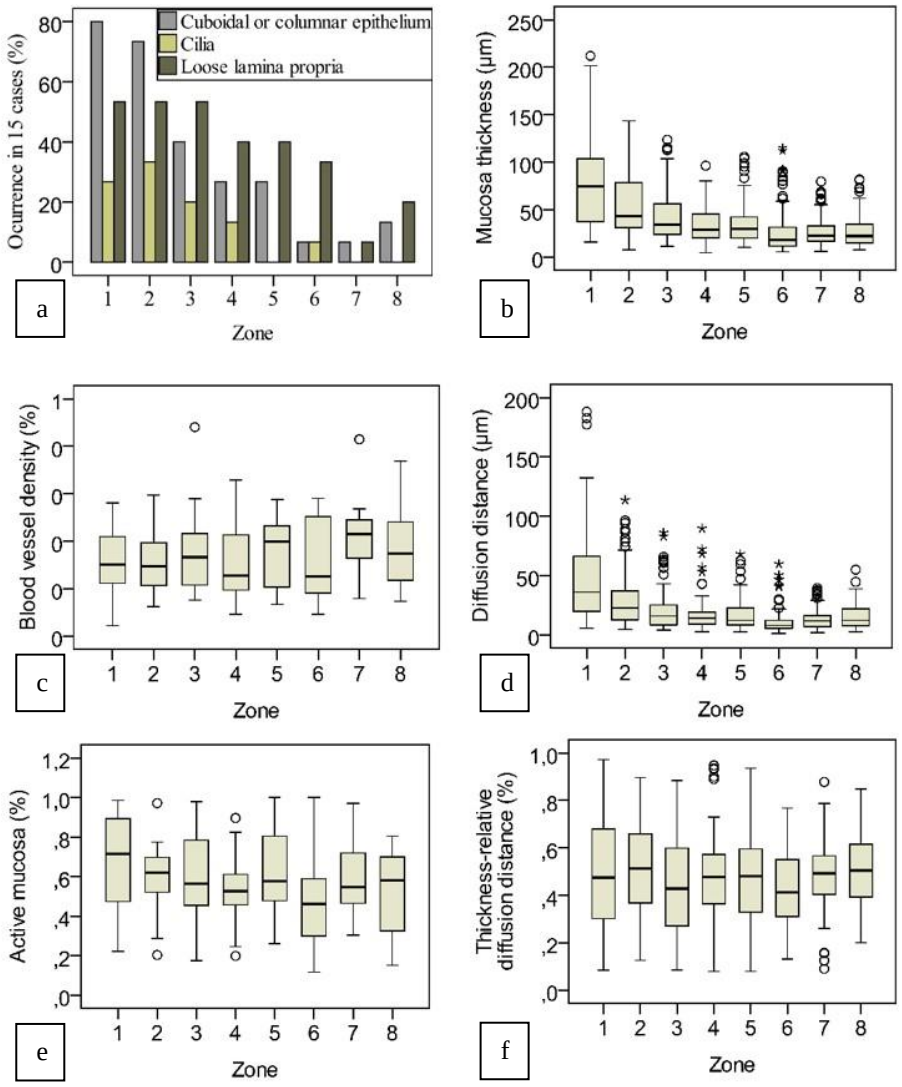

Figure 4-4. Statistics of mucosa morphology and morphometry. The boxplots illustrate the middle 50% of the morphological measurements. The horizontal lines within the boxplots are the medians, whereas the whiskers represent the 95% confidence interval. Outliers are represented with different signs for different cases.

The depth to the blood vessels was significantly larger in the anterior and inferior TC, averaging 28.73 µm, compared to the rest of the TC and the MACS, whereas the superior MACS had the most superficial blood vessels, with a mean depth of 7.91 µm. There was a significant positive correlation between the overall mucosa thickness and

the depth to blood vessels: Spearman's ρ = 0.819, p = 0.000. The thickness of mucosa can indicate also the predilection for gas exchange.

Cilia were often described in TC (20 – 30% of samples), whereas only occasionally in antrum and MACS. They accompanied the respiratory-type epithelium, i.e. cubical or cylindrical, was identified in both TC and mastoid. However, it was identified in the TC in most samples (70-80 %), in antrum in about 25% of samples, and in the MACS in only under 15% of samples. The remaining proportions were flat epithelium

Mucosa in about half of the TC samples and under half of MACS samples presented a loose lamina propria. Mucosa was generally looser when the sample contained more filled blood vessels either in TC or in MACS.

4.3.2. MORPHOLOGICAL CHANGES DURING OTITIS MEDIA (PAPER 5)

Results and interpretation

The average proportion of bone in lateral versus medial samples in normal mastoids was respectively of 39 % (SD 10) and 36 % (SD 7), whereas in the sclerotic mastoid it increased to respectively 75 % (SD 7) and 59 % (SD 8). Foci of new bone formation were frequent. The sclerotization seemed to occur by new bone formation within the air space or at the margins of extended soft tissue accumulations (Figure 4-5).

