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Exploring the genetic landscape of otosclerosis: current understanding and future perspectives

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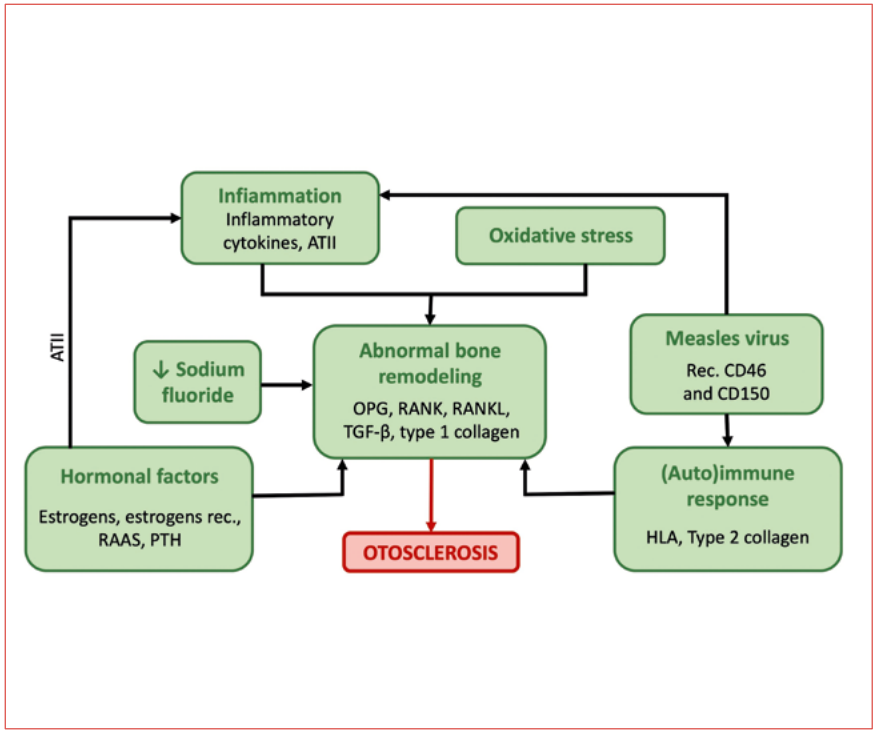

Cover figure. Model for the pathophysiology of otosclerosis. ATII: angiotensin II; OPG: osteoprotegerin; RANK: receptor activator of nuclear factor-kappa B; RANKL: ligand of RANK; RAAS: renin-angiotensin-aldosterone system; PTH: parathyroid hormone; HLA: human leukocyte antigen; TGF: tumour growth factor; CD: cluster of differentiation.

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Summary

Otosclerosis is characterised by abnormal bone remodelling in the otic capsule, leading to progressive hearing loss. Unlike many genetic disorders, the causative genes for otosclerosis remain largely unidentified despite extensive research using linkage analysis and genome-wide association studies (GWAS). Inheritance patterns in otosclerosis suggest a multifactorial model involving genetic predisposition and environmental triggers, a model applied to other common diseases, such as age-related hearing loss, coronary artery disease, and Alzheimer's disease. Linkage analysis has identified nine loci associated with monogenic forms of otosclerosis, yet the specific causative genes and variants remain elusive. Promising insights have emerged from GWAS, with strong associations identified for novel candidate regions, including the *RELN* gene. Recent studies using next generation sequencing have identified several candidate genes such as *SERPINF1, ACAN*, and *MEPE*. SERPINF1, encoding pigment epithelium-derived growth factor, is linked to regulation of angiogenesis in bone remodelling. ACAN, associated with the OTSC1 locus, encodes aggrecan a crucial component of the extracellular matrix in cartilage, showing a range of variants with varied

effect sizes and frequencies. MEPE, involved in bone homeostasis, has been significantly associated with otosclerosis in large family-based and case-control cohorts. While considerable progress has been made in identifying potential genetic contributors, the precise genetic architecture of otosclerosis remains to be fully elucidated. An integrated approach combining genetic data and clinical information, such as audiometric testing and temporal bone imaging, is essential for a comprehensive understanding of otosclerosis.

Key words: genetics, otosclerosis, genome-wide association studies, susceptibility, next generation sequencing

Introduction

Otosclerosis is a disease of bone remodelling that results in localised bone dysplasia within the otic capsule, causing progressive conductive hearing loss in 90% of affected patients, with a sensorineural or mixed component in the remaining 10% [1]. Reports of hereditary conductive hearing loss, consistent with clinical otosclerosis, date back to the late 19th century [2]. Subsequent studies have identified patterns of inheritance, including autosomal dominant with incomplete penetrance (about 25-40%) [3] and a digenic pattern [4]. Nevertheless, families exhibiting a clear Mendelian-like autosomal dominant inheritance of otosclerosis are uncommon, as the majority of cases of otosclerosis with a positive family history (50-60%) do not adhere to clear Mendelian patterns, while the remainder (40-50%) are sporadic with no family history of the disease [5].

Among the numerous models to explain the inheritance patterns of otosclerosis, the most likely explanation lies in complex (multifactorial) inheritance, entailing a combination of genetic susceptibility genes and environmental factors, and their interactions, a model proposed for many other diseases, such as age-related hearing loss, Alzheimer's, and coronary artery disease [1]. However, monogenic and complex inheritance models should not be considered as separate entities, but as part of a continuum spectrum where the different genetic variants can be ordered, ranging from very rare ones with a large size effect and an almost perfect Mendelian segregation, to common variants with low effect sizes that act as susceptibility factors in a more complex and multifactorial aetiological pattern. In this model, genetic susceptibility factors interact with environmental triggers to generate the pathological phenotype. Among the possible environmental risk factors for the development of otosclerosis, infection with measles virus, low sodium fluoride in drinking water, and endocrine factors are under investigation (Cover figure).

Epidemiologically, the incidence of otosclerosis is higher in Caucasian patients of European descent, showing a prevalence of 0.2-1% in the general population [5], whereas it is rare among Africans, Asians, and American Indians [6]. This disparity may reflect differences in genetic contributions and environmental risk exposures. Additionally, otosclerosis is more prevalent in females than males, at a ratio of about 2:1. This observation suggests a potential role for sex hormones in the development of pathologic otic capsule dysplasia.

