On the biology of the bony otic capsule and the pathogenesis of otosclerosis

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The present PhD thesis is based upon the following publications. These will be referred to by their roman numerals:

I. Bloch SL, Sørensen MS. Three-dimensional reconstruction of the otosclerotic focus. Acta Otolaryngol 2009;130:429-34.

II. Bloch SL, Sørensen MS. The spatial distribution of otosclerosis: A quantitative study using design-based stereology. Acta Otolaryngol 2009;130:532-9.

III. Bloch SL, Kristensen SL, Sørensen MS. The viability of perilabyrinthine osteocytes: A quantitative study using bulk-stained human temporal bones. Anat Rec 2012;295:1101-8.

IV. Bloch SL, Sørensen MS. The viability and spatial distribution of osteocytes in the human labyrinthine capsule: A vector-based stereological study. Hear Res 2010;270:65-70.

BACKGROUND

The pathogenesis of otosclerosis has been a mystery for centuries. Otosclerosis is characterised by abnormal focal bone remodeling in the bony otic capsule or, in rare cases, the ear ossicles. In the active initial phase, the otosclerotic lesion is histologically dominated by resorptive spaces and a high cellularity (otospongiosis). In the late stages, the lesions may become fibrous or sclerotic [1].

Otosclerosis can develop anywhere in the bony otic capsule. However, the sites of predilection are the window regions, cochlea and apical-medial wall of the internal ear channel. Otosclerosis in the window regions may cause conductive hearing loss by ankylosis of the stapes footplate or obliteration of the round window opening.

Approximately 0.2-0.5% of populations with light skin types has hearing loss as a consequence of otosclerosis. However, up to 7-10% of cadaver temporal bones have otosclerotic lesions [2,3]. The incidence of otosclerosis varies worldwide with the highest rate reported in the Southern India and the lowest rate in Africa [4-6].

Half of the patients have a family history of otosclerosis and almost twice as many women than men are clinically affected and recruited for surgery [5,7]. However, the incidence of histological otosclerosis in high ages seems to be the same between genders [8].

Despite extensive research, the pathogenesis of otosclerosis remains unknown. Consequently, the disease cannot be predicted, stopped, or treated medically. Previously genetic research has failed to identify a specific otosclerosis-gene, and theories of virus infections [9-11], autoimmunity [12,13], enzymatic disorder [14-16] or association to generalized bone diseases [17,18] have been unable to explain why otosclerosis only occurs in the bony otic capsule while the rest of the skeleton remains completely normal.

Bone dynamics and the inner ear OPG/RANKL/RANK signaling system

During the growth period our skeleton is sculpted by *modeling*. This occurs by formation of new bone at one site and the removal of bone from another site within the same bone.

Bone *remodeling* occurs throughout all stages of life and does not change the shape of the bone. It is an important process for repair of skeletal damage such as microcracks that result from repeated stress, prevention of the accumulation of old bone, which can loose its flexibility and become brittle and finally for the function of the skeleton as reservoir for calcium and phosphorus.

Bone remodeling is a spatially coordinated process dictating the skeletal mass, structure, and strength via the integrated and sequential actions of osteoclasts and osteoblasts by their individual roles in bone resorption and formation. This coupled process occurs at separate bone sites within basic multicellular unit (BMU). In the first step of bone remodeling, bone is resorbed by osteoclasts. This resorptive phase is followed by a reversal formative phase where osteoblasts lay down osteoid lamellae, which eventually become mineralized. During this process, a fraction of the osteoblasts is trapped within the new bone matrix and persists as osteocytes while untrapped osteoblasts may transform into bone surface lining cells.

In the bony otic capsule, bone metabolism differs fundamentally from the rest of the skeleton since growth, modeling, and remodeling are virtually absent close to the inner ear spaces. Primary fetal bone is simply compacted, highly mineralized and generally preserved [19,20]. Previous studies on time-labeled undecalcified human and animal temporal bones discovered that the bone turnover rates in the healthy adult temporal bone increase centrifugally from a mere 0.1% in the innermost perilabyrinthine zone enclosing the inner ear space towards a normal rate of 10% per year at the capsular periphery (Figure 1 and 2) [21-23].

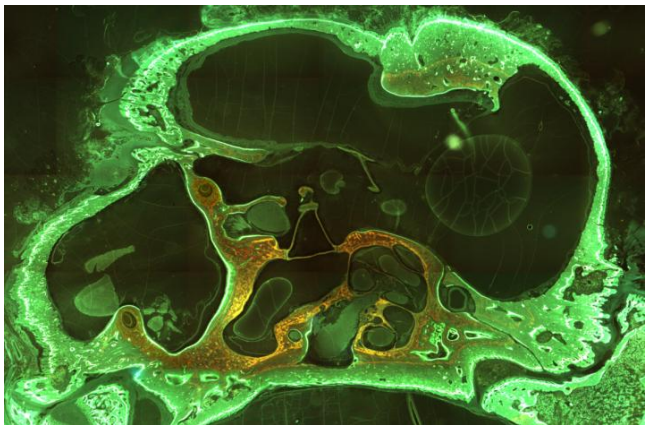

Figure 1
Epifluorescence of a rabbit temboral bone time-labelled with demeclocycline (yellow) 7 days before birth, alizarin complexone (red) 2½ weeks after birth and calcein (green) 5½ weeks after birth. The overview shows persistence of early fetal (yellow) perilabyrinthine bone around the inner ear space. Original magnification x 4 [34].

These observations predicted the existence of a local inner ear mechanism that inhibits perilabyrinthine bone resorption and remodeling. The candidate signaling system responsible for this centripetal inhibition of bone resorption is the inner ear OPG/RANKL/RANK signaling system discovered at Massachusetts Eye & Ear Infirmary, Harvard, Boston, USA [24]. Osteoprotegerin (OPG) is an anti-resorptive cytokine named from its ability to "protect" bone against bone resorption. OPG is expressed in high levels (>1000 x normal bone levels) by inner ear structures to inhibit perilabyrinthine osteoclast formation and functions in competition with receptor activator nuclear-kβ ligand (RANKL) for the receptor activator nuclear-kβ (RANK) receptor (Figure 3). The candidate anatomical route for inner ear OPG is the lacunocanalicular porosity accommodating the osteocyte syncytium (Figure 3).