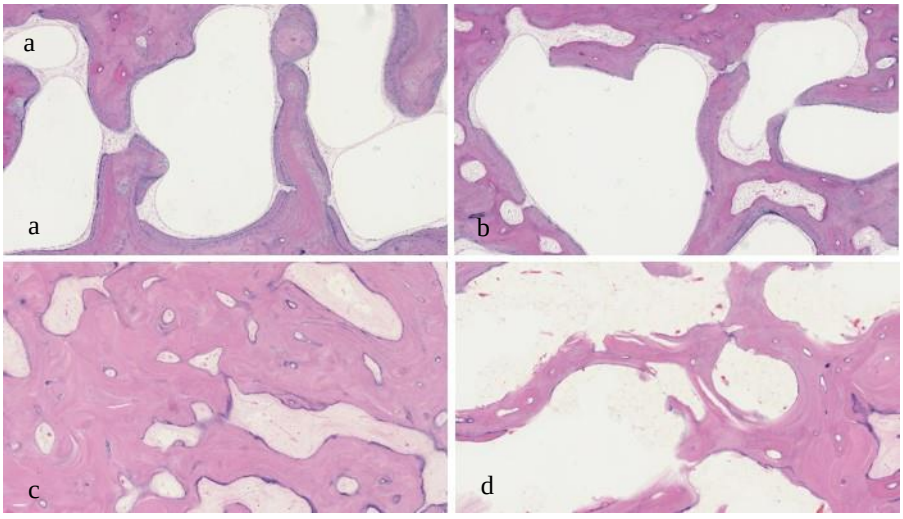

Figure 4-5. Two cases of paired samples of normally pneumatized mastoid (upper panel) and sclerotic mastoid (lower panel). Lateral samples are to the left, and medial samples to the right (magnification 5x).

The proportions of mucosa and air did not present differences in the mastoids with normal pneumatization, but their difference tended to increase in diseased mastoids, where the lateral air cells presented soft tissue expansions replacing the air space.

There has been described instances where the mastoid air cells were filled out with soft tissue or fat tissue or a mixture of both, thus the thin mucosa layer with flat epithelium and short distance to blood vessels described in PAPER 4 disappeared completely. The gas exchange cannot occur when the gas exchange interface disappears, as well as changes in mucosal congestion are less effective in altering the ME pressure.

4.3.3. IMMUNOHISTOCHEMISTRY OF MIDDLE EAR MUCOSA (PRELIMINARY RESULTS)

Results and interpretation

The success of different staining methods was limited, due to high number sections detached from the glass slides. However, the HE staining was successful, as well as APS several of the immunohistochemical analyses after increasing the baking time before staining. Results are presented in Table 4-1, and illustrated in Figures 4-6 and 4-7.

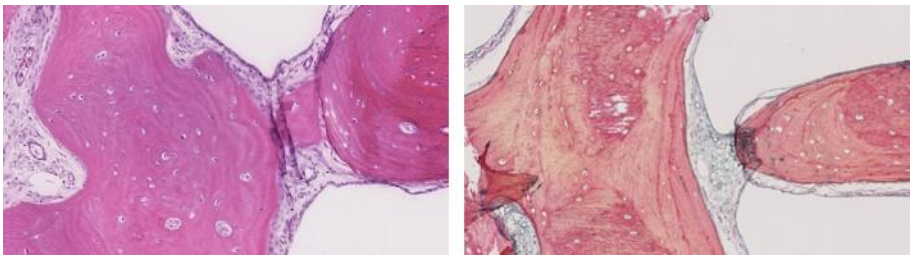

Figure 4-6. (left panel) HE staining for overview of the sections stained by immunohistochemical methods in Figure 4-6; (right panel) APS staining of collagen fibers (magnification 20x).

Briefly, the ME mucosa stained positively for CD31 in all cases (5/5), and revealed a rich vascular content; it stained also positively for LAM, revealing apparently continuous basement membranes around the blood vessels, and moreover for ASMA (2/2), emphasizing the smooth muscles around the blood vessels. However, it stained weak positive for nerve structures for NF (2/2), S100 (2/2), SYP (4/4), and for smooth muscles by DES (4/4). No staining was obtained for SOX10 for peripheral nerves. The results of ERG-combined staining failed to visualize the blood vessels, but stained positively for D240 and PCK, emphasizing respectively the lymphatic vessels and the epithelium. Staining for CD 3, CD20, and CD68 stained only occasionally positive among five cases.

