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MORPHOLOGY AND BIOCHEMISTRY OF THE TYMPANIC MEMBRANE

Johan Knutsson

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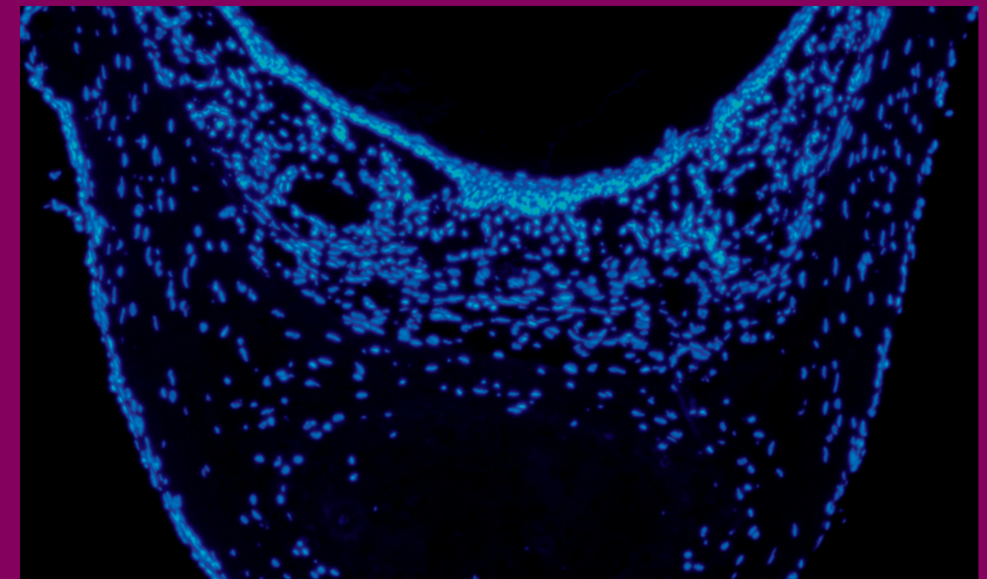

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Cover image:
A healthy human tympanic membrane sectioned through the umbo. Fluorescent staining of the cell nuclei with the use of DAPI.

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ABSTRACT

Otitis media is very common during childhood and may predispose for tympanic membrane (TM) pathology later in life. Therefore, it is important to investigate the possible changes in TMs subjected to otitis media.

There is evidence of a relationship between previous otitis media and TM retraction disease. A weakening of the collagen fibers, that form the backbone of the TM, is a prerequisite for the genesis of a retraction. Secretory otitis media results in a TM stiffness loss in animals but no loss of collagen fibers has been found. Different collagen types have different tensile strength. An altered collagen type distribution due to otitis media could possibly explain the stiffness loss. The relative distribution of the different collagens in the TM in normal and pathologic states needs further clarification. Secretory otitis media also results in a thickening of the outer keratinizing epithelium. In cholesteatomas, as well, the outer keratinizing epithelium is thickened but to a much greater extent.

The overall aim of the study was to further elucidate the possible relationship between otitis media and retraction pathology. The specific aims were to identify the different collagen types in the normal TM in rats and humans. Thereafter, the collagens and the morphology were investigated in cholesteatomas and human TMs subjected to secretory otitis media. Furthermore, the human keratinizing epithelium was investigated regarding the presence of possible stem cells.

This was done with the use of healthy TMs from rats and humans, biopsies from human TMs subjected to long-standing secretory otitis media and cholesteatomas.

The methods used were immunohistochemistry with DAB and immunofluorescent techniques using antibodies against the four most common collagen types and different stem cell markers. Visualization was achieved using light microscopy, laser confocal microscopy and transmission electron microscopy.

Collagen type II was found to be the predominant collagen type in the lamina propria of TMs from both rats and humans. In the human TM, a distinction could be made between the outer and inner layers of the lamina propria. The inner layer proved to contain collagen type III to a large extent. Collagen type IV was found in the basal laminas. In the TM biopsies, all four types of collagens could be identified but no quantification could be performed. The cholesteatomas proved to contain remnants of collagens and were positive for all collagen types except for collagen type III.

The outer keratinizing epithelium displayed thickness variations in the normal human TMs, especially regarding the basal layer, which was markedly enlarged in the umbo, along the handle of the malleus and in the annular region. In these areas the basal layer cells were elongated perpendicularly to the basal lamina, a finding contrasting to the findings in the adjacent regions. The basal layer cells in the thicker parts of the TM biopsies and the cholesteatomas were perpendicularly elongated in a similar fashion. It is hypothesized that this is a sign of an increased cellular proliferation. The healthy human TMs were investigated for stem cell markers that proved to be positive in the areas with a thickening of the basal layer of the keratinizing epithelium.

Key words: Tympanic membrane, retraction pathology, secretory otitis media, cholesteatoma, pars tensa, lamina propria, keratinizing epithelium, collagen type, progenitor cells, stem cells, immunohistochemistry, electron microscopy

LIST OF PUBLICATIONS

This thesis is based on the following papers.

I. **Knutsson J**, Bagger-Sjöbäck D, von Unge M. Distribution of different collagen types in the rat's tympanic membrane and its suspending structures. *Otology & Neurotology. 2007 Jun;28(4):486-91.*

II. **Knutsson J**, Bagger-Sjöbäck D, von Unge M. Collagen type distribution in the healthy human tympanic membrane. *Otology & Neurotology 2009 Dec;30(8):1225-9.*

III. **Knutsson J**, von Unge M, Rask-Andersen, H. Progenitor cells and stem cells in the human tympanic membrane *(submitted)*

IV. **Knutsson J**, Bagger-Sjöbäck D, von Unge M. Histological studies of inflamed human tympanic membranes and cholesteatomas *(in manuscript)*

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LIST OF ABBREVIATIONS

DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
EGF	Epidermal growth factor
FA	Fibrous annulus
IHC	Immunohistochemistry
KGF	Keratinocyte growth factor
PBS	Phosphate buffered saline
PT	Pars tensa
SOM	Secretory otitis media
TEM	Transmission electron microscopy
TM	Tympanic membrane

1 INTRODUCTION

The tympanic membrane (TM) separates the outer ear from the middle ear. The TM not only acts as a protector of the middle ear, but also plays a crucial role in the physiology of hearing. In addition, the TM often offers the clinician a possibility to evaluate the middle ear status; either directly if the TM is transparent, or indirectly when inspection of the TM reveals clues about the middle ear status. In cases of middle ear infection and inflammation, the TM is affected by the subsequent spreading of the inflammation to the TM. The long-term effects are unclear.
There is evidence that previous otitis media may predispose for retraction pathology later in life.[1-3] Since otitis media is very common during childhood it is important to investigate its relationship with possible structural changes of the TM.

1.1 ANATOMY

The ear is traditionally divided into the outer ear, the middle ear and the inner ear with the TM as the dividing zone between the two former and the foot-plate of the stapes as the dividing zone between the two latter. The anatomy of the TM is presented in this thesis in detail whereas the anatomy of the other parts of the ear is only briefly presented, since these are not the scope of this thesis.

1.1.1 External ear

The external ear is composed of the auricle and the outer ear canal. The auricle is made up of fibroelastic cartilage and it is covered with skin. The human outer ear canal is 2,5-3 cm long. Its lateral part is composed of cartilage and covered with skin including hair follicles and sebaceous glands that produce cerumen. The medial part of the outer ear canal is located in the temporal bone and it is lined with skin without hair follicles and glands.

1.1.2 Tympanic membrane

1.1.2.1 Anatomy

The TM is a cone-shaped diaphragm that is suspended between the handle of the malleus and the tympanic sulcus in the temporal bone. In the greater part of the circumference of the TM there is a thickening most peripherally called the fibrous annulus that inserts into the tympanic sulcus and thereby attaching the TM to the temporal bone.[4]
The shape of the TM is slightly oval with its longest diameter in humans measuring 9-10 mm and its shortest diameter measuring 8-9 mm resulting in a total surface area of approximately 64 mm^2.[4]
Rodents are often used in otologic research. In rats, the TM is substantially smaller than in humans, approximately 3-4 mm in diameter and in mice the TM is even smaller.[5] The surface area of the rat TM is approximately 11 mm^2.[6] The TM consists of two parts; the pars tensa and the pars flaccida (Shrapnell´s membrane). The human pars tensa is roughly ten times larger than the pars flaccida while in many other species the pars flaccida is relatively larger, up to half the size of the pars tensa.[5]

The thickness of the human pars tensa is approximately 30-90 μm, being thicker most peripherally and along the handle of the malleus. The human pars flaccida is thicker than the pars tensa, measuring 30-230 μm.[4] The rat pars tensa is significantly thinner, only 5-10 μm.[7]

1.1.2.2 Histology

The tympanic membrane consists of three different principal layers; the outer keratinizing squamous epithelial layer, the lamina propria and the inner mucosal epithelial layer.[4, 8] See Fig 4c in the fourth paper.

1.1.2.2.1 Outer epithelium

The outer epithelium is a stratified squamous cell epithelium of keratinocytes. It can be further subdivided morphologically into several layers: the basal layer (adhered to the basal lamina); the spinous layer (Malphigian layer); the granular layer; and most superficially the stratum corneum (the keratin layer). In the stratum corneum the cells have lost their nuclei. Under normal conditions the human outer epithelium consists of 5-10 cell layers.[8] In rodents the cell layers are fewer.[7] The epithelium of the pars flaccida is more heavily keratinized.[5]
The histological picture of the outer epithelium has many similarities with that of the skin and the outer epithelium is sometimes in the otologic literature referred to as the epidermis of the TM. There are also several histological differences. The TM lacks hair follicles and adnexal glands and the skin keratinocytes exfoliate perpendicularly, whereas in the TM the keratinocytes are transported centrifugally towards the fibrous annulus and the external ear canal.[9-11] The average migration velocity has been estimated to 0,05 mm per 24 hours. In most mammals, including humans and rats, the migratory direction is peripherally towards the fibrous annulus and superiorly along the handle of the malleus.[9, 12] In guinea-pigs another direction of the epithelial migration has been observed; from the inferior to the superior.[13]

1.1.2.2.2 Lamina propria

The lamina propria of the pars tensa consists of two main layers of connective tissue that are predominantly composed of collagen fiber bundles. The outer layer of the lamina propria has a radial arrangement of the collagen bundles while the inner layer consists of circularly arranged collagen fibers.[4, 5, 14] The inner layer is more pronounced peripherally.[15] Parabolic, transverse and crescent formed collagen bundles have been described.[15-17] These fibers are according to some authors located between the two main collagen layers while other authors have found parabolic and crescent formed collagen bundles within the inner circular collagen layer. Between the collagen layers there are also vessels. Within the collagen layers there are scattered fibroblasts that produce collagen.[4]
Collagen is the principal and most abundant connective tissue component. The fibers are flexible and have great tensile strength. The collagen fibers are composed of three polypeptide chains, so called alpha chains, which are arranged as a triple helix. Differences in the structure of the alpha chains determine the type of collagen.[18] There are many different types of collagens in the connective tissues with collagen

types I-IV being the most common ones. There is evidence of collagen type II present in the lamina propria.[19] The possible presence of other types of collagens and their relative distribution in the TM is unclear.
In the pars flaccida, there is an abundance of elastic fibers and loose connective tissue. There are also collagen fiber bundles.[20] The bundles are fewer as compared to the pars tensa, and are irregularly arranged.[4] In addition, the pars flaccida harbors numerous mast cells.[21] The vascular supply of the pars flaccida resembles that of the pars tensa.[20, 22]
According to comparative studies of different species no major differences are found regarding the content of the lamina propria of the TM. An exception is the guinea-pig, which is reported to have a lamina propria consisting of only one layer of coarse collagen fiber bundles.[7] Other differences between mammals are the guinea-pig having a very small pars flaccida and the gerbil having a more cell dense pars flaccida.[5]

1.1.2.2.3 Inner epithelium

The inner epithelial layer consists of a single-lined cuboidal or squamous epithelium, depending on species, and is continuous with the mucosa of the middle ear cavity. In humans both a cuboidal and squamous epithelium have been described and even pseudo-stratified columnar epithelial cells have been described in the pars tensa of human TMs [4, 23-25] The cells are non-ciliated and no goblet cells are found in the inner epithelium of the human TM.[8]

1.1.2.2.4 Vessels

The blood vessels of the TM are mainly located in the loose connective tissue compartments (more predominant on the lateral side) but are not penetrating the basal lamina of the epithelia on either side.[8] The vascular supply to the lateral part of the human TM derives from the manubrial artery from the deep auricular artery, a branch of the internal maxillary artery, which in turn is a branch of the external carotid artery. The medial part of the TM is supplied by a branchlet from the middle meningeal artery and a vascular circle formed by the anterior and posterior tympanic arteries. The anterior and posterior tympanic arteries branch off from the internal maxillary artery. The posterior tympanic artery enters the middle ear along with of the chorda tympani while the anterior tympanic artery enters the tympanic cavity where the chorda tympani exits, i.e. through the petrotympanic fissure.[26, 27]

1.1.2.2.5 Nerve supply

On each side of the lamina propria there is a thin layer of loose connective tissue that harbors fibroblasts and nerve fibers.[7, 14, 22] The lateral part of the TM is innervated by the auriculo-temporal branch of the trigeminal nerve and the auricular branch of the vagus nerve. The medial part of the TM is innervated by the tympanic branch of the glossopharyngeal nerve.