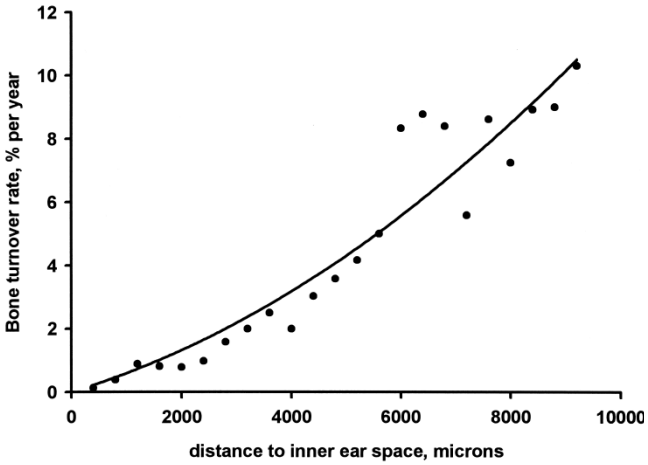

Figure 2
Fluorochrome labeling of dogs revealed an overall capsular bone turnover of 2.1% per year with a range of 0 to 0.13% per year for the innermost perilymphatic zone, through a centrifugal increment toward 8% to 10% per year in the capsular periphery. The underlying individual bone remodeling units exhibit a similar centrifugal pattern in numerical density and size. These findings indicate an inhibition of remodeling presumed to emanate from the perilymphatic spaces, and affecting both the activation of osteoclasts and the extent of resorption by the osteoclasts [23].

Except from the spiral ligament, other major biological sources to OPG production are not well-established although osteoblasts [25], endothelial cells [26], and vascular smooth muscle cells [27] all have been demonstrated to produce OPG. RANKL is produced by osteoblasts or cells of that lineage [28,29], endothelial cells [26], activated T lymphocytes [30,31] and other cell types such as bone marrow stromal cells, fibroblasts, chondrocytes, mammary epithelial cells [32]. Cells of the osteoblast lineage can express RANKL on their surface in a manner that facilitates osteoclastogenesis *in vitro* via cell-to-cell contact with osteoclast precursors [33]. RANK is expressed by osteoclasts, osteoclast precursors, dendritic cells, chondrocytes, endothelial cells, fibroblasts and macrophages [32].

The importance of inner ear OPG is emphasized by the fact that OPG-knockout mice develop excessive and disorganized capsular bone remodeling, stapes fixation and progressive loss of hearing [35-37].

AIM OF THE STUDY

Accumulation of dead osteocytes is highly expected in bone, which is not constantly replenished by normal remodeling. Dead osteocytes may cause aberration in the anti-resorptive OPG signaling system with potential implications to bone resorption, bone remodeling and otosclerosis.

The unique spatial distribution and the exact rates of otic capsular bone modeling and remodeling have previously been documented and quantified in time-labeled undecalcified human and animal temporal bones. However, the possible impact on the health and distribution of the capsular osteocyte population and the distribution of pathological capsular bone remodeling has not been determined. This is the subject of the present thesis, which aims to identify a possible spatial link between the viability of capsular osteocytes and the location of otosclerosis.

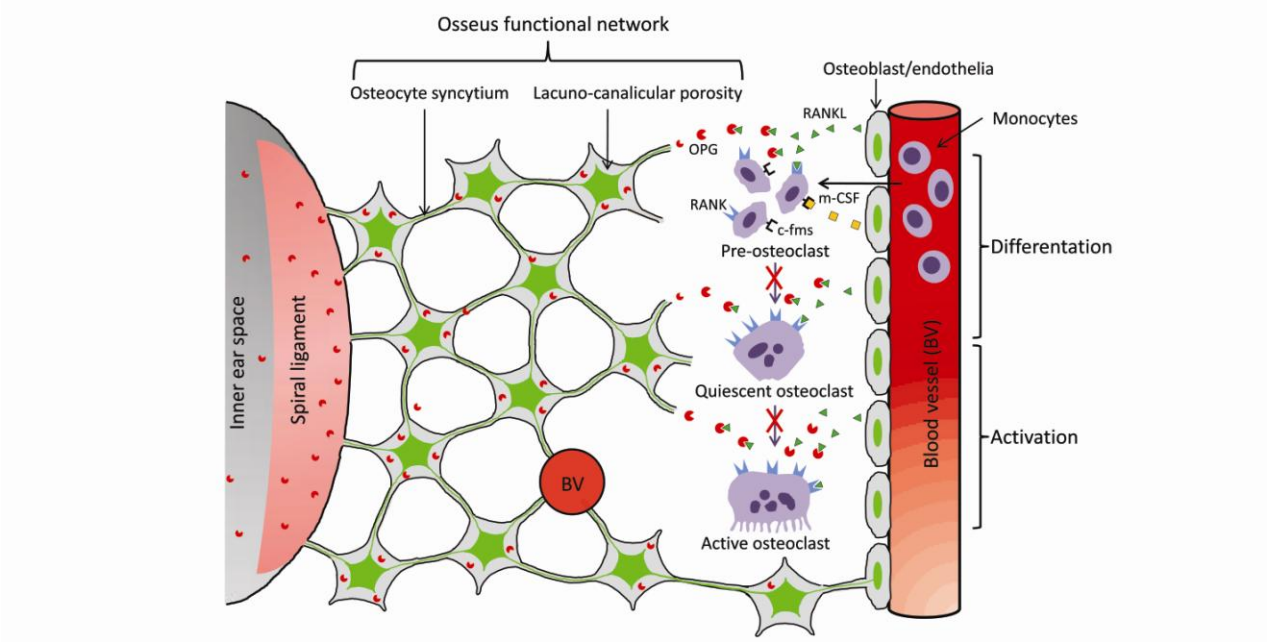

Figure 3
A schematic overview of the OPG/RANKL/RANK signaling system. RANKL mediates a signal for osteoclast formation through RANK expressed on osteoclast progenitors. High levels of inner ear OPG counteracts this effect by decoy of RANKL. Macrophage colony stimulating factor (M-CSF) acts on its receptor (c-fms) to increase the number of precursors available to form osteoclasts

METHODS

A brief review of stereological principles is presented and how these methods are applied to the current studies of the bony otic capsule.

Stereology and its applications in temporal bone research

Despite disagreement about nomenclature, most authors consider morphometry a two-dimensional (2-D) quantitative method that uses a direct measurement of objects under microscopic observation. In contrast to this, stereology does not perform a direct measurement and the analyses are always three-dimensional (3-D)-analyses. These may be either quantitative or qualitative, for instance reconstruction of 3D-objects from serial sections.

Stereology (Greek; stereos = solid, ology=the study of) is commonly defined as the science of estimating higher dimensional information from lower dimensional samples. Stereology is a powerful tool that provides practical techniques for extracting quantitative information about size, shape, and number of 3-D structures from measurements made on essential 2-D sections of the structure. The quantitative information of 3-D structure is revealed by utilizing test-systems of probes such as points, lines or cycloids. Events that result from random interactions of the measuring probes and the structures under study are counted. The average counts for the full population of probes are estimated and connected to a geometric property of the structure being sampled. Estimating the geometric quantities is derived by combining basic geometric facts and probability statistics (mainly survey sampling inference; statisticians regard stereology as a form of sampling theory for spatial populations).

In traditional *model-based* stereology, a section is considered "typical" for the sample by assuming the material is homogeneous. To extrapolate from the section sample to the 3-D material, *a priori* assumptions about the shape of the investigated structures are required in order to apply appropriate unfolding algorithms [38,39]. Methods that postulate a statistical model based on approximation of material structures are considered biased because the assumptions are hard to prove. The method only works if the models truly represent the actual objects. If reality differs, bias (systematic error) is introduced into the parameter estimates.

