

Systematic Review

On Molecular Factors in Tympanosclerosis: A Systematic Review with Clinical Implications for Diagnosis and Treatment

Barbara Parol ^{1,*}, Julia Zapart ¹, Grzegorz Ciamciak ¹, Manuela Makiola ², Noemi Boguslawska ², Alicja Florczak ², Zygmunt Domagala ^{2,3} and Karolina Dorobisz ^{1,*}

¹ Department and Clinic of Otolaryngology, Wrocław Medical University, 50556 Wrocław, Poland; julia.zapart@student.umw.edu.pl (J.Z.); grzegorz.ciamciak@student.umw.edu.pl (G.C.)

² Division of Anatomy, Department of Human Morphology and Embryology, Wrocław Medical University, 50368 Wrocław, Poland; manuela.makiola@student.umw.edu.pl (M.M.); noemi.boguslawska@student.umw.edu.pl (N.B.); alicja.florczak@student.umw.edu.pl (A.F.); zygmunt.domagala@umw.edu.pl (Z.D.)

³ Walbrzych Branch, Wrocław Medical University, 58300 Walbrzych, Poland

* Correspondence: barbara.parol@student.umw.edu.pl (B.P.); karolina.dorobisz@umw.edu.pl (K.D.)

Abstract

Tympanosclerosis is a chronic middle ear disorder characterized by fibrosis, hyalinization, and calcification of the tympanic membrane and ossicles, which often results in conductive hearing loss. The objective of this systematic review was to synthesize current evidence on molecular pathways and genetic susceptibility factors contributing to the development of this condition. PubMed, Embase, Web of Science, and Google Scholar were searched up to December 2024. Eligible studies were original peer-reviewed articles in English investigating gene expression, genetic polymorphisms, or molecular signaling pathways in human or animal models. Risk of bias was assessed using standardized tools, and results were synthesized narratively due to heterogeneity. Twenty-five studies were included from 1815 screened records. Reported findings implicated inflammatory cytokines, such as TNF- α and IL-6, oxidative stress-related enzymes, including CAT, and iNOS, and bone remodeling pathways involving Wnt signaling, TGF- β 1, and osteopontin. Polymorphisms in *TLR4*, *NOS2* and *NAT2* were associated with increased susceptibility or severity. Evidence remains limited but highlights potential biomarkers and therapeutic targets.

Keywords: tympanosclerosis; genomics; gene expression regulation; inflammation; Wnt signaling pathway

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1. Introduction

Tympanosclerosis is a chronic pathological condition characterized by fibrous, calcified plaques with hyaline degeneration appearing within the tympanic membrane and the mucosa of the middle ear. These histopathological changes result in thickening and reduced elasticity of the affected tissues, which may lead to conductive hearing loss, depending on the extent of ossicular chain involvement [1–5].

This condition is a well-known consequence of chronic otitis media [6], which is one of the most common diseases in children. By age 3 years, 70% of children have experienced at least one episode of acute otitis media, with 33% experiencing it three or more times [7]. This condition can also develop secondary to the ventilation tube insertion

in the treatment of chronic secretory otitis media [8]. The prevalence of tympanosclerosis varies across populations and clinical settings. In a study involving 775 patients with chronic suppurative otitis media, tympanosclerosis was identified in 11.6% of cases [9]. Temporal bone studies have reported a prevalence of up to 14.1%, with the majority of cases found in individuals over 40 years [1]. In another series of studies, the incidence of tympanosclerosis ranged from 7% to 33% among patients with chronic middle-ear infections [10]. Myringosclerosis, which involves sclerotic changes restricted to the tympanic membrane without middle-ear involvement, is more common than tympanosclerosis, which involves the entire middle ear, and is reported unilaterally in 24% to 51% and bilaterally in around 40% to 60% of patients with chronic otitis media [11]. It can develop at any age and has been documented even in children as young as two years old [12]. Its incidence is highest in pediatric populations, largely due to the prevalence of secretory otitis media and the frequent use of ventilation tubes. Among children who underwent tube insertion, myringosclerosis was observed in 28% to 61% of cases [13], while general population estimates suggest a prevalence of 5% to 7% in children aged five to six years [14]. In contrast, intratympanic tympanosclerosis is more prevalent in adults—it has been reported that 90% of patients with this condition were over 30 years of age and had a history of ear disease spanning more than two decades [15]. Although myringosclerosis is considerably more common, it is a distinct condition limited to the tympanic membrane and should not be confused with tympanosclerosis, which involves the tympanic membrane, middle ear mucosa, and ossicular chain.

Tympanosclerosis is commonly associated with chronic inflammatory processes in the middle ear [3], which damage the layer of connective tissue. The risk factors of this condition have not been thoroughly investigated; however, it is widely considered to be a result of damage to the middle ear, such as acute or chronic otitis media, injuries, and surgical procedures, as well as a consequence of genetic predisposition, changes in immunological reaction, ongoing inflammation, and local changes in metabolism [9,16–20]. While it is a well-established risk factor, recent studies suggest a significant genetic component in its pathogenesis. Multiple genetic polymorphisms have been implicated in tympanosclerosis, particularly in genes regulating immune response, oxidative stress, bone metabolism, and extracellular matrix remodeling.

The aim of this study is to provide a comprehensive review of the current understanding of the genetic mechanisms underlying the development of tympanosclerosis. By synthesizing the existing literature on molecular and genetic contributors to tympanosclerosis, this review seeks to highlight the complexity of tympanosclerosis pathophysiology and underscore the potential of genetic markers for future diagnostic and therapeutic strategies. A better understanding of these mechanisms may pave the way for more precise interventions and improve outcomes for patients affected by this middle ear disorder.

2. Materials and Methods

2.1. Study Protocol and Registration

This systematic review adhered to a pre-designed protocol (see Supplementary File S1) and followed the rigorous standards of conducting systematic reviews outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (see Figure 1 for PRISMA 2020 flow diagram) (see Supplementary Files S2 and S3) [21]. The review protocol was not pre-registered. This article is a review and did not involve human participants or animals; therefore, Institutional Review Board approval was not required.

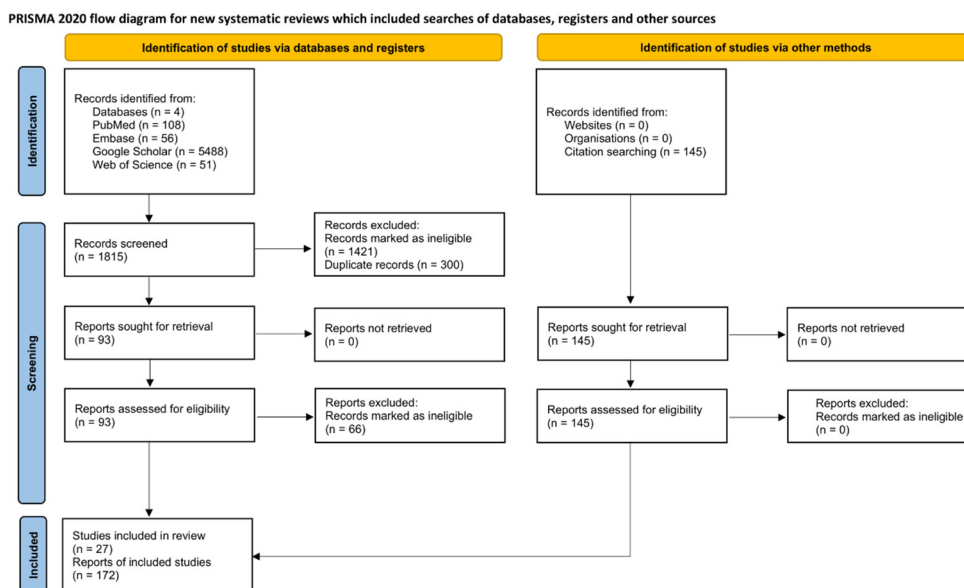


Figure 1. This PRISMA flow diagram visualizes the flow of information through the different phases of our systematic review [21].

