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On the connection between the tympanic membrane and the malleus

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Abstract

Background: *The tympano-malleal connection (TMC) is the soft-tissue connection between the tympanic membrane (TM) and the manubrium of the malleus. Some studies suggest that its mechanical properties may have a substantial influence on the mechanics and transfer function of the middle ear. However, relatively little is known about the dimensions of the TMC and its variability among individuals.*

Method: *Thirteen samples were collected from human temporal bones, consisting of only the malleus and the TM. They were imaged using μ CT without contrast enhancing agent. From the μ CT images, the TMC dimensions were measured in both anterior-posterior direction (TMC width) and medial-lateral direction (TMC thickness). Three selected samples were examined using histological microscopy.*

Results: *Both TMC width and thickness featured a large variability among individuals. The minimal TMC width along the manubrium for different individuals covered a range between 83 and 840 μ m. The minimal thickness ranged from 48 to 249 μ m and the maximal thickness from 236 to 691 μ m. Histological sections showed that the TMC consists of a narrow core of dense regular connective tissue, surrounded by loose connective tissue. In some samples, either of these two components was absent in the TMC at some manubrium locations. The configuration of these components varied among the samples as well.*

Conclusion: *Our data confirm that a large inter-individual variability exists in the properties of the TM-malleus connection in humans in terms of its dimensions, tissue composition and configuration. Average data and their variability margins will be useful input for testing the importance of the TMC in finite element models.*

Abbreviations: TMC: Tympano-malleal connection – TM: Tympanic membrane – LPM: Lateral process of the malleus – (μ)CT: (Micro-)computed tomography – UC: Uncertainty – SD: Standard deviation – Ant: Anterior – Post: Posterior – Med: Medial – Lat: Lateral – DRCT: Dense regular connective tissue – LCT: Loose connective tissue

Key words: middle ear; manubrial fold; stria mallearis; plica mallearis; eardrum; manubrium; collagen fibers; connective tissue

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Highlights

- The tympano-malleal connection (TMC) was studied by micro-CT and histology.
- Both its width and thickness vary among people by (almost) an order of magnitude.
- The TMC has a thin, dense collagenous core at most locations along the manubrium.
- The histological configuration varies considerably among individuals.

1 Introduction

The tympanic membrane (TM) and the manubrium (or handle) of the malleus are connected by a soft tissue structure, the tympano-malleal connection (TMC). In the literature, the TMC has been assigned different names, including manubrial fold (De Greef et al., 2014; Ferrazzini, 2003), stria mallearis (Hoffstetter et al., 2010), plica mallearis (Gea and Decraemer, 2010; Gulya and Schuknecht, 1995) or simply TM-malleus attachment (Koike et al., 2002). Multiple studies have attempted to investigate the functional role of the TMC through finite element modeling, yet their conclusions are not aligned. For instance, Koike et al. (2002) concluded that *“the modification of the properties and dimensions [of the TMC] did not greatly influence the vibration mode and displacement of the TM”*, whereas some more recent studies do predict an important role for the TMC in middle ear mechanics (De Greef et al., 2014; Hoffstetter et al., 2010).

Literature data on the morphology of the TMC is limited and inconsistent as well. Most published studies are based on light microscopy of histological sections (Ferrazzini, 2003; Graham et al., 1978; Gulya and Schuknecht, 1995; Politzer, 1892), and to date, one study was published based on micro-computed tomography (μ CT) (Gea and Decraemer, 2010). All studies agree that the connection between TM and malleus is tight at the levels of the umbo (inferior tip of the manubrium) and the lateral process of the malleus (superior end of the manubrium) (LPM). Both anatomical locations are indicated in Figure 2. However, they disagree substantially on the TMC morphology in the middle part of the manubrium. At this level, the histology-based studies describe the TMC as “a mere ‘stalk’ with a fibrous core and covered by mucosa” (Graham et al., 1978) or “only a narrow bridge” (Politzer, 1892, p. 224), that is clearly much narrower than the manubrium of the malleus. In contrast, Gea and Decraemer (2010) observed on μ CT images that the TMC was almost as wide the manubrium at mid-manubrium level. Gea and Decraemer (2010) suggested that *“this discrepancy in width could be caused by (1) shrinking effects during the histological preparation or (2) moisture that had collected in the constriction and showed up on the CT images with a contrast similar to that of the tissue of the PM”*. In other words, they assumed that the difference was to be attributed to systematic errors of the sample preparations and imaging techniques, rather than to real variability between individuals. Given the possible importance of this structure, these speculations need to be validated.