Bone remodelling is a fundamental biological process that is essential for repairing bone damage, preventing the accumulation of aged bone – which may lose its flexibility and become brittle – and creating a reservoir for calcium and phosphorus. The efficacy of bone remodelling lies in the dynamic equilibrium between bone resorption and deposition. This balance is achieved through the spatially coordinated, integrated, and sequential actions of osteoclasts and osteoblasts, which collectively form a basic multicellular unit. This process is tightly regulated by various factors, including osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B (RANK), and its ligand (RANKL) [7].

Mesenchymal-derived bone-forming osteoblasts express and secrete RANKL in response to a variety of hormones, cytokines, and mechanical stimuli. Upon secretion, RANKL binds to its receptor RANK on monocyte progenitor stem cells (MPC), initiating their differentiation into mature, active osteoclasts. These osteoclasts, which fuse into multi-nucleated cells, are responsible for bone resorption by secreting lysosomal enzymes, such as collagenases, and hydrochloric acid, capable of dissolving hydroxyapatite, one of the main acellular components of bone tissue. The activation and differentiation of osteoclasts through RANKL are neutralised by the decoy receptor OPG, also secreted by osteoblasts [8]. Conversely, bone repair is initiated by osteoblasts that secrete the osteoid seam in the lacunae created by osteoclasts. Calcium and phosphate ions then deposit in the form of hydroxyapatite, effectively trapping the secreting osteoclasts, which differentiate into osteocytes. An imbalance of the OPG/RANKL ratio has been implicated in various bone disorders, such as osteoporosis, rheumatoid arthritis, and bone metastases [9], while inactivating mutations in the OPG gene have been demonstrated to cause juvenile Paget's disease of bone [10].

The unique biology of otic capsule

The dense tissue of the petrous temporal bone that surrounds the membranous labyrinth of the inner ear is known

as the osseous labyrinth or otic capsule. It has long been recognised as a histologically unique bone, characterised by the highest bony density in the body [11], and a rate of growth, modelling, and remodelling that is minimal compared to other bones, and virtually absent close to inner ear spaces [12-14]. The primary foetal bone in the otic capsule is compact, highly mineralised [15], and formed by endochondral ossification, a process that involves a cartilaginous precursor which is resorbed and replaced by dense lamellar bone. Bone turnover rates in the adult temporal bone increase centrifugally from 0.1%/year in the innermost perilabyrinthine zone – where epifluorescence demonstrated a persistence of early foetal bone around the inner ear spaces in a rabbit animal model [16] – to about 10%/year at the capsule periphery, a rate comparable to other bones in the body [13,16,17]. These observations suggest the existence of a local inner ear mechanism that inhibits perilabyrinthine bone resorption and remodelling.

In 2010, Stankovic and colleagues [18] used real-time quantitative polymerase chain reaction (RT-PCR) and in-situ hybridisation to compare gene expression between the healthy adult murine otic capsule and other bones in the body, particularly the tibia (formed by endochondral ossification like the otic capsule) and the parietal bones (formed directly by intramembranous ossification without an intermediate tissue). They found that the molecular profile in the otic capsule significantly differs from the other bones, involving a reduction of pro-inflammatory cytokines, an increased expression of anti-inflammatory cytokines, and a distinct pattern of expression of bone morphogenic proteins (BMP). The key predictors of otic capsule bone were OPG, bone morphogenic protein receptor 1B (BMPR1B), and bone morphogenic protein 3 (BMP3).

The increased expression of OPG is the most characteristic marker of the otic capsule, as established in numerous studies [19-23], and is expressed 1600 times more in the spiral ligament and 800 times more in the fluid-filled inner ear space than in other bones in the body [19] and associated with a marked inhibition of bone remodelling within the otic capsule. OPG is expressed at high levels by cochlear neurons of the inner ear and then diffuses into the surrounding otic capsule, being a central regulator not only of bone resorption inhibition but also of neurite growth stimulation. A possible explanation for the central role of the OPG-RANK-RANKL pathway in the dynamic interaction between the skeletal and nervous systems is provided by the hypothesis that RANKL may activate the expression of neurite growth inhibitor A (NOGO-A), which, when expressed, has been associated with a dramatic shortening of spiral ganglion nuclei neurites [23,24]. Therefore, the competitive inhibition of RANKL by OPG has the potential to inhibit bone remodelling and favour nerve growth, aiming to prevent nerve compression by bone growth in the otic capsule. Conversely, during the early postnatal development of the inner ear, bone remodelling is active in otic capsule development and nerve growth is inhibited [23]. OPG knockout mice (OPG-/-) demonstrated a degeneration of the cochlear nerve with progressive sensorineural hearing loss (SNHL) that was superimposed on a form of earlier conductive hearing loss due to resorption of ossicles in the middle ear [19,21,25]. The mechanism of apoptosis caused by the loss of OPG in spiral ganglion neurons likely involves the ERK signalling pathway, an important regulator of myelination that makes neuron cells more sensitive to oxidative stress [25]. In OPG-/-, this process can be rescued with medical therapies involving the administration of exogenous OPG, ERK inhibitors, or bisphosphonates [25]. Alterations in the expression of OPG have been associated with diseases such as osteopetrosis [26], osteoporosis [27], otosclerosis [19,28], juvenile and adult Paget's disease of bone [29], and celiac disease characterised by high-turnover osteoporosis [30].

BMPs play an essential role in skeleton development and repair, and have been reported to be important for otic capsule formation and maintenance of the membranous labyrinth [31,32]. Conversely, inhibition of BMP by noggin has been associated with a loss of the otic capsule and membranous epithelium in an avian model [32]. BMPR1B is histologically unique to the otic bone, characterised by endochondral formation, while it is absent in parietal bones, intramembranous bones whose development does not involve a cartilage intermediate [18]. A lower expression of BMP3 is characteristic of the otic capsule compared to other bones [18]. It is the most abundant BMP in adult trabecular bone, being a negative regulator of bone density [33]; considering that the otic capsule is the densest bone in the body, the down-regulation of BMP3 is coupled with the reported increase in bone density. However, when comparing BMP3 knockout mice [33] with BMP3 over-expressor mice [34], no gross abnormalities within the otic capsule or the cochlear membranous labyrinth were reported, concluding that BMP3 has a minimal role in controlling bone remodelling in the otic capsule [18].