2.2. Search Strategy

We searched all the literature on the changes in gene expression and gene expression profiles that can possibly result in the development of tympanosclerosis or the progression of this condition. We searched PubMed, Embase, Google Scholar and Web of Science up to December 2024 with the use of the following combination of keywords: ‘tympanosclerosis’ AND (‘genetics development’ OR ‘genetics factors’ OR ‘genetics etiology’ OR ‘gene factors’ OR ‘gene development’ OR ‘gene etiology’ OR ‘gene’ OR ‘expression’). All the results of the selected databases were revised, except for Google Scholar, where we have screened the first 200 results, which is sufficient according to the literature [22]. At this point of the review, we did not make any strict restrictions during the search and have been selecting all the works that seemed to include the scope of the study based on their title, abstract and keywords. Four authors independently screened all the articles found using abstracts, titles, and keywords. All references of the found studies were also screened; if any were deemed valid, we included them in our review.

2.3. Selection Criteria

To be included in the systematic review, the article had to meet the specific criteria as follows:

1. The record is an original, peer-reviewed and published study.
2. The full text is available.
3. The full text is in English.
4. The full text is relevant to the topic of our review—it describes or mentions genes, their modifications, molecules, enzymes, molecular processes and pathways that are connected to the development, progression and pathomechanism of tympanosclerosis.

Four independent reviewers screened the studies simultaneously; all the reviewers were familiar with the selection criteria and were instructed to include studies for which they needed clarification. A fifth reviewer was consulted to resolve any problems and to validate the selection results. Excluded records were compiled and summarized (see Supplemental File S4).

2.4. Risk of Bias Assessment and Critical Appraisal

To assess the risk of bias and conduct a critical appraisal of the studies, we have used the JBI's critical appraisal tools for case-control, cohort, and Randomized Controlled Trial studies [23], as well as the SYRCLE's Risk of Bias tool for experimental animal studies [24] (see Supplementary File S5). Two reviewers assessed the data using the tools. A third reviewer validated the outcomes. To minimize reporting bias, we searched multiple databases, without restrictions on publication date, and screened the reference lists of included articles. Only peer-reviewed studies published in English were included, which may have introduced language or publication bias. No assessment of reporting bias was performed, as few studies were included, and we did not perform statistical analysis.

2.5. Data Extraction

The data was extracted using a designed data extraction form (see Supplementary File S6). The data was extracted independently by four authors, then revised by the fifth author. We extracted all reported outcomes.

The primary data we collected was the first author's name, title of the work, year of publication, name of country where the study was conducted, type of study, eventual presence of interventions, aim of the study, type of collected samples, source of collected samples, affected or discovered genes, type of observed alterations, and results of those alterations, as well as the potential limitations and financing source. During the extraction and synthesis process, more precise additional data was searched for if needed. Any discrepancies were resolved by discussion between the team members until a conclusion was reached.

2.6. Outcome Analysis

This article is divided into four sections: introduction, materials and methods, results, and discussion. As no meta-analysis was conducted, no quantitative effect measures were calculated, and the results were synthesized narratively. We grouped the studies according to the molecular pathways they explore to structure the synthesis. No special data transformations were required, and the extracted results were presented as reported in the original studies. In the results, we discuss all the factors involved in the pathogenesis and/or pathomechanism of tympanosclerosis according to the reviewed studies, and their possible roles in this process. Summarized findings are supported with tables and descriptive pathway figures. The certainty of the evidence was not assessed, as most included studies were preclinical and heterogeneous.

3. Results

3.1. TNF- α and Inflammatory Progression

Since no single factor is solely responsible for inducing inflammation, researchers have investigated cytokines that might contribute to the transition from regular inflammation to tympanosclerosis. TNF- α , primarily produced by macrophages and mast cells, has been detected in patients with chronic serous otitis media [25,26].

Studies confirm that TNF- α expression in the tympanic cavity mucosa and tympanic membrane is significantly higher in tympanosclerosis-affected ears than in healthy ears, with expression levels correlating with disease severity [25–27]. A statistically significant association was shown between mRNA TNF- α levels and both the frequency of tympanostomy tube placement and the duration of secretory otitis media, while no such significance was shown between mRNA TNF- α levels and antibiotic use or episodes of acute otitis media [25,26]. TNF- α levels were significantly lower in patients with multiple tube insertions in comparison to patients with a single tube insertion [25,26,28]. Moreover, it

was observed that the longer the duration of secretory otitis media (lasting over three months), the higher the TNF- α levels. Antibiotic therapy and episodes of acute otitis media did not seem to have much influence on the TNF- α levels. These results indicate that TNF- α expression is increased in patients with chronic serous otitis media who are at risk of developing tympanosclerosis. The level of this cytokine is additionally influenced by the nature and duration of inflammation and by the presence of a ventilation tube, which may possibly allow for assessing the severity of patients' condition and prediction of tympanosclerosis risk [25,26].

3.2. Oxidative Stress and Enzymatic Antioxidant Defenses

Oxidative stress, which results from an imbalance between ROS production and antioxidant capacity, is a key factor in tympanosclerosis pathogenesis. Forséni et al. suggest that the expression of inducible nitric oxide synthase (iNOS) is a risk factor in the formation of tympanosclerosis. It was found that iNOS was more often present in tympanosclerosis than in secretory otitis media, and it was mostly expressed in mucosal surface cells. Cytokine presence during the inflammation process leads to the secretion of nitric oxide (NO) by osteoblasts via iNOS—NO reduces osteoclasts' activity and enhances bone remodeling processes taking place in tympanosclerotic tissue [29].

Forséni Flodin et al. have also explored the role of iNOS—its expression in rats was detected in the ear mucosa and tympanic membrane at days 3 and 6 since *Streptococcus pneumoniae* inoculation. Thereafter, iNOS levels were observed to gradually decline, becoming undetectable after three months, meaning that the expression of iNOS appeared much later in inflammation in acute otitis media than the simultaneously studied IL-6. The iNOS-presenting cells had the appearance of macrophages and were present in a similar location and quantity to the cells detected in the macrophage staining, and since the NO-producing enzyme can be expressed by induction in activated macrophages, it is possible that macrophages are activated continuously in the middle ear mucosa as a consequence of exposure to environmental antigens. Unlike IL-6, iNOS expression was high in tympanosclerosis cases, but absent in serous otitis media cases in human biopsies [30]. It remains unclear whether the absence of iNOS causes serous otitis media or whether the fluid associated with this condition inhibits iNOS production. Kucuktag et al. have also detected significant expression of iNOS in tympanosclerotic tissues of rats [31]. At the same time, the use of antioxidants, such as N-acetyl cysteine and ascorbic acid, was discovered to reduce the expression of iNOS, decrease inflammatory cell infiltration, and decelerate the development of myringosclerosis in guinea pigs treated with those substances. Significantly lower expression of VEGF and TGF- β was detected as well. Decreased expression of IL1- β was observed only in animals treated with N-acetyl cysteine [32].

In addition to the role of iNOS as an inflammatory mediator, it is closely associated with oxidative stress, which appears to play a crucial role in the pathogenesis of tympanosclerosis. Hyperoxia and inflammation contribute to the excessive generation of free oxygen radicals, which can induce irreversible injury, leading to hyaline degeneration and calcification in the middle ear [2,12,33,34]. Catalase (CAT) and NOS2 (gene encoding iNOS) polymorphisms were investigated as the key antioxidant enzymes, which may be particularly important in protecting tissues against oxidative damage. In this study, three groups were compared: patients with tympanosclerosis, patients with chronic otitis media and a control group. Among the analyzed variants, the present discussion focuses on the GG and AA genotypes of the NOS2 -277A>G and the TT genotypes of the CAT -21A>T polymorphism.