The main aim of this study is to extend the knowledge base for the morphology of the TM-malleus connection. For this purpose, we visualized the TMC with both μ CT and histology. This way, we can also investigate whether the reported discrepancy in MF dimensions based on μ CT imaging (Gea and Decraemer, 2010) and light microscopy of histological sections (Ferrazzini, 2003; Graham et al., 1978; Gulya and Schuknecht, 1995; Politzer, 1892) is a side-effect of either of the two imaging techniques, or whether it represents real inter-individual variability.

2 Materials and methods

2.1 Sample preparation

Thirteen human temporal bones were acquired from the University of Antwerp. Microscopic examination of the tympanic membrane was performed by an accredited Otorhinolaryngologist (VVR) before and after fixation. No otoscopic abnormalities were observed, i.e. we did not observe myringosclerosis, (healed) perforation of the tympanic membrane, attical retraction, atelectasis, attical or pars tensa cholesteatoma. A circumferential incision in the ear canal skin enabled the elevation of the tympanic membrane (TM) by means of endoscopy (Caremans et al., 2015; Van Rompaey et al., 2013). All suspensions of the malleus (anterior malleolar ligament, tensor tympani tendon, incudo-malleolar joint and chorda tympani) were disconnected surgically. Hence, samples consisting only of the malleus, the TM, the tympanic annular ligament and some ear canal skin were obtained. Figure 1 shows one of the samples mounted on a stage for μ CT. Immediately upon dissection, the samples were submerged into a 4% paraformaldehyde solution (0.1M Phosphate buffer, pH 7.4). Samples were kept in paraformaldehyde for 1 to 9 days before μ CT. No contrast-enhancing staining was applied to the samples to avoid shrinkage effects (see section 4.4.2).

2.2 μ CT scans

To prepare for μ CT scanning, the samples were placed in a small custom-made closed container made of PMMA (Plexiglas). The sample laid on a small plateau in the middle of the container, surrounded (but not in contact with) water. This design prevented dehydration of the sample during the long recordings of 1-3h at a temperature of approximately 30°C. Figure 1 shows the holder without its wall and cover lid.

The samples were positioned obliquely so that the TM and manubrium were oriented at an angle of approximately 45° with the X-ray direction. This orientation provided the best trade-off between sample stability and image quality. To realize this positioning, the superior part of tympanic annular ligament rested on a synthetic foam platform at the edge of the centered PMMA-platform, as shown in Figure 1. This material was chosen because of its ideal balance between low X-ray absorption (Gea et al., 2005) and mechanical stability.

μ CT-scans were performed using a Skyscan 1172 micro CT scanner (Bruker, Kontich, Belgium). Scanner settings were optimized for good contrast between the soft tissue of the TMC and the surrounding air, without image saturation. The most important scanning and reconstruction parameters are listed in Table 1. Sample TMC1 was scanned using different settings than the rest because it was the pilot sample of the experiment and the optimal protocol was not yet established at the time of recording this sample.

Figure 1: Sample on its measurement stage for μ CT recording in an oblique position. During scanning, this stage was closed off by a fitted Plexiglas cylinder and a cover lid that are removed for this picture.