1.1.3 Middle ear

The middle ear is located within the temporal bone and harbors two air-filled compartments: (i) the mastoid cells and (ii) the tympanic cavity, which are connected

via the antrum. The tympanic cavity is limited laterally partly by the TM and medially by the promontory of the cochlea and is connected to the rest of the upper airways through the Eustachian tube. The tympanic cavity contains the three ossicles; the malleus, the incus and the stapes. The long process of the malleus is called the handle of the malleus and is attached to the superior part of the pars tensa of the TM. The incus forms a bridge between the malleus and the stapes. The footplate of the stapes connects to the inner ear via the oval window.

The malleus is attached to the tympanic cavity walls with three ligaments; the anterior, the superior and the lateral mallear ligaments. The superior and posterior incudal ligaments attach the incus.

The middle ear is lined with a single-layer of cuboidal or flat epithelial cells. There are also descriptions of a pseudo-stratified epithelium in the middle ear.[8, 14] Cilia has been described in some locations of the tympanic cavity.[23]

Two muscles are found in the tympanic cavity; (i) the stapedial muscle which origins from the pyramidal eminentia on the posterior tympanic cavity wall and inserts into the superstructure of the stapes. (ii) The tensor tympani muscle origins from the Eustachian tube and enters the tympanic cavity at the cochleariform process. The muscle inserts into the neck of the malleus.

The middle ear is also a passage for the chorda tympani nerve which transverses the tympanic cavity after leaving the facial nerve. The chorda tympani runs between the handle of the malleus and the long process of the incus and further on through the petrotympanic fissure to the lingual nerve into which it is incorporated.

1.1.4 Inner ear

The inner ear is located in the temporal bone and consists of the cochlea, the vestibule and the semicircular canals. The cochlea serves for the hearing. The vestibule, containing the sacculus and utriculus, and the semicircular canals are responsible for the sensory inputs regarding linear and rotatory motion changes.

The cochlea is spiral-shaped and is divided into three compartments; the scala vestibuli, the scala media and the scala tympani. The former two are separated by the Reissner's membrane. The latter two are separated by the basilar membrane which is attached to the organ of Corti. Here the mechanical inputs are transformed into nerve excitations that are conveyed to the central nervous system by the vestibulo-cochlear nerve which exits the temporal bone at its apex.

1.2 EMBRYOLOGY AND DEVELOPMENT

The ear has an early developmental onset in the fetal period. The first signs of an ear are seen at three weeks of gestation.

1.2.1 Tympanic membrane

At four to five weeks, a primitive TM is formed by the fusion of the ectodermal meatal cord and the endodermal tubo-tympanic recess from the first pharyngeal pouch. At eight weeks of gestation the lamina propria is formed by mesenchymal ingrowth between the two above mentioned layers.[28] Thus, the three layers of the TM have

different embryologic origins; the outer epithelium has an ectodermal origin; the middle layer has a mesodermal origin; and the inner epithelium has an entodermal origin.[29]
During the following eight weeks the tympanic ring is developed. At birth the TM has almost reached the size of an adult TM but its position is different, almost horizontal, while in adults the TM is nearly vertical with its posterior and superior parts positioned more laterally.

1.2.2 Middle ear

The tympanic cavity has an endodermal origin, deriving from the first pharyngeal pouch as it has grown laterally. The distal part of the pharyngeal pouch forms the tubotympanic recess and when it widens it forms the primitive tympanic cavity. The proximal part of the first pharyngeal pouch remains narrow and forms the Eustachian tube.[30]
The formation of the mastoid cells starts later in fetal life and they are formed when the tympanic cavity expands dorsally and by this initially forming the tympanic antrum. After birth the developing mastoid is invaded by epithelium from the tympanic cavity and epithelial lined sacs are formed. The continuing development results in pneumatization of most of the sacs by connecting them to the tympanic antrum and the tympanic cavity.[29, 31]
The ossicles appear in the first part of fetal life. They derive from cartilage from the first two pharyngeal arches. The muscles of the middle ear also origin from the pharyngeal arches. Initially the ossicles are embedded in mesenchyme and remain so until the third trimester. At the time when the mesenchyme dissolves, the ligaments of the ossicles are formed. The malleus and the incus derive from the first pharyngeal arch. The tensor tympani muscle and its innervation, the mandibular branch of the trigeminal nerve, correspondingly derive from the first pharyngeal arch. The stapes and the stapedial muscle origin from the second pharyngeal arch which explains why the muscle is innervated by the facial nerve via the stapedial nerve, since the facial nerve is a derivative of the second pharyngeal arch.[32]

1.2.3 External ear

The external ear canal develops from the dorsal portion of the first pharyngeal cleft as it invaginates to meet with the tubo-tympanic recess. At approximately 10 weeks of gestation the epithelial cells at the bottom of the external ear canal start to proliferate. By the seventh months, these cells participate in the formation of the definitive TM.
The auricle develops from six mesenchymal proliferations in the first and second pharyngeal arches and the first pharyngeal cleft. The mesenchymal proliferations fuse and gradually the normal form of the auricle is shaped. During this process the auricle also ascends from its original position in the lower neck region.[29]

1.3 PHYSIOLOGY

The TM has two main functions; (i) protection of the delicate middle ear structures and (ii) sound transmission. In addition, the TM may as an indicator for middle ear diseases.
The protective role of the TM is solely a barrier protection. By being a barrier, the TM protects the middle and inner ears from microbes entering through the external ear canal.

1.3.1 Energy transmission

The sound-transmitting role of the TM is much more delicate than the protective role. The sound wave reaches the TM after being collected by the auricle and modulated and amplified by resonance in the external ear canal. When hitting the TM, some of the energy carried by the sound wave is deflected back through the outer ear canal and some energy is collected at the umbo and transmitted to the ossicular chain. The sound wave acts on the middle ear in two ways: (i) direct transmission of energy, which reaches the round window. This route of energy transmission is negative for the hearing since the energy conveyed to the cochlea via the round window is interfering with the pressure waves of the inner ear fluids. (ii) The indirect transmission of energy via the ossicles is much more important since the energy is led to the inner ear via the oval window.
The role of the TM in sound transmission is more intricate than it seems at first. According to time-averaged hologram studies the sound-transmitting role of the TM is varying with the frequency of the incoming sound wave. The TM has specific vibrating patterns for different frequencies and intensities of the incoming sound.[33, 34] At frequencies above 3 to 4 kilohertz the volume displacement of the TM becomes independent of frequency. It has been speculated that the incoming sound from the above-mentioned frequencies would exert its force directly on the handle of the malleus and that the contribution of the TM becomes negligible. The TM would in this situation be completely uncoupled, vibrating at only very small amplitudes.[34]
When the sound energy has reached the oval window the mechanical wave is transformed into a motion in the inner ear fluid that reaches the inner hair cells attached to the basilar membrane. Subsequently the energy is transformed into a nerve excitation and the sound information is transmitted via the cochlear nerve and synapses to finally reach, and be recognized by, the central nervous system.

1.3.2 Energy enhancement

The TM and the ossicles transmit sound energy. Moreover, they modulate and amplify the sound. The amplification is greatly needed since the incoming sound wave is carried by air whereas the energy reaching the hair cells is carried in fluid. Through its high mechanical impedance (determined by density and elasticity) the fluid needs much more energy to build up a pressure wave than air does. The TM performs a major energy wave enhancement by its much larger area than that of the oval window. Thus the energy from a larger area is concentrated onto a smaller area. The anatomical area ratio is 21:1.[35] Since the vibrating area of the TM is approximately only two thirds of its surface area, the functional area ratio is however 14:1.[35]

The ossicles acts as a lever system that further increases the force of the sound wave by a factor of 1,3.[34] Subsequently the TM and ossicles together increase the energy reaching the oval window by a factor of 18.

1.3.3 Modulation of sound transmission

The two muscles of the middle ear are considered to have sound modulating tasks. The tensor tympani muscle acts upon the neck of the malleus by stretching the TM slightly and thereby enhancing its sound transmitting properties. The stapedial muscle on the other hand can reduce the sound transmitting capacity by stiffening the ossicular chain movements. Hence, the stapedial muscle might have a role in the tuning of the middle ear acoustic system.

1.3.4 Middle ear pressure regulation

The position of the TM is greatly affected by the middle ear pressure. When the middle ear pressure becomes lower than the air pressure in the external ear canal (i.e. the ambient atmospheric pressure) the TM is subjected to an inward pressure force. The middle ear pressure is affected by two major factors; the resorption of gases from the tympanic cavity and the passage of air through the Eustachian tube.

1.3.4.1 Gas resorption

Gas exchange through tissues depends on the trans-barrier partial pressure gradient and the specific physiochemical properties of the gas exchanging tissue.[36] Exchange to and from the middle ear occurs through different passive pathways. Gas exchange through the mucosa to blood vessels is considered the most important pathway. There is an ongoing dispute whether the gas diffusion is limited by diffusion properties or by vascular perfusion. There is also evidence of gas exchange to the inner ear via the round window membrane and exchange of oxygen and carbon dioxide through the TM.[37, 38] The resultant of the gas exchanges is a flow of gases from the middle ear. With this loss of gases, the pressure of the tympanic cavity is lowered. This is compensated by gas entering through the Eustachian tube.[39, 40]

1.3.4.2 The Eustachian tube

The physiology of the Eustachian tube is of great interest for the understanding of the middle ear pressure changes that affects the TM. The Eustachian tube is a tube-like formation with a ciliated single layer mucosa lining. It connects the middle ear to the nasopharynx. The Eustachian tube is formed by a cartilage that, in cross-section, is hook-like and forms a firm wall of the lateral and uppermost medial parts of the tube. The rest of the medial wall is flaccid and subsequently the tube is collapsed most of the time.

The closure of the Eustachian tube protects the middle ear from the extensive pressure changes taking place in the upper respiratory tracts.[41] It also protects against autophonia since a person’s own voice would otherwise take the shortcut through the Eustachian tube. The sound would thus reach the middle ear too early compared to the sound reaching the ear via the external ear canal.