The downregulation of inflammation is a key molecular feature of the otic capsule, and is possibly important in the maintenance of normal hearing. Proinflammatory cytokines in the cochlea are generally considered markers of disease and have been associated with hearing loss, with anatomical communication between the otic capsule and the cochlea perilymph [19], and with pathological new bone for-

mation within the membranous labyrinth (labyrinthitis ossificans) [35]. Consistently, when comparing the healthy adult murine otic capsule and other bones in the body, Stankovic and colleagues [36] reported a lower expression of pro-inflammatory cytokines (TNFα, IL1α, IL1β, IL6, NFKβ1) and an increased expression of anti-inflammatory cytokines (particularly IL11) in the otic capsule.

Monogenic and familial forms of otosclerosis

Gene identification in otosclerosis depends on the mode of inheritance, distinguishing between confirmed familial (possibly monogenic) cases and those with a complex (likely multifactorial) inheritance pattern. For large families with numerous members affected, gene identification relies on linkage analysis to pinpoint the disease-causing variant by highlighting the chromosomal region shared by all affected individuals within a family. The effectiveness of this method hinges on the size and structure of the family selected for study. Typically, hundreds to thousands of genetic markers, which segregate in a Mendelian fashion, are analysed in a chromosomal region common to all affected family members, effectively acting as a monogene. Once the region of interest is identified, it is possible to refine the candidate loci using additional markers or genetic databases, and suspected genes can be examined through mutation analysis. Although this approach has identified nine different loci (Tab. I), pinpointing causative genes has been challenging, with only two candidate genes: T-cell receptor beta (TRB locus) identified in the OTSC 2 region on chromosome 7 [36] and FOXL1 recently identified on chromosome 16 in OTSC 11 locus [37]. However, even in this instance, the variants responsible for the disease remain unidentified.

OTSC 1

The first locus associated with familial otosclerosis was reported by Tomek and colleagues in 1998 [38]. In their analysis, they explored the impact of age on disease progression, noting that the sensorineural component of HL worsened in older subjects, whereas conductive HL did not significantly differ between younger and older subjects. Further genetic linkage analysis using short tandem repeat polymorphisms (STRPs) yielded a maximum multipoint logarithm of odds (Lod) score of 3.4 [38]. The Lod score, a statistical method used to assess linkage, evaluates the likelihood of a given sequence of genetic events occurring if the loci are linked as compared to its occurring if the loci are not linked. Linkage is considered to be established if the Lod score exceeds 3. Subsequent genetic analyses restricted the linked region to a 14.5 centimorgan (cM) segment between the far and near centriole segments of the long arm of chromosome 15 (D15S657). This region contains the gene for aggrecan (ACAN), the primary non-collagenous component of the cartilaginous extracellular matrix [39,40].

OTSC 2

The OTSC 2 region on chromosome 7 harbours some known genes, such as TIF1a (transcription intermediary factor 1-alpha), and PLOD3 (procollagen-lysine, 2-oxyglutarate, 5-dioxygenase 3)41. TIF1 is a growth suppressor required for the activity of retinoic acid, which has been shown to disrupt the development of the otic capsule [42]. PLOD3 takes part in the biosynthesis of collagen, and in vitro expression studies have shown that PLOD3 hydroxylates lysyl residues in collagen sequences in non-triple-helical conformation. Moreover, PLOD3 activity is enhanced by tumour necrosis factor-alpha (TNFα), which is a key mediator in the pathogenesis of arthritis, causing cartilage degradation and joint destruction [43]. Moreover, the region harbours T-cell receptor beta (TRB locus), which is one of the candidate genes for which evidence of association with otosclerosis was provided by Schrauwen and colleagues [1,36], describing a lower mRNA expression of TCR-beta and a decreased percentage of circulating TCR-alpha/beta-positive T cells in patients with otosclerosis linked to OTSC2 compared to controls and patients with a complex form of the disease. Further analysis showed significant disturbances in specific T-cell subsets, including an increased population of CD28null T cells in OTSC2 patients, which are considered senescent cells and whose higher proportion may indicate an altered T cell development or aging in OTSC2 patients. Overall, these findings may contribute to elucidate a possible immunological contribution to the development of otosclerosis. However, the pathological TCR-beta variant responsible for this phenotype could not be identified.

OTSC 3

The region 6p21.3-22.3 on chromosome 6 (identified as OTSC 3) includes candidate genes such as RING1 and COL11A2 [44]. Overall, these genes may provide insights into the association of otosclerosis with collagen abnormalities. RING 1 together with Yin Yang 1 binding protein (RYBP) interacts with Yin Yang 1 (YY1) [45], a transcription factor activator of the COL1A1 (collagen type 1) promoter in fibroblasts [46]. An abnormal COL1A1 transcription may impact the normal stoichiometry of COL1A1 and COL1A2 in the

Table I. Loci associated with monogenic and familial forms of otosclerosis.

Locus	Position	Study	Investigated candidate genes	Family countries of origin
OTSC 1	15q25-26 (14.5 Mb)	Tomek et al., 1998	/	Southern India, Tunisia
OTSC 2	7q34-36 (16 Mb)	Schrauwen et al., 2010; Van Den Bogaert et al., 2001	TRB locus, ATP6V0A4, CLEC5A, EPHA1, EPHB6, HIPK2, KLRG2, LUC7L2, MKRN1, PIP, PRSS2, SSBP1, TRIM24, TRPV5, TRPV6	Belgium, England
OTSC 3	6p21.3-22.3 (17.4 Mb)	Ali et al., 2007; Chen et al., 2002	/	Cyprus, Tunisia
OTSC 4	16q21-23.2 (10 Mb)	Brownstein et al., 2006	/	Israel
OTSC 5	3q22-24 (15.5 Mb)	Van Den Bogaert et al., 2004	PCOLCE2, CHST2	The Netherlands
OTSC 7	6q22.3-6q23.3 (16.5 Mb)	Thys et al., 2007	COL12A1, COL9A1	Greece, The Netherlands
OTSC 8	9p13.1-q21.11 (34.16 Mb)	Bel Hadj Ali et al., 2008	TJP2, TRPM3, KLF9	Tunisia
OTSC 10	1q41-44 (26.1 Mb	Schrauwen et al., 2011	TGFB2, AGT	The Netherlands
OTSC 11	16q24.1 (9.96 Mb)	Abdelfatah et al., 2022	FOXL1	Canada

production of collagen trimers, as exemplified by osteogenesis imperfecta, a disease also characterised by fixation of the stapes footplate.