Modern *design-based* stereology is based on selecting "representative" sections at random, according to a specified random sampling protocol and using test probes, which are positioned uniformly and randomly (UR) in the structure [40]. For surface and length estimates using test-lines, isotropy (I) are also required. Since the sample scheme and probes are defined *a priori* "designed", the method is independent of any geometrical assumption of the structures investigated and potential sources of systematic errors in the calculations are eliminated. "Unbiased quantitative data may mean the difference between `interesting observations´ and real knowledge" [41].

Stereology permits researchers to effectively and efficiently gather data. The aim of the sampling design is to obtain a sufficient amount of quantitative structural information and to capture most of the biological variation at a given total cost and effort. The precision of stereological estimates can be expressed by the coefficient of error (CE), which gives a hint of how far the true value deviates from the estimate. The size of CE depends on the sampling intensity and how sections are produced. More details regarding this issue is described in paper II, appendix II.

Biological specimens consist of elements that are highly ordered into spatial relationships, which are required for execution of biological processes [38]. In fact, there are very few biological samples for which isotropy or uniformity can be assumed. This non-randomness or anisotropy (having a preferential direction) can influence the results of quantitative histological estimates depending on the plane of sectioning. For example, in a cross-

section through a femur shaft more cross-sectional osteon profiles are found than in a longitudinal section. Previously, the problem of measuring anisotropic structures was solved by introducing isotropy in the planes of section by "destruction" of the internal structural orientation of an organ. Methods capable of making "isotropic uniform random, IUR" sections are the orientator [42], the isector [43] or vertical sections [44]. Vertical sections have been used to estimate the surface density in trabecular bone biopsies [45]. The isector is a simple method for obtaining uniformly oriented sections by embedding small specimens in spherical capsules and then rolled on a table before further embedding and sectioning. Although this method might be applied to temporal bones, the normal sectional orientation is lost as well as the ability to use a global coordinate system to interrelate structures within the specimen. In addition, many of these methods are unpractical to use on plastic-embedded undecalcified temporal bones because of technical difficulties of rotating and cutting the hard tissue at different planes in the saw. Introducing isotropy to an anisotropic material is required for most stereological procedures for instance estimation of cell size, surface area, length or membrane thickness [46]. However, two exceptions from this are estimation of volume and number [41].

Three dimensional reconstructions (I)

It is straightforward to generate 3-D reconstructions from a stack of images. However, potential pitfalls and sources to misinterpretations of the reconstructed specimens must be taken into account. Poor manual alignment of the sections may thus cause distortion, disproportions and false anatomical interrelations of the reconstructed object. Three-dimensional reconstruction is the visual result of a preceding process of mathematical algorithms extrapolating the boundary profiles between consecutive sections into a distinct surface. Consequently, visualization and information about finer anatomical structures "hiding" in between sections remain unknown although they may prove biological important. Visualization of 3-D data sets typically involves too much data to interpret on the screen at once. Discarding parts that are not of interest and highlighting structures of interest (e.g. otosclerotic lesions) help to produce interpretable displays. Rotating movie sequences, which change the point of view, transparency of selected structures and altered position of a virtual light source are all effective tools to enhance the perception and identification of spatial patterns from 3-D reconstructions.

Estimation of spatial volume distributions (II)

Volume estimation of otosclerosis can successfully be accomplished with any plane of sectioning (avoiding IUR sections) by using the principle of Cavalieri and point grids because points themselves have no direction [47]. It is sufficient to randomly place a set of points through the structure with an equal probability of sampling all regions of the structure. However, we have gone one step further by estimating the spatial volume distribution of otosclerosis taking the non-randomness of the system into consideration. To measure and describe non-randomness (non-uniformity and anisotropy) is a challenging task. The methodological approach requires a very specific experimental design based on substantial *a priori* knowledge of the structural system under consideration and of the scientific purpose of the study.

The challenge was to access data on the spatial volume distribution of otosclerosis in radial directions from the inner ear space towards the capsular periphery, without violating the stochastic conditions for random interaction between test probes and structures of interest when using the principle of Cavalieri. This was achieved by obtaining the position of each test point as well as the boundary profiles of the inner ear space by tangential delineation (Figure 4 and 5). The shortest (perpendicular) distances between the inner ear line-segments and test points were automatically estimated by a series of mathematical hierarchical steps (Paper II, Appendix I). The wide range of estimated distances were gathered within predetermined iso-wide zones throughout the bony otic capsule, and the volume fraction of otosclerosis estimated as a function of the distance to the inner ear space.

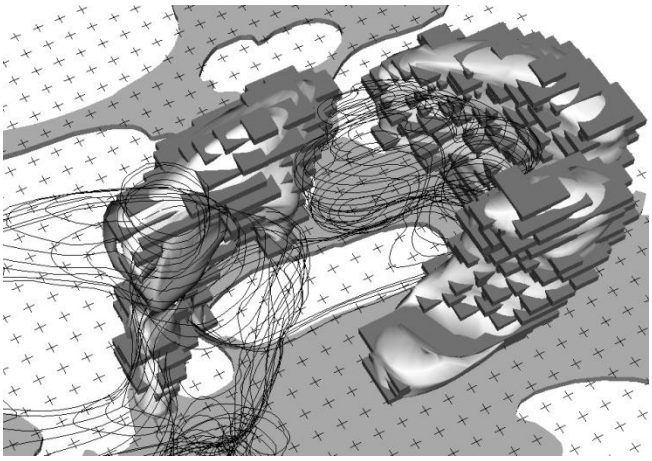

Figure 4
Otosclerotic lesions are decomposed into small bricks of known volume by using the principle of Cavalieri.

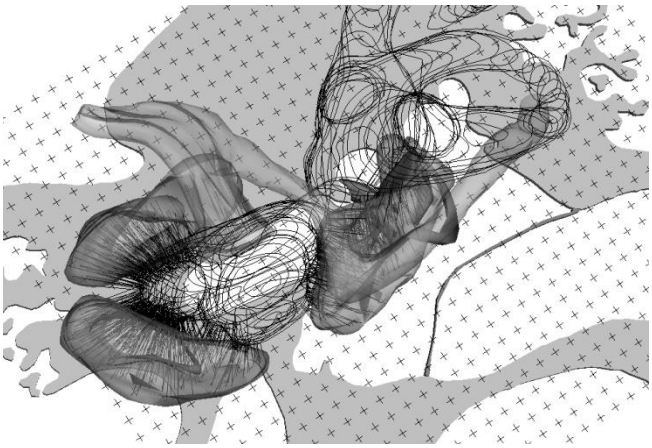

Figure 5
The shortest distance (2-D) from each test point (brick of bone) to the inner ear space boundary profile was estimated and the volume data plotted in radial directions within iso-wide virtual zones of bone. The stereological approach may be regarded as a hybrid-method.