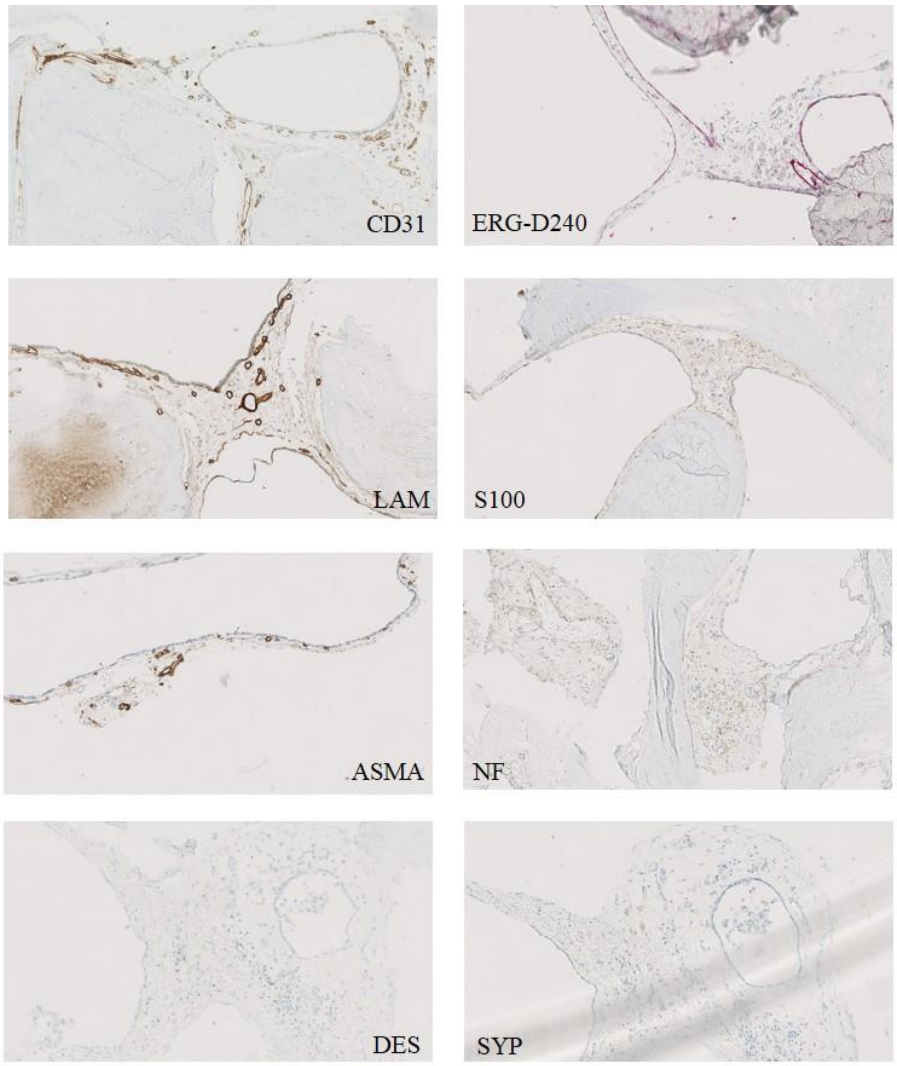

Figure 4-7. Immunohistochemical staining of paraffin-embedded mastoid mucosa of the same biopsy and at the same magnification (20x). CD31: strong positive; ERG-D240: negative for ERG and positive for D240; LAM: strong positive; S100: weak positive; ASMA: strong positive; NF: weak positive; DES: weak positive; SYP: weak positive.

"Having a good idea is not the end;
it's not the beginning of the end;
it is the end of the beginning."

- Winston Churchill

CHAPTER 5. DISCUSSION

The success rate of the treatment of OME, including the surgical treatment strategies, is still conditioned by the limited knowledge about the mechanisms of underpressure and the overall MEP regulation (Alper et al., 2017; Luers and Hüttenbrink, 2016). The present thesis approached some of the requested parameters related to each of the three main contributors to ME pressure regulation (Alper et al., 2017). They are being discussed in the following subsections.

5.1. STUDY I. CONTRIBUTION OF THE EUSTACHIAN TUBE

The aim of Study I was to describe the ET openings, in order to better understand how they contribute to the counterbalance of ME pressure changes. The value of this study resided in the method used to access the pressure changes produced by ET openings directly from inside the ME with an intact tympanic membrane.

The mean opening time of 0.34 s obtained by this method was in good agreement with the means of 0.36 s and 0.43 s obtained by sonotubomety, an indirect technique based on sound stimulation by a nostril and the acquisition of the transmitted sound through the ET to the ipsi-lateral external ear canal concomitant with different types of swallow attempts (Asenov et al., 2010; Mondain et al., 1997).

Moreover, the direct approach of pressure measurements enabled us to measure the ME pressure gradient and the relative pressure change by ET openings. The lack of correlation between the opening time and the ME pressure gradient lead us to the conclusion that the ET openings may behave like a reflex, having a relatively stable duration in larger and in smaller pressure gradients. Thus, larger gradients were rather counterbalanced by successive ET openings of the same short duration, until the pressure equilibrium with the ambient pressure was re-established. The observation of successive ET openings was in agreement with other reports, which relied on the number of swallowing-related ET openings for the estimation of the ME counterbalancing ability consecutive experimental inflation or deflation tests (Asenov et al., 2010; Elner et al., 1971).

The hypothesis of a reflex ET opening has been mentioned in several studies, and associated with the swallowing reflex (Alper et al., 2012; Asenov et al., 2010; Mover-Lev et al., 1998; Pau et al., 2008). However, a more recent study in a cohort of unconscious patients with paralysis of their pharyngeal muscles, and thus, unable to swallow, indicated that 50% did not develop ME underpressure, as otherwise expected. This casted doubt on either a common reflex mechanism of ET opening and of swallowing, or on an probably overestimated role of the ET openings in the overall ME pressure regulation (Cinamon et al., 2017).

To this question mark we tried to answer by counting the number of ET openings recorded in physiological conditions in conscious subjects, by direct continuous long-term recordings. The mean number of daily ET openings detected in our study was 84/day (Nielsen et al., 2018), which was significantly lower than the mean of 1228 daily swallowing events reported in other studies (Ars, 2008; Cinamon et al., 2017; Crary et al., 2013; Rudney et al., 1995; Sadé and Ar, 1997). This indicated that the mechanism underlying the reflex of ET opening might be different than that of swallowing (Nielsen et al., 2018). Whereas the swallowing reflex is typically triggered by saliva accumulation in the pharynx (Denk et al., 1998; Rudney et al., 1995), the ET openings are expectedly triggered by pressure gradients between the ME and the ambient pressures, which may be sensed by mechano-receptors within the TM or ME cavity (Eden et al., 1990; Hentzer, 1969; Lim, 1970; Nagai and and Tono, 1989; Rockley and Hawke, 1992; Sami et al., 2009; Songu et al., 2009). However, the two possibly different reflex mechanisms include common effector elements, consisting of the muscles surrounding the ET, i.e. tensor veli palatini and levator veli palatini, involved in both swallowing and ET opening (Alper et al., 2012; Poe et al., 2000). Nevertheless, their respective involvement in each of the two reflex actions may be quite different, as there could be concluded, by both the present study and other studies in literature that not every swallowing is able to generate an ET opening (Asenov et al., 2010; Mondain et al., 1997; Nielsen et al., 2018; Sadé and Ar, 1997). However, the ET may also open passively at sudden large ME – ambient pressure gradients, as well as by voluntary actions like yawning, aspects not included in the present Study.