COL11A2 is a putative collagen-modulating element gene expressed in the otic capsule that causes autosomic dominant non-syndromic HL at the DFNA13 locus [47]. Moreover, the region contains human leukocyte antigens (HLA) genes, consistent with the reports of a significant association of certain HLA-A and HLA-B antigens with otosclerosis [48].

OTSC 4

The OTSC 4 region on chromosome 1649 involves several genes related to bone homeostasis or immune development, including members of the cadherin superfamily (transmembrane proteins that mediate cell recognition and cell-cell adhesion), of the conserved oligomeric Golgi (COG) multiprotein complexes, involved in intracellular membrane trafficking and expressed in the immune system, and of members of the DEAD (Asp-Glu-Ala-Asp) box proteins, involved in RNA transcription, translation, export and turnover, and ribosome and spliceosome assembly [49].

OTSC 5

The OTSC 5 region on chromosome 3 involves two candidate genes: procollagen COOH-terminal proteinase enhancer protein 2 (PCOLCE2) and carbohydrate sulfotransferase 2 (CHST2). The *PCOLCE2* gene product is a glycoprotein that binds the COOH-terminal propeptide of type I procollagen and is highly expressed in non-ossified cartilage in developing tissues. The *CHST2* gene product is a Golgi-associated sulphotransferase, with a possible role in intercellular communication. However, mutation analysis of the coding region and the intron-exon boundaries of both genes did not reveal any disease-causing mutation [50].

OTSC 7

The candidate gene in the OTSC 7 region in chromosome 6 is represented by COL12A1 (collagen type II alpha 1) which belongs to the fibril-associated collagens with discontinuous triple helices, and is expressed in the cochlea, while further mutation analyses failed to reveal any disease-causing mutation [51].

OTSC 8

Among the genes in the OTSC 8 region on chromosome 9,

Bel Hadj Ali and colleagues [52] described three possible candidates: tight junction protein 2 (TJP2), transient receptor potential cation channel, subfamily M, member 3 (TRPM3), and kruppel like factor 9 (KLF9). TJP2 belongs to the family of membrane-associated guanylate kinase (MAGUK) homologues, which take part in epithelial and endothelial intracellular junctions. TRPM3 is a cation-selective channel important for cellular calcium signalling and homeostasis and for osteoclast activity. KLF9 is a strong activator of activating enhancer binding protein 2 alpha (AP-2), which is a fundamental regulator of the mammalian craniofacial development.

OTSC 10

The region on chromosome 1 identified by Schrauwen and colleagues [53] and named OTSC10 contains 306 gene predictions, including two candidate genes: transforming growth factor beta 2 (TGFB2) and angiotensinogen (AGT), selected due to their known role in bone remodelling and on the basis of previously found associations with otosclerosis [51,55].

OTSC 11

In a recent study, Abdelfatah and colleagues [37] identified on chromosome 16 a novel OTSC locus (OTSC 11) in a Caucasian family of English extraction with a form of autosomal dominant otosclerosis who had previously tested negative for shared OTSC loci haplotypes and susceptibility genes (*COL1A1, COL1A2, NOG*), and for rare variants associated to the SERPINF1 gene. Sanger sequencing for 12 positional candidate genes identified an in-frame deletion in FOXL1 (NM_005250.3: c.976_990del) associated with the phenotype in the affected family, resulting in a significant loss of the protein's helical structure. FOX proteins are a superfamily of transcription factors with a wide range of functions at the junction of multiple signalling pathways, with crucial roles in regulating gene expression in cell metabolism, proliferation, differentiation, and apoptosis [56].

Unsuccessful sequencing of functional candidate genes

To identify candidate genes of monogenic forms of otosclerosis, direct sequencing of positional candidate genes has been applied. The selected genes were prioritised based on phenotypic similarities between otosclerosis and related diseases. For example, NOG mutations cause several syndromes which share the presence of stapes ankylosis [57], while COL1A1 and COL1A2 encode alpha chains of collagen type 1, and when mutated cause osteogenesis imperfecta [58], which is characterised by a form of HL resembling otosclerosis (progressive, developing from the second-third decade of life, often both conductive and sensorineural). However, despite the resemblance with otosclerosis, this approach did not identify any disease-causing mutation [58].
Despite the 20-year lapse since the mapping of OTSC1, the *OTSC* genes remain refractory to discovery due to the rarity of monogenic families, diagnostic challenges, and reduced penetrance. However, the application of new approaches (such as positional cloning and next generation sequencing, NGS) as applied in the recent work by Abdelfatah et al. [37]. on OTSC 11, may produce successful results in identifying all *OTSC* genes, which remains a fundamental step in clarifying the genetic landscape of otosclerosis.

Complex forms of otosclerosis

Although otosclerosis appears to follow a Mendelian-like autosomal dominant pattern in some isolated families, most hereditary forms of the disease result from a complex transmission. In these cases, no single genetic susceptibility factor is either necessary or sufficient to develop the phenotype; rather, it is the combination of all factors that is crucial. The typical research method to identify genetic variants of complex diseases involves candidate gene-based association studies using a case-control design. This approach, which identifies variants of selected genes that are significantly more frequent in patients than in matched controls, has been performed in numerous studies. Some genes have been found to be significantly associated with otosclerosis in more than one study, while associations with other candidate genes have not been replicated. However, lack of replicability does not necessarily rule out an association, as the sample size might have been inadequate, or different disease-causing variants may be present in different populations. The most recent alternatives to candidate gene-based association studies are genome wide association studies (GWAS) and microarray gene expression studies, which compare gene expression in diseased tissues to that in controls. With the latter method it was possible to identify different pathways to which otosclerosis susceptibility factors seem to belong, including bone remodelling and immunological, inflammatory, and endocrine pathways.