Estimation of cell numbers by the optical dissector (III & IV)

When 3-D objects are cut into thin 2-D histological sections, the number of profiles per unit of area (N_A) is not equal to the number of objects per unit of volume (N_V). This is known as the "Wicksell's corpuscle problem" emphasizing that larger cells, cells with complex shapes and cells with their long axis perpendicular to the sectioning plane have a higher probability of being hit and appear as profiles on the cross-section [48]. Wicksell proposed a correction formula for spherical objects, $N_V = N_A / 2r$. However, this formula relies on geometrical assumptions of the studied objects. If reality differs, bias occurs.

The corpuscle problem can be eliminated by using 3-D counting probes. In its simplest form it is composed of two sections or disector pairs [49]. The disector counting procedure eliminates the potential biases associated with assumption-based methods as it uses direct counting of objects in a well-defined volume of tissue. To avoid oversampling, half of the surfaces of the 3-D counting probe are designated as exclusion surfaces and the other half as inclusion surfaces. By reducing the object to a unique sampling point, consideration of the size, shape and orientation of the object is eliminated [50]. By using certain counting roles, objects are counted only once, avoiding oversampling and the edge effect.

Estimation of spatial densities gradients of osteocytes (IV)
Again, the non-randomness of the specimen was considered in order to detect a possible radial orientated density gradient of viable and non-viable osteocytes. The virtual 3-D disector probes were randomly and uniformly spread throughout the structure of interest. By registration the spatial position of disector probes and the boundary profiles of the inner ear space by punctual delineation, a global matrix of 3-D coordinates was collected. From this large data set, the shortest distance from the surface of the inner ear space to the 3-D lattice of disector probes was estimated by hundreds of surface normal vectors. This outer-pointing vector field of normals is not truly perpendicular to the inner ear surface but closely approximated. The reason is that we only used every third section, punctual delineation and the fact that no zero-thickness of physical sections exists. Finally, the average numerical densities of viable and non-viable osteocytes were estimated within concentric capsular shields and presented as a function of age and distance to the inner ear space.

RESULTS AND DISCUSSION

Location, shape and spatial distribution of otosclerosis
Three-dimensional reconstructions demonstrated how the majority of otosclerotic lesions are located in the central parts of the bony otic capsule close to the inner ear space and with a smooth demarcation against the surrounding bone. Larger otosclerotic lesions are anisotropic with a bulky end facing the inner ear space and a volumetric decline towards the capsular periphery (Figure 6). The 3-D findings suggest a general centripetal distribution of otosclerotic bone remodeling (Paper I).

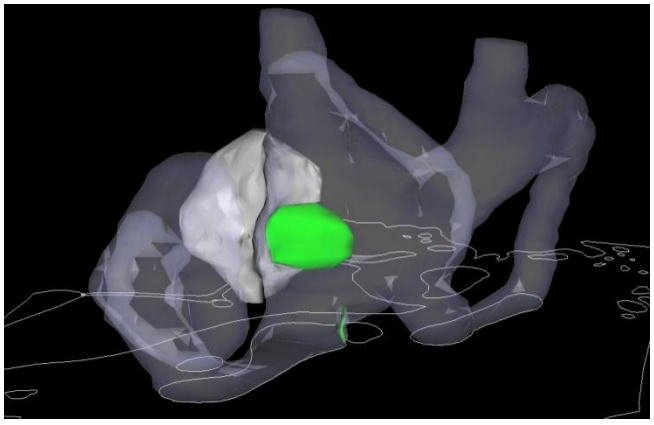

Figure 6
Three-dimensional reconstruction of an otosclerotic lesion (white structure) in the anterior oval window region. Note the close approximation of the focus to the inner ear space (transparent) and volumetric decline towards the capsular periphery. Stapedial footplate and round window membrane are labeled in green.

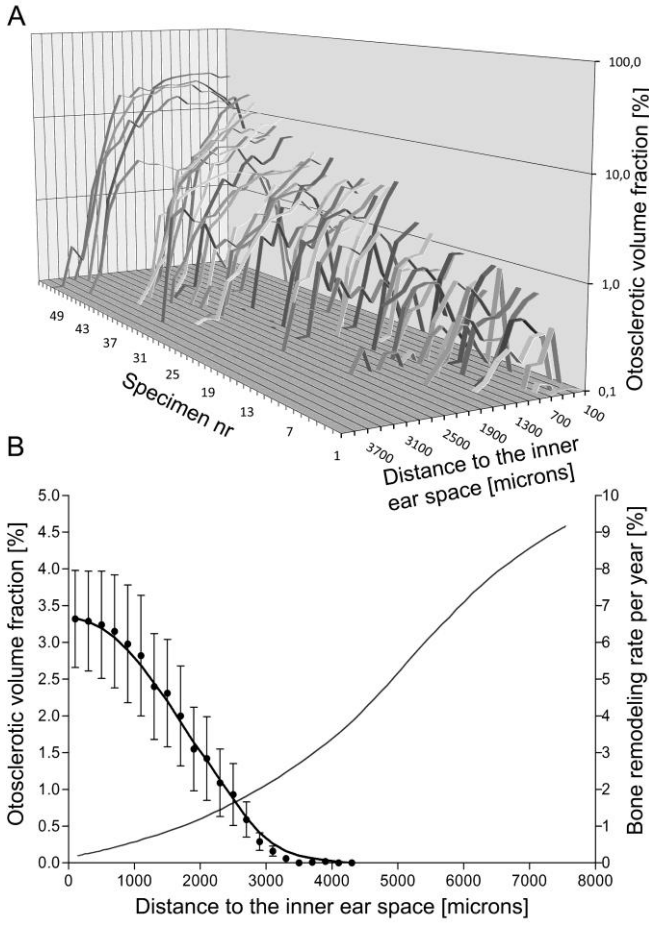

Figure 7
(A) The individual volume fraction of otosclerosis and (B) the average volume fraction of otosclerosis as a function of the distance to the inner ear space (Left axis, bars ±SEM, n=53). External data (Frisch T et al., 2000) of the normal distribution of bone remodeling in the bony otic capsule (right axis). Note the inverse spatial relation between otosclerotic and normal bone remodeling.

By using vector-based stereology, we discovered how the volume of otosclerotic bone was heterogeneously distributed in space with a general volumetric decline from the inner ear space towards the capsular periphery (Paper II) (Figure 7).

The viability and spatial distribution of labyrinthine osteocytes
The bony otic capsule is an exceptional environment for studying osteocyte survival as a function of age since bone remodeling is virtually absent in perilabyrinthine bone. We discovered bi-phatic osteocyte kinetics of viable and non-viable osteocytes in perilabyrinthine bone (Figure 8).

The first phase may correspond to the cancellous compaction and periosteal apposition during the growth period (bone modeling) of the bony otic capsule. The second phase reflects how the lack of bone remodeling affects the viability of osteocytes. Data were compared with age-matched control rib segments. Despite a notable biological variation, the average density of non-viable osteocytes was 40000 cells/mm^3 in perilabyrinthine bone compared to 5000 cells/mm^3 in ribs at 80 years of age (Paper III).