The NOS2 gene is located at chromosome 17q11.2-q12 [35,36]. The GG genotype of the NOS2 polymorphism was found to be more frequent in patients with

tympanosclerosis than in the control group (34.7% vs. 23.4%). The authors suggested that the AA genotype of the *NOS2* -277A>G polymorphism may be associated with an increased risk of tympanosclerosis compared to chronic otitis media [37]. The exact impact of this polymorphism on *NOS2* gene regulation and transcription remains unclear, but increased NO production has been implicated in the pathogenesis of tympanosclerosis. Karlıdağ et al. reported significantly higher concentrations of NO and malondialdehyde in the middle ear tissues of patients with tympanosclerosis compared to those without tympanosclerosis, indicating increased oxidative damage and iNOS involvement in the affected tissue [38]. Furthermore, in a rat model, Forséni et al. demonstrated increased immunohistochemical expression of inducible nitric oxide synthase (iNOS) in tympanosclerotic middle ear tissues. These findings support the hypothesis that nitric oxide plays a role in the inflammatory response and fibrotic changes characteristic of tympanosclerosis [39].

The TT genotype of the *CAT* -21A>T polymorphism in the promoter region, which can potentially reduce the transcriptional activity of the gene [40], was more frequent both in patients with tympanosclerosis (22.2%) and in patients with chronic otitis media (16.6%) than in healthy controls (8.5%). It has been hypothesized that the T allele may decrease catalase activity, weaken antioxidant defense mechanisms, and increase susceptibility to oxidative stress-related conditions, including tympanosclerosis [37]. These findings suggest that genetic variation in oxidative stress-related genes may affect susceptibility to tympanosclerosis.

Polymorphisms in the N-acetyltransferase 2 (*NAT2*) gene, whose products are involved in detoxification and management of oxidative stress, have also been studied in relation to tympanosclerosis. The analysis revealed that mutations in *NAT2*, particularly the *NAT2**6A mutant genotype, are linked to a significantly higher tympanosclerosis risk [41]. *NAT2* is a phase II xenobiotic-metabolizing enzyme that contributes significantly to defense against oxidative stress by reducing reactive oxygen species [42]. Genetic polymorphisms in *NAT2* can disrupt antioxidant balance. Two phenotypic variants of this enzyme—rapid and slow acetylators—have been identified [43]. The *NAT2**6A slow acetylator phenotype seems to correlate with an increased risk of developing tympanosclerosis when compared to control subjects [41].

3.3. PAI-1 in Early Stage Inflammation in Tympanosclerosis

Shin et al., using mice as a research material, have demonstrated a correlation between PAI-1 (type 1 plasminogen activator inhibitor), responsible for regulating inflammatory cell migration, and infectious otitis media with typical characteristics of tympanosclerosis [44]. According to their study, the expression of the *Serpine1* gene, encoding PAI-1, was significantly upregulated during the first hours of non-typical *Haemophilus influenzae* (NTHi) middle ear infection, which indicates that PAI-1 plays a crucial role in early stages of inflammatory processes in otitis media by being an acute phase response mediator. Furthermore, PAI-1 induces the migration of neutrophil cells to the site of an active inflammatory process. PAI-1 upregulates the expression of inflammatory genes and pro-inflammatory cytokines, such as TNF- α , IL-6, IL-10, IFN- γ , keratinocyte-derived chemokine, and macrophage inflammatory protein 2, in lungs [45]. Moreover, PAI-1 regulates infectious wound healing processes by inhibiting the *Plat* and *Plau* genes, encoding the tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) [46], respectively. It has been revealed that the *Plat* expression is highly upregulated in NTHi infection, with later rapid downregulation. On the other hand, *Plau* showed no significant upregulation of expression, but its expression was downregulated at a later stage of otitis media. The study has also shown that the deficiency of *Serpine1* in mice with no PAI-1 production resulted in a lack of effective inflammatory response in the early phase

of inflammation. The study was performed on mice with no *Serpine1* expression, and the results have revealed that the inflammatory response of these mice in an early stage of otitis media induced by NTHi was ineffective, leading to delayed resolution of inflammation. Additionally, the prolonged inflammation of the middle ear caused the development of pathological changes associated with tympanosclerosis [44]. All of that implies the positive role of PAI-1 in the otitis media inflammatory process, by inducing the expression of inflammatory mediators and migration of the neutrophils, and its role in the prevention of the development of tympanosclerosis.

3.4. Cathepsins in Mucosal Homeostasis and Inflammation

Recent studies have examined the role of the cathepsin family genes, which primarily encode lysosomal cysteine proteases involved in intracellular digestion, proenzyme activation, antigen presentation, bone resorption, and apoptosis [47,48] in the development of tympanosclerosis. It is hypothesized that the expression of the cathepsin genes *CTSK* and *CTSL* helps maintain middle ear mucosal homeostasis, while *CTSB* may act as an inflammatory factor in the pathway leading to acute otitis media. According to a study on rats, local epithelial cells may release *Ctsb* and other pro-inflammatory factors, contributing to lymphocyte migration, inflammatory mediator synthesis, and activation of the mitochondrial apoptotic pathway [47]. Evidence suggests that *CTSB* is present in exudates from both acute and chronic otitis media [49]. Its activity has been shown to correlate with inflammation severity, neutrophil recruitment, activation of pro-inflammatory caspases, and synergy with TNF in promoting apoptosis [50–52]. If the protease–inhibitor balance is disrupted and *Ctsb* expression remains elevated, middle ear mucosal regeneration may be impaired, leading to permanent pathological changes, such as tympanosclerosis [47].

3.5. Polymorphisms of TLR4 and Chronic Inflammation

The *TLR4* Asp299Gly polymorphism, characterized by the substitution of aspartic acid with glycine at the 299th amino acid position in the Toll-like receptor 4 (*TLR4*) gene [53–55], has been associated with increased susceptibility to recurrent otitis media and tympanosclerosis [56]. *TLR4* plays a crucial role in innate immunity by recognizing pathogen-associated molecular patterns and modulating inflammatory cytokine responses [54,55].

Studies have reported that individuals carrying the *TLR4* Asp299Gly variant exhibit an increased risk of invasive diseases, particularly during *Streptococcus pneumoniae* infections [57], due to altered immune responses. In the context of tympanosclerosis, the presence of this polymorphism has been linked to chronic inflammation, as it leads to reduced expression of key cytokines, including IL-6, IL-10, and TNF- α . This reduction contributes to prolonged inflammation, macrophage-to-osteoclast transformation, and, as a result, plaque formation characteristic of tympanosclerosis. Moreover, a higher frequency of the *TLR4* Asp299Gly polymorphism has been observed more frequently in patients with tympanosclerosis than in those without this condition, suggesting it may be a potential genetic predisposition and a triggering factor in tympanosclerosis, as it leads to an unregulated inflammatory response [58].

3.6. Interleukin IL-6 as a Contributor to Calcification

IL-6 and its receptors are known to mediate bone remodeling and osteoclast differentiation [30,59]. Moreover, according to a study on rats, IL-6 may be involved in calcium homeostasis and NO production; therefore, these two mediators are in close relationship with each other [60]. IL-6 is produced by various cell types, including macrophages and epithelial cells [61]. Previous research has demonstrated that inhibition of IL-6 production can mitigate the development of tympanosclerosis.