Altered bone metabolism

Collagens

The first study to hypothesise a common genetic basis between otosclerosis and osteogenesis imperfecta type 1

(caused by mutations in type 1 collagen) was published in 1998 by McKenna and colleagues [59]. They provided a rationale for this association based on the similarities in histopathology and HL features between the two diseases. This initial case-control genetic association study demonstrated a significant association of clinical otosclerosis with mutations of COL1A1 in a small population of European descendants in Massachusetts. These findings were later confirmed by the same group [60], which further identified an association with otosclerosis for polymorphisms in the first intron of the Sp1 binding site of COL1A1, a finding also recently reported by Zhang and colleagues [61]. In 2007, Chen et al. [62] confirmed these results and reported an association with polymorphisms that alter the binding of transcription factors regulating COL1A2, leading to an increase in COL1A1 homotrimers and a subsequent abnormal bone deposition in the otic capsule. Normally, collagen type 1 triple helices are composed of COL1A1 and COL1A2 in a 2:1 ratio, whereas COL1A1 homotrimers are rare. Associations of otosclerosis with COL1A1 have been reported in numerous studies [63,64], but not in others [65,66], and, more relevantly, were not reported in a meta-analysis of GWAS studies of otosclerosis in three population-based biobanks comprising 3,504 cases and 861,198 controls [67]. Instead, this meta-analysis reported a significant association with a subunit of collagen type 4 (COL4A2), a collagen form located in the basement membrane and highly conserved across species. Mutations in other subunits of collagen type 4 have been linked to Alport syndrome, which is characterised by progressive SNHL, nephritis, and histologically by an abnormal basement membrane and dysmorphogenesis of the organ of Corti. Future studies will need to determine the role of COL4A2 as a structural or signalling element in the context of otosclerosis.

Transforming growth factor-beta (TGF-β) superfamily

The TGF-β superfamily is composed by cytokines playing a crucial role in embryonic development and maintenance of the otic capsule [68], as demonstrated by its influence on the expression of glycosaminoglycans, fibronectin, and collagen in the extracellular matrix [69]. The most relevant member of the TGF-β superfamily in the context of otosclerosis is TGFβ1, a major osteogenic cytokine involved in regulating bone mass and bone matrix. TGFβ1 is expressed in the otosclerotic foci and the hyalinised spiral ligament [70]. It induces several processes in connective tissues, including the promotion of collagen type 1 and fibronectin formation [71], interference with potassium circulation by affecting fibrocytes in the spiral ligament [72], induction of chondrogenesis in the otic capsule mesenchyme, and the promotion of otic capsule growth during early stages of inner ear development [73]. A large association study [54] reported the Thr263Ile substitution to be significantly more expressed among otosclerosis patients than controls both in a Belgian-Dutch and in an independent French sample, and these results were replicated also in Tunisian [74], Hungarian [66] and British studies [67], but not in a black South African population [75]. Additionally, sequencing the exons and intron-exon boundaries of TGFβ1 in 755 patients with otosclerosis and 877 controls revealed three rare nonsynonymous variants in four patients [76], which were not present in the controls (c.G86A, p.Gly29Glu; c.G86C, p.Gly29Ala; c.C722T, p.Thr241Ile). An analysis in an Indian sample linked the c.-509C > T single nucleotide polymorphisms (SNP) with otosclerosis, as well as a specific G-T-T-G haplotype constructed from four SNPs [77]. A de novo mutation in the promoter region was also discovered in one patient, leading to decreased expression of TGFβ1 [77]. A study on proteomic analysis and immunostaining of temporal bones, conducted by Richard and colleagues [78], identified TGFβ1 as being expressed in patients affected by cochlear otosclerosis and hyalinisation of the spiral ligament.

The mechanism by which TGFβ1 contributes to the pathogenesis of otosclerosis remains unclear. However, one theory suggests that TGFβ1 may influence the globuli interossei within the otic capsule (residual cartilage rests within the dense bone of the otic capsule, a unique feature of the inner ear), potentially targeting these structures for an immune reaction that results in otosclerosis [78].

A meta-analysis of GWAS studies involving 3,504 cases and 861,198 controls [67] confirmed the association between TGFβ1 and otosclerosis, identifying the intronic variant rs8105161 as the strongest. Additionally, the analysis highlighted the potential roles of other genes in the TGFβ1 signalling pathway. These include RUNX2, a transcription factor essential for osteoblast and chondrocyte differentiation, regulated by TGFβ1 [79]. RUNX2 was found to be expressed only during the development of the otic capsule and not in its mature state. This is a critical finding, as otosclerosis may be triggered by globuli interossei, embryonic remnants within the otic capsule. Other significant genes are SMAD3 (a downstream transcription factor), CD109 (a TGFβ1 co-receptor acting as a negative regulator of the TGFβ1 pathway), LTBP3, which regulates the latency and activation of TGFβ1 through direct extracellular binding, and AHSG, which antagonises TGFβ1 signalling and directly affects the mineralisation process by inhibiting calcium phosphate precipitation. Mutations in AHSG can cause multiple-syn-

ostosis syndrome, which is characterised by stapes fixation, an otologic presentation that mimics otosclerosis [57]. In a microarray analysis of otosclerotic stapedial footplates and controls, Ealy and colleagues [80] identified two other genes within the TGFβ1 pathway that are significantly expressed in both groups: PF4, which selectively prevents TGFβ1 from binding to its type I receptor and may inhibit bone resorption if overexpressed by downregulating TGFβ1 signalling, and IBSP, which is influenced by TGFβ1 signalling in rats and has been found to be downregulated in otosclerosis, along with TGFβ1.

Bone morphogenic proteins

BMP2 and BMP4 also belong to the TGFβ1 signalling network and are crucial in various molecular processes, including bone homeostasis [81]. A study by Schrauwen [82] identified a correlation between otosclerosis and specific SNPs: rs3178250T > C in the 3' UTR of BMP2 and rs17563, p.(Val152Ala) in BMP4. These SNPs were analysed in an Indian population, with only the BMP4 SNP showing a significant association [64]. However, when these SNPs were examined in Tunisian and Hungarian populations, no association was found, likely due to insufficient study power [66,74]. Further research by Ealy et al. [83] in a German cohort found no link between common variants in BMP2 and BMP4 and otosclerosis, although 4 rare variants – including 2 missense mutations, one large deletion, and one synonymous variant – were exclusively found in affected individuals. Functional assays revealed that the large deletion in BMP2 and the missense mutation p.(Asn150Lys) in BMP4 led to decreased Smad receptor phosphorylation [83]. Additional studies in an Indian cohort demonstrated elevated levels of BMP2 and BMP4 in otosclerotic stapes tissues, reinforcing the involvement of these proteins in otosclerosis [64].