The spatial distribution of viable and non-viable osteocytes was presented in a mesh plot by plotting the numerical densities as a function of age and distance to the inner ear space (Figure 9). The density gradients of viable and non-viable labyrinthine osteocytes were inversely related in time (age) and space (the distance to the inner ear space). (Paper IV).

Since bone remodeling is low, the average bone age increases toward the inner ear space and more so with age compared to the capsular periphery simply because the bone is not renewed. Actually, the age of perilabyrinthine bone is just as old as the individual +5months/-3 years. For this reason, osteocytes which die by senescence accumulate towards the inner ear space with age. Thus, the bony otic capsule might be expected to be less viable than other bones of the human skeleton. However, the data revealed how the density of viable osteocytes was actually higher in the bony otic capsule compared to ribs until the age of 70-80 years of age.

Histological observations (Paper III and IV) demonstrated how the accumulation of non-viable osteocytes is not homogenously distributed in the capsular wall. Empty or mineralized osteocyte lacunae tend to cluster in local regions of varying size. Moreover, these regions were surrounded by the viable and intact parts of the osteocyte network (Figure 11).

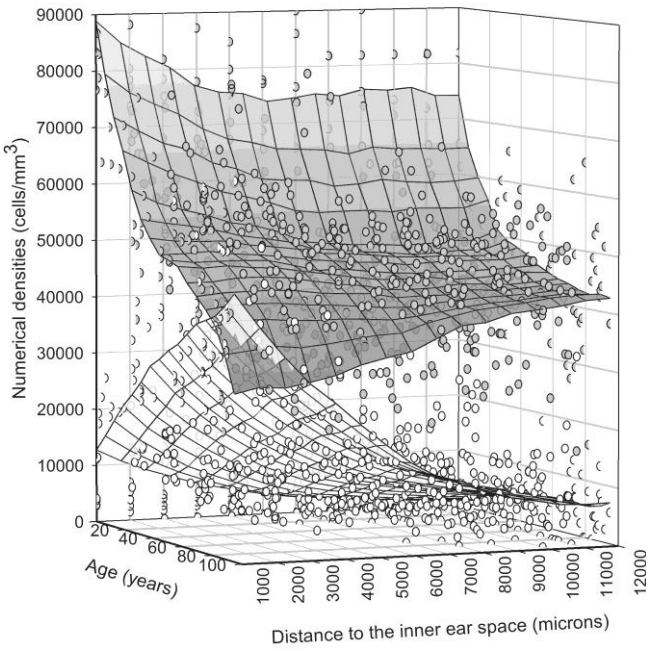

Figure 9
Density gradients of viable (dark surface) and non-viable (white surface) labyrinthine osteocytes as a function of time and space. The dots are the raw data. In young individuals, the density of viable osteocytes is high and distributed centripetally around the inner ear space. This density gradient declines with age and changes into a centrifugal distribution with the highest density of viable osteocytes at the capsular periphery. Contrary to this, non-viable osteocytes accumulate centripetally around the inner ear space with age. The visualization of raw data reveals a considerable biological variation in osteocyte densities.

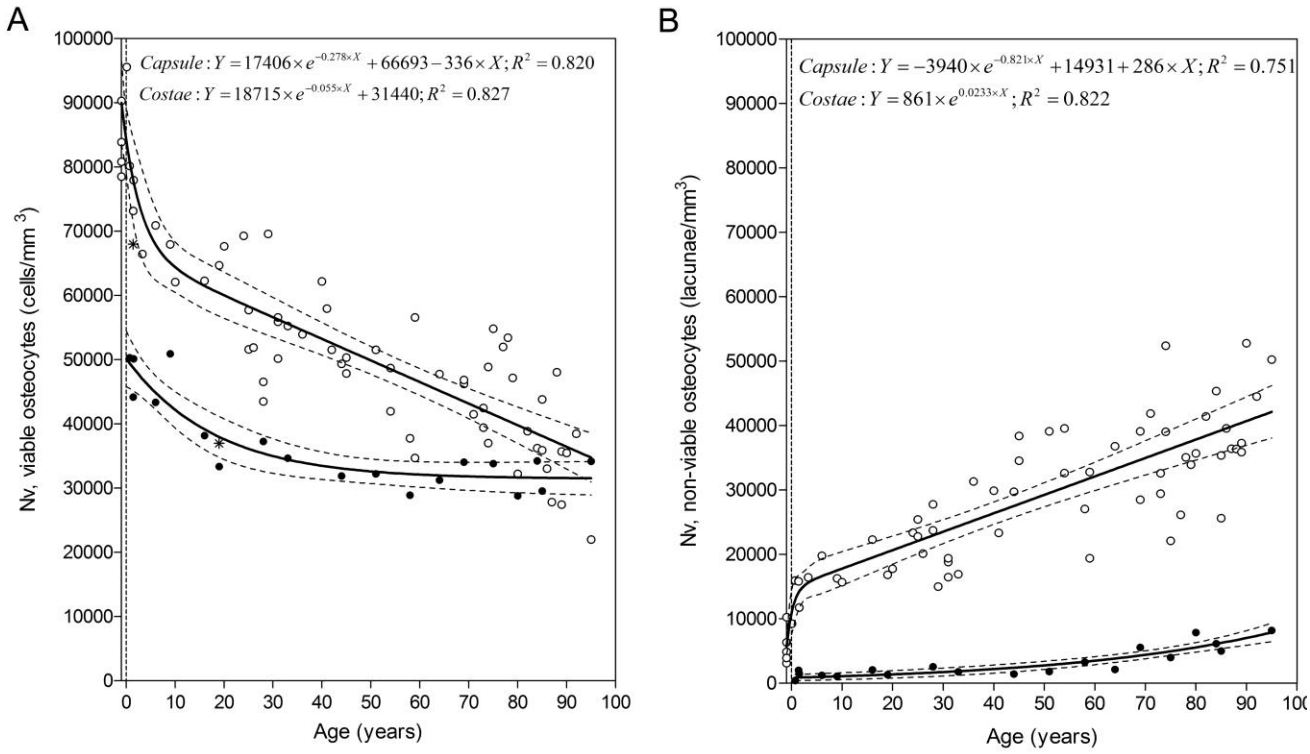

Figure 8
Bi-phatic osteocyte kinetics of viable (A) and non-viable (B) osteocytes in perilabyrinthine bone (hollow legend) and ribs (solid legend). Note the increasing density of non-viable osteocytes in perilabyrinthine bone compared to ribs where the normal rate of bone turnover nearly balances osteocyte death throughout life.

GENERAL DISCUSSION AND PERSPECTIVES

In the present PhD thesis, the spatial distribution of otosclerosis and viability of osteocytes were reconsidered in view of new knowledge about inner ear bone metabolism and regulation [20,24].

When nonlinear directions of variation are present, the situation becomes very complex and requires a great of knowledge about the specimen to design a proper stereological method [47]. We applied 3-D reconstructions and vector-based stereology on archival and dedicated materials of human bulk-stained temporal bones and ribs to obtain quantitative data. The strength of stereology is the ability to provide data for 3-D structures in 3-D quantities. This imposes a better degree of objectivity and reproducibility when measuring structural features.