Table 1: μ CT scanning and reconstruction parameters

Parameter	Value for TMCs 2-13	Value for TMC 1
Source voltage	65 kV	52 kV
Source current	153 μ A	188 μ A
X-ray sensor size	2664x4000	2664x4000
Exposure time	1.77 s	1.77 s
Rotation step	0.25° - 0.4°	0.2°
Total scan duration	74-119 min (depending on field of view)	179 min
Physical filter (beam hardening)	0.5 mm Aluminum	0.5 mm Aluminum
Frame averaging	3	4
Reconstruction voxel size	4.98 μ m	3.36 μ m

2.3 Histological preparation

Three samples of our dataset were selected after examining the μ CT reconstructions: one representing an ‘average’ TMC (sample 1), one with a very narrow TMC (sample 9) and one with a very wide TMC (sample 5). These samples were prepared for histological examination after the μ CT scans. The samples were decalcified in a 10% ethylenediaminetetracetic acid (EDTA) solution for 8 to 15 days to enable slicing through the malleus. After embedding the samples in paraffin, 5 μ m sections were cut on a microtome (Leica Microsystems, Diegem, Belgium). Transverse sections (i.e. perpendicular to the direction of the manubrium, see Figure 2) were made. Every 50 μ m a section was stained using Heidenhain's AZAN trichrome stain. Finally, the sections were digitized by a CCD camera (Olympus DP70, Center Valley, PA, USA) connected to an optical microscope (Zeiss Axiophot, Zeiss, Zaventem, Belgium).

2.4 Morphometrics

2.4.1 Metric definitions

To facilitate interpretation of the following paragraphs, Figure 2 indicates some anatomical and mathematical directions.

Figure 2: Indication of anatomical and mathematical directions around a manubrium (blue). The depicted slice is a transverse slice, similar to all slices shown in this paper (from both μ CT and histology).

Two quantitative metrics were defined to characterize the morphology of the TMC: TMC width and TMC thickness. Both are indicated in Figure 3A. TMC width quantifies the anterior-posterior dimension, TMC thickness quantifies the lateral-medial dimension. The frame of reference for this context is defined by the direction of the manubrium towards the LPM (x-axis), the direction perpendicular to the manubrium towards the middle ear (y-axis) and the direction perpendicular to x- and y-axes, towards the anterior side of the malleus (z-axis) (Figure 2).

Figure 3: A. Visual indication of both TMC width and thickness on a transverse μ CT slice of sample 6. Note that the actual calculations took the 3D shape of the boundaries into account; B. A transverse slice of sample 10 showing a 'tight coupling' between the TM and manubrium. Here, the determination of TMC width is ambiguous, as explained in section 2.4.4.

We define the TMC width as:

$$w_{ant-post}(x) = \min_y \{ \min(d = \|\overrightarrow{p_{ant}}(x, y) - \overrightarrow{p_{post}}(x', y')\| | \overrightarrow{p_{post}}(x', y') \in S_{post}) \}, \quad (1)$$

where, $\overrightarrow{p_{ant}}(x, y)$ is the 3D vector representation of the points that describe the anterior surface of the TMC, $\overrightarrow{p_{post}}(x', y')$ is the equivalent for the posterior TMC surface and S_{post} is the posterior surface itself. In other words, this metric quantifies the shortest distance in three dimensions from any point on the anterior surface to the opposite, posterior surface for every position x along the manubrium. This distance is not necessarily measured parallel to any of the anatomical directions, but in the direction of shortest distance.

TMC thickness is defined similarly to the width, for the medial and lateral boundary surfaces of the TMC. The medial surface is defined by the interface between the manubrium bone and the TMC soft tissue and the lateral surface by the lateral surface of the TM. Mathematically, we define the TMC thickness by:

$$t_{med-lat}(x) = \min_z \{ \min(d = \|\vec{p}_{med}(x, z) - \vec{p}_{lat}(x', z')\| | \vec{p}_{lat}(x', z') \in S_{lat}) \}. \quad (2)$$

2.4.2 Image segmentation

To obtain the geometry of the boundary surfaces of the TMC (anterior, posterior, lateral and medial), the μ CT images were imported into Amira® 5.3 (FEI Visualization Sciences Group, Hillsboro, Oregon, USA) for segmentation. In a first step, the images were virtually resliced so that the slices were perpendicular to the manubrium. Next, segmentation (by DDG) was performed by a combination of automatic and manual tools. In most cases, automatic segmentation tools, including threshold-based region growing, edge detecting region growing and slice interpolation, produced satisfactory results. On a minority of the virtual slices, where a lower contrast or worse SNR did not enable satisfactory segmentation using automatic tools, a minimum of manual intervention was applied using drawing and eraser tools.