About half of the subjects included in the inflation-deflation experiments of the current study (five out of nine cases) presented no or reduced ET activity and predominantly smooth counter-equilibration of ME pressure changes (Gaihede et al., 2010, 2013,). This seems to agree with the observation of Cinamon et al. (2017), that half of the subjects unable of ET opening did not develop underpressure, thus other ME mechanisms fulfilled the counter-equilibration role. Moreover, this may indicate that there can be situations where ET openings are less required.

Furthermore, sonotubometry studies have noticed that most pathological ears presented also ET openings in association with swallowing, but of reduced duration, magnitude, and frequency (Asenov et al., 2010; Poe and Pyykkö, 2011). This may indicate that the ET functionality is not ON – OFF, but rather that it may gradually decrease under inflammatory conditions.

Thus, a certain degree of ET functionality might be sufficient after surgical reconstruction resulting in smaller ME cavities when the diseased mucosa is removed and the source of underpressure eliminated. This would actually agree with the findings of the current study in that the pressure change by ET openings correlates significantly with the ME-ambient pressure gradient, so as a smaller gradient correlate with a lower pressure change i.e. an ET opening of lower magnitude.

A pressure counterbalance of lower magnitude may be realized by a smaller opening of the ET lumen, whereas a larger opening would increase the magnitude of the pressure change, as well as of the rate of change, due to the relatively constant opening time.

For situations where a long-lasting ME pathology results in reduced ability of the ET lumen to open (Takahashi et al., 1987), new techniques of increasing its compliance have been employed in the recent years with the insertion of a catheter into the ET lumen and dilating it by means of an inflatable balloon. This forced dilatation of ET proved to be efficient in increasing its permeability (Wanscher and Svane-Knudsen, 2014).

5.2. STUDY II. CONTRIBUTION OF THE TYMPANIC MEMBRANE

The aim of Study II was to obtain a quantification of the buffer capacity of the TM in daily physiological conditions, and to verify whether it correlated with the ME volume.

The long-term recordings provided us with sequences of the pressure change during elevator trips (Figure 4-2). This material was a suitable example of physiological pressure deviations at low pressure changes in ambient atmosphere (± 40 daPa), and a slow pressure change rate (2 daPa/s), at which the buffering role of the TM was expected to be easily detectable. By contrast, the ET openings analyzed in PAPER 1 were produced by mean pressure gradients of -325 to 390 daPa, which triggered ET openings in several cases.

The effect of the TM deformations was calculated by the difference between the pressure changes inside the ME and in the ambient during both elevator ascent and descent. These deformations reflected the buffer capacity of the TM in tandem with the ossicular chain attached to it (Cheng et al., 2007). The results showed that the TM counterbalanced in average 23.3% of the created pressure gradient, and this was in good agreement with 25% assessed in experiments on fresh temporal bones (Decraemer and Dirckx, 1998), whereas the artificial ME models obtained slightly larger results but still within range (Cinamon and Sadé, 2003).

The individual values for the buffer capacity ranged between 5 and 55%. As expected, they correlated negatively with the ME volumes, which ranged between 5 and 11 mL (r^2 = 0.92, p = 0.000). This suggested an interdependence between the TM and the mastoid, regarding the buffering role, even in rapid pressure changes in the order of seconds. The mastoid might intervene as buffer, "diluting" the pressure gradients, and having a protective effect over the TM. This becomes relevant in pathologic states, where smaller ME volumes has been found to correlate with a higher degree of TM retraction (Sadé et al., 1996). This suggests, in line with our study that the smaller ME's are more exposed to overload in pathologic ME's. Thus, it may be expected that the surgical eradication of the diseased ME mucosa, followed by leaving a radical cavity instead of mastoid air cells would be enough to eliminate the risk of underpressure,

rendering a TM reconstruction safe. However, this method has proven ineffective, due to frequent chronic discharge and recurrence of underpressure (Luers and Hüttenbrink, 2016; Takahashi, 2017).

By contrast, obliteration of the eradicated mastoid, introduced in recent years, has provided a considerable reduction of the cholesteatoma recurrence, but the mechanisms responsible for its higher success rate are not clear yet (Alper et al., 2017; Csakanyi et al., 2014; Luers and Hüttenbrink, 2016; Takahashi, 2017). The present study suggests that in smaller ME's, as long as the gas exchange is not impaired, the pressure gradients may be efficiently buffered by the TM. Moreover, even if the ET is most often impaired in chronic ME's, it might still function at a reduced efficiency, however enough to face the reduced need for aeration of a much smaller ME cavity, as discussed in the previous section (Asenov et al., 2010; Sadé and Luntz, 1989). Together, these two factors might support the success of mastoid obliteration.