Under normal conditions the Eustachian tube opens shortly during every day life activities such as swallowing, chewing and yawning. The opening of the Eustachian tube can also be forced by the Valsalva maneuver. This maneuver may alleviate a negative middle ear pressure, which is often the result of impaired opening function of the Eustachian tube. The most common reason for such impairment is an upper respiratory infection when the mucosa of the Eustachian tube is temporarily swollen. In a prospective study, 75% of pre-school children had tympanometric evidence of a reduced Eustachian tube function during an upper respiratory infection. The rate was only 13% in the tympanograms of children without clinical signs of an upper respiratory infection. In most of the cases the Eustachian tube dysfunction was evident already on the first or second days of the disease.[42]
The Valsalva maneuver is also useful in situations of sudden changes in the ambient pressure as in multi-stories elevators, air-plane ascents and descents and when travelling by high-speed trains through tunnels, although these sudden pressure changes are unphysiological for humans. Animals that are physiologically subjected to even greater pressure changes have developed special features to adjust the middle ear pressure to the ambient pressure in order to prevent the pressure gradients from rupturing the TM. Deep-water diving mammals and birds are the animals exposed to the greatest physiological pressure changes. These animals have a special venous organ to regulate the middle ear pressure. A corpus cavernosum like structure is located under the middle ear mucosa. When the venous structure is filled with blood, the middle ear volume decreases and thus the middle ear pressure increases in accordance with Boyle's law.[43, 44] Similar structures located in the external ear canal, in order to close it off and thereby protect the TM from the elevated pressure, has also been developed by some animals.[45]

1.4 INFLAMMATORY MIDDLE EAR DISEASE

The middle ear is susceptible to microbial invasion. Both bacteria and viruses can invade the tympanic cavity. Normally, the Eustachian tube is the only route for such an invasion if the TM is intact. A hematogeneous spreading of a bacterial disease to the tympanic cavity might occur, but would most likely be very rare and would of course be difficult to prove.

1.4.1 Purulent otitis media

Bacterial infection of the middle ear may result in an empyema since the tympanic cavity is a more or less closed off environment. Often the disease is preceded by a viral infection making the middle ear mucosa less resistant to the infecting bacteria. The most common pathogens are *Streptococcus pneumonie* followed by *Hemophilus influenzae* and *Moraxella catarrhalis*.[46] Most often the patient is in the lower age pediatric spectrum. The classical signs are aural pain, low-grade fever and impaired hearing. The physical examination reveals either a bulging TM or a red TM with decreased mobility on pneumatic otoscopy unless the TM has ruptured resulting in pus in the external ear canal.
The impact the purulent otitis media has on the TM has been studied in experimental set-ups with rats and Mongolian gerbils.[47, 48] Impedance and moiré interferometry

studies showed changes in the acoustic impedance and a reduced TM stiffness already in the first days of the disease.[49] Early histological examinations showed dilated vessels, followed by thickening of the sub-epithelial layer which was most pronounced in the periphery of the TM. A slight accumulation of keratin on the external pars tensa surface was seen on the third day of the purulent otitis media. The overall thickness of the TM was threefold, including all TM layers. The lamina propria appeared intact when studied with transmission and scanning electron microscopy.[49]

1.4.2 Secretory otitis media

Transudate in the middle ear may be termed secretory otitis media (SOM) but is also commonly known as otitis media with effusion. The pathogenesis is thought to be multifactorial. Infection, obstruction of the Eustachian tube, anatomical factors, impaired immunological status, allergy, familiar predisposition, environmental factors and gastro-esophageal reflux have all been suggested to play a role in the pathogenesis of SOM.[50-52] In clinical cases of SOM, viruses as well as bacteria have been cultured from the effusions but the pathogenetic significance of these microbes in regard of the persistence of the SOM is not known.[53]
Incompetence of the Eustachian tube plays an important role in SOM.[54] Due to the resorption of middle ear gases to the surrounding tissues, the middle ear pressure decreases continuously - at least to a certain level.[55] As a result of the negative middle ear pressure, a hydrostatic force develops that affects the middle ear mucosa. Subsequently a transudate is drawn into the tympanic cavity from the surrounding tissues and thus the SOM is formed. The effusions have a complex composition and major components include secreted mucus glycoproteins, proteins, lipids and inflammatory mediators and enzymes.[56] In advantageous situations the effusion is believed to be cleared via the Eustachian tube.[54] Temporary SOM is often seen after a purulent otitis media, but diminishing over time.[57]
SOM can be induced in animals by different means. Plugging of the Eustachian tube with a foreign material results in a mild SOM.[58] Electro-cauterization of the nasopharyngeal orifice induces a more severe form of SOM with a mucoid effusion.[59] Bisection of the tensor veli palatini muscle is an alternative way of inducing a SOM as is inoculation of non-viable bacteria.[60, 61]
The histological changes of the TM incurred by SOM have been examined in animal models. First the lamina propria thickness is increased and later on a substantial sub-epithelial thickening is developed on the lateral side of the lamina propria. There is also an increased cell content in the lamina propria and on scanning electron microscopy there are signs of disintegration of the inner circular collagen fiber bundles of the lamina propria.[62]

1.5 TYMPANIC MEMBRANE PERFORATIONS

The TM can rupture from direct trauma, barotrauma and middle ear infection, the latter being the most common. The healing of the TM starts at the edges of the perforation with an increased mitotic activity in the basal layer of the outer epithelium. The epithelium thereby thickens and starts to send out spurs of epithelial cells towards the defect, although with an initial lack of obvious organization. Thus a thin epithelial layer is produced. This thin layer acts as a scaffold for fibroblasts that invade the area and

produce collagen fibrils which re-creates the lamina propria. The epithelial proliferation continues and ultimately the outer epithelial layer resumes its normal structure.[63] The regulation of the epithelial proliferation is still unclear. There is evidence of keratinocyte growth factor (KGF) and epidermal growth factor (EGF) playing regulatory roles, but it is uncertain to what extent they regulate the regeneration.[64-66]
The collagen layer of the pars tensa consists of different types of collagen. In the acute healing phase after a myringotomy or an infection, there are initially collagen types I and III present. The collagen content of the TM is later modified during the inflammatory and healing processes towards higher concentrations of collagen type II.[67]

1.6 TYMPANIC MEMBRANE RETRACTION

A tympanic membrane retraction is a result of persistent negative pressure in the middle ear and is by some researchers believed to be the result of sequelae of previous otitis media episodes, especially long-standing secretory otitis media.[1, 68-70] If there is a loss of stiffness in the TM, pronounced retractions can appear. The retractions can be either focal or more general and can appear in the pars tensa as well as in the pars flaccida, the latter being more common. If present in the pars tensa, the retraction is most commonly located in the superior posterior quadrant.[68, 71] In a study of 100 ears with TM retractions, 40% of the retractions were located in the pars flaccida whereas 60% were located in the pars tensa.[68] Other authors have reported a higher incidence of TM retractions in the pars flaccida than in the pars tensa and that there can be multiple retractions in a TM simultaneously.[72]
Another theory on the genesis of TM retractions has been proposed. It is based on the Eustachian tube being persistently open. In order the close it to relieve an unpleasant feeling in the ear, the patient develops a sniffing behaviour resulting in repetitive barotrauma to the TM. This causes a weakening of the TM, which predisposes for retraction pathology.[41, 73]
In minor retractions in the posterior quadrants of the TM, the retraction can adhere to the incudo-stapedial joint whereas larger retractions can reach and adhere to the promontory of the cochlea. A total collapse of the TM is termed an atelectatic ear or adhesive otitis. Histological studies of atelectatic ears have shown a lack of the collagenous backbone of the TM. The outer epithelium showed thickening and hyperkeratosis.[74]
TM retractions can result in a conductive hearing loss due to ossicular chain erosion and if the above mentioned hyperkeratosis fails to desquamate adequately the risk of cholesteatoma development is obvious.

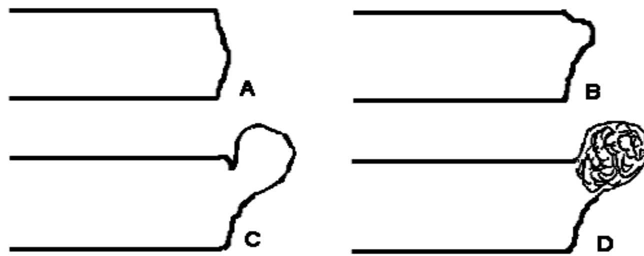

Fig A. Schematic drawing of the outer ear canal and the tympanic membrane. The schematic drawing shows the development of a tympanic membrane retraction and the development of a cholesteatoma according to the retraction pocket theory.

A. Tympanic membrane in normal position.
B. Small focal retraction.
C. Larger retraction, retraction pocket.
D. Epithelial debris retained in the retraction pocket and a cholesteatoma is thus formed.

1.7 CHOLESTEATOMA

A cholesteatoma is an accumulation of squamous epithelium behind the normal position of the TM. The development of cholesteatoma is not fully understood and several theories on the genesis of cholesteatoma have been proposed.
According to the retraction pocket theory a cholesteatoma develops due to epithelial debris in a retraction pocket.[75, 76] The migration theory states that the keratinizing epithelium of the TM grows into the middle ear through a TM perforation. It has also been hypothesized that the keratinizing epithelium of the TM can, under an inflammatory stimulus, develop papillae growing invasively through the lamina propria and result in a cholesteatoma. Yet another theory has been put forward stating that cholesteatomas can origin from metaplasia of the middle ear mucosa, also due to an inflammatory stimulus.[77, 78]
Cholesteatomas have proven difficult to induce in animal models. The Mongolian gerbil is most commonly used since it readily develops a cholesteatoma if the outer ear canal is ligated.[79] There are also reports on the same procedure in rabbits resulting in cholesteatoma development.[59, 80] Based on findings of metaplasia in chole-steatomas, there have been efforts to induce cholesteatomas in rats using a carcinogenic agent.[81]
Histological examinations of cholesteatomas have revealed a discontinuation of the lamina propria. In some areas the collagen fibers were missing while in other parts intact collagen fiber bundles were seen.[82, 83] The outer epithelial layer has shown marked hyperproliferation with evidence of increased mitotic activity in both the basal and the suprabasal layers.[84-86]

1.8 CLINICAL CONSIDERATIONS AND MANAGEMENT

Purulent and secretory otitis media are common diseases and are often diagnosed and treated by primary care physicians. The more severe forms of TM disease are usually cared for by otolaryngologists.

1.8.1 Purulent otitis media

Otitis media is one of the most common diseases during childhood. Apart from being a medical problem it is also a social problem and results in numerous health care visits. Although generally self-limiting, purulent otitis media is in most countries treated with antibiotics to prevent the serious complications that appear in a limited number of cases. The more feared complications are mastoiditis, sigmoid sinus thrombosis, intracranial spreading of the infection and labyrinthitis with a subsequent sensorineural hearing loss. According to a consensus from the year 2000, physicians in Sweden are no longer obliged to treat purulent otitis media with antibiotics if certain criteria are met.[87]

In case of repetitive episodes of purulent otitis media, transmyringeal insertion of ventilation tubes are often used although the evidence-base for this treatment in lacking.[88] Nevertheless, parents often report a positive effect on recurrent episodes of purulent otitis media by the ventilation tube treatment. The decreased number of otitis media episodes might as well be due to the natural history of the disease, which is rapidly becoming less common as the child becomes older.