TNFRSF11B

The gene *TNFRSF11B* is responsible for coding OPG, which acts as a decoy receptor for the RANKL. Research has highlighted OPG's involvement in otosclerosis, with studies showing reduced OPG mRNA expression in stapes tissue from patients compared to normal tissue [8].

Alterations in the expression of OPG have been associated with diseases such as osteopetrosis [26], osteoporosis [27], otosclerosis [19,28], juvenile and adult Paget's disease of bone [29], and celiac disease characterised by high-turnover osteoporosis [30].

In genetic studies focusing on otosclerosis, a SNP in TNFRSF11B, rs1485286, displayed marginal significance in a Belgian-Dutch male population (p value 0.049) [82]. Meanwhile, analysis of 12 Italian patients from otosclerosis-affected families did not reveal pathological mutations; however, the polymorphism rs2073618 was present in 10 of these patients. Further sequencing of the polymorphism in 98 unrelated patients did not show an association with otosclerosis [84]. However, in an Indian male population, SNP rs2073618 was significantly linked to otosclerosis [8]. Meta-analyses incorporating data from Italian and Indian populations affirmed the association of this SNP with the condition [8]. A subsequent meta-analysis involving Tunisian, Indian, and Italian samples also supported this association [85].

Role of inflammation and oxidative stress

The molecular profile of the otic capsule is markedly different from other bones in the body, characterised by a down-regulation of proinflammatory cytokines, and an up-regulation of anti-inflammatory cytokines [18], to the point that proinflammatory cytokines in the cochlea are generally considered markers of disease and have been associated with HL, with anatomical communication between the otic capsule and the cochlea perilymph [19], and with pathological new bone formation within the membranous labyrinth (labyrinthitis ossificans) [35]. TNFα and its receptor were reported to be over-expressed during active otosclerosis [86], and because TNFα promotes bone resorption, it may act as a potential catalyst for the dysregulation of bone metabolism in otosclerosis, and may also be a potential contributor to the development of SNHL in otosclerosis [87]. Moreover, angiotensin II is a key regulator element for the production of proinflammatory cytokines, it has been reported to be a key factor for inflammation and bone remodelling in otosclerosis, providing a link between the inflammatory and the endocrine pathways in the context of the disease [88].

Oxidative stress has the potential of impacting several cell signalling pathways, and it has been linked to other forms of HL. In otosclerotic patients, immunohistochemical studies have demonstrated an increase in 4-hydroxynonenal (HNE)-protein adducts in comparison with controls. 4-HNE protein adducts are a major bioactive marker of lipid peroxidation which act also as second messengers of free radicals. Although 4-HNE protein adducts were also present in control samples, the primary difference lies in their distribution: they are confined to the periosteal region in controls, whereas in otosclerotic samples HNE-product positive areas are multifocal and irregular [89].

Role of the immune system

It has been suggested that the immune system, particularly an autoimmune reaction targeting the otic capsule, might play a significant role in the development of otosclerosis, and is supported by the fact that immune cells and immune-regulatory factors were discovered in the regions impacted by otosclerosis [87]. Initial studies speculated that an autoimmune reaction against type II and other less prevalent collagens could be a potential trigger for the disease [90]. The COL2A1 gene, which codes for type II collagen, was targeted for study because this type is plentiful in the globuli interossei, and it has been linked to localised chondrodysplastic lesions [91]. Nonetheless, further research involving genetic studies, histological examinations, and immunohistochemical tests have failed to confirm the hypothesis that an autoimmune reaction to collagen is the leading cause of otosclerosis [92].

Additionally, it has been postulated that otosclerosis may be significantly influenced by an autoimmune response initiated by persistent infection with measles virus [87], though definitive proof is still pending.

HLA is an essential part of the human major histocompatibility complex, crucial for presenting antigenic peptides to T cells and controlling the immune response. HLA has been linked to various diseases with an immunologic basis. Although some studies have found associations between certain HLA antigens and otosclerosis, these associations have not been consistent across various studies [87]. Nevertheless, the evidence suggesting a significant relationship between HLA and otosclerosis points to an immunological component in the disease, with certain HLA markers potentially affecting susceptibility within specific populations [87]. Further investigation is needed to confirm any substantial connections between HLA antigens and otosclerosis.

Role of the endocrine system

Oestrogen

Numerous studies have explored the influence of the endocrine system on the development of otosclerosis, particularly given its more frequent occurrence in females and reports of its manifestation or progression during pregnancy. However, the link with pregnancy is still subject to debate. For instance, a retrospective study did not establish a correlation between the number of pregnancies or children and the progression of otosclerosis-induced HL [93]. Proposed mechanisms include the possibility that variants of the estrogen receptor might mediate abnormal bone remodelling in response to oestrogen. Furthermore, oestrogen promotes hyperprolactinaemia, which has been linked to increased bone resorption. This process could counteract the effects of oestrogen itself on bone by diminishing the osteoclast response to RANKL, thereby reducing bone resorption [94].

Renin-angiotensin-aldosterone (RAA) system

The RAA system regulates blood pressure, but is also involved in bone resorption and formation, and specifically angiotensin II has been implicated in key events of inflammation and bone remodelling by its interaction with various growth factors and cytokines [88,89]. The hypothesis that the RAA system plays a role in the development of otosclerosis may have been influenced, to some extent, by the observed activation of this pathway during pregnancy [95], coupled with the notion that otosclerosis often appears during or soon after pregnancy. In a 2008 candidate gene-based association study on a French population, polymorphisms in the angiotensin and angiotensinogen-converting enzyme genes associated with higher plasma concentrations of angiotensin II were associated with an increased relative risk of developing otosclerosis [55]. Moreover, the same study reported that angiotensin II enhances the secretion of interleukin 6 (IL6) and reduces alkaline phosphatase activity in vitro exclusively in otosclerotic cells, indicating that angiotensin II may play a part in disrupting bone remodelling processes, contributing to the onset of otosclerosis [55]. However, these results were not replicated in another candidate gene-based study in a large Belgian-Dutch population [96], and in a study on a Hungarian population [66].