Several new aspects of the spatial distribution of otosclerosis and viability of labyrinthine osteocytes were identified. A threshold concept of the pathogenesis of otosclerosis emerged.

Control of bone remodeling

Osteocytes are the most abundant cells in bone constituting up to 95% of all bone cells [51]. The cells are considered to be directly involved in control of bone remodeling by their central position in the tissue. Osteocytes form an elaborating "osseus functional system" involved in metabolic traffic [52], maintaining of the bone matrix [53,54], damage censoring [55], and targeted bone remodeling [56,57] but the dynamic aspects of these functions are not exclusively defined.

With an annual bone turnover rate of 10%, there must exist a general drive towards bone remodeling. Evidence in numerous disease models and settings suggests that the ratio of RANKL:OPG represents an important determinant of bone resorption and ultimately control of skeletal remodeling and bone mass [58-60]. It has been suggested that a certain balance of RANKL and OPG maintains a sufficient pool size of active osteoclasts at steady state [61]. The sites of bone remodeling are not considered stochastic. For example, specific bone areas adapt in response to physical exercise. A possible mechanism is altered flow of fluid through the lacuno-canalicular porosity in response to mechanical forces [55]. This generates fluid shear stress that activates calcium fluxes via voltage-sensitive or stretch-activated channels on the osteocytes as well as PGE2 release that enhance cell-to-cell communication through cellular gap junctions [62,63]. It has also been suggested that osteocytes modulate targeted bone remodeling by their own apoptosis next to microdamage by secreting osteoclastogenic factors [56,57,64,65]. However, the regulation and trafficking of biochemical signals between osteocytes, osteoblasts and osteoclasts are poorly understood.

Otosclerosis: A perilabyrinthine threshold phenomenon

The molecular profile of the bony otic capsule is clearly different from that of other bone [66]. Inner ear OPG is exceptionally upregulated in the spiral ligament (x1600 normal bones levels) and high levels have been detected in the fluid filled inner ear space (x800) [24]. Hence, OPG plays a pivotal role by suppressing the action of perilabyrinthine osteoclasts and in the end bone remodeling. Despite a high average bone age and extensive accumulation of non-viable osteocytes and fatigue microcracks, which both are in favor of bone remodeling, the remodeling response remains subtle with sporadic attempts of small BMUs, which are only just getting started before they are "turned off" [23]. The biological purpose of this perilabyrinthine inhibition of bone remodeling might serve to protect the sensori-neural epithelium against noxious effects from adjacent BMUs [16] or to conserve the size and spatial configuration of the cochlear space during the growth period of the expanding cranium. Although these questions remain unanswered at this stage, it remains that nature has provided us with a strong anti-resorptive system in the inner ear. In most cases this will prevent significant bone remodeling, except in otosclerosis.

This study demonstrated how otosclerotic bone remodeling and dead osteocytes are located toward the innermost and oldest bone zones of the bony otic capsule (Figure 10).

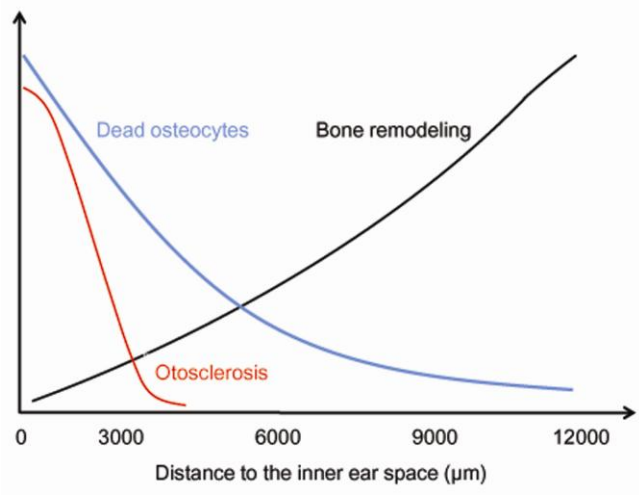

Figure 10
The spatial distribution of otosclerotic bone (red) and dead perilabyrinthine osteocytes (blue). Note the comparable distributions and inverse spatial relation to normal capsular bone remodeling (black).

We suggest that clusters of non-viable osteocytes may cause local aberrations in the anti-resorptive signaling system because the osteocyte syncytium and lacuno-canalicular porosity is deteriorated or lost within these regions (Figure 11). As a result, the regional flow of nutrients, oxygen and signaling molecules such as OPG will decrease. Below a critical density of viable osteocytes, the region becomes vulnerable to focal bone resorption through the absence of anti-resorptive OPG. Osteoclast precursors from the blood circulation may uninhibited enter the region below a critical level of OPG if they are activated by endothelia cells, osteoblasts, stromal cells or activated T-cells.

Another option is reactivation of quiescent osteoclasts within the vicinity of the affected region. Consequently, BMUs are not necessarily targeted via specific osteoclastogenetic signals from the affected region but rather by the absence of inhibitory signals (OPG). When the osteoclasts are activated, these may uninhibited resorb fragile bone within the "atypical" bone region devoid of modulating osteocytes and anti-resorptive OPG. The resorptive front may progress until a barrier of viable osteocytes and anti-resorptive OPG is reached. If the barrier consists of ageing osteocytes with few connections and consequently low flow of OPG, a well-established resorbing front may carry on its progression. However, if the barrier is well-defined by a dense network of osteocytes and lacuno-canalicular connections (sealed OPG), this may stop the advancing BMU, or the rejected BMU may physically

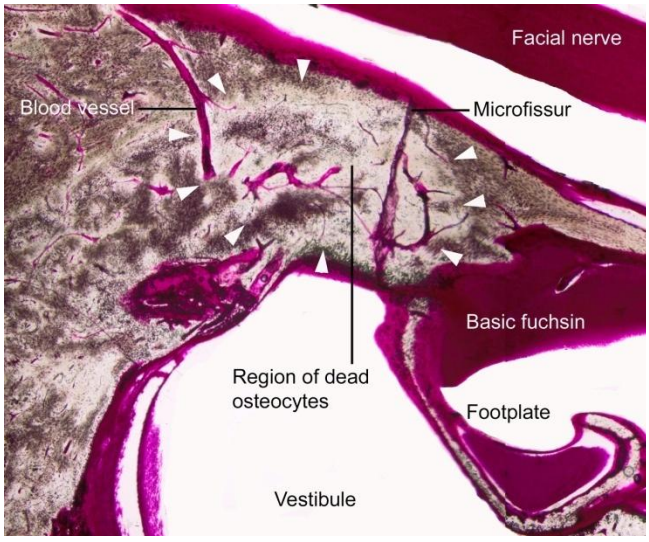

Figure 11
Anterior oval window region occupied with dead osteocytes and a single microfissur. Note how the region is surrounded by the intact and viable parts of the osteocyte network (arrows).

bounce back from the vital border and continue towards lower OPG levels (Figure 12). The regional lack of anti-resorptive OPG and modulating osteocytes combined with high levels of OPG at the surrounding area may account for the disturbed and irregular BMU patterns of the otosclerotic lesion. This hypothesis may explain the anisotropy of individual otosclerotic lesions, general centripetal distribution of otosclerosis and mosaic-like histology.