Vaccination against pneumococci has been studied in patients with recurrent episodes of purulent otitis media but the results are discouraging.[89, 90] Recently vaccination against several pneumococcal strains was introduced in the Swedish vaccination program. The main purpose of the program is to prevent invasive pneumococcal disease. A study of possible positive side effects of the pneumococcal vaccine program noted a decline in ventilation tube insertions. In that study the first vaccine dose was given at 2 month of age and then repeatedly during the following year.[91] There are also indications of a change in the pattern of pathogens causing purulent otitis media, which may be due to the introduction of the pneumococcal vaccine.[92]

1.8.2 Secretory otitis media

Eustachian tube incompetence is common. In a study of subjectively normal human ears, only 72% of the test persons could equilibrate negative ear pressures completely, although not everyone develops a transudate.[93] In cases of mucosal swelling in the Eustachian tube, most often due to an upper respiratory infection, the ability to transport air into the middle ear is impaired and subsequently a transudate may develop.[54]

SOM normally resolves over time but in long-standing cases with impaired hearing, long-term antibiotics are sometimes tried. Amoxicillin had in a placebo-controlled randomized trial a positive effect on middle ear effusions after two weeks but there was no significant effect after four weeks. Other types of antibiotics in that study did not have any effect at all.[94] Another double-blind placebo-controlled randomized study has shown a permanent positive effect on the middle ear effusion after the use of amoxicillin-clavulanate.[95]

Insertion of transmyringeal ventilation tubes most often resolves the hearing impairment within the immediate postoperative period.[96] Numerous types of ventilation tubes are available in different materials, coatings and forms. The T-tube was in a randomized study shown to result in an increased risk of retention of the tube and a persistent perforation.[97] Apart from that study, experimental studies are lacking and thus the knowledge regarding the results of the different types of tubes comes from observational studies. Recently a large randomized study was launched testing four different types of transmyringeal tubes.[98]
The adenoid has a negative effect on the Eustachian tube function and adenoidectomy has in some studies proved to be as effective as ventilation tube treatment for secretory otitis media.[99, 100] A temporary hearing aid is also an alternative.
With increasing age the pediatric patient develops a more competent immune defense system and usually a better function of the Eustachian tube. Hence, secretory otitis media is ordinarily a problem diminishing with age.

1.8.3 Tympanic membrane perforation

Small TM perforations do not result in any recordable hearing loss. Larger TM defects result in a conductive hearing loss with a positive correlation between the size of the perforation and the extent of the hearing loss. The hearing loss is most pronounced in the lower frequencies. I most cases the TM heals spontaneously within a few weeks and often faster than that. There is no exact definition of the time-lapse until a perforation is considered chronic. Six weeks to five years are the extremes of time-intervals used when defining a perforation as chronic.[101]
Since the TM is a barrier protection of the middle ear, a perforation increases the risk of middle ear infection. To protect the ear or to stop it from running, myringoplasty may be performed. In case of a large TM perforation, a successful myringoplasty can also be expected to reduce the hearing level threshold.
Myringoplasties are most commonly performed with an underlay technique where a graft is placed medially to the remnant TM. Several types of graft materials are used with muscle fascia and cartilage being the most commonly used. During the healing process the graft acts as a scaffold for the outer epithelium which heals both from the center of the TM and the periphery whereas the graft itself integrates with the lamina propria.[102]
Fat graft myringoplasty may be an alternative to a conventional myringoplasty. The surgery is faster and has in selected cases shown similar success rates as a conventional myringoplasty.[103, 104] It is not an uncommon finding that the keratinizing epithelium has grown around the edge of a perforation. To ascertain that no squamous epithelium will remain medial to the lamina propria, the fat graft myringoplasty includes a resection of the edges of the perforation. This is followed by an autologous patch of fat being inserted into the perforation. The fat serves as a scaffold for the regenerating outer epithelium and subsequently the perforation may heal.

1.8.4 Tympanic membrane retraction

A TM retraction that comes in contact with the ossicles may lead to erosion of the ossicular chain. The long process of the incus is the most susceptible site. Disruption of the ossicular chain may result and as a consequence of that, various degrees of

conductive hearing loss can occur. The retracted TM often bridges the distance between the undamaged parts of the ossicles resulting in a less pronounced conductive hearing loss than what would otherwise be expected from a discontinuation of the ossicular chain.
TM retractions can sometimes be treated by transmyringeal ventilation tube insertion, especially if the negative middle ear pressure is expected to be temporary. Without signs of secretory otitis media the hearing will not improve with a tube insertion. The hearing loss can be treated with a hearing aid or surgery which includes a myringoplasty and an ossiculoplasty. The surgery can often give a good hearing result but the risk of new TM retractions is imminent in case the Eustachian tube function does not improve. Performing the myringoplasty with cartilage, which is more resistant to the pressure gradients than fascia, may in these situations be advantageous.
Except for the risk of conductive hearing loss, the main concern with TM retractions is the risk of accumulation of inadequately desquamating epithelia subsequently forming a cholesteatoma.

1.8.5 Cholesteatoma

A cholesteatoma often grows in a tumor-like fashion in the middle ear cleft and thereby erode the ossicles leading to a conductive hearing loss. Cholesteatoma may also act as a base for bacterial infections leading to chronic or intermittent secretions to the external ear canal. The infection can spread to the mastoid causing mastoiditis and can in severe cases result in intracranial complications. This is however unusual in highly developed countries since cholesteatomas can be successfully treated with adequate surgery. Another complication to a cholesteatoma is a peripheral facial palsy due to a pressure effect or an endotoxin mediated toxic effect on the middle portion of the facial nerve. The lateral semicircular canal can also affected by a cholesteatoma resulting in severe vertigo.
Cholesteatomas are surgically removed unless it is quite small and the bulk of the cholesteatoma is easily evacuated. The main goal of the surgery is to eradicate the middle ear from all keratinizing epithelium which otherwise is likely to produce a residual cholesteatoma. The canal wall down technique is often preferred to optimize visualization of the middle ear. A mastoidectomy is performed if necessary. In severe cases the middle ear is left for a future second look or magnetic resonance imaging of possible residual disease, but most often the middle ear and the posterior external canal wall can be reconstructed. Depending on what parts of the ossicles that are intact, either a remnant ossicle or a prosthesis can be used for the restoration of the ossicular chain. If a conductive hearing loss is not managed completely, a hearing aid may be useful.

2 AIMS OF THE STUDY

The stiffness changes of the TM found in experimental otitis media have not been fully explained. The collagen in the lamina propria is the physical backbone of the TM. There are different types of collagens with differing tensile strengths. Long-standing inflammation of the TM can possibly change the collagen content of the TM with a shift towards less deformation resistant collagen types.
There is evidence of a relation between previous otitis media and cholesteatoma development. In cholesteatomas the lamina propria is disrupted. The collagen content of the remnant collagen fibers is unknown.
The outer epithelium of the TM is the first structure to close a TM perforation. The epithelium is also affected in cases of inflammation, which can increase the epithelial layer thickness. Under normal conditions, there is a continuous centrifugal transferal of keratinocytes from the umbo. It is plausible that there is a source of new cells in the umbo, a source that can be referred to as stem cells.

The aims of the study were:

- *to clarify the distribution of different collagen types in the normal tympanic membrane and the adjacent structures. (First and second studies)*

- *to assess the collagen distribution changes in human TMs subjected to long-standing secretory otitis media and cholesteatomas. (Fourth study)*

- *to test the hypothesis of epithelial stem cells being located in the umbo. (Third study)*

- *to investigate the histology of the outer epithelium of TMs subjected to secretory otitis media. (Fourth study)*

3 MATERIALS AND METHODS

3.1 ANIMAL SPECIMENS

The first study was performed using adult healthy female Sprague-Dawley rats weighing approximately 250 g. The rats were delivered from the animal department at Karolinska Institutet. After intraperitoneal injection of a lethal dose of pentobarbital hydrochloride, decapitation was undertaken. The TMs were inspected with the use of an operation microscope and all TMs appeared normal without signs of middle inflammation. The temporal bones were dissected and the tympanic bulla opened. The TMs were immediately isolated together with the bony rim and the handle of the malleus. Ethical approval had been obtained from the institutional animal care committee before the start of the study (N344/02 Stockholms Norra Djurförsöksetiska Nämnd, Stockholms Tingsrätt).

3.2 HUMAN SPECIMENS

The second and third papers are based on normal human TMs. One of the standard surgical procedures for the removal of an acoustic neuroma is the translabyrinthine approach. This approach is used in cases of a preoperatively non-serviceable residual hearing on the affected ear. As part of the standard procedure the TM, the malleus and the medial part of the external ear canal skin is removed and wasted.
All patients were adult and none of the patients had a recent history of otitis media. All TMs had normal appearances at otomicroscopy preoperatively without signs of retractions. All middle ears were found to be normal at surgery. The TMs were removed including the malleus, the fibrous annulus and the medial part of the external ear canal skin. (Fig B)

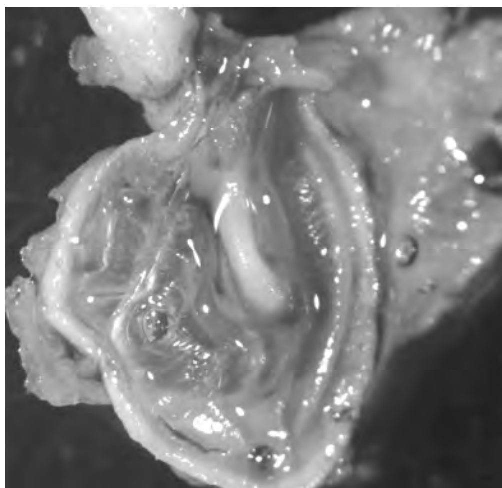

Fig B. A seldom seen view: the normal human TM from the medial side. In the background the medial part of the external ear canal skin is seen.

In the fourth study two different kinds of human TM material were used; TM biopsies from patients with long-standing secretory otitis media and cholesteatoma matrix from surgery. The TM biopsies were harvested with the use of a custom made biopsy instrument. The patients were scheduled for insertion of a transmyringeal ventilation tube because of long-standing secretory otitis media with hearing impairment. A written informed consent was obtained from the caregiver prior to surgery. Under general anesthesia a myringotomy was performed in the antero-inferior quadrant. The 1 x 1 millimeter biopsy was harvested followed by insertion of the ventilation tube. The cholesteatoma specimens were collected at surgery for the disease.
Ethical approvals had been obtained from the local ethics committee (Karolinska Institutets forskningsetikkommitté Nord 03-523, Regionala etikprövningsnämnden Stockholm 04-349) prior to study start.

3.3 SPECIMEN PROCESSING

In the first study the rat specimens were fixated in 4% paraformaldehyde for 1 hour. Decalcification of the malleus and the bony rim was obtained by the use of 0.1M EDTA (pH 7.4) during 4 weeks. This was followed by dehydration in serial alcohol concentrations and paraffin embedding. By the use of a microtome, 15 µm sections were cut perpendicularly to the handle of the malleus. The sections were put on Superfrost slides, five consequently cut sections on each.
The human TMs for the second and third studies were immediately after the removal fixated in fresh paraformaldehyde (4%) followed by decalcification in EDTA, dehydration in serial alcohol concentrations and embedding in paraffin. 5 µm sections were cut using a microtome perpendicular to the handle of the malleus.
For the fourth study the specimens were fixated in different agents depending on what investigative method that was supposed to be following. For the light microscopy including immunohistochemistry the specimens were immediately fixated in formaldehyde (4%). This was followed by dehydration in serial alcohol concentrations, paraffin embedding and microtome cutting into 5 µm sections. The sections were put on Superfrost slides.
In all studies the deparaffinization of the paraffin embedded sections were done with the use of Xylene and this was followed by rehydration with the use of graded alcohol series.
The specimens intended for transmission electron microscopy were immediately fixated in 2.5% glutaraldehyde + 0.5% paraformaldehyde in phosphate buffer. After rinsing in phosphate buffer and post-fixation with 1% osmium tetroxide, the specimens were dehydrated in graded alcohol series and propylene oxide. The specimens were embedded in agar 100 resin and heat incubated for polymerization. Staining was performed using 2% uranylacetate in 70% ethanol and Reynolds lead. Ultrathin sections were cut using an ultrotome.