5.3. STUDY III. EVIDENCE FOR THE CONTRIBUTION OF THE MUCOSA

The largest proportion of the ME mucosa lies within the MACS having an average surface area of 194 cm^2 for an average mastoid volume of 9.4 cm^3, which corresponds to a surface-to-volume ratio (S/V ratio) of 22.4 cm^{-1} (Cros et al., 2016). By contrast, the TC is an approximately 0.7 cm^3 compartment, covered by only 7.8 cm^2 mucosa (S/V ratio of 11,8 cm^{-1}) (Swarts et al., 2010).

Traditionally, the mastoid has been regarded as a passive reservoir of gas attached to the physiologically active TC (Alper et al., 2011), but the recent calculation of its huge mucosa surface has drawn an increased attention onto the probability that it might be an active organ in the ME pressure regulation (Alper et al., 2017; Magnuson, 2003). This hypothesis has also been emphasized by interesting observations during in vivo monitoring of the ME pressure in daily conditions (Nielsen et al., 2018; Tideholm et al., 1996) as well as in inflation – deflation experiments (Gaihede et al., 2010). Such long-term monitoring demonstrated that ME pressure had a very dynamic behavior, represented by continuous slow gradual fluctuations (Figure 3-1, c), which could not be explained by ET opening (Figure 3-1, d) or by gas exchange. Moreover, the ME response to induced over- and underpressure was, in several cases, a further accentuation of the induced pressure change instead of counter-regulation towards baseline, or a crossing over the baseline (Gaihede et al., 2010). Thus, the hypothesis of pressure counterbalance by changes in mucosa thickness was adopted as reasonable (Magnuson, 2003). This mechanism might further have an hypothetical active control, which should be considered for future investigations (Eden et al., 1990; Nagaraj and Linthicum, 1998).

As the physiology of the ME mucosa is not readily accessible, we have designed histological investigations in order to gather morphometric and structural evidence for its potential functions.

By the histo-morphometric study (PAPER 4), we found that the blood vessel density, as well as the surface of active mucosa did not differ significantly between different regions of the ME (Figure 4-4), indicating that the mucosa of eight sampled regions within both the TC and the MACS were structurally equipped in quite the same degree for gas exchange. However, the identification of blood vessels was limited to the open vascular structures containing erythrocytes due to the general HE staining. A more precise quantification is expected from immunohistochemical staining of the blood vessels, which enables the identification of all blood vessels including those with collapsed lumen. A parallel staining with D2-40 of neighbor sections, enabling the identification and exclusion of the lymphatic vessels, can increase the accuracy in blood vessel identification. Our preliminary immunohistochemical investigations demonstrated the feasibility of these staining methods on mucosa from EDTA-decalcified and paraffin-embedded mastoid samples (Section 4.3.3 and PAPER 4, Figure 6).

The mucosa thickness as well as the diffusion distance were lower in the posterior and superior TC and in all the MACS samples, with lowest values in the superior MACS, suggesting a facilitation of the gas exchange in the posterior and superior part of the ME. This observation is in agreement with the conclusion of a previous similar study, which included fewer ME regions (Ars et al., 1997).

The morphological observations presented in PAPER 4 concluded that in the anterior and the inferior regions, where the mucosa was thickest and the diffusion distance longest, the epithelium was also higher and often ciliated, and the lamina propria of the mucosa was usually looser than in the other regions. This brought one more evidence for the specialization in clearance of the anterior and inferior TC, in agreement with previous histological studies (Ars, 1998; Hentzer, 1970; Sadé and Facs, 1966; Tos, 1984).

We found also that the thickness of the lamina propria differed significantly with the presence versus absence of the blood-filled vascular lumen sections, resulting in a mean difference of 4 μm. This brings evidence about the contribution of the changes in mucosa thickness to ME pressure changes. Our measurements bring evidence which is in agreement with the empirical calculations of Magnuson (2003), who found that a mere difference in mucosa thickness of 6 μm in an average mastoid can give a ME pressure increase of 100 daPa; thus, it seems acceptable that changes in mucosal thickness based on our histological samples (4 μm) are able to participate to pressure changes required for the physiological pressure regulation.

The basic concept of mucosal congestion as a regulatory factor in physiological processes has been described earlier for instance in nasal mucosa (Widdicombe, 1997), as well as in the ME of the diving animals (Odend'hal and Poulter, 1966; Sadé et al., 2008; Sassu and Cozzi, 2007). In all these examples, a high content of sinusoids has been described ensuring a high capacity of engorging blood, and thus a high ability of pressure counterbalance by mucosa changes in thickness (Figure 1-6). We sought to investigate whether the mucosal blood vessels had features of sinusoid type, such as discontinuous basal membrane. However, our preliminary LAM staining failed to show that.

Another important indication of the special role of the vascularization in the ME physiology is the numerous lateral micro-channels especially at the level of the mastoid (Cros et al., 2013). They could be identified on all the histological preparations included in the current study, including a rich blood supply from the lateral surface of the temporal bone towards the MACS, supplementing the vascular supply coming from the internal large arteries such as carotid artery, internal maxillary artery, accessory meningeal artery (Merchant and Nadol, 2010). The increased vascularization of the ME in the mastoid compartment suggests a highly demanding physiology, which may be justified for both an efficient gas exchange as well as effective changes in mucosa thickness.

However, we have noticed in PAPER *5* that the lateral micro-channels tend to fill up with fat tissue and become smaller until disappearance in sclerotic mastoids, where the entire air cell is gradually replaced by effusion, soft tissue and later fat tissue. Moreover, an intense new bone formation has been noticed in the sclerotic mastoids apparently starting at the peripheral air cells, where the increase in bone mass is more accentuated in the lateral mastoid than in the medial mastoid. This seems to associate with the disappearance of the micro-channels, which might emphasize their importance in the normal ME physiology, and further on the need for future research on the role of the mastoid in the ME pressure regulation.