2.4.3 Algorithm

The segmented volumes were exported from Amira to MATLAB (Release 2012b, The MathWorks, Inc., Natick, Massachusetts, USA). The following algorithm returned the TMC width as a function of manubrium position (for TMC thickness, the algorithm was equivalent):

- Find the anterior and posterior boundaries of the TMC from the segmented volumes. These are now represented as two 2D matrices with rows in the dimension along the manubrium (x -direction) and columns perpendicular to them in the lateral-medial direction (y -direction).
- For every individual point on the anterior surface, calculate the three-dimensional distance to all points on the posterior surface in an area of 100×100 pixels (corresponding to $500 \times 500 \mu\text{m}$) around the anterior surface point. These values represent d from equation (1).
- Find and store the minimum of these distances in a matrix. The result of this step for a single sample is shown in Figure 4.
- For each column of the matrix, i.e. each x -position, find and store the minimum along this column in a new 1D array. In Figure 4, the white line indicates the location of these minima for every column of the matrix. This minimum represents the TMC width $w_{ant-post}(x)$ at the corresponding location along the manubrium.

Figure 4: Distance between the anterior and posterior surfaces of the TMC as a function of position on the manubrium. The white line indicates the ‘valley’ of minimal width for every position. The color value along this valley is the TMC width as defined in equation (1). Data is only available at (x, y) -positions where the anterior surface was segmented.

2.4.4 Tight coupling

The above algorithm was applicable at all locations where a clear minimum can be identified, as in Figure 3A. However, a subset of CT-slices showed a shape for which the above rationale does not yield an objective value, as in Figure 3B. Therefore, at these locations, the TMC width was not

quantified but categorized as ‘tight coupling’. The width value in these areas was chosen to be equal to the width at the nearest location that was not tightly coupled. For some samples, there was a tight coupling along the entire manubrium. The TMC width was then measured manually at the base of the TMC using a native measuring tool in Amira on multiple slices until a minimum was found. The width along the entire manubrium was set to this measured value.

2.4.5 Uncertainty on 3D distance

The measurement uncertainty on all three coordinates x , y and z of the segmented surfaces was estimated to be equal to the voxel size, i.e. $4.97 \mu\text{m} \approx 5 \mu\text{m}$ for samples 2-13 and $3.36 \mu\text{m}$ for sample 1. By performing statistical error propagation, it follows that the uncertainty (UC) on the 3D distance between two of these surface points is equal to $\sqrt{2} \times 4.97 \mu\text{m} \approx 7 \mu\text{m}$ for samples 2-13 and $\approx 5 \mu\text{m}$ for sample 1.

3 Results and discussion

3.1 μCT scans

Figure 5 shows for every sample the virtual transverse μCT slice (i.e. perpendicular to the manubrium) with the smallest TMC width. This visual comparison indicates a large variation in minimal TMC width.

Figure 5: Virtual transverse μCT slices at the position where the TMC is the narrowest. All images have the same scale. In these images, the gray values were optimized for soft tissue contrast. The gray value range of the original images was larger than in these images.

3.2 Morphometric results

3.2.1 Tympano-malleal connection width

Figure 6 presents the TMC width as a function of the manubrium position for all samples. Again, a large inter-individual variability is clearly apparent. Table 2 presents statistical parameters of the data. In most samples the width featured a local minimum in the middle of the manubrium and

higher values at the edges. Some samples exhibited a second local maximum in the middle. Because of the mixture of values representing normal TMC width and tight coupling width, calculating an average and standard deviation on these data was not meaningful and therefore avoided.

Figure 6: Tympano-malleal connection width (anterior-posterior) as a function of manubrium position for different individuals. Solid lines indicate the quantified TMC widths of the different samples. Dotted lines indicate areas of ‘tight coupling’ between the manubrium and TM.