Inflammatory mediators that could lead to the development of tympanosclerosis were investigated in a study by Flodin et al. (2002) [30], based on a model of acute otitis media with development of tympanosclerosis in rats, biopsies from children with secretory otitis media, and patients with already-developed tympanosclerosis. In the rat acute otitis media model induced by an intentional inoculation of *Streptococcus pneumoniae* into the middle ear, IL-6 expression appeared very early. IL-6 mRNA was detected in the middle ear mucosa, and protein expression was observed as early as one hour after bacterial inoculation. In human tissues, IL-6 expression was higher in serous otitis media than in tympanosclerosis [30]. These findings suggest that IL-6 reaches its peak concentration early in the immune response and may initiate a cascade of reactions that lead to tympanosclerosis. This is supported by other studies that have demonstrated that tympanosclerotic lesions can form rapidly during the early stages of inflammation [30,62]. Moreover, macrophages were present in all groups: rat tissues with developed acute otitis media, human biopsies of secretory otitis media, and tympanosclerosis [30]. Macrophages have the ability to differentiate into osteoclasts, and, at the same time, IL-6 is involved in bone remodeling; thus, by increasing the RANKL expression in osteoblasts, IL-6 may indirectly affect osteoclastogenesis [59,63]. This is supported by another study on rats that reports macrophages differentiating into osteoclasts in the bullar bone during inflammation in tympanosclerosis [64]. Thus, it is possible that IL-6 expression peaking early in inflammation influences macrophages to trigger tympanosclerosis [30].

IL-10 was also analyzed in samples obtained from patients with chronic otitis media and tympanosclerosis—its expression was elevated by 50% compared with samples from healthy tympanic membranes. In the same samples, IL-6 expression was also elevated in tympanosclerosis, but by a much higher value of 80% [65]. It is believed that the outcome of the interleukins' activity depends on IL-6 and IL-10 balance in inflammatory tissue.

3.7. *TGF- β 1 in Inflammation, Fibrosis and Calcification*

Transforming growth factor β 1 (TGF- β 1) is a growth regulatory protein that participates in embryonic development, cell proliferation, immune response, and tissue repair, as well as collagen deposition [66–69]. There are some reports on how the upregulation of TGF- β 1 influences the frequency and prognosis of otitis media, which is widely believed to be one of the main causes of tympanosclerosis [70–73]. Some studies reveal an increased expression of the *TGF- β 1* (or *Tgfb1*) gene in tympanosclerotic tissues of both humans and guinea pigs [65,67]. Additionally, Guo et al. (2012) observed that the expression of TGF- β 1 was nearly undetectable in healthy tissue, while in tympanosclerosis it was significantly higher [67]. The expression of *TGF- β 1* is increased in patients with inflammation of the middle ear [74], as well as *Tgfb1* in rats with acute otitis media [75]. Furthermore, it was revealed that the increased expression of TGF- β 1 correlated with the course of the disease—not only was the level of TGF- β 1 significantly increased in the mucous membrane of the middle ear in tympanosclerosis, but also the longer the tympanosclerosis lasted, the greater the TGF- β 1 expression was [67].

Furthermore, several studies reveal that the upregulation of TGF- β 1 stimulates fibrosis in many parts of the body, which resembles pathological changes in tympanosclerosis [70–73], suggesting a connection between fibrosis and tympanosclerosis [76]. That hypothesis is confirmed by another study, where the LRRC25 (Leucine Rich Repeat Containing 25) expression and TGF- β 1 expression were examined. The *LRRC25* is a gene coding a protein responsible for, among other functions, protecting the heart from pathological remodeling by inhibiting NF- κ B activation, which is related to cardiac hypertrophy [77]. In the study, the loss of activity in LRRC25 was found to promote TGF- β 1 expression, which led to an increased activity of SMAD3, an intracellular mediator of TGF- β 1 signaling, resulting in cardiac fibrosis in mice. Suppression of TGF- β 1 expression was observed to

withdraw the fibrosis induced by the lack of LRRC25 and has also weakened the inflammatory response by decreasing Col1, α -SMA, pSmad2/3, p-NF- κ B, TNF- α , and IL-1 β activities. The opposite of this situation, thus analogous to the cardiac fibrosis, is likely to appear in tympanosclerosis due to TGF- β 1 upregulation (see Figure 2).

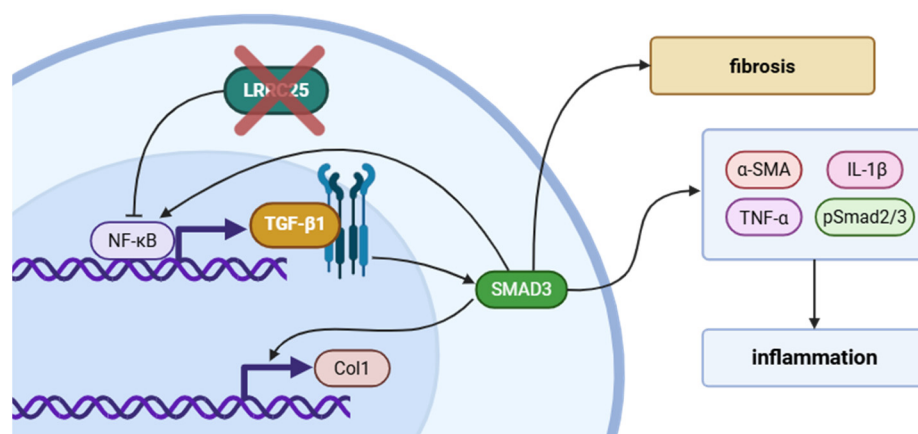


Figure 2. Inhibition of LRRC25 inhibitory activity leads to upregulation of TGF- β 1 by NF- κ B. TGF- β 1 promotes the activity of SMAD3, which then increases the activity level of pro-inflammatory mediators and fibrosis associated molecules—Col1, α -SMA, pSmad2/3, p-NF- κ B, TNF- α , and IL-1 β —resulting in fibrosis and inflammation in cardiac fibrosis, and possibly tympanosclerosis. (LRRC25 – Leucine Rich Repeat Containing 25, TGF- β 1 – Transforming Growth Factor β 1; NF- κ B – Nuclear Factor kappa-light-chain-enhancer of activated B cells; Col1 – Collagen type 1; SMAD3 – SMAD Family Member 3; α -SMA – α Smooth Muscle Actin; TNF- α – Tumor Necrosis Factor α ; pSmad2/3 – phosphorylated SMAD Family Member 2 and 3; IL-1 β – Interleukin-1 β). Created in BioRender. Mazurek, M. (2025) <https://BioRender.com/ftlpx67>, accessed on 11 April 2026.

A study on rats infected with inactivated *Streptococcus pneumoniae*—for establishing a rat model of tympanosclerosis—examined the influence of the expression of *Tgf β 1* on the development of tympanosclerosis. The results further confirmed the hypothesis that TGF- β 1 contributes to transitioning from otitis media to tympanosclerosis by promoting fibrosis during ongoing inflammation [78,79]. Another study was conducted on guinea pigs and rats to investigate the effect of captopril and losartan in attenuating the progression of *Streptococcus pneumoniae*-induced tympanosclerosis by exploring mechanisms related to the suppression of TGF- β 1, as the used drugs have been proven to suppress the fibrosis progression at least partially because of inhibiting the expression of *Tgf β 1* [80–83]. Animals in the experimental groups were infected with type-3 *Streptococcus pneumoniae*, which led to the appearance of lesions typical of tympanosclerosis. The expression of TGF- β 1 was upregulated in the tympanosclerosis groups compared to that in the control groups, where it was nearly undetectable. It was reduced, however, in the captopril- and losartan-treated groups. The tympanosclerosis group was also characterized by a thickened membrane, inflammatory infiltration, and degeneration of hyaline that appeared in the mucosa of the middle ear at week 6. Thus, we suspect that overexpression of TGF- β 1 leads to hyalinization, calcification, and inflammation in the middle ear mucosa—the processes that typically occur in tympanosclerosis. Captopril- and losartan-treated groups showed a reduction of calcification and hyalinization in the mucous membrane of the middle ear, and tympanosclerosis occurred less frequently in them. Moreover, the expression of TGF- β 1 (*Tgf β 1*) was reduced at protein and mRNA levels. This study demonstrated that combined treatment with captopril and losartan suppresses the overexpression of

TGF- β 1 and, consequently, inhibits the progression of tympanosclerosis in animal models [76].