5.4. OTOSURGICAL IMPLICATIONS

In cholesteatoma surgery, obliteration of the mastoid cavity has gained more attention during the last two decades, because clinical data has demonstrated a significant reduction in recidivism. The explanation for this is basically unknown or limited to statements about the reduction in the amount of diseased mucosa (Alper et al., 2017; Csakanyi et al., 2014; Luers and Hüttenbrink, 2016; Takahashi, 2017).

However, based on our current findings, a smaller ME volume corresponding to the tympanum results in a larger TM buffer capacity (Padurariu et al., 2016). This situation is quite similar to obliterated cases, where only the tympanum remains open. Moreover,

vast amounts of diseased mucosa that may work as a gas sink or favor gas absorption is removed, which may decrease the demand for gas supply. In addition, fibrosis is common after recurrent or chronic infections, and such changes may reduce the functional properties of the mucosa, so that variations in volume and congestion are limited; this means that any possible pressure regulation by changes in mucosal congestion may vanish (PAPER *5*).

Surgical reconstruction of the TM with cartilage grafts has become popular within the last two decades; compared with fascia grafts, cartilage grafts provide the reconstructed TM with higher stiffness without any notable effects on the hearing levels. The increased stiffness has been claimed to reduce the risks for recurrent retractions and cholesteatoma formation, but long-term evidence is still lacking. Thus, if sustained underpressure prevail in the ME, cartilage is still a living tissue that inevitably will yield at some point (Figure *5*-1) (Zahnert et al., 2000).

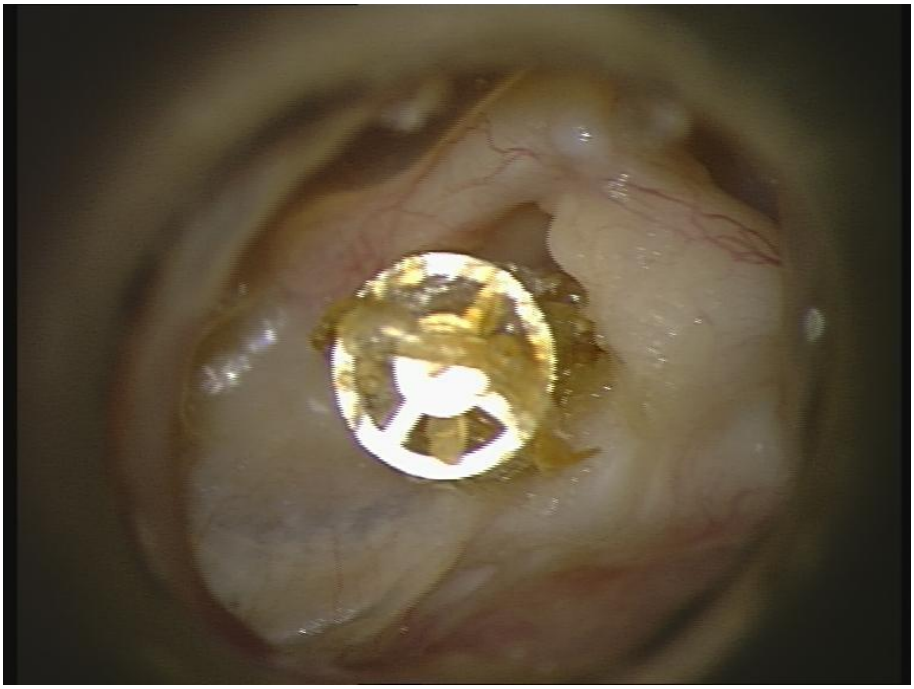

Figure 5-1. Extrusion of clip prosthesis through the reconstructed TM (by courtesy of Dr. Michael Gaihede)

The function of the ET may be improved by balloon dilatation, which has become increasingly common during the last decade (Wang et al., 2018). Such dilatation seems to increase the ability to ventilate the ME on a short term basis, but the results have not been documented by long-term observations, as well as randomized studies are still in demand (Wanscher & Svane-Knudsen, 2014). However, the impairment of the ET

function can be relative, and thus, if the demand for gas supply has been reduced by a mastoid obliteration, then even an ET with an impaired function may be capable of sufficient ventilation of such a reconstructed ME.

The overall ME physiology is of immense importance for successful reconstruction of the ME structures. Thus, any perfect reconstruction of the TM and the ossicular chain for optimal sound transfer and good hearing is still challenged by the demand for normal pressure regulation with sufficient ventilation of the ME; otherwise, all reconstructions are doomed to fail (Figure 5-1). Thus, further improvements of our basic understanding of the ME's structural and functional properties are still important, and the best treatment strategies need to combine both optimal acoustic transfer as well as regulation of the static ME pressure. Ultimately, such treatments aim at a safe and dry ear with long-term optimal hearing.