3.4 STANDARD HISTOLOGICAL STAINING

In the third and fourth papers, sections were stained with fresh Mayer's hematoxylin. Hematoxylin is a basic dye that colors basophilic structures purple. Nucleic acids are

basophilic and therefore areas rich in nucleic acids are stained. Usually the cell nucleus has the highest content of nucleic acids and thus hematoxylin is considered to be a cell nucleus staining.
Counterstaining was performed with eosin, which stains eosinophilic structures bright pink. The eosinophilic structures are often extracellular proteins. Subsequently eosin was used to stain the extracellular matrix.
After the hematoxylin and eosin staining the sections were dehydrated in graded alcohol series and the slides were mounted with cover glass and examined with the use of a light microscope.

3.5 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) was utilized in all four studies. IHC is a procedure based on the ability of an antibody to bind specifically to an antigen. The antibody is detectable and can by different means be visualized. The primary antibody can be labeled for direct visualization, which is termed direct IHC. Indirect IHC is more often employed, which was the case in all four studies.
In indirect IHC the antibody binding to the antigen is called a primary antibody. The secondary antibodies are antibodies directed towards the Fc-region of the primary antibody. Since the secondary antibody is labeled, it can be visualized by the following procedures.
Monoclonal antibodies against the specific antigen detected for, were used when available. Monoclonal antibodies are derived from a single cell line and therefore have a higher specificity. Polyclonal antibodies are produced by injecting an antigen into a mammal. This induces the B-lymphocytes to produce IgG immunoglobulins specific for the antigen. The polyclonal immunoglobulins are purified from the mammal’s serum. If monoclonal antibodies were not available, polyclonal antibodies were chosen.

3.5.1 Antigen retrieval

The fixation process impairs or totally destroys the immunoreactivity of many antigens. This negative effect can sometimes be reversed. Pretreatment of a deparaffinized tissue section with the enzyme pepsin improves the immunoreactivity of many antigens. Pepsin solution for enzyme-induced epitope retrieval was used in the second and fourth papers. After blocking of the endogenous peroixadase activity and washing in phosphate buffered saline (PBS), the sections were covered with the pepsin solution for 10 minutes at 37°C in a humidified chamber. This treatment was followed by further washing with PBS.

3.5.2 DAB

DAB (3,3'-Diaminobenzidine) is one common technique to make the secondary antibody visible under the microscope and it was used in the first, second and fourth studies. The DAB-IHC procedure is based on biotin, which is a low molecular weight vitamin. Biotin can be conjugated to secondary antibodies. Avidin is a large glycoprotein with a very high affinity for biotin and thus readily forms complexes with biotin. When using the DAB-technique, the avidin molecule is labeled with an enzyme,

peroxidase, which can be developed by DAB to produce a consistently brown precipitate.
Tissue sections can have an endogenous peroxidase activity. If not blocked, the endogenous peroxidase can give an unspecific brownish color to the section, hampering the evaluation of the antigen-specific results. Therefore the endogenous peroxidase activity was blocked using 0,3% hydrogen peroxide for 30 minutes followed by repeated washing with PBS.
Before the application of the primary antibodies, non-specific sites were blocked with bovine serum albumin, also in order to reduce background staining.
The appropriate antibody concentration was found through repeated tests to find the best antigen visualization. The antibody solution was diluted with PBS and applied to cover all sections but one on each glass. One section was used as a negative control and it was covered with PBS instead. The incubation took place overnight in 8°C in a humidified chamber. After washing with PBS, the sections were incubated with the appropriate biotinylated secondary antibody for 45 minutes followed by washing in PBS and the application of the peroxidase conjugated avidin for 30 minutes. After further washing DAB was applied. The coloration process was stopped after 5 minutes by the use of distilled water. The slides were mounted with cover glass and examined with the use of a light microscope.

3.5.3 Immunofluorescence

Immunofluorescent IHC employs fluorescence-labeled antibodies to visualize the antigens, a technique that was employed in the third paper. Fluorescence is the emission of visible light from a substance when it has absorbed light of another wavelength. Usually the absorption of light of a specific wavelength induces an emission with a larger wavelength which carries less energy.
The fluorescence microscope is equipped with a light source that illuminates the specimen with light of a specific wavelength. This light causes the fluorescent antibody to emit light of its specific wavelength. The illumination light is separated from the much weaker emitted fluorescence through the use of an emission filter and thus only the emitted light is acknowledged by the microscope.
A significant problem with immunofluorescence is photo bleaching, meaning that the fluorescent intensity decreases continuously. The phenomenon is much accelerated by light exposure. In order to reduce photo bleaching, the time-span of light exposure must be kept at a minimum.

3.5.3.1 Fluorescent secondary antibodies

The basic technique with the immunofluorescence is the same as when DAB is used, but the blocking of the endogenous peroxidase activity can be omitted. The main difference is that the secondary antibodies are labeled with fluorescent agents. Using the filters of the fluorescence microscope, multiple antigens can be detected in the same section if primary antibodies of different species are used. In the third paper primary antibodies of two different species were used; mouse and rabbit. Thus two fluorescent-labeled secondary antibodies could be used; an anti-mouse antibody emitting a green light when absorbing light at a wavelength around 488 nm and an anti-rabbit antibody emitting an orange light when hit by light with a wavelength around 555 nm.

The secondary antibodies were applied for two hours followed by washing with PBS.
To prevent photo bleaching, the application of the secondary antibodies as well as all subsequent procedures were done under darkened conditions. After application of DAPI (see below), the glasses were covered with Vectashield and a cover glass. Visualization was done with a microscope (Nikon TE2000, Japan) equipped with a fluorescence unit. A digital camera was linked to a computer system including image-merging software. Separate images of the different wavelengths could thus be obtained and merged images could be produced and digitally stored. In all, three colors were captured; the two from the secondary antibodies and an additional one from DAPI.

3.5.3.2 DAPI

In the third study DAPI was used to visualize cell nuclei. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that can pass through intact cell membranes and binds strongly to DNA. When DAPI is bound to double-stranded DNA it can be excited with ultraviolet light and appears blue in the fluorescence microscope. Double-stranded DNA is generally speaking only found in the cell nucleus. Therefore DAPI is used to visualize the cell nucleus.
DAPI can also bind other intracellular substances like RNA, which is found not only in the cell nucleus but also in the cytoplasm. The binding to RNA is however less fluorescent and the emission wavelength shifts when bound to RNA. With the use of the wavelength filter of the fluorescence microscope, RNA-related fluorescence can be excluded.
In the third study, the specimens were incubated with DAPI for 5 minutes after the application of the secondary antibodies. After repeated washing, Vectashield and a cover glass was applied. Visualization was done with a microscope as mentioned above.

3.6 CONFOCAL MICROSCOPY

To further examine the fluorescent results of the third study, confocal microscopy was used. In conventional fluorescence microscopy the entire specimen is exposed to the wavelength-specific light. The fundamental difference of confocal microscopy is that a focused beam of light, usually from a laser, achieves the illumination. The laser scans across the specimen point by point. The emitted light from each point is registered and light that is not in focus can be filtered, which results in a contrast enhancement. Since the images are obtained in multiple optical planes, three-dimensional images can be reconstructed, a feature that is not achievable in the conventional fluorescence microscope.
In the third study confocal microscopy was performed with the use of a Nikon TE300 microscope, equipped with a laser imaging system with three different filters. A computer-based program was utilized for image storing and reconstruction (ImageJ, NIH, USA).

3.7 TRANSMISSION ELECTRON MICROSCOPY

In addition to light microscopy, transmission electron microscopy (TEM) was used in the fourth paper to achieve greater magnification possibilities than the light microscope offers. An electron microscope employs a particle beam of electrons to illuminate the specimen and thus a magnified image of the specimen is created. The use of electrons gives the microscope a greater resolving power than the light microscope. This can be achieved because the electrons have wavelengths about 100,000 times shorter than the photons of visible light. Under optimal conditions the electron microscope can magnify up to one million times, while the light microscope is limited to a possible maximum magnification of 1000 times.
The TEM is the original type of electron microscope. Later several other types have been developed. TEM uses a high voltage electron beam, which is focused by electrostatic and electromagnetic lenses. When the beam of electrons hits the specimen, some of the electrons pass through it while others are scattered. The deflected electrons carry information about the structure of the specimen. This information is converted by the microscope's objective lens system into a grey-scale image. Since many biological materials do not scatter the electrons well, the specimens are stained with heavy metals. Lead, uranium and tungsten are often used in standard TEM since these heavy metals are excellent deflectors of electrons, thus contrasting between the different structures depending on how much staining the different parts of the specimen retains.
The specimens in the fourth study intended for TEM were stained by uranylacetate and lead after the microsectioning. Microscopy was performed with the use of a Jeol 1230 transmission electron microscope and the images were digitally stored.

3.8 QUANTIFICATION OF RESULTS

In the third and fourth studies the results were descriptive. In the first and second studies the results were semi-quantified in addition to descriptive. A semi-quantification was performed assigning the staining intensity a score. Four levels of staining intensity were used; 0 (no staining), 1 (light staining), 2 (moderate staining) and 3 (intense staining). The scoring was performed by a colleague in a blinded fashion, i.e. not knowing what type of collagen the section had been stained for. To validate the semi-quantification method, the scoring was later re-evaluated with the evaluator blinded in regard to the previous results.

4 RESULTS

The results are presented divided into two parts. The first part concerns the lamina propria and here the basal lamina is included. The second part is referring to the outer, keratinizing epithelium.

4.1 COLLAGENS IN TYMPANIC MEMBRANES

The distribution of different collagen types was analyzed in normal TMs from rats as well as from healthy human TMs by the use of immunohistochemistry (IHC). The same method was used in addition to transmission electron microscopy (TEM) for the investigation of collagens in human cholesteatomas and human TMs subjected to long-standing secretory otitis media (SOM).

4.1.1 Collagen distribution in normal rat TMs

In the first study the distribution of different collagen types was analyzed in healthy rat TMs. The specimens were investigated regarding four parts of the TM and its suspending structures; the peripheral pars tensa (PT), the central PT, the inner portion of the fibrous annulus (FA) and the outer portion of the FA. In addition three adjacent structures were also investigated serving as controls; the bone, the periosteum and the epidermis.

4.1.1.1 Results with respect to structure

The peripheral portion of the PT was mainly stained for collagen types II and IV. The results were the same for the central portion of the PT, although less marked as compared to the peripheral PT. The inner portion of the FA was mainly stained for collagen type II. The outer portion was stained the strongest for types III and IV. The bone of the outer ear canal presented the strongest staining for type I, whereas the periosteum was most intensely stained for types I and III. The epidermis proved to stain intensely for all four kinds of collagen but most markedly for type III.

Table 1.

	Peripheral PT	Central PT	Inner AF	Outer AF	Bone	Periosteum	Epidermis
Collagen I (n=24)	1,63	1,71	0,96	0,83	2,25	2,46	2,17
Collagen II (n=35)	2,31	2,00	1,91	1,74	1,51	1,77	1,94
Collagen III (n=30)	1,47	1,60	1,20	2,53	1,27	2,87	2,87
Collagen IV (n=22)	2,45	2,27	1,27	2,64	0,82	1,45	2,36

Legend: Mean staining intensity scores on a scale from 0 to 3. 0 (no staining), 1 (light staining), 2 (moderate staining) and 3 (intense staining). Results based on observations in light microscopy of stained sections.

4.1.1.2 Results with respect to collagen type

Collagen type I had the highest scores in the bone and the periosteum and the lowest in the outer portion of the FA. Type II was found to the greatest extent in the peripheral and central portions of the TM. Type III had the highest mean values in the epidermis and the periosteum as well as the outer portion of the FA. The staining for type IV was most intense in the outer portion of the FA and in the central and peripheral portions of the PT and was least intense in the bone.
In the blinded re-evaluation, eleven percent of the observations were scored one level higher than previously and eight percent were scored one level lower. Eighty-one percent of the observations were scored the same in both observations. In no case did the score change by more than one level.