3.8. *Evi1* Mutation and Mucin Overproduction

EVI1 (Ecotropic Viral Integration Site 1) is a protein expressed during inflammation that affects middle ear epithelial cells and fibroblasts [84]. In tympanosclerosis, it seems to contribute to an upregulation of *Tgfb* expression. The study on mice revealed that the mutation in one of the zinc-finger domains of the *Evi1* gene, caused by A2288T transversion in the *Evi1* locus, impairs its function. While the functional EVI1 can control cell development by repressing the TGF- β /SMAD signaling pathway [85,86], the mutation may lead to a reduction in the TGF- β pathway inhibition, thereby enhancing the effusive processes in the middle ear. Additionally, this mutation leads to an upregulation of MUC2 expression, which may increase effusive processes [84]. MUC2 is responsible for mucin secretion in middle ear effusion, and it has been documented that the *MUC2* gene is increased in patients with otitis media [87]. Mucins increase the viscosity of the effusion, which may impair normal mucociliary clearance and potentially lead to pathological changes [88]. As a result, the mutation in the *Evi1* gene may predispose to the development of inflammation and effusion via upregulating TGF- β and MUC2 expression, which can lead to tympanosclerosis [29,44,65].

3.9. Phosphate Regulation and *Enpp1* with *Rpl38* in Calcification

Disrupted phosphate homeostasis may lead to systemic inflammation, resulting in, among others, calcification, as observed in mouse models [89]. Although *Rpl38* (ribosomal protein L38) has not been proven to participate in phosphate regulation yet, it was observed to increase organic phosphate levels in *Rpl38* tail-short (*Rpl38*Ts) mutant mice [90], which indicates a possible role of *Rpl38* in phosphate homeostasis [89]. Mineralization disorders caused by elevated phosphate levels have been connected to inflammation in many diseases, such as chronic kidney disease [91,92] and cardiovascular [93]. The presence of calcified tissue in the middle ear cavity could interfere with hearing in multiple ways, contributing to conductive hearing loss.

ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1), an enzyme encoded by the *Enpp1* gene, produces inorganic pyrophosphate, an important inhibitor of calcification. ENPP1 controls bone mineralization and calcification of soft tissues [94]. The *asj* mutation in mice (the “ages with stiffened joints” mutation) is a single nucleotide substitution of T to A in exon 7 of the *Enpp1* gene, causing an amino acid substitution from valine to aspartic acid at residue 246 (p.V246D) of protein Reference Sequence NP_03283. It is a recessive missense mutation in the *Enpp1* gene, which leads to suppression of its expression [89,94,95].

Decreased ENPP1 levels have been observed to correlate with increased FGF23 levels (fibroblast growth factor 23) in the serum of *Enpp1* mutant mice [96,97]. Pathological serum levels of FGF23 contribute to systemic disorders in humans, such as renal phosphate wasting diseases and hyperphosphatemia, as this enzyme plays an important role in the homeostasis of phosphate and calcium and in controlling bone mineralization [96–98]. Mice with *asj* mutation—with suppressed expression of *Enpp1* and increased FGF23 levels—showed significant mucoperiosteum proliferation and presented ectopic mineralization and bone deposition in the otic capsule. On this account, we suspect that abnormally elevated levels of FGF23 in the middle ear caused by ENPP1 deficiency may result in mucoperiosteum proliferation leading to the development of otitis media, which is then complicated by tympanosclerosis due to unregulated mineralization. Stapes fixation was not detected in the *asj* mutant mice group. Instead, they noticed a white patch on the tympanic membrane and fusion of the malleus and incus—symptoms typically indicating

tympanosclerosis. They also observed thickening of a round window membrane with reduced ENPP1 levels [89], which may increase the stiffness of the membrane and impair physiological cochlear fluid flow and hair cell stimulation [99]. Since mice with decreased *Enpp1* expression have significantly reduced levels of inorganic pyrophosphate (PPi), it is expected that abnormal mineralization and calcification may occur in their middle ear [94]. Similar pathological processes have been observed in many tissues of mice with other *Enpp1* mutations, such as “tiptoe walking” missense mutation, which appears spontaneously [100], a genetically engineered knockout mutation [97], and a C397S nonsense mutation induced by a chemical mutagen—ENU (N-ethyl-N-nitrosourea) [101]. The tissues, which showed the most extensive defects in mineralization, included arteries, long bones and spinal ligaments. It is worth mentioning that the stapedia artery undergoes atrophy early in human development, while it remains throughout the whole life in mice [102]—in this study, thickening of the stapedia artery wall in *Enpp1 asj* mutant mice, was observed—likely due to calcification, which could restrict movement of stapes—because the artery runs through its ring structure—thereby contributing to the observed hearing loss, which would not occur in humans. This is not the sole possible explanation of the hearing loss and other pathologies observed in the studied mice, and the exact linkage between pathological mineralization and the development of otitis media has to be further researched, especially with the use of human tissues. However, pathological mineralization is already known to contribute to the pathogenesis of otosclerosis and tympanosclerosis, which usually cause hearing loss in human patients—this makes these results significant in the context of human disease [89].

3.10. Wnt Signaling Pathway and Sclerostin in Osteogenesis and Calcification

One of the key stages in the development of tympanosclerosis is osteogenesis and calcification of middle ear structures—a consequence of regeneration of tissues damaged by the ongoing inflammatory process. These processes have been investigated in a study on rats [103]. Wnt proteins can be divided into two classes: the Wnt1 class (consisting of Wnt1, Wnt2, Wnt6, Wnt7a, Wnt7b, Wnt9a, Wnt9b, Wnt10b) and the Wnt3 class (including Wnt3 and Wnt3a) [104]. The Wnt pathway plays a crucial role in several biological processes, including cell proliferation and tissue regeneration [105,106]. The signaling cascade of the Wnt pathway begins with the Wnt protein binding to the LRP5/6-Fzd receptor on the cell surface, leading to the accumulation of cytoplasmic β -catenins. β -catenins then migrate to the cell nucleus, where they form a complex with TCF/LEF (T-cell factor/lymphoid enhancer factor) and stimulate the expression of target genes [103,107], in this case, the osteoprotegerin (*OPG*) gene in mature osteoblasts, which is supposed to inhibit osteoclast activity and, thus, reduce bone resorption and promote bone formation [108]. *OPG* is a decoy receptor belonging to the TNF receptor superfamily, which blocks the connection of RANKL (Receptor Activator of Nuclear Factor κ B Ligand) to RANK (Receptor Activator of Nuclear Factor κ B), the activator of kappa factor B located on cells of the osteoclast lineage, which stimulates their activity [109]. Therefore, *OPG*/RANK ratio is used to determine the direction of bone remodeling. As Wnt can regulate the *OPG*/RANK ratio by increasing osteoprotegerin expression and promoting osteogenesis, it is believed to be implicated in the formation of tympanosclerotic plaques. Wnt pathways are key in regulating many processes, including cell proliferation, tissue regeneration, and embryonic development, but it also has a variety of other local and systemic effects [103]. One of the most notable proteins in this pathway is Wnt3a, which promotes osteoblast proliferation and early differentiation, while inhibiting the transformation of pre-osteoblasts into adipocytes [103,110]. Studies on rats have shown that during the formation of calcified lesions in tympanosclerosis, Wnt3a and β -catenin expression levels in the middle ear are

significantly higher compared to an unaltered middle ear, which suggests an important role of this pathway in the pathogenesis of the disease [103].