Parathyroid hormone (PTH)

PTH is secreted by the parathyroid glands in response to decreased blood calcium levels, stimulating osteoblasts to release RANKL, which in turn increases bone resorption and liberates more free calcium into the blood [94]. Given the significant role that PTH plays in bone metabolism, its involvement in the development of otosclerosis has been suggested. Research has shown that higher concentrations of PTH are necessary to enhance adenylate cyclase activity [97]. Additionally, in otosclerotic stapes cell cultures, there is a decreased expression of PTH-PTH-related peptide receptor mRNA accompanied by a reduced cyclic AMP response [98]. These findings suggest that a dysfunctional response to PTH may contribute to the abnormal bone turnover observed in otosclerosis.

Vitamin D receptor

Vitamin D stimulates intestinal absorption of calcium, which is associated through an increase in calcitonin, with a decrease in bone resorption [99]. Due to their role in bone

metabolism, vitamin D and its receptor were proposed as contributing factors to the development of otosclerosis. Yildirim and colleagues [100] genotyped four polymorphisms of the vitamin D receptor gene in a small Turkish population, and found that 3 (Bsm I-rs1544410, Apa I-rs7975232, and Taq I-rs731236) were associated with otosclerosis. However, these results have not yet been replicated in a larger cohort, and therefore it is not possible to draw conclusions on the possible role of vitamin D receptor in the pathophysiology of otosclerosis.

Measles virus

Over the past 30 years, many authors investigated the potential role of measles virus in the development of otosclerosis. The first account of this theory was published in 1986 by McKenna and colleagues [101] who identified filamentous structures resembling measles virus nucleocapsid in osteoblast-like cells in otospongiotic tissue specimens. Over the years, several techniques have been used to investigate this association, including electron microscopy, immunohistochemistry, perilymph analysis, reverse transcription polymerase chain reaction (RTPCR), reverse transcription-quantitative polymerase chain reaction (RTQPCR), and glyceraldehyde 3-phosphate (GADP) to detect mRNA of measles virus in otosclerotic stapes and control samples [102-105]. However, other studies could not find evidence of measles virus or of a reaction to it in otosclerotic samples [106,107]. The organotropism demonstrated by the measles virus for the otic capsule in humans and primates is due to the complementary cell surface structures CD46 and CD150, which act as virus receptors [108,109]. Some studies showed the existence of novel splice variants of CD46 present exclusively in otosclerotic stapes footplates [110-112]. An unresolved question lies in the temporality of this relationship: does measles virus trigger the production of new CD46 splicing variants, or do pre-existing unique isoforms enhance virus affinity and facilitate virus replication? The solution might be found in the activity of various regulatory proteins for alternative splicing, which result in distinct expression patterns and modified functions of CD46. In a study by Schrauwen and colleagues [82], 2 (rs2796267 and rs2796270) out of 7 SNPs in CD46 were significantly associated with otosclerosis in males of Belgian-Dutch origin, but the results were not replicated in a French population analysed in the same study. However, the exact role of measles virus in the pathogenesis of otosclerosis and the contribution of genetic factors to this process remain to be described.

Genome wide association studies

One of the limitations of association studies lies in the fact that candidate genes to be tested must be selected in advance based on their potential role in the pathophysiology of otosclerosis. Different from association studies, GWAS is free from the need of hypotheses formulated in advance, and can therefore identify genes associated with the disease that had not been previously considered. GWAS aim to identify association between genetic variants and specific phenotypes (in this case, clinical otosclerosis) by surveying the genome of individuals affected by the disease and controls, and looking for genomic variants that occur more frequently in cases than in controls.

The first GWAS was published in 2009 by Schrauwen and colleagues [113], performed in a population of 1,149 Belgian, Dutch, and French patients and 1,174 matched controls, and identified two regions on chr7q22.1 and chr11q13.1 associated with otosclerosis. The chr7q22.1 region is located near the RELN gene and harbours an intronic SNP rs3914132 found to be strongly associated with the disease. In the same study, a notable correlation with otosclerosis was identified for the SNP rs670358 located on chromosome 11q13.1, a finding that was later confirmed in two separate subpopulations. Additionally, another SNP in this region, rs494252, showed a significant link to otosclerosis in a work by a Tunisian group [114]. This SNP is positioned intronically within the CDC42BPG gene and close to EHD1 and MEN1 genes. Both EHD1 and MEN1 are known to be important in the development of bone and cartilage [115,116]. However, no subsequent research has definitively shown that these genes play a role in the pathogenesis of otosclerosis.

In 2023, Rämö and colleagues [67] published the largest meta-analysis of GWAS on otosclerosis, utilising data from 3 population-based biobanks that comprised 3,504 cases and 861,198 controls. This study identified 27 risk loci, 23 of which were new, and confirmed associations with otosclerosis for the *RELN* gene and 3 previously reported candidate genes or linkage regions: TGFβ1, MEPE, and OTSC7. Most of the loci identified were situated near protein-coding genes that are implicated in bone remodelling and mineralisation, which are already associated with severe skeletal disorders such as diaphyseal dysplasia (TGFβ1) and osteopetrosis (CLCN7, TNFSF11).

RELN

The region chr7q22.1, which includes an intronic SNP rs3914132 within the *RELN* gene, has been consistently identified as having a strong link with otosclerosis, from

the first GWAS [113] to subsequent studies across diverse populations [114,117-120]. However, some studies lacked sufficient power to reliably detect this association [66,119]. Priyadarshi et al. [117] conducted a meta-analysis using a cumulative population from multiple studies, comprising 2,670 cases and 2,812 controls, and reported a significant association of the rs3914132 polymorphism with otosclerosis across different populations and genetic models.

The *RELN* gene, responsible for producing the reelin protein, plays a crucial role in neural migration and positioning in the developing brain. Reelin is produced exclusively by neural tissues and is implicated in the development of several neurological disorders [121]. Disruptions in reelin signalling have been reported in diseases such as bipolar disorder, schizophrenia, and autism [122-124].