4.1.2 Collagen distribution in normal human TMs

The distribution of collagens in healthy human TMs was investigated in the second study. A similar semi-quantification as in the first study was performed. Regarding the human TMs, a distinction could be made between the outer and the inner collagen layers. In addition, the staining of the outer and inner portions of the FA was performed.

4.1.2.1 Results with respect to structure

Collagen type II was found to be the most abundant collagen present in the lamina propria. The radially oriented fiber layer stained mainly for collagen type II, but also for types III and I. The circularly oriented fiber layer stained most intensely for type III, but also stained for types II and I. The basal lamina of the tympanic membrane stained mainly for type IV. The inner portion of the FA was stained mainly for types II and I, while the outer portion stained mainly for types III and I.

Table 2.

	Radial fibers	Circular fibers	Outer annulus	Inner annulus
Collagen type I (n=30)	1.57	1.80	1.93	1.72
Collagen type II (n=38)	2.37	2.05	1.39	1.97
Collagen type III (n=28)	1.89	2.28	2.26	1.37
Collagen type IV (n=27)	0.81	1.15	1.58	0.88

Legend: Mean staining intensity scores on a scale from 0 to 3. 0 (no staining), 1 (light staining), 2 (moderate staining) and 3 (intense staining). Results based on observations in light microscopy of stained sections.

4.1.2.2 Results with respect to collagen type

Collagen type I scored highest in the outer part of the FA and the lowest in the radial fiber layer of the lamina propria. For type II the distribution was the opposite, with the highest scores in the radial fiber layer of the lamina propria and the lowest scores in the outer portion of the FA. Type III scored highest in the circular layer of the lamina

propria and the outer portion of the FA and lowest in the inner part of the FA. Type IV stained mainly in the basal lamina.
In the blinded re-evaluation, no score changed more than one level. Seventeen percent were scored one level lower, whereas seven percent were scored one level higher than in the first evaluation. Thus seventy-six percent were scored the same in both evaluations.

4.1.3 Collagen in biopsies from TMs subjected to SOM

In the fourth study the lamina propria was investigated by the use of TEM which revealed collagen bundles in the outer radial layer as well as the inner circular layer appearing intact. Medial to the lamina propria, the inner subepithelial layer of loose connective tissue was thickened carrying fibroblasts and vessels.(Fig C) The outer subepithelial layer of loose connective tissue was not significantly affected. The immunostainings for collagen types I-III were positive in the lamina propria. Staining for collagen type IV was positive in the basal laminas.

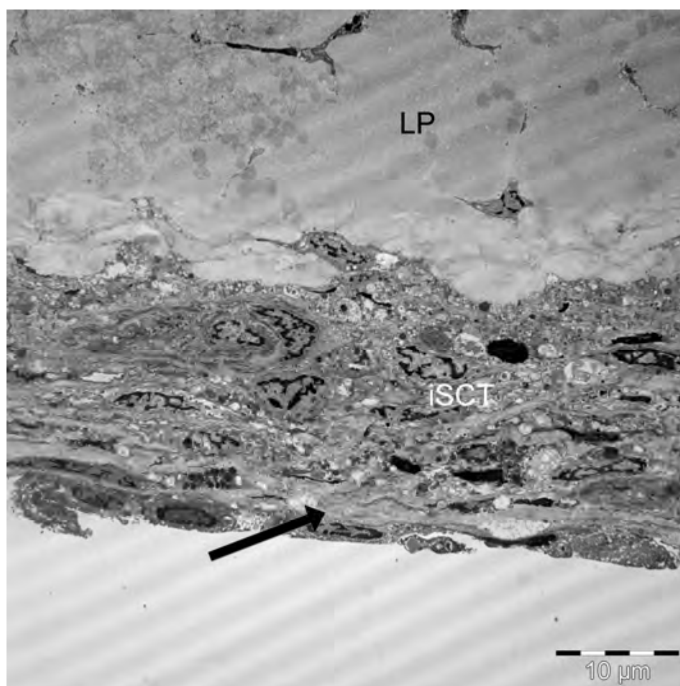

Fig C. Transmission electron microscopy of a human TM subjected to long-standing secretory otitis media. LP=lamina propria, iSCT=inner subepithelial connective tissue layer (thickened), arrow at inner basal lamina. A single-lined epithelium is seen outside of the basal lamina.

4.1.4 Collagen in cholesteatomas

The fourth study used TEM for high magnification visualization of the cholesteatomas. In the cholesteatomas, remnants of a lamina propria were found that appeared to have intact collagen fiber bundles. In other parts only scattered, disorganized collagen fibers were found. In the cholesteatomas the collagen staining was positive in scattered areas where remnants of collagen fibers were found. These areas stained for types I and II and but not for type III. Staining for type IV was positive in the basal laminas.

4.2 THE OUTER, KERATINIZING EPITHELIUM OF THE TM

The keratinizing epithelium was evaluated in the third and fourth studies. This included both normal human TMs and TMs subjected to middle ear disease.

4.2.1 The keratinizing epithelium in normal human TM

The normal human TM was investigated in the third study. With the use of hematoxylin and eosin staining, the outer epithelium was observed consisting of flat epithelial cells. The thickness of the epithelium varied between 5-10 µm in the intermediate portion of the pars tensa.

The epithelium was thicker in the umbo, in the annular region and along the handle of the malleus. The general thickness of the umbo epithelium was approximately 20 µm, but in relatively small, localized spots the thickness was even larger, up to 40 µm. Notably, the basal layer accounted for most of the overall thickness increase at the localized spots. In these areas the cells were elongated, appearing tightly packed and their cytoplasm being relatively small. Several mitotic events were observed in the thickenings.

The epithelium along the handle of the malleus was more uniform with a thickness of approximately 25 µm. The cells of the epithelial basal layer were tightly packed, but the cell nuclei were not elongated. The subepithelial layer near the handle of the malleus was rich in blood vessels.

In the annular region, the keratinized epithelial layer showed a relatively uniform thickness of approximately 40 µm. In the basal layer the epithelial cells were elongated, with an increased number of densely packed cells with elongated nuclei and very little cytoplasm. More laterally, the epithelium was thinner as it transitioned into the epidermis of the outer ear canal.

4.2.2 The keratinizing epithelium in TMs subjected to SOM

The fourth study revealed thickness variations of the keratinizing epithelium in the intermediate pars tensa region in TMs subjected to SOM. The outer epithelial layer appeared normal in configuration. The thickness was normal or increased to between 15 and 25 µm. The TEM investigation also visualized the cells of the basal layer. These cells were mainly elongated parallel to the basal lamina but in the thicker parts the cells were elongated perpendicular to the basal lamina. In one of the specimens the epithelium was markedly thickened and showed signs of an interstitial edema with the epithelial cells seemingly separated by fluid.

4.2.3 The keratinizing epithelium in cholesteatomas

The outer, keratinizing epithelium of the cholesteatoma specimens was investigated with both hematoxylin and eosin staining and TEM in the fourth study. The epithelium showed great variations regarding the thickness, measuring between 30 and 250 µm when the desquamating keratin was excluded. The four layers (stratae basale, spinosum, granulosum and corneum) were clearly visible and markedly enlarged in many areas. The basal layer cells had notably large nuclei. (Fig D, see also Fig 2b in the fourth paper).

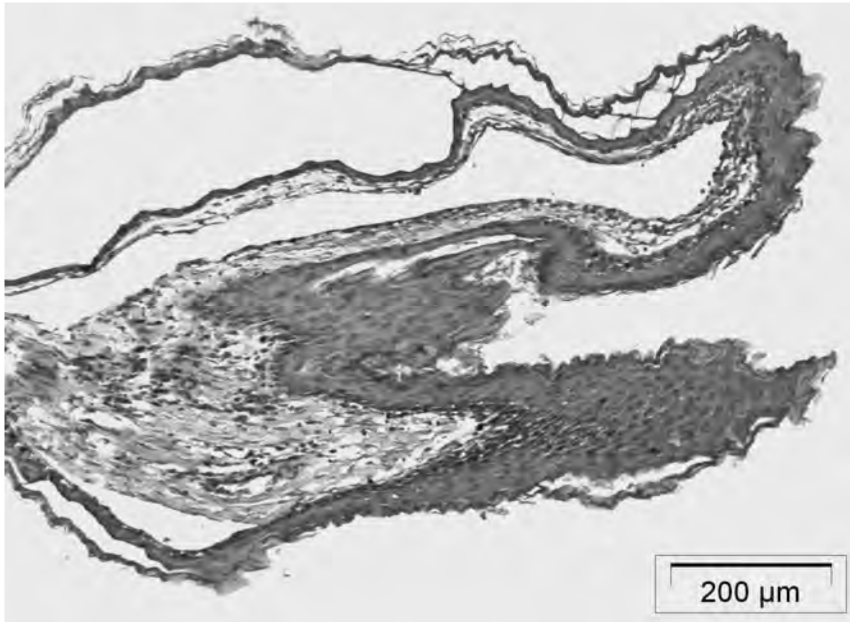

Fig D. Hematoxylin and eosin staining of a cholestetoma

In TEM, the thickened epithelium showed an increased number of cell layers with an edematous separation of the cells similar to what was observed in one of the biopsies. The cells of the basal layer were elongated perpendicularly to the basal lamina. (Fig E) The superficial layers cells were flat and lacked nucleus.

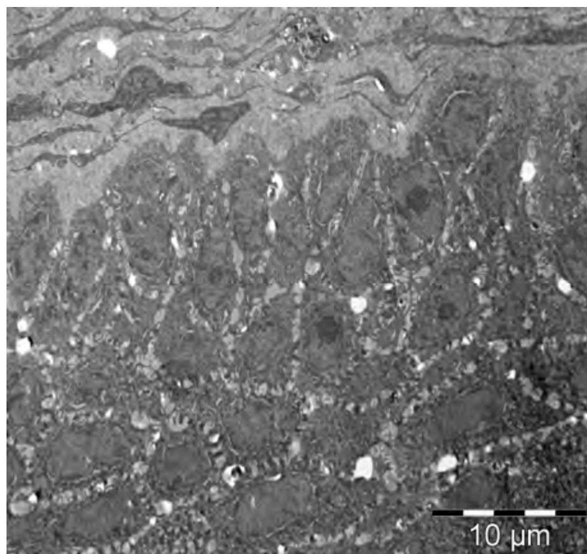

Fig E. TEM of the basal layer of the keratinizing epithelium of a cholesteatoma.

4.2.4 Possible presence of stem cells in the normal human TM

In the third study, three different stem cell markers were used for the identification of possible stem cells in the outer epithelium.
In the intermediate region of the pars tensa, the immunofluorescent staining was negative for β1 integrin, CK19 and α6 integrin.
In the ear canal skin, the stainings for β1 integrin and CK19 were positive in the epidermis whereas the staining for α6 integrin was positive only in the basal layer of the epidermis.
In the outer epithelium of the umbo, the stainings for β1 integrin and CK19 were positive in the major parts of the epithelium, whereas the staining for α6 integrin was positive only in its basal layer. The α6 integrin-positive cells in the umbo were elongated and orientated slightly inclined from perpendicular to the basal lamina. The inclinations of these cells were investigated in more detail with the use of laser confocal microscopy but the slightly skewed direction of these cells did not show any obvious pattern.
Along the handle of the malleus the immunostainings were positive for β1 integrin and CK19 in all layers of the epithelium and for α6 integrin only in the basal layer.
The epithelium in the annular region was positively stained for β1 integrin and CK19, whereas the staining for α6 integrin was positive only in the basal layer of the epithelium.
The negative controls did not show any fluorescent staining.