Table 2: Statistical parameters of TMC width and thickness. For the width, the maximal value along the manubrium could not be determined unambiguously because it always corresponded to a location of ‘tight coupling’ (see section 2.4.4). UC: uncertainty; SD: standard deviation

Unit: μm	Minimum among samples	Maximum among samples	Average
Minimal TMC width along manubrium	83 ± 7 UC	840 ± 7 UC	470 ± 220 SD
Minimal TMC thickness along manubrium	48 ± 7 UC	249 ± 7 UC	128 ± 71 SD
Maximal TMC thickness along manubrium	236 ± 7 UC	691 ± 7 UC	470 ± 130 SD

3.2.2 Tympano-malleal connection thickness

In Figure 7, the TMC thickness is shown as a function of manubrium position for all samples except one. The μCT scans of the absent sample (nr.2) exhibited slight motion artifacts that prevented unambiguous segmentation of the medial and lateral TMC surfaces, so this sample was omitted from the thickness results.

The TMC thickness, like the TMC width, exhibits a rather large inter-individual variability. The steep descent at the umbo and steep ascent at the lateral process of the malleus (LPM) are caused by the rounding of the bone at its edges. These steep edges were disregarded for the calculation of the minimal and maximal thicknesses by excluding the first 5% and last 10% of the data. The values of these extrema are included in Table 2.

The locations of both the minimal and maximal thickness are clustered for all samples (except one sample’s minimum): between 5% and 20% for the minimum (i.e. close to the umbo) and between

70% and 90% for the maximum (i.e. close to the LPM). In between the minimum and maximum thickness, a more or less linear increase can be observed in most samples and in the average curve. Between the maximum and the LPM, the thickness decreases and finally increases again towards the edge of the bone.

Figure 7: Tympano-malleal connection thickness (medial-lateral) as a function of manubrium position for different individuals. Gray lines indicate the quantified TMC thicknesses of the different samples. The thick blue line indicates the average and the light blue area the region between the average plus and minus one standard deviation. The blue and red dots indicate the locations of the minima and maxima of individual samples.

3.2.3 Correlation between width and thickness

Figure 8 presents TMC width and thickness curves for every individual sample, except for sample nr. 2 (for reason see section 3.2.2). In most samples where the TMC is tightly coupled along the entire manubrium (full length straight red line, samples 3, 4 and 5), the overall TMC thickness is smaller (blue). Sample 11 is an exception on this. Furthermore, where the TMC clearly becomes locally thicker, it tends to become narrower as well, as can be seen in samples 6, 7, 8, 9, 10 and 13. In samples 1 and 12, both width and thickness are fairly constant along the manubrium.

Figure 8: TMC width and thickness for every sample (except one).

3.3 Interpretation of the histological sections

After obtaining the morphometric results, three samples were selected for histological preparation: one representative for an ‘average’ TMC (sample 1), a very narrow TMC (sample 9) and a very wide TMC (sample 5).

3.3.1 Histological examination of an ‘average’ tympano-malleal connection

Figures 9 and 10 show transverse histological sections of sample 1 (average TMC). Figure 10A1-B1 (top row) are higher magnifications of Figure 9C-D. Figure 10A2-B2 (bottom row) present schematic views of the most important tissues identified in the top row.

In sample 1, the connection between the TM and manubrium appears to be most tight at the umbo (Figure 9A), gradually becoming looser and more distant (Figure 9B-E), before becoming tighter again towards the LPM (Figure 9F), consistent with the morphometric observations in this and most other samples. In Figure 9F, the cartilaginous cap of the LPM of the malleus is visible.

Figure 10A indicates that the TMC consists of a core of dense regular connective tissue (DRCT), containing tightly packed collagen fibers, surrounded on both sides by loose connective tissue (LCT). Both the TMC and the malleus perichondrium and periosteum are lined by simple squamous epithelium that is continuous with the most medial TM layer. The DRCT layer runs obliquely from lateral-posterior to medial-anterior. It is continuous with collagenous fibers in the lamina propria layer of the TM at its lateral-posterior end and merges with the perichondrium of the malleus at its medial-anterior end. The DRCT core is present Figure 9A and B as well, albeit with different widths.

In Figure 10B1-2 (corresponding to Figure 9D), the dense core is no longer present. Here, the TMC solely consists of LCT that is continuous with the internal tissue of the TM. This is the case in Figure 9E as well.

Figure 9: Transverse histological sections of sample 1 at different positions along the manubrium. C and D are magnified in Figure 10.