Since we consider otosclerosis as an osteo-dynamic threshold phenomenon, several local factors and stochastic events are involved:

Bone remodeling becomes the dominant process by the time that bone reaches its peak mass at 20-40 years of age [67]. Thus, increased osteoclast activity may trigger the onset of otosclerosis, which usually becomes noticeable in the second and third decades of live [68]. Pregnancy is considered to increase the risk of otosclerosis or accelerate its progression [69]. This might be related to the extra calcium mobilization during pregnancy and lactation or estrogen-induced hyperprolactinemia, which is known to down-regulate OPG [70]. Environmental factors may also be involved. For example measles virus has been associated with otosclerosis [9-11]. A virus infection may trigger the process via RANKL on activated T-lymphocytes. However, a vulnerable "OPG poor" region must still exist to reach a ratio of OPG for RANKL in favor of bone remodeling.

In the human skeleton and especially in the old bone matrix of the bony otic capsule, microcracks accumulate with age when they not are removed by bone remodeling [71-74]. Fatigue microcracks physically disrupt the osteocyte syncytium and lacunocanalicular porosity. The bone surrounding microcracks is dominated by low numbers of osteocytes and deprived of fluid flow [75,76]. The identification of osteocyte apoptosis and increased osteoclast activity near microcracks support the idea of targeted bone remodeling [64,77]. For these reasons fatigue microcracks may well be involved in generating OPG-"unplugged" capsular regions and/or trigger osteoclastogenesis via apoptosis of viable osteocytes.

Numerous growth factors, hormones, cytokines and drugs are known to influence the expression of OPG and RANKL. It is beyond the scope of this thesis to discuss all regulators of OPG and RANKL. Nevertheless, the issue is highly relevant because 50% of the patients have a familiar predisposition and the incidence of otosclerosis varies between populations. Epidemiological differences in gene expressions may affect the levels of these cytokines and the settings of the OPG/RANKL/RANK signaling system and consequently the vulnerability of the bony otic capsule.

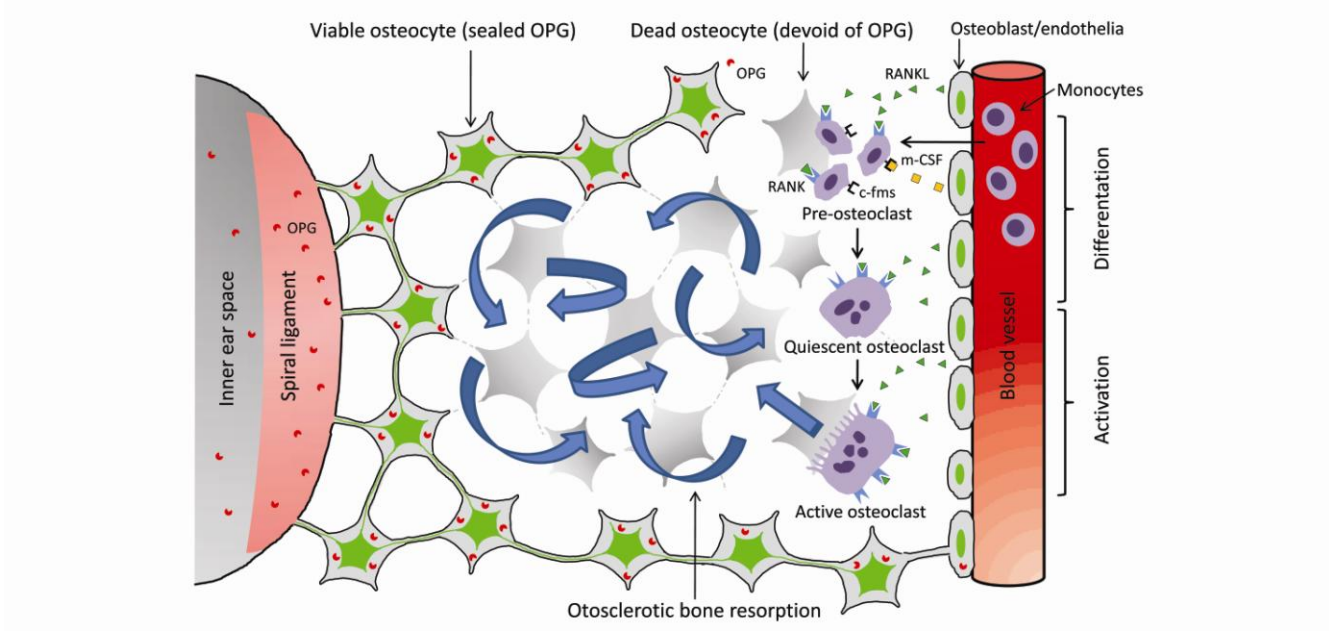

Figure 12
Uncontrolled focal bone resorption in a region devoid of viable osteocytes and anti-resorptive OPG signals. Progression of the process is restricted by the surrounding network of viable osteocytes and sealed OPG.

Predilection sites of otosclerosis: Connectivity and OPG exposure

Connectivity

We speculate that the probability of cellular disconnections and consequently unplugged capsular regions is higher in narrow capsular bone walls.

If microcracks and non-viable osteocytes are detrimental for lacuno-canalicular transport, the balance between cellular demand and supply may be affected adversely. This may cause a further decrease in the local viability of osteocytes. In a slender bony wall, the impact of even small amounts of microcracks and dead osteocytes may have substantial effect on the local connectivity and viability because these lesions more easily can isolate the region from the surrounding functional network. Contrary to this, in a bulky capsular wall, local cellular disconnections by osteocyte death or matrix microcracks may occur with very limited consequence because the fluid flow may bypass the affected region via the pronounced functional network in the surroundings (Figure 13).

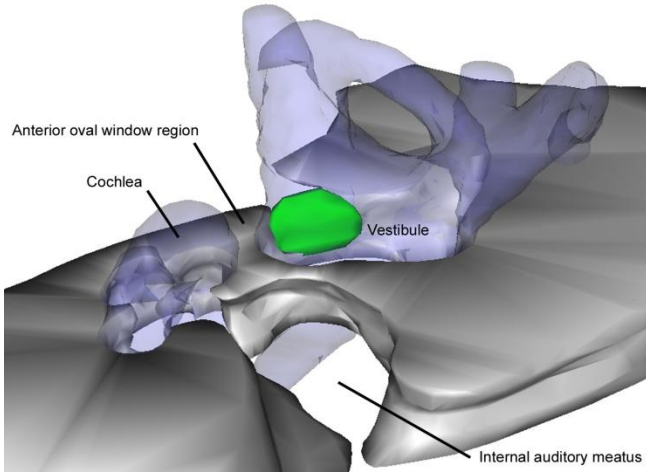

Figure 13
Three-dimensional reconstruction of the inner ear space (transparent) and surrounding perilabyrinthine bone at the level of the footplate (green). Note the slender capsular walls in the central part of the bony otic capsule.