The pathomechanism underlying the formation of calcified plaques in tympanosclerosis comprises three phases: inflammation, fibroplasia repair, and bone remodeling toward osteogenesis [2,13,14,111,112]. The first step in the formation of calcified plaques is inflammation. Increased expression of sclerostin (SOST), a glycoprotein produced mainly in osteocytes and known for its involvement in the pathomechanism of many diseases related to bone metabolism, such as rheumatoid arthritis and ankylosing spondylitis [106,113], has been shown to play a key role in this process [103]. There are two pathways in which SOST participates in the development of tympanosclerosis—the first involves the inflammatory cascade through the activation of ERK1/2 during the inflammatory phase [103,114], and the second is through antagonizing the Wnt1 and Wnt2 proteins and upregulating Wnt3 and Wnt3a in the osteogenesis phase [115] (see Figure 3).

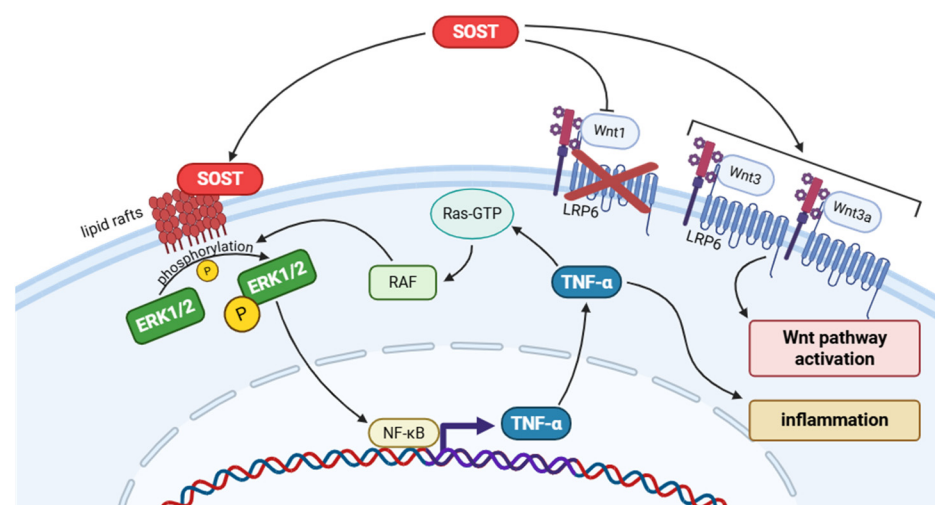


Figure 3. SOST binds to Raft, which induces the phosphorylation and activation of ERK1/2 and leads to enhanced transcription of TNF- α via NF- κ B, resulting in inflammation. TNF- α then activates the RAS-Raf pathway and boosts phosphorylation of ERK1/2 to further increase the TNF- α expression. At the same time, SOST inhibits the binding of the Wnt1 protein to LRP6 and simultaneously activates the binding of Wnt3 and Wnt3a to LRP6. The balance of those two processes ultimately leads to the activation of the Wnt pathway (Raft—lipid raft; SOST—Sclerostin; ERK 1/2—Extracellular signal-Regulated Kinases 1 and 2, also MAPK3/1 / MEK; NF- κ B—Nuclear Factor kappa-light-chain-enhancer of activated B cells; TNF- α —Tumor Necrosis Factor α ; Ras-GTP—Ras GTPase, RAF—RAF kinase, LRP6—low-density lipoprotein receptor-related protein 6, Wnt1/3/3a—Wnt protein 1/3/3a). Created in BioRender. Mazurek, M. (2025) <https://BioRender.com/b84p8u8>, accessed on 11 April 2026

ERK1/2 are extracellular signal-regulated kinases belonging to the MAP kinase (MAPK) group, whose signaling pathways induce inflammation by increasing the expression of inflammatory cytokines (e.g., TNF- α) [116]. Activation of the ERK1/2-MAPK pathway begins with membrane transfer and activation of Raft (also known as lipid rafts—cholesterol-rich membrane microdomains), which then phosphorylates ERK1/2 (p-ERK1/2) [103]. P-ERK1/2 can activate transcription factors recruited to the TNF- α promoter, such as NF- κ B, which activates the transcription of TNF- α , one of the major cytokines mediating inflammatory responses [116,117]. The pathway is closed in a p-ERK1/2-NF- κ B-TNF- α -Ras-RAF-P-ERK1/2 circle. In a study where experimental tympanosclerosis was induced in a rat model after inoculation with meticillin-resistant *Staphylococcus aureus* (MRSA), the expression of SOST and the concentration of p-ERK1/2 were found to be

increased in the tympanic membrane and middle ear mucosa of a group of rats after the inoculation. Their tympanic membranes were calcified and their middle ear mucosa was thickened, rich in inflammatory cells, and showed signs of fibroplasia, while in the control group, no such pathologies were observed. Thus, these results suggest that this signaling pathway may play a role in the inflammatory phase of tympanosclerotic plaque development [103].

In addition, SOST is also a strong antagonist of Wnt1 class proteins, which bind to propeller 1 of LRP6 (LDL receptor-related protein 6) and block Wnt1 protein binding. At the same time, SOST slightly increases Wnt3 and Wnt3a activation, which may be due to LRP6 dimerization. The enhancement of Wnt3 and Wnt3a signaling and the resulting osteogenesis may lead to the formation of tympanosclerotic plaques, thereby representing the second pathway involving SOST leading to the development of tympanosclerosis [115]. An increased expression of SOST, Wnt3a and β -catenin in post-infection calcifications of the tympanic membrane in rats was detected, as well as the expression of these molecules was upregulated in the thickened, fibrotic middle ear mucosa of the experimental group [103], thereby suggesting the involvement of these proteins in the last phase of formation of tympanosclerotic lesions, caused by osteogenesis and calcification, as these are among processes mediated by the Wnt pathways [103,115].

3.11. Osteopontin as a Link Between Inflammation and Calcification

Understanding that tympanosclerotic lesions may result from chronic middle ear inflammation led researchers to look for a linking factor [4,118]. Osteopontin (OPN) was identified as a key candidate due to its role in both physiological and pathological tissue calcification (e.g., atherosclerosis), as it has a high hydroxyapatite-binding capacity [59,119].

OPN has been detected in the microcalcifications in chronic otitis media, particularly in tympanosclerosis-related calcifications within the tympanic membrane and middle ear mucosa [31,118]. This is supported by a study, in which the *SPP1* gene, which encodes OPN, has been found elevated in tympanosclerotic tissues in comparison to healthy tympanic membranes [65], while the results of Sakowicz-Burkiewicz et al. (2017) indicate even higher levels of the *SPP1* in tympanosclerosis membranes compared to healthy ones [120]. Further research identified macrophages (CD68+ cells) as the main source of OPN expression. Normally, monocytes and macrophages in healthy tissue do not express OPN, but during inflammation, tissue macrophages secrete OPN, which binds to hydroxyapatite formed by degenerating connective tissue collagen. The calcium-sensing receptor (CaSR), which regulates calcium homeostasis and mineral deposition, was also found to be overexpressed in tympanosclerosis [31]. These findings strongly support the hypothesis that OPN regulates calcification formation in tympanosclerosis following chronic middle ear inflammation [118].