Figure 10: Top row: Sections 4 (A1) and 5 (B1) of Figure 9 in close-up. A2&B2: schematic representation of A1 and B1; Legend: B: bone; C: Cartilage; P: Perichondrium/periosteum; LCT: Loose connective tissue; DRCT: dense regular connective tissue; E: squamous epithelium.

3.3.2 Histological examination of two 'extreme' tympano-mallear connections

Figure 11 displays sections at the middle of the manubrium of samples 5 (A) and 9 (B), which were the samples with the largest and second smallest TMC width of our population, respectively. In both samples, the same tissues can be identified as in the average sample (see Figures 9 and 10). However, the configuration is different, especially for the widest TMC (sample 5, Figure 11A). There, the DRCT in the lamina propria layer of the TM splits up both anteriorly and posteriorly of the manubrium and merges at different locations with the perichondrium/periosteum of the manubrium. A portion of the DRCT fibers passes lateral to the manubrium. The space between the DRCT layers and the manubrium is larger on the anterior side than on the posterior side and is filled with LCT. Further towards the umbo (not shown), the anterior space filled with LCT becomes smaller and filled with DRCT instead, while the posterior part of the connection remains largely similar. At the umbo, the images are similar to the average sample (sample 1). Further towards the LPM, the collagen fiber layer of the TM and its connection to the manubrium become thinner, on both anterior

and posterior sides of the manubrium. At the LPM, the tissue configuration is again similar to sample 1.

Figure 11B (sample 9) shows that the very narrow TMC consists at mid-manubrium level of a dense collagenous core similar to the average sample (sample 1), connecting the TM to the perichondrium/periosteum of the manubrium. Only on the anterior side of the TMC, a small area of LCT can be identified. In this sample as well, the observations at the umbo and LPM are similar to samples 1 and 5.

Figure 11: Top row: Histology sections at the middle of the manubrium of samples 5 (A) and 9 (B), the samples with the largest and smallest TMC width respectively. A2&B2: schematic representation of A1 and B1; Legend: B: bone; C: Cartilage; P: Perichondrium/periosteum; LCT: Loose connective tissue; DRCT: dense regular connective tissue; E: squamous epithelium.

4 Discussion

4.1 Morphometric results

At both the umbo and the LPM, we observed a tight coupling between the manubrium and the TM in all samples. This is consistent with published literature describing the TMC. At locations between the umbo and the LPM, however, large differences between individuals were observed. In this zone, both TMC width and TMC thickness range over (almost) an order of magnitude among samples. The samples exhibiting a narrow, stretched fold in the middle of the manubrium are consistent with histological studies (Ferrazzini, 2003; Graham et al., 1978; Gulya and Schuknecht, 1995; Politzer, 1892), while the samples with a wide, tightly connected TMC are to some extent consistent with Gea and Decraemer (2010), a study based on μ CT imaging. This observation indicates that the

discrepancy in the literature on TMC morphology could represent real inter-individual variability, rather than being caused by imaging artifacts.

Gea and Decraemer (2010) reported measurements of TMC dimensions based on μ CT-scans of one temporal bone. In the reported case, the TMC thickness (in medial-lateral direction), going from umbo to LPM was reported at 7 evenly spaced locations: 150 - 270 - 330 - 680 - 815 - 440 μ m. The trend in these values is similar to our data, although none of our samples reaches a thickness of 815 μ m. The TMC width was reported at two locations near the middle of the manubrium: 540 μ m and 680 μ m. Both values are well inside our range of values.

In surgical myringectomy interventions, the TM is surgically detached from the manubrium. This detachment is experienced as being more difficult at the umbo and the LPM than in the middle of the manubrium in many patients (Marquet, 1987). This is in agreement with the findings of the narrow TMC's of this study, but not with the most tightly coupled TMC's.

The locations of both minimal and maximal TMC thickness along the manubrium are rather consistent among samples. This is an interesting starting point for further investigation of the implications of the detailed TMC anatomy. It could be used to construct a statistical 'average' TMC model, along with models of the thin and thick extrema.