OPG exposure

Histological observations (Paper III and IV) indicated that the viability of osteocytes was higher around the semi circular canals throughout life compared to the central parts of the bony otic capsule. We propose that this reflects regional differences in the OPG exposure and thereby bone remodeling activity, a notion supported by fluorochrome labelling of BMU in rabbits and dogs [78]. The OPG exposure might be higher in the central parts of the bony otic capsule by a closer proximity to the OPG-rich cochlear space and spiral ligament. The 3-D reconstructions showed how the anterior oval window region is located between the large surface area of the cochlea and the vestibule (Figure 13). This might facilitate an intense OPG exposure by "cross fire" of the region (imaging a unit of bone from a stereotaxic perspective). Finally, the fissula ante fenestrum (if not ossified) directly connects the anterior oval window region with the inner ear space of the vestibule.

The presented suggestions of connectivity and OPG exposure need further studies in order to confirm/reject these hypotheses.

CONCLUSIONS

In this PhD thesis, we demonstrated how dead osteocytes accumulate excessively towards the inner ear space with age in a spatial distribution very similar to that of otosclerotic bone.

Clusters of dead osteocytes are devoid of metabolic traffic and anti-resorptive OPG signals. Paradoxically, these previously highly controlled regions may consequently become subject to renewed bone remodeling activity by circulating resorptive cells. Peri-focal osteocyte survivors from the infantile high-density population, also discovered by our studies, may sustain a surrounding anti-resorptive signal, which may distort remodeling morphology and kinetics to produce the histological image of human otosclerosis.

Several local, systemic and possibly also environmental factors may be involved through their influence on settings of the OPG/RANK/RANKL signaling system and the state of the bony otic capsule. Therefore otosclerosis might best be regarded as an osteo-dynamic threshold phenomenon in a biological system with unique initial conditions.

FUTURE PERSPECTIVES

The discovery of the OPG/RANL/RANK signaling system has greatly increased our understanding of how bone remodeling is controlled although several aspects remain undetermined. Novel publications constantly provide new perspectives and information about this system. For instance, the blood concentration of OPG gradually increases with age suggesting that OPG may protect against age-related bone loss [79,80].

An interesting research project could be to measure the OPG:RANKL ratio in otosclerotic and non-otosclerotic ears. This could be done by collecting samples of perilymph from the drilling hole of the footplate during stapedotomy and the round window opening before inserting the electrode of cochlear implants. Another possibility is to compare the OPG:RANKL ratio between populations with a high and low incidence of otosclerosis. Other projects of interest are:

To quantify the surface density of microcracks around the inner ear space at different ages and to examine for anisotropy of the crack-network by measurements on three orthogonal surfaces and applying polar plots.

To quantify the regional viability of osteocytes at the predilection sites of otosclerosis and compare those data with less affected labyrinthine areas.

To determine if non-viable osteocytes within a region are distributed at random, clustering or self avoidance by measuring the nearest neighbor distance.

To bulk-stain temporal bones from populations with a high and low incidence of otosclerosis and quantify the spatial distribution of viable and non-viable osteocytes.

ABBREVIATIONS AND DEFINITIONS

µm	Micrometer = 10^{-6} meter
MW	Molecule weight
n	Number of specimens or sections
D	Dalton
SD	Standard deviation
CE	Coefficient of error
SEM	Standard error of the mean
CV	Coefficient of variation
N_A	Number of cells per area
N_V	Number of cells per volume
V_V	Volume density
Q^-	Number of objects (cells)
BMU	Basic multicellular unit. Collaborative cellular team of osteoclast and osteoblasts
OPG	Osteoprotegerin
RANK	Receptor activator of nuclear factor-kappa β
RANKL	Receptor activator of nuclear factor-kappa β ligand
Hybrid	Combination of two or more different things aimed at achieving a particular objective or goal
COV	Covariance
Lost caps	Object that are physically lost or optically not visible in a thin section at the upper and lower surface
Perilabyrinthine	Bone surrounding the vicinity of the inner ear space
Centripetal	Latin: centrum = "center", *petere* "tend towards", directed or moving inward towards the centre
Centrifugal	Latin: centrum = "center" and *fugere* = "to flee"), tending to move away from the center
Limaciform	Limax or slug-like shape
SURS	Systematic Uniform Random Sampling
IUR	Isotropic Uniform Random
Random	Without preferences
Isotropy	The property of being identical in all directions. Refers to structures within material or the condition where the orientation of the probe does not affect the mean.
Anisotropy	Preferred orientation in space. The property of being directionally dependent, as opposed to isotropy, which implies homogeneity in all directions
Isotropic section	A completely random section in which all directions are equally probable
Uniformly	No variation in measures with position
Non-uniformity	Variation in measures of structures with position
Morphometry	Measurement of morphological structures but not necessarily in three-dimensions

SUMMARY

In human otosclerosis, focal pathological bone remodeling occurs in significant amounts inside the normally anti-resorptive perilabyrinthine domain of the bony otic capsule. Otosclerosis causes hearing loss in 0.2-0.5% of the population by ankylosis of the footplate. The disease cannot be predicted, avoided or medically reversed as the pathogenesis remains unknown. Previously genetic research has failed to identify a specific otosclerosis-gene and earlier theories of virus infections, autoimmunity or association to generalized bone diseases have been unable to explain why otosclerosis only occurs in the bony otic capsule while the rest of the skeleton remains completely normal.

Studies from the otopathological laboratory (RH) have revealed how the bone turnover rates increase centrifugally from a subnormal 0.1% adjacent to the inner ear space towards a normal 10% per year at the capsular periphery. This graded restriction of bone remodeling is most likely caused by the anti-resorptive action of the cytokine osteoprotegerin (OPG), which is expressed in high levels (1000 x normal bone levels) by inner ear structures to inhibit perilabyrinthine osteoclast formation and function. OPG knockout mice develop excessive, irregular bone remodeling, stapes fixation and progressive hearing loss. The lacunocanalicular porosity is the candidate anatomical routes for the transmission of OPG-derived signals to the surrounding bone. This extracellular signaling pathway depends crucially on the viability of individual osteocytes. When bone remodeling is low, the average age of the bone matrix and osteocytes increases. We detected a high fetal density of labyrinthine osteocytes, which may secure a life-long anatomical route for inner ear OPG despite accumulation of non-viable osteocytes. Moreover, 3-D reconstructions and vector-based stereology revealed a co-existence between non-viable osteocytes and otosclerosis. We suggest that bone remodeling may commence when the effect of anti-resorptive OPG fails locally within regions of non-viable osteocytes. A sustained OPG signal from surrounding osteocyte survivors might distort the process and account for the otosclerotic morphology.

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