An inhibitor of the Wnt signaling pathway is Dickkopf-1—it suppresses Wnt signaling mostly through binding to LRP5/6 [121,122]—which was found to be downregulated in rat tympanosclerosis models [123]. Dickkopf-1 (DKK1) is a secreted glycoprotein whose expression occurs not only in osteoblasts and maturing osteocytes [124], but also in the endothelium and in other cells [125]. In the study by Zhang et al. (2017) [123], the statistical analyses of the results showed that DKK1 expression was significantly downregulated in rats with tympanosclerosis in relation to the control group. This discovery proves that DKK1 deficiency plays an important role in the pathogenesis of tympanosclerosis in animal models. Decreased expression of DKK1 protein seems to have a lesser inhibitory effect on Wnt/ β -catenin signaling [123]. In consequence, the bone formation and density of the bones are pathologically increased [126,127], which may lead to many disorders, including tympanosclerosis [128–130].

3.12. Matrix Metalloproteinases and Extracellular Matrix Degradation

Matrix metalloproteinases (MMPs) are responsible for the degradation of all of the extracellular matrix (ECM) components. The MMP-9—one of the members of the MMP family—is known for its role in the degradation of type IV collagen and gelatin [67]. Studies have shown a significantly higher activity of MMP-9 in the tympanosclerotic tympanic membrane in comparison to the healthy one in humans and rats [65,131]. In addition, the other members of the MMP family, MMP-3 and MMP-12, have been observed to be upregulated in rat tympanic membrane tissue during the inflammatory phase, while MMP-9 was upregulated during the remodeling phase [131]. Moreover, an overexpression of MMP-9 is correlated with the degradation of ECM components, leading to fibrosis visible in tympanosclerosis [67]. Notably, TGF- β 1 is able to upregulate and downregulate the expression of MMPs, thereby affecting the degradation of ECM [132,133]. One of the studies, conducted on guinea pigs, was designed to analyze changes in the expression of TGF- β 1 and MMP-9 in tympanosclerosis, as well as their synergistic effects and interactions. The results showed that the expression of these two cytokines has increased in tympanosclerosis, so we may conclude that the upregulation of TGF- β 1 and MMP-9 contributes to the formation of tympanosclerotic changes [67]. We may also suspect that the expression of MMP-9 was regulated by TGF- β 1 [67], due to the fact that TGF- β 1 may regulate the expression of MMP-9 and its activity to a certain extent, in order to prevent excessive or insufficient remodeling of the ECM, thereby maintaining tissue homeostasis [134]. Figure 4 shows molecular mechanisms of the upregulation and downregulation of the expression of MMP-9 by TGF- β 1, as well as mutual interactions between these cytokines (see Figure 4) [135–143].

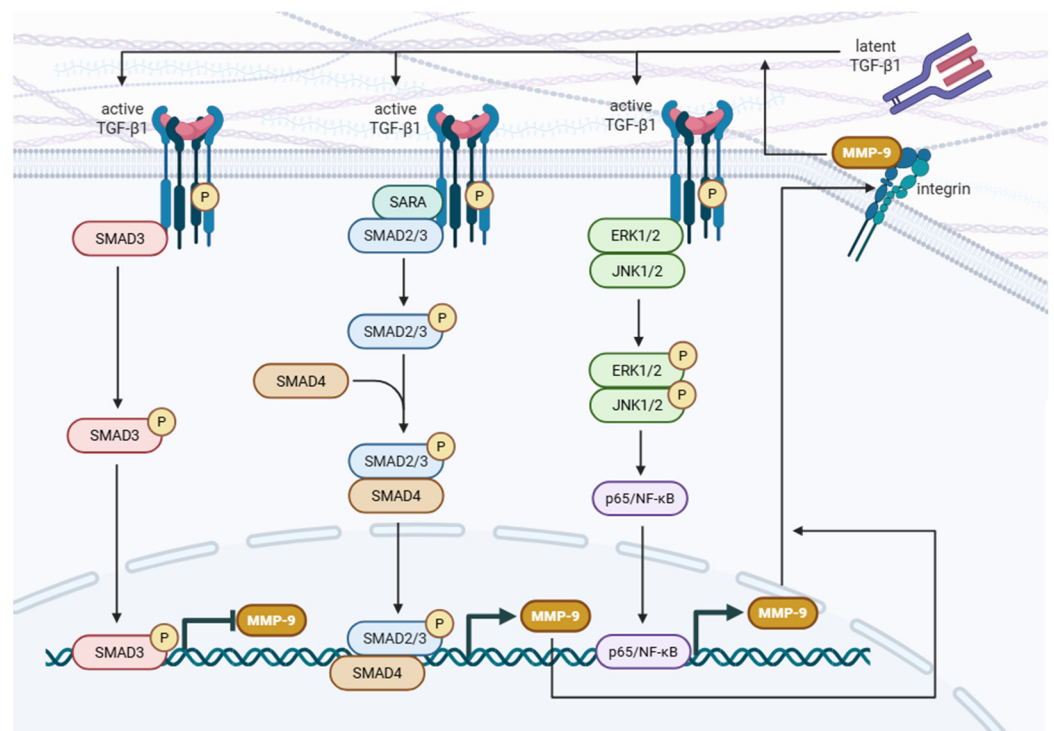


Figure 4. TGF- β 1 induces the phosphorylation and migration of SMAD2/3 from the cytoplasm to the cell nucleus, resulting in the upregulation of MMP-9 expression. At the same time, TGF- β 1 induces phosphorylation of the ERK1/2 and JNK1/2 kinases, which then stimulate the translocation of p65/NF- κ B from cytoplasm to the nucleus, resulting in p65/NF- κ B also upregulating the expression of MMP-9. At the same time, TGF- β 1 can downregulate the expression of MMP-9 by inducing the phosphorylation and translocation of SMAD3 from the cytoplasm to the cell nucleus. The latent TGF- β 1 is released from the ECM and is proteolyzed by MMP-9, resulting in activation of the TGF-

β 1 (TGF- β 1—Transforming Growth Factor β 1; ; SARA—Smad Anchor for Receptor Activation; SMAD2/3—SMAD Family Member 2 and 3; SMAD 4—SMAD Family Member 4; ERK 1/2—Extracellular signal-Regulated Kinases 1 and 2; JNK1/2—c-Jun N-terminal kinases 1 and 2; p65 – transcription factor p65/RELA; NF- κ B—Nuclear Factor kappa-light-chain-enhancer of activated B cells; MMP-9—Matrix Metalloproteinase-9; ECM—extracellular matrix). Created in BioRender. Mazurek, M. (2026) <https://BioRender.com/8nj0vg9>.

3.13. Bone Morphogenetic Proteins Alter the Connective Tissue

Bone morphogenetic proteins (BMPs) are part of the TGF- β family. BMP genes are expressed in numerous tissues and are known for inducing bone formation [144]. In a study by Nemati et al. (2021) [145], researchers have marked the levels of expression of BMP-8a in tympanosclerotic tissue. They revealed the upregulation in the expression of the BMPs in tympanosclerosis and atherosclerosis, with the higher level of expression in tympanosclerosis, which indicates a possible difference in the calcification process between those two diseases [145].