5.5. CONCLUSION OF THE STUDIES

The present work included a collection of studies, which contributed to an improved understanding of the roles of respectively the ET, TM, and the ME mucosa to the overall ME pressure balance with the ambient atmosphere. We found that the ET contributes to the ME ventilation by intermittent openings of 0.34s of mean duration and approximately 84 times per day; the openings seem to be represented by a distinct reflex, but a swallowing reflex is also a prerequisite. The TM buffers approximately 23 % of the low-range pressure changes, but its relative contribution may increase beyond 50 % in MEs with smaller air volume. The ME mucosa presents a morphology appropriate to a dynamic physiology which seems adapted to both an efficient gas exchange as well as effective volume changes by variations in mucosal congestion. In pathological MEs, the ET openings may decrease considerably in magnitude and frequency, depriving the ME of gas supply and generating by this underpressure, which the TM will buffer by extended displacements. Further, the mastoid seems to undergo sclerotic changes starting from its lateral side, by the disappearance of the microchannel-related blood supply and the progressive filling of the air space with soft tissue, fat tissue, and new bone.

CHAPTER 6. PERSPECTIVES

6.1. PRESSURE PHYSIOLOGY

Much research has been made and still many questions remain to be answered. The physiological studies of the ME pressure by direct approach trough mastoid puncture have offered some insight into the dynamics of the ME pressure regulation in vivo and in physiologic conditions, but they could not distinguish between overlapping pressure regulating mechanisms, such as the contribution of the mucosa by either gas exchange or volume changes. However, the study opened up for improvements to the method, which could come closer to the individual mechanisms. Thus, a new series of physiological recordings by mastoid puncture in healthy and intact MEs can combine the ME pressure measurements with human microvascular imaging, in order to measure the closer investigate blood flow. Possible joint techniques could be Laser Doppler for relative measurements of flow, as well as videomicroscopy for estimation of the vascular density flow, which have been developed for monitoring of microcirculation in intensive care (De Backer et al., 2010; Wright et al., 2016).

Moreover, the TM buffer capacity can be monitored simultaneously using one of the various available techniques such as laser Doppler vibrometry (Whittemore et al., 2004), stroboscopic holography (De Greef et al., 2014), moiré topography (Dirckx and Decraemer, 1991), or optical coherence tomography combined with a film patch on the TM integrated with a strain sensor (Just et al., 2011). This would complete the TM patterns as seen from inside with the image of TM buffer contribution as seen from outside.

A longer recording time of the ME response to experimental volume or pressure deviations is needed. This would allow a better quantification of the exponential decrease or increase better, which in the current experiments seemed almost linear over 10 minute-frames. Furthermore, it may be possible over longer time-frames to distinguish the contributions of gas absorption and mucosal congestion, since pressure decrements may represent both gas absorption and mucosal decongestion working in the same direction, whereas pressure increments may represents gas absorption and mucosal congestion working in opposite directions.

Another interesting aspect would be to analyze the correlation between the high-frequency oscillations presented in Figure 1-5 with the blood pressure and pulse. It might be hypothesized that changes in amplitude correlate with changes in blood flow and in mucosa congestion, and further on with pressure changes.

6.2. IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a valuable tool for further clarification of the mechanisms by which mucosa contributes to the ME pressure regulation. The structure of the human ME mucosa so far has not been investigated by immunohistochemical staining methods, except few studies focusing on the autonomic innervation at the level of the TC mucosa (Nagaraj and Linthicum, 1998), on the cholesteatoma (Kim and Jung, 2004; Shin et al., 2015) or the innate immune response in otitis media (Val et al., 2016, 2015). This is probably due to the technical challenges related to histological preparations of biological material including both hard and soft tissue (Kang et al., 2003).

Our primary aim included an investigation of the microscopic structure of the ME mucosa by immunostaining methods targeting specifically vascular, neural and smooth muscle structures, and it could only partly be achieved. There main challenges resided in the lack of attachment of the biopsy sections on the glass slides, even if they were electrostatically treated and baked for one day. This may be related to the long decalcification time in 10% EDTA (1-2 years), which affected also the antigenicity in immunohistochemical staining. However, the results were improved by prolonging the baking of the slides to two days and by the application of the routine antigen retrieval procedures.

Neutral EDTA was chosen for the advantage of maintaining the best tissue morphology and antigenicity in immunohistochemistry, with the expectation of a slower decalcification time compared to acid agents (An and Martin, 2003; Rolls, 2012). However, the process took much longer than expected, and might be due to the fact that the mastoid biopsies included cortical bone, which is harder and requires longer decalcification than the air cells part. We have purposely included cortical bone because we have been interested in the transition between the lateral part with high density of micro-channels to the MACS mucosa. Thus, by excluding the cortical bone and concentrating only on mastoid samples including air cells, the decalcification time and quality of samples might be improved in the future studies for mastoid mucosa histology.

It remains open for further studies to perform a systematic study of immunohistochemical staining of the blood vessels, neural and muscular structures, as well as a APS staining for collagen fibers. Combined with digital image analysis, proportion calculations may be obtained and used in a detailed comparison between the ME regions.

Mucosal vascular endothelium stained positively for CD31, but failed to stain for ERG. Mucosal lymphatic vessels stained positively for D2-40, and this can be used as the differential marker in parallel with CD31 for a more specific differentiation and quantitation of the blood vessels.

Mastoid mucosa includes smooth muscular structures in the walls of the blood vessels, which stained positively with ASMA, and very weak with DES. If further studies using ASMA emphasized the presence of smooth muscle fibers also within the lamina propria, this would evidence that the changes in mucosa thickness are actively controlled.

Mastoid mucosa stained weakly positive with SYP and NF, and positive but rather unspecific with S100, whereas it did not stain with SOX10. This preliminary results have been difficult to interpret, whether there was a positive staining weakened by the poor antigenicity of the long-decalcified samples, or just unspecific staining. However, a new analysis using SYP and NF might emphasize the presence of nerve fibers in the vicinity of the blood vessels, suggesting that their opening and closing might be under neural control, casting light on the role of the mastoid mucosa in the ME pressure regulation.