The role of reelin in bone metabolism remains largely unclear. However, research by Dou and colleagues [125] on multiple myeloma suggests that reelin significantly influences bone formation and the balance between osteolysis and osteogenesis. High levels of reelin are expressed by osteocytes, the bone's mechanosensing cells [126], and the protein may contribute to the mechanosensory adaptation mechanism of bone remodelling, as it is detected with elevated expression in limbs compared to skull bones [127]. Moreover, distinct expressions of reelin have been observed in the stapes tissues of humans with otosclerosis [120], and a recent study demonstrated the role of RELN variation in familial ankylosing spondylitis, further strengthening the role of this gene in disorders of bone remodelling [128]. However, the exact mechanism of RELN in the pathogenesis of otosclerosis remains unclear.

Next generation sequencing

By massive parallel DNA sequencing (sequencing millions of fragments simultaneously per run), NGS allows a high-throughput sequencing of the complete genome, the exome (meaning all coding regions), or selected custom-panels of genes. By being relatively fast and cost-effective, this approach has broken down the limitations associated with sequencing only targeted genes, and has allowed the identification of new genes involved in the pathogenesis of otosclerosis: *SERPINF1, ACAN*, and *MEPE*.

SERPINF1

In 2016, Ziff and colleagues [129] identified multiple missense mutations in the serpin peptidase inhibitor-clade F (SERPINF1) gene using various genetic techniques, including whole exome sequencing (WES), on 4 families that exhibited an autosomal dominant inheritance pattern of otosclerosis. SERPINF1 encodes pigment epithelium-derived growth factor (PEDF), a potent angiogenesis inhibitor and a known regulator of bone remodelling. Angiogenesis is a key feature of otosclerosis, and is associated with both Schwartze's sign and the increased promontory blood flow observed in Doppler flowmetry [130]. Moreover, mutations in SERPINF1 are linked to the rare type 4 osteogenesis imperfecta, another bone remodelling disorder [131]. However, a larger study conducted in 2019 by Valgaeren et al. [132] on 1,604 unrelated patients, 62 probands from large families, and 1,538 controls, only found 3 missense variants previously reported by Ziff et al. [129] (c.167C > G, c.331G > A, c.392C > A) in 5 patients and 4 controls. Familial analysis identified 12 variants in all affected family members; however, these variants were also frequently found in the control population, which complicates establishing a pathogenic role. None of the variants reported by Ziff and colleagues [129] were found in any of the 62 large families studied. Additionally, a study by Richard et al. [78] in 2015 using proteomic analysis and immunostaining found decreased expression of SERPINF1-012 in otosclerosis patients, with or without SERPINF1 mutations; however, these results have not been replicated.

ACAN

The ACAN gene, located at the OTSC1 locus, encodes aggrecan, the primary non-collagenous component of the cartilaginous extracellular matrix, which is essential for cartilage function and skeletal development [38]. The potential role of ACAN in otosclerosis was first suggested by Dawson in 2018 at the Molecular Biology of Hearing and Deafness meeting. He reported the findings from 19 probands from large otosclerosis families who were tested using WES, followed by targeted NGS of 61 candidate genes in 160 familial otosclerosis patients. Notably, more than 20% of these patients carried rare variants of the ACAN gene. In a subsequent study in 2021, Højland and colleagues [133] sequenced the entire *ACAN* gene – including all coding regions, exon-intron boundaries, and untranslated regions (UTRs) – in 1,468 unrelated patients, 29 familial cases, and 1,437 unscreened controls. This study identified 14 nonsense and missense variants. ACAN is distinguished by a remarkable spectrum of variants in terms of number, effect size, allele frequency, and direction of effect. Specifically, some variants showed strong effects but low frequency, resembling the transmission pattern of monogenic diseases, while others had minimal effect sizes and were more common, serving as susceptibility factors.

MEPE

The *MEPE* gene encodes a matrix extracellular phosphoglycoprotein with a role in bone homeostasis, suppression of renal calcification, and regulation of serum phosphate. In a study on a large Turkish family affected by hereditary congenital facial paresis and a mixed form of HL similar to otosclerosis, a mutation of the MEPE gene segregating with the phenotype was reported [134]. To confirm this finding, a large case-control study was performed, including 123 members from 62 families, 1,604 cases, and 1,538 controls. This study identified 6 heterozygous frameshift and nonsense variants in 19 patients and 3 unscreened controls, indicating a relatively low frequency of these variants in the population but a high effect size. These results were replicated in the large GWAS meta-analysis by Rämö et al. [67], which also reported the dynamic changes in MEPE expression throughout postnatal development in the murine inner ear by immunostaining, going from a more diffused expression at 2 days of life to a limited expression to mature osteocytes at 3 months of age.

Conclusions

Overall, the hereditary pattern in otosclerosis is predominantly complex, involving both environmental and genetic factors, a model already described for other relatively common diseases such as age-related HL and coronary artery and Alzheimer's disease. Unlike other genetic disorders, where linkage analysis, positional cloning, and GWAS have led to the identification of many causative genes, the genetics of otosclerosis remain largely unidentified. Linkage analysis of monogenic forms of otosclerosis has led to the identification of 9 loci, but the genes responsible and their variants have yet to be extensively described. So far, the most promising results have come from GWAS, which identified strong associations with novel candidate regions. The use of NGS to zoom into these candidate genes in search of causative variants has recently led to the identification of large-effect risk factors in MEPE [134] and variants with high variation in frequency and effect size in ACAN [133], highlighting the potential of this approach.

Apart from genetic studies, epigenetic analyses could provide valuable insights into the pathophysiology of otosclerosis by correlating the impact of environmental factors on local gene expression, as has been described in numerous other complex diseases. A limitation of this approach is the need to use samples of stapes tissue, in contrast with genetic studies that mainly use DNA drawn from blood samples. Additionally, future studies should include clinical data, especially audiometric testing and temporal bone imaging, for better correlation with genetics and pathophysiology, as most large databases have not included such information. An integrated approach, utilising various genetic and epigenetic techniques in conjunction with clinical data and possibly aided by new bioinformatic techniques, will likely provide a better understanding of the pathophysiology of otosclerosis.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

SC, FL, LB: conceived the initial idea for the narrative review and developed the structure of the article; GF, FF: performed the literature review and critically analyzed the sources. All authors contributed significantly to drafting the manuscript, revising it for important intellectual content, and approving the final version to be submitted. Each author has read and agreed to the published version of the manuscript.

Ethical consideration

Not applicable.

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