The fact that the tightly coupled TMC's represent the samples in the lower range of TMC thicknesses is not surprising. If there is only a small distance between the TM's lateral surface and the malleus's lateral surface (small TMC thickness), there is no space for the TMC to become very narrow between the TM and malleus.

4.2 Interpretation of the histological sections

The histological examination of three of our samples demonstrated that the variability among samples is not merely a question of dimensions, but also of configuration and composition. Consistent with our morphometric results, the histological properties of the TMC are the same for all samples at the umbo and the LPM. At the umbo, the TMC can be described as a splitting collagenous layer of DRCT of the TM (i.e. its lamina propria) that merges with the perichondrium or periosteum of the malleus. At the LPM, the cartilaginous cap of the malleus attaches to the DRCT layer of the TM. In between, the properties are very divergent. In both the narrowest and average TMC's, at mid-manubrium level the TMC has a core of DRCT. The difference between both is a much smaller amount of LCT surrounding the dense core in the narrow sample.

In the widest TMC, however, a qualitatively different connection is observed. Instead of one, there are four locations at which the DRCT layer of the TM attaches to the manubrium, because it splits anterior and posterior to the malleus. The connection was asymmetrical because on the anterior side, large volumes of LCT fill up the space between the manubrium and the DRCT layers.

The observations in the narrowest TMC samples are consistent with the literature based on light microscopy of histological sections (Ferrazzini, 2003; Graham et al., 1978; Gulya and Schuknecht, 1995; Politzer, 1892). However, the majority of TMC's in this study are wider than reported in these sources. The current study therefore clearly shows that the generally accepted narrow TMC shape represents only a portion of the population.

The variation of histological configurations and composition between samples and manubrium positions could have mechanical implications, as DRCT and LCT have significantly different

mechanical properties. In DRCT, collagen bundles are aligned in order to provide maximum tensile strength, whereas in LCT, the collagen fibers are loosely packed and separated by abundant matrix, resulting in a more deformable tissue that mainly serves to occupy space and support other structures (Kerr, 2009).

4.3 Implications in the clinical context and for middle ear modeling

In a clinical context, the findings of this study will be valuable in understanding middle ear injuries such as malleus fractures and their effect on middle ear mechanics and conductive hearing loss. Our results suggest that treatment in scenarios such as malleus fracture, malleovestibular prosthesis placement or tympanoplasty could be improved by following a patient-specific approach, based on the type of TMC.

In the context of middle ear modelling, it was emphasized by Funnell et al. (2013, p.7 of chapter) that a priori knowledge of material parameters is always better than “simply adjust[ing] parameters to fit particular experimental results”. It is reasonable to assume that the same is true for anatomical details that could have a significant influence on the system’s mechanics and transfer function (e.g. (De Greef et al., 2015)).

Some recent finite element models of the middle include a simplified geometry for the TMC (Aernouts et al., 2012; De Greef et al., 2014; Hoffstetter et al., 2010; Koike et al., 2002; Volandri et al., 2012; Yao et al., 2013). Others either do not include the TMC as a separate structure or are unclear about whether or not they do (Cai et al., 2010; Gentil et al., 2014; Higashimachi et al., 2013; Kelly et al., 2003; Koike et al., 2002; Liu et al., 2014; Zhao et al., 2009). Volandri et al. (2012) stated that “a more realistic representation of the complex connection between the TM and the malleus certainly could improve the present model in finding more meaningful parameters”. Our measurements now deliver data of average TMC geometry and its upper and lower dimensional boundaries.

4.4 Method considerations

4.4.1 Comparison between μ CT and histology

The results show that μ CT and histology are a powerful and complementary combination to fully understand the morphology of a delicate biological component such as the TMC. From unstained μ CT images, morphometric parameters can be extracted, because the imaging results in isotropic voxels and misalignment between slices is ruled out completely. However, it is impossible to identify all different tissue types that are present from μ CT. This can be seen by comparing the histological sections in Figures 9, 10 and 11 to the corresponding μ CT images in Figure 5. On the other hand, histology can provide detailed insight into the tissue composition and configuration, but is not as suitable as μ CT images for morphometry because of the more aggressive (biochemical) tissue preparation, the anisotropic voxels and the difficult section alignment. Moreover, histology requires irreversible physical sectioning of the sample and is more laborious and expensive.