Altogether, future investigations with more detailed immunohistochemical mapping of the mucosal structures may further elucidate the role of the mastoid mucosa in the overall pressure regulation.

6.3. PROTEOMICS

Proteomics has been anticipated for more than one decade ago to open up for a new horizon in the ME research by facilitating access to quantitative analyses of the MEs molecular profiling (McGuire and Casado, 2004; Palmer-Toy et al., 2005). The feasibility of mass spectrometry has been proven in the study of cholesteatoma (Britze et al., 2014; Kim and Jung, 2004; Shin et al., 2015), acute and chronic otitis media (Harrison et al., 2016; Val et al., 2016), as well as various inner ear disorders (Alawieh et al., 2015; Chiarella et al., 2012; Randall et al., 2015). However, the potential of the new method has only sparsely been explored so far.

In addition to the histo-morphological comparison of the ME mucosa in different regions presented in Study III (Padurariu et al., 2018), a MS analysis may add a more robust comparison at the proteomic level, with the advantage of much smaller samples compared to histology.

We have conducted a preliminary study in two samples from the same human ear, aiming at testing the feasibility of proteomics in ME mucosa comparison in fresh frozen as well as formalin-fixated samples. The preliminary results showed that proteomics could be employed in the study of ME mucosa and anticipated significant differences in the proteome comparing different locations of the same ME (Figure 7-1, manuscript in preparation).

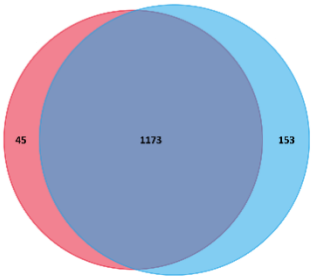

Figure 6-1. Diagram of the number of proteins identified in a pair of samples from the same ear, one sample from the tympanic mucosa (blue circle), and the other from the mastoid air cells (red circle).

Secondly, we have tested the protein recovery and optimal sample preparation strategy from each step in the histologic processing in a set of biopsies from the same donor, i.e. fixation, decalcification with EDTA, decalcification with formic acid, paraffin-embedding including dehydration, compared with fresh frozen sample. The number of the identified proteins in processed samples varied between 50 and 80% compared with the fresh sample (Figure 7-2, manuscript in preparation).

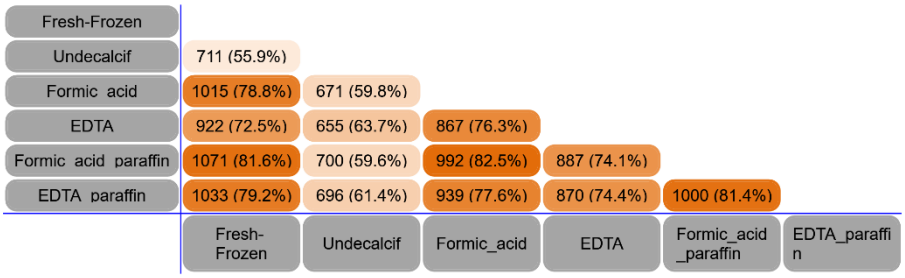

Figure 6-2. Protein recovery after different steps in tissue processing

Thirdly, we have performed a comparison between formalin fixation, RNAlater preservation and the use of fresh frozen samples regarding the proteome recovery in four anonymized sets of human samples, according to Bennike et al. (2016). Results indicated that the proteome data quality was high and did not differ significantly among the three preservation methods. However, for practical considerations, RNAlater might be most convenient for future studies based on patient material from operation.

While proteomics has been applied primarily for investigations on diseases, the content of structural proteins may also point to regional differences in the functional properties of the ME mucosa, and hence, further elucidate the physiological aspects of the ME including the pressure regulation. Thus, topics for further studies could be:

- differences in the collagen content of the mucosa layer, possibly suggesting a higher potential for volumetric changes of the mastoid mucosa compared to TC mucosa;

- differences in the vascular structures, possibly specialized capacitance vessels or sinusoids in the mastoid mucosa, able of an important contribution to the volumetric changes;
- differences in the neural content of the two regions, possibly suggesting an active neural control of the vascular structures within the mastoid.

A newer and promising MS application for the systematic study of the ME mucosa would be MALDI Imaging technology, which makes now possible to perform proteomics analyses directly on histological slides (Fujino et al., 2016; Papp et al., 2016). Similarly, the use of laser micro-dissection-based microproteomics from fresh or formalin-fixed paraffin-embedded tissue enables detailed insight into the ME regions (Longuespée et al., 2016; Roulhac et al., 2011).

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SUMMARY

One of the most frequent otological diseases is otitis media with effusion. This condition consists of inflammatory changes within the middle ear, and accumulation of an under-atmospheric pressure. The consequences are often serious when the under-pressure persists for a long time, becoming chronic. In certain cases, the condition results in the formation of retraction pockets in the tympanic membrane, bone erosion, and cholesteatoma formation. This loss of structural integrity of the middle ear leads inevitably to hearing loss. The treatment of chronic Otitis media with effusion requires often surgery. The success of surgical reconstruction depends on the restauration of the pressure balance between the middle ear and the ambient atmosphere. Therefore, the understanding of the basic mechanisms of the pressure regulation within the middle ear is essential.

The following work contributes evidence to the role of the main compliant structures of the middle ear, known to or hypothesized to influence the middle ear pressure.

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