3.14. Collagen Deposition, Fibrosis and the Role of Collagen Type II

Fibrosis is accompanied by collagen deposition—the *COL2A1* is a gene that participates in this process by overseeing the synthesis of the pro- α 1(II) chain of type II collagen. Sakowicz-Burkiewicz et al. (2017) [120] showed that the expression of *COL2A1* in the tympanic membrane in patients with tympanosclerosis is significantly higher than in healthy tissues. The increased expression of *COL2A1* was found to correlate with the severity of the tympanosclerosis. According to this study, the levels of *COL2A1* mRNA in healthy tympanic membranes are extremely low, but are highly increased in tympanosclerotic tissues. On the other hand, the expression of *COL1A1* was observed to be significantly lower in patients with tympanosclerosis than in healthy individuals. Moreover, the *COL1A1* mRNA levels were inversely correlated with the severity of tympanosclerosis [120]. A study by Pawelczyk et al. (2014) confirms those results—they observed enhanced expression of *COL2A1* in tympanosclerosis in comparison to the healthy tympanic membrane, as a consequence of increased fibroblast sensitivity to mast cell stimulation [65]. Furthermore, both of the studies indicate increased levels of *COL4A1* expression in the group of patients suffering from tympanosclerosis in comparison to healthy individuals [65,120].

3.15. T-Cell Receptor Specificity and Autoimmune Response to Collagen Type II

The molecular mechanisms underlying tympanosclerosis, an immunological condition among others, can be explored through insights from studies of type II collagen (CII)—induced autoimmune diseases. It is widely known that T-cell receptor (TCR) gene specificity plays a pivotal role in immune responses to CII, with a relatively limited repertoire of TCRs being activated in response to this antigen. TCR V β refers to specific variable regions of the β -chain of T-cell receptors that are expressed by subsets of T lymphocytes involved in the autoimmune response to type II collagen. A study by Lee et al. (1994) demonstrated that lymphocytic infiltration of the tympanic membrane lesions in mice immunized with type II collagen was associated with the expression of TCR V β -6, whereas TCR V β -8 was predominantly found in arthritic joints [146]. This differential expression suggests that distinct T-cell clones, characterized by their TCR V β usage, mediate immune responses in different tissues. The presence of V β -6-positive T cells in tympanic lesions in mouse models supports the role of cell-mediated immunity in the development of tympanosclerosis and its role in the pathogenesis of this condition [147].

The importance of TCR gene specificity in autoimmune diseases is further supported by the notion that blocking these critical TCRs can lower the immune response below a threshold required for disease induction or maintenance [148]. Additionally, the

localization of CII in the lamina propria of the tympanic membrane [149], and the observation of tympanosclerotic changes in guinea pigs immunized with CII [150], suggest that type II collagen plays a significant role in the pathogenesis of tympanosclerosis. These studies collectively emphasize the involvement of TCR-mediated immune responses in the tympanosclerosis development and provide a basis for further exploration of targeted therapies for this condition.

3.16. *NBPF* Genes and Chromosome 1q21 Microduplications

A case report study showed that the genes *NBPF15* and *NBPF16*, which are located in 1q21.1 and 1q21.2 chromosomal regions, respectively [151–153], may be the cause of mild developmental delay and facial dysmorphism, as well as bilateral tympanosclerosis, which were the symptoms observed in a studied patient [154]. Microduplications, as well as bilateral microdeletions of chromosome 1q21.1 and/or 1q21.2, have been associated with many different clinical symptoms, without identifying the exact pathological mechanism. When extensive repeats appear in the DNA on chromosome 1q21.1 and 1q21.2, the regions are more likely to rearrange recurrently through non-allelic homologous recombination. The rearrangement results in microduplication and microdeletion events. Patients with these mutations present various symptoms, such as delayed development [155], congenital heart disease [156], mental illnesses [157–160], microcephaly or macrocephaly [161], and neuroblastoma [155]. In the discussed patient, a 1q21.2 microduplication involving two *NBPF* genes occurred. Both of these genes were duplicated in the patient, and not duplicated in either of their parents; they also did not present any of the symptoms. This case observation supports an association between *NBPF* genes and 1q21 microduplication and microdeletion syndromes. Even though it is possible that other genes are involved in the pathomechanism of the observed symptoms, we suspect that duplication of *NBPF* genes is associated with tympanosclerosis. Further studies are required to confirm that hypothesis [154].

4. Discussion

Tympanosclerosis occurs most often as a result of chronic and secretory otitis media. The ongoing process can result in the fixation of the auditory ossicle chain in up to 66% of cases [162]. This is particularly important, as stapes fixation can result in a conductive hearing loss, which will progress as the fixation advances. Despite the lack of solid evidence or a detailed description of the pathomechanism of this disease, we managed to create a picture of changes at the molecular level in the inflammatory process, according to the reviewed studies. Such changes manifest in alterations in the expression levels of factors and enzymes involved in the inflammatory process, which lead to the development of lesions characteristic of tympanosclerosis (see Table 1).

Table 1. Summary of the upregulation and downregulation of the genes or factors and the consequences of these processes.

Gene/Factor	Study Model	Direction of Expression Change	Functional Consequence
TNF- α	Human	Upregulation	Increasing inflammation; contributing to chronic serous otitis media
<i>NOS2</i> /iNOS	Human	Upregulation	Increasing the production of NO
NO	Human, Animal	Upregulation	Inhibiting osteoclasts; in the long term, exacerbating oxidative stress and tissue remodeling
IL-6	Animal, Human	Upregulation	Exacerbating inflammation and inducing iNOS activity; increasing

			osteoclastogenesis; inducing calcification and fibrosis processes
			Inflammation; inducing the migration of neutrophils to the site of an inflammatory process; upregulating the expression of inflammatory genes and pro-inflammatory cytokines; inhibiting tPA and uPA
PAI-1	Animal	Upregulation	
tPA	Animal	High upregulation in the early stage of otitis media with late rapid downregulation	Unspecified
uPA	Animal	Downregulation in the later stage of otitis media	Unspecified
<i>Ctsb</i>	Animal	Upregulation	Lymphocyte migration, inflammatory mediator synthesis, activation of the mitochondrial apoptotic pathway and apoptosis by caspases; sustaining the inflammation process
TGF- β 1	Human, Animal	Upregulation	Stimulating fibrosis and collagen deposition; leading to hyalinization and degeneration of the mucosa; affecting the expression of MMP-9
FGF23	Animal	Upregulation	Mucoperiosteum proliferation; ectopic mineralization; the development of otitis media
Wnt3a	Animal	Upregulation	Stimulating calcification and osteogenesis
SOST	Animal	Upregulation	Activating the ERK1/2 pathway, leading to chronic inflammation; inhibiting Wnt1 and Wnt2 proteins, affecting mineralization
<i>SPP1</i>	Human	Upregulation	Binding of the osteopontin to hydroxyapatite formed by degenerating connective tissue collagen; regulating calcification formation in tympanosclerosis
DKK1	Animal	Downregulation	Lesser inhibitory effect on Wnt/ β -catenin signaling and osteogenesis; causing excessive bone formation and ear deposits
MMP-9	Human, Animal	Upregulation	Degradation of ECM components—leading to fibrosis
BMP-8a	Human	Upregulation	Promoting osteogenesis
<i>COL2A1</i>	Human	Upregulation	Excessive fibrosis
<i>COL1A1</i>	Human	Downregulation	Affecting severity of tympanosclerosis
<i>COL4A1</i>	Human	Upregulation	Excessive fibrosis and pathological remodeling

Increased expression of SOST activates the ERK1/2 pathway, which enhances TNF- α production through the activation of NF- κ B, leading to chronic inflammation. SOST also

inhibits the Wnt1 and Wnt2 proteins, affecting mineralization. At the same time, the expression of Wnt3a and β -catenin is increased, which stimulates osteogenesis and calcification. The Wnt pathway is further modulated by Dickkopf-1 (DKK1), whose reduced expression reduces its inhibitory effect on osteogenesis, leading to excessive bone formation and ear deposits. It has been established that epigenetic mechanisms have a significant impact on the modulation of SOST expression, particularly through DNA methylation of the promoter region, which could also be the case in tympanosclerosis; however, this has not been studied yet [163].

Among the cytokines that are the most active in tympanosclerosis are