5 DISCUSSION

The present study is discussed from three overall points of view;

- material
- methodology
- results

5.1 MATERIAL

In total, four kinds of materials were used in the studies that form the basis of this thesis; normal rat TMs, normal human TMs, human TMs subjected to long-standing secretory otitis media and human cholesteatomas.

5.1.1 Rat TMs

The aim of the first study was to evaluate the collagen distribution in healthy TMs in general. The Sprague-Dawley rat was chosen since it is a commonly used animal in otologic research showing many resemblances with the human TM. The weight of the animals was approximately 250 g, which is the normal weight of healthy adult female Sprague-Dawley rats. Female rats were chosen because they are considered easier to handle than males. No intersex differences seemed plausible regarding the collagen distribution in the TM. Neither was any effect of the menstrual cycle anticipated.
All studies on mammals need an ethical approval, which was obtained prior to the start of the study. In the planning of every animal study, three ethical considerations have to be made; replacement, reduction and refinement.[105]

5.1.1.1 Replacement

First of all, one has to re-evaluate whether the animals can be replaced by computer models, cell cultures or animals of a lower developmental level. The first two alternatives were not applicable, since the first study aimed at investigating collagens in morphologically intact tissues. An animal of a lower developmental level was not considered reasonable due to the fact the rat is often used in otological research.

5.1.1.2 Reduction

If replacement of the animal is not achievable, the number of animals must be kept at a minimum, which is the second ethical consideration regarding laboratory animals. The number of animals used must, however, not be reduced to an extent that the reliability of the study becomes at risk. If it did so, the animal sacrifice would be of no use and the study would thus be considered unethical.
In the present study ten TMs were used. The figure was considered sufficient since no disease was investigated. In pathological states, tissues of different individuals may react in different ways and to a different extent. Therefore, the number of specimens needed for the investigation of pathology is generally higher.
No intervention was done and subsequently there was no need for the contralateral ear to be left untouched as a control ear. Thus the number of rats could be further limited.

5.1.1.3 Refinement

The third ethical consideration is refinement of the animal handling standards. The animals in the first study were delivered from the animal department at Karolinska Institutet and were without delay killed in order to reduce the stress the animal may perceive when transferred from the animal department to a new environment. The killing of the animals was undertaken by a standard method; pentobarbital hydrochloride injected intraperitoneally followed by decapitation.

5.1.2 Healthy human TMs

Healthy human TMs were used in the second and third studies. The TMs were obtained from surgery by the translabyrinthine approach for an acoustic neuroma, an approach where the TM is routinely wasted. All TMs had normal appearances at otomicroscopy preoperatively and all middle ears were found to be normal at surgery. The surgical specimens included the malleus and the fibrous annulus to reduce the risk of damaging the TM and facilitate the orientation of the material. None of the patients had a recent history of otitis media. A weakness of especially the second study is that it is unknown whether the patients had recurrent episodes of purulent otitis media or long-standing otitis media during childhood. There was no possibility to check medical records for previous otitis media. In theory, the collagen structures investigated in the second study could be altered due to otitis media in childhood. The fact that the middle ears were found normal at surgery speaks against a history of advanced otitis media.

5.1.3 Human TMs subjected to SOM

In the fourth study TM biopsies were taken from patients with long-standing secretory otitis media (SOM). According to the Act concerning the Ethical Review of Research Involving Humans (Swedish Code of Statutes, 2003:460) ethical approval is needed when a physical intervention is performed on a patient for the sake of research. Accordingly, the protocol was approved by the local ethics committee before the start of the fourth study.

A weakness of the fourth study is that it is impossible to know the exact length of time the patient had suffered from SOM. An otolaryngologist had seen the patients regularly and the SOM diagnoses had been confirmed at least at a three-month interval for at least six months. Episodes of improvement of the SOM cannot nevertheless be ruled out, since the disease often has a variable course. Improvement of the hearing within the three-month interval was denied by the parents. It must however be acknowledged that parental assessment of the hearing is not a reliable tool for the identification of hearing impairment in children.[106]

The TM biopsies were harvested under general anesthesia. The impact of anesthesiology is evident in research with physiological aspects and must not be neglected but we have no reason to believe that the TM morphology and biochemistry was altered by the anesthesia.

The biopsies were harvested in the antero-inferior quadrant. A weakness of the study is that the collagens of the TM may theoretically be differently affected in different parts of the TM. There is evidence of the normal morphology being different in the postero-

superior quadrant.[71] Biopsies from that part of the TM are not achievable because of the risk of accidentally affecting the ossiclular chain.
The TM biopsies were obtained with the use of a custom made instrument after a myringotomy. It is a clinical observation that myringotomy sometimes produces an almost immediate TM reaction with swelling of the TM. Such fast reaction might be related to hemorrhage or a change in blood flow. An effect on the collagen type distribution is not plausible. Neither is an effect on the number of cell layers in the keratinizing epithelium.
A more troublesome problem was the mechanical damage to the specimens produced by the biopsy instrument that to some extent obscured the assessment. For better morphology possibilities, the construction of a new instrument would have been needed. The instrument used in the fourth study had been used before by another research group, whereas a new instrument would not have been tested before on humans.[107] Furthermore, it cannot be ruled out that a new instrument would produce similar mechanical damages to the biopsies and since the current biopsies were acceptable, no further effort was put in achieving new biopsies. The decision was also influenced by the difficulties in achieving parental consent.
Eleven TM biopsies were used in the fourth study. More biopsies would have been desirable for the reliability of the study, but the parents were often unwilling to allow the harvesting of a biopsy. The parents often expressed a positive attitude towards research but were reluctant since the procedure would affect their child.
The small size of the biopsies rendered problems with the orientation of the specimens in the embedding process. There are instruments for obtaining larger biopsies but that would inflict greater trauma to the TM than what would be necessary for tube insertion. Thus larger biopsies were considered to be unethical.

5.1.4 Human cholesteatomas

The cholesteatomas were collected at surgery for the disease. Cholesteatomas emanating from the pars flaccida were excluded since an aim of the fourth study was to conduct a comparison with the TM biopsies, which were harvested from the pars tensa of patients with long-standing SOM. It is not possible to obtain biopsies from the pars flaccida from patients with SOM.

5.2 METHODOLOGY

The fixation of the material was immediate in all studies except for the first study where the TM and the surrounding structures were dissected immediately post-mortem followed by the fixation. Time to fixation is important since degrading of a biological tissue starts as soon as the nutritive blood flow stops. In a study of human TMs, it was found that if the fixation took place within three hours post-mortem, the specimens remained well preserved. Longer times to fixation resulted in structural damage of the cell organelles.[8]
The collagen content of the TM was analyzed in the first, second and fourth papers, using a well-described immunohistochemical method for the subtyping of collagens.[108] Collagen types I-IV were focused on since they are the most common collagen types. The primary antibodies for these antigens have a high specificity for

each of the collagen types tested for, exhibiting less than ten percent cross reactivity with other collagen types.
For the visualization of the secondary antibody, the DAB-technique was used since it is easier to quantify such results than when immunofluorescence is used, although immunohistochemistry is primarily a qualitative method rather than quantitative. The DAB-procedure has the advantage of preserving the possibilities of a histological overview of the specimen, whereas with immunofluorescence the histology cannot be assessed more than briefly and only in the fluorescent areas.
All specimens except the negative controls showed some degree of non-specific background staining, but using hydrogen peroxide for blocking of the endogenous peroxidase activity and a normal blocking serum reduced this. In addition, tissues can have an endogenous biotin activity, which results in a non-specific background staining. In such cases, the endogenous biotin activity can be reduced by pre-treatment with avidin that is not enzyme-labeled. Thus the endogenous biotin activity is blocked. This blocking procedure was used in the initial tests of the first study but did not further reduce the background staining.
In the first study, the staining of adjacent structures was also scored as a control and a validation of the method. Collagen type I is a major constituent of bone and was strongly stained. The epidermis stained intensely for collagen types I, III and IV. The latter is interpreted as staining of the basal lamina of the epidermis.
For the quantification of the DAB staining in the first and second studies, a semi-quantitative method was used giving a score for the staining intensity of each of the structures in each specimen. Four levels were used; no staining, light staining, moderate staining and intense staining. The scoring was performed by one of the co-workers who was blinded from what kind of collagen the specimens had been stained for in order to reduce the risk of bias.
The semi-quantification method was validated by performing a second semi-quantification of the first part of the material. The test-retest reliability was 81% in the first study and 76% in the second. More importantly, a change of more than one scoring level did never occur in the second semi-quantification. Therefore, the results can be considered reasonably reliable.
In the third study, immunofluorescent secondary antibodies were used for the visualization of the primary antibodies bound to the antigens. The reason for choosing the immunofluorescence technique was that it enables visualization of several antigens simultaneously. With the use of primary antibodies from different animal species, two different antigens can be detected at the same time. Using image-merging software, co-expression of antigens can be demonstrated. In the assessment of the stained antigens, the DAPI staining is helpful in defining the cell nuclei, as was performed in the third study.
The study of the outer epithelium was to some extent negatively affected by the fixation and sectioning, since some of the desquamating epithelium detached in the fixation process, a problem that has been reported before.[8] Therefore, the thickness of the outer epithelium is difficult to measure exactly. The outermost layers of the epithelium contain metabolically inactive cells but nevertheless accounting for some of the thickness. If alternative modes of processing are not possible, one way of dealing with the problem could be measuring only the epithelium to the level where the nuclei are still visible.

5.3 RESULTS

5.3.1 Collagen types II and IV in the rat TM

Collagen type II has been found in many different parts of the rodent's ear, including the TM.[109] Collagen type II has been reported to be a major constituent of the TM.[110] No analyses has however until recently been undertaken concerning other types of collagens.
The finding of collagen type II in the TM was in the first study confirmed in the central as well as the peripheral portions of the TM. Collagen types I and III was also found in the pars tensa of the TM, although to a lesser extent, which has recently been reported by other authors.[67] Staining for collagen type IV was also noted. Collagen type IV is mainly found in basal laminas and the positive staining for collagen type IV is interpreted as staining of the basal lamina in the TM.[111] No regular hematoxylin staining was performed to confirm this. The basal laminas do not stain well with standard staining methods. Instead PAS stain or specific silver stains could have been used for this.

5.3.2 Collagen type III in the lamina propria of the human TM

The pars tensa of the human TM generally stained the strongest for collagen type II, which is consistent with the findings of the first study. The human TM is five to ten times thicker than that of the rat thus enabling the two different collagen layers to be assessed separately with regard to the staining intensity.[5] The outer radial fiber layer had the strongest staining for collagen type II. The inner, circular layer was also strongly stained for collagen type II but in fact even stronger for collagen type III. This finding has not been reported before and the clinical and physiological impact is unclear. The TM has to resist significant pressure gradients but simultaneously transmit the subtle pressure variations of the sound waves. Since collagen type III provides less strain resistance than collagen type II, it can be speculated that the presence of collagen type III plays a role in the mechano-acoustic properties of the TM that have been studied in detail be other authors.[33, 34]
A previous rat study found a change in the collagen fiber distribution during the reparative process of a healing TM.[67] Initially collagen types I and III is produced, whereas the collagen content later is shifted towards type II. In the surgical healing of a TM perforation a graft is often used, which acts as a scaffold for the outer epithelium. Later the graft is integrated into the healed TM.[102] Muscular fascia is commonly used for myringoplasties and consists primarily of collagen type I.[112] Cartilage is sometimes used in myringoplasties. Since both the lamina propria and cartilage are primarily made up of collagen type II, cartilage grafting is, from a theoretical point of view, attractive. Several other types of grafts have however also been used for myringoplasties with successful results indicating that not one single specific tissue is needed for the postoperative healing of a chronic perforation.[103, 113, 114]

5.3.3 Two portions of the fibrous annulus

The fibrous annulus has been less extensively investigated than the TM itself. Using TEM, the fibrous annulus (FA) in the guinea pig TM has been shown to be amorphous but also containing collagen fibers.[115] In the first and second studies it was noted that the FA can be divided into two portions based on the immunohistochemical staining pattern; an outer and an inner portion. The distinction between the two portions was however less obvious in the second study.