4.4.2 Omission of biochemical X-ray contrast-enhancement

Acquiring good contrast on mixed-tissue type samples (both hard and soft tissue) traditionally requires heavy-element staining to enhance the X-ray absorption of the soft tissue. However, Buytaert et al. (2014) demonstrated that many conventional types of staining induce a significant shrinkage of all soft tissue types that were included in the study. Because soft tissue morphometry was the main property of interest of this study, we avoided staining of our samples. Therefore it was

necessary that we fully optimized the contrast in other ways. One of these ways was sample orientation, the other is the removal of excessive bone tissue from the sample.

4.4.3 Paraformaldehyde preservation

The removal of excessive temporal bone enabled us to optimize the X-ray scanner settings for optimal contrast in the samples at hand and hence to omit sample staining. The disadvantage of the dissection is the removal of the in-situ mechanical suspension of the eardrum. Preliminary CT-scans taught us that the membrane is prone to motion artifacts during the long scan durations if no chemical fixation was applied. This is the reason for our choice to preserve the samples in paraformaldehyde. The literature suggests that the influence of tissue shrinkage due to formaldehyde preservation is non-existent or marginal (Kerns et al., 2008; Miller and Dark, 2014).

4.4.4 Segmentation operator bias

As described in section 2.4, some degree of manual intervention during the segmentation was indispensable in order to get correct segmentation results. In Buytaert et al. (2014), the influence of the operator on segmentation results on μ CT images was quantified. The authors concluded that morphometric results following from a manual segmentation process are only marginally dependent on the operator. Although operator bias depends on the image quality and inherent image contrast of the study at hand, the cited conclusion provides an indication that subjectivity has a small to negligible effect on results of morphometric analysis of μ CT images through image segmentation.

5 Conclusions

The tympano-malleal connection exhibits a very large variability between individuals, regardless of the imaging technique used (μ CT or histological microscopy).

Morphometrically, we assessed the TMC in two dimensions: the TMC width (in anterior-posterior directions) and the TMC thickness (in medial-lateral direction). In general, at the umbo and LPM, the TMC width was larger and the TMC thickness was smaller than in the region between these two points.

We found that minimal TMC width along the manubrium for different individuals covered a range between $(83 \pm 7) \mu\text{m}$, and $(840 \pm 7) \mu\text{m}$. In some samples, the TMC is a thin, stretched fold at the middle of the manubrium (e.g. samples 7 and 9 in Figure 5), whereas in others, the TMC is entirely different in shape and the malleus is tightly embedded into the TM along the entire manubrium (e.g. samples 5 and 11 in Figure 5). The minimal TMC thickness ranged from $(48 \pm 7) \mu\text{m}$ to $(249 \pm 7) \mu\text{m}$ and the maximal thickness from $(236 \pm 7) \mu\text{m}$ to $(691 \pm 7) \mu\text{m}$. The locations of both minimum and maximum thicknesses are well clustered near the umbo and the LPM, respectively. The TMC width and thickness seem to be roughly correlated in the sense that wide TMC's are thinner (these are the tightly coupled cases) and narrow TMC's are thicker (these are the stretched fold cases).

Histologically, we observed that the TMC consists of loose connective tissue (LCT), dense regular connective tissue (DRCT) or both, and is covered by simple squamous epithelium. An average TMC consisted of a core of dense tissue surrounded by loose connective tissue on both sides and the dense core disappears when moving towards the LPM. In the narrow TMC, much smaller amounts of surrounding loose connective tissue were observed, while in the sample with the wide TMC, the tissue configuration was different with dense fibers attaching to the manubrium's anterior and posterior surface while still passing by laterally.

The large variability in morphometry and histology of the TMC suggests that treatment in various clinical scenarios could be improved by following a patient-specific approach, based on the type of TMC. It also suggests a variability in basic mechanics and ME functioning. Therefore, in future work, results of this study will be incorporated in an existing finite element model of the TM-malleus complex (De Greef et al., 2014) to study the functional effect of differences in TMC geometry.

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