The inner portion stained mainly for collagen type II and seemed to form a continuation from the lamina propria of the TM. Collagen type III was dominating in the outer portion. Collagen type III is normally found in the connective tissue of some organs and smooth muscle. The role of collagen type III is to provide structural support and elasticity. The presence of smooth muscle fibers in the attachment of the FA to the tympanic bone has been reported, which could explain the finding of collagen type III in the outer portion of the FA.[116, 117] Collagen type IV was also found in the outer portion of the FA, which can be correlated to the presence of small vessel with a basal lamina.[117]

5.3.4 Intact collagens in biopsies from TMs subjected to SOM

Experimentally induced SOM in Mongolian gerbils does not initially affect the lamina propria.[62] In ears with a highly viscous form of SOM, a disintegration of the inner circular collagen bundles is later seen but this is not the case in low viscosity forms of SOM.

In the fourth study, the TM biopsies had no signs of collagen fiber disorganization when observed using TEM. It could be argued that the human form of SOM more resembles the gerbil’s low viscosity type of SOM. It is of course difficult to assess if the human form of SOM in the fourth study shares features with an experimentally induced SOM in a laboratory animal. Furthermore, the low number of specimens, due to the problems in obtaining research material, decreases the possibilities of drawing valid conclusions.

The different collagen types in the TMs subjected to long-standing SOM were investigated in the fourth study with similar methodology as in the first and second papers. Presence of collagen types I-III in the lamina propria and collagen type IV in basal laminas were found, but no semi-quantification of the results could be performed due the mechanical damage from the biopsy instrument.

5.3.5 Remnant collagen in cholesteatomas

Previous studies on the collagen fibers in cholesteatomas have found areas of abundant collagen fiber bundles, but have also found collagen fibers to be scarce in other areas.[82, 83] These findings are consistent with those of the fourth study where the cholesteatomas showed presence of collagen only in smaller parts. In other areas the light microscopy and TEM was not able to identify any organized collagen fiber bundles, finding only desolate collagen fibers. In TM retractions, the collagen fibers of the lamina propria are stretched and the cross-links between the fibers break down.[118] With a continuing negative middle ear pressure, further disintegration evolves. Ultimately the outer and inner epithelial layers come in contact. Another

theory on the disintegration of the lamina propria has been presented regarding a possible necrosis in the perimatrix, but this has been disputed by other authors.[83, 119] In the areas where collagen was present in the fourth study, the stainings were positive for collagen types I and II but not for collagen type III. Collagen type IV was found in basal laminas. Collagen type III plays a role in the reparative process and therefore the absence of collagen type III might be of importance regarding the reparative processes in a developing cholesteatoma.[67]

5.3.6 The basal epithelial layer of the normal human TM

The thickness of the outer keratinizing epithelium proved to be different in different parts of the TM ranging from 5-10 μm in the intermediate portion of the pars tensa to 40 μm in parts of the umbo and the annular region. Notably, it was the basal layer of the epithelium that accounted for the major thickness variations.
In the intermediate region of the pars tensa the basal layer cells are elongated and arranged parallel to the basal lamina.[8] This was confirmed in the third study except for the areas with the marked thickenings where the cells of the basal layer were elongated perpendicular to the basal lamina. These cells had large, elongated nuclei and were densely packed. Mitoses were observed in the umbo and here the cells seemed to form papillary extensions reaching into the underlying connective tissue layer. This reminds of the rete of the skin epidermis that is a localization of interfollicular epidermal stem cells.[120] The elongated cells arranged almost perpendicular to the basal lamina might indicate an increased cell division activity, possibly with an increased cellular turnover because this cell arrangement allows for more cells being present per area.

5.3.7 The keratinizing epithelium in SOM and cholesteatoma

The SOM biopsies in the fourth study exhibited a normal to a slightly increased thickness of the keratinizing epithelium with variations within the specimens. The thickness increase is in accordance with previous studies of Mongolian gerbils with SOM showing a thickness increase in the outer epithelium.[62]
Using TEM, the four epithelial layers were clearly distinguishable and in some areas they were markedly enlarged. Notably, the basal layer cells in the thickened parts were elongated almost perpendicular to the basal lamina.
The keratinizing epithelium of the cholesteatomas was in some parts extremely thickened with great variations, ranging from 30 to 250 μm in the same specimen. The epithelium was on the TEM images showing signs of an interstitial edema. This finding was also noted in one of the SOM biopsies. It can be speculated that the edema is resulting from inflammatory mediators.
The cells of the basal layer of the cholesteatomas were elongated almost perpendicular to the basal lamina. These findings strengthen the theory that the perpendicularly arranged basal layer cells indicate a cell proliferation activity.
MIB-1, also referred to as ki-67, is a marker of cellular proliferation. MIB-1 has been reported to be increased in the basal as well as the suprabasal layers in cholesteatomas, although these findings have been disputed by other authors.[84-86, 121] Factors that possibly regulate the growth of the keratinizing epithelium have been investigated. Epidermal growth factor (EGF) is up-regulated in cholesteatomas. Under normal

conditions EGF and the EGF-receptor are found in the basal layer of the epithelium. In cholesteatomas EGF has been found in the suprabasal layers.[122, 123] Keratinocyte growth factor (KGF) regulates the keratinocyte proliferation and is expressed in cholesteatomas. The KGF-receptor is found mainly in the suprabasal layers.[124] The EGF-receptor has been found to be associated with the more proliferative parts of the cholesteatomas whereas the KGF-receptor is dominating in more differentiated areas.[124]

5.3.8 Progenitor cells in the normal human TM

Since there is a continuous centrifugal transferal of keratinocytes from the umbo, it is plausible that there is a source of new cells in the umbo that can be referred to as stem cells. The hypothesis of stem cells in the TM was tested in the third paper.

A stem cell has been defined as a "…cell in a tissue which, under normal circumstances, maintains its own population, undiminished in function and size, and furnishes daughters to provide new functional cells of that tissue".[125] Stem cells can be either totipotent, pluripotent, oligopotent or unipotent, according to the number of different cell types they can differentiate into. Unipotent stem cells can only differentiate into one type of cell, but has the ability to maintain its own population. The term "progenitor cell" is not clearly defined but is often used in the context of cells that can divide only a limited number of times, whereas stem cells can divide infinitely.[126] The term "progenitor cell" is nevertheless sometimes equated with stem cells, which can be a source of confusion.

Very little research has been done on stem cells in the TM, whereas the skin is one of the main fields in stem cell research. There are several similarities between the outer epithelium of the TM and the epidermis of regular skin. Therefore, applicable knowledge from dermatological research was transferred to the TM studies in the third paper.

In the skin, stem cells are found in close proximity to hair follicles, in interfollicular locations and in sebaceous glands.[120, 127, 128] The interfollicular stem cells were focused on, since the TM lacks hair follicles and glands. For the detection of interfollicular stem cells, β1 integrin and α6 integrin are the most commonly used markers, in addition to cytokeratins 10, 15 and 19.[129] There is however no marker that singularly pinpoints epithelial stem cells. α6 integrin is considered to have the highest sensitivity and specificity among the interfollicular stem cell markers.[130, 131]

Based on the epithelial thickenings noted by the hematoxylin and eosin staining, three areas were chosen for further investigations; the umbo and the annular region because of the apparent thickening of the basal layer and the region along the handle of the malleus due to reports of epithelial migration along the handle of the malleus.[9, 12] These areas were compared with the intermediate portion of the pars tensa and the skin of the external ear canal.

Three stem cells markers were used; α6 integrin, β1 integrin and CK19. α6 integrin was used because it is reported to have the highest sensitivity and specificity.[130, 131] The other two were chosen because there is a report of β1 integrin and CK19 positivity in the umbo and annular region in rat TMs.[132]

The staining for α6 integrin was positive in the basal layer in the umbo, the annular region and along the handle of the malleus. In the same locations β1 integrin and CK19 were found in all layers of the epithelium. The staining of several layers demonstrates the lack of specificity when using β1 integrin and CK19 since the stem cells are supposedly located in the basal layer.[133]

The results of the third study suggest the presence of stem cells in the TM and this is supported by BrdU-studies on rats where BrdU-labeled cells have been observed in the same regions as the stem cell markers in the present study.[134]

In the umbo region, the positively stained cells appeared to have a somewhat uniform orientation. This finding could contradict the stem cell theory, since the keratinizing cells migrate towards the periphery in all directions.[9, 12] Laser confocal microscopy was used for further clarification but no obvious directionality was detected.

The α6 integrin positive cells were abundant in the basal layer, raising questions about the specificity of the method since it has been estimated that less than 10% of the cells in the basal layer of the epidermis are stem cells.[130, 135] The rest of the basal layer cells are believed to be progenitor cells called transit amplifying cells, which are also α6 integrin positive. The interpretation of the findings of α6 integrin positive cells in the TM is that they are progenitor cells rather than stem cells. A progenitor cell must, however, originate from a stem cell and stem cells are therefore likely to be located in the same areas. Further investigations are needed to determine the exact location of the individual stem cells.

6 CONCLUSIONS

The following conclusions are drawn based on the findings in the four studies of this thesis:

- The main collagen in the pars tensa of the TM is collagen type II. The inner collagen fiber layer of the human TM is dominated by collagen type III. Collagen type IV is found in the basal laminas.

- The outer portion of the fibrous annulus has collagen type III as its major collagen constituent while the inner portion is mainly made up of collagen type II.

- Collagen types I-III are present in the lamina propria and collagen type IV in the basal lamina in TMs subjected to long-standing secretory otitis media. Collagen type I and II are found in some areas of cholesteatomas.

- Possible progenitor cells are found in the umbo, in the annular region and along the handle of the malleus. Further studies are needed to pinpoint the source of the progenitor cells, a source that is likely to be stem cells.

- TM biopsies from patients with long-standing secretory otitis media show a variable thickening of the outer epithelium. The outer epithelium in cholesteatomas exhibits immense thickness variations.

7 FUTURE PERSPECTIVES

The present study warrants further investigations. More studies on the lamina propria are needed, primarily regarding the role of the fibroblasts and their regulation in retraction disease. The significance of the fibroblast growth factor in cholesteatomas has only rudimentary been investigated before.

Regarding the outer epithelium, a number of questions have been raised. The studies of the progenitor cells can be further developed. The investigation of progenitor cells in cholesteatomas would be of great interest and could possibly be done using the present cholesteatoma material. In TM perforations, it can be hypothesized that progenitor cells are multiplied in the border of the perforation, a matter that could be investigated in a rat model of TM perforations. Furthermore, it would be of utmost interest to elucidate the regulation of the progenitor cells and ultimately pinpoint the stem cells that are supposedly located among the progenitor cells. One possibility to achieve this could be BrdU-pulsing of either rats or human TMs cultured in vitro and study the BrdU-label-retaining cells and compare with the $\alpha 6$ integrin-positive cells.

To shed further light on the properties of the outer epithelium, the EGF- and KGF-receptors could be investigated in the human normal TMs, which has not been done before. The results could be compared to the expression of the EGF- and KGF-receptors in healing perforations as well as in cholesteatomas. This could be done in conjunction with studies of proliferation markers. Further important information could be obtained if EGF and KGF would be used to stimulate experimentally induced perforations and cholesteatomas. Studies of the cell proliferation activity in TMs subjected to SOM would be of great interest to further elucidate the possible relationship between SOM and the development of cholesteatoma.

If the stem cells could be pinpointed and the regulation of the stem cells and the keratinizing epithelium would be understood, there would be new possibilities for alternative and better treatments of TM perforations as well as cholesteatoma. Continuing research using a combination of human and rat material is needed to reach that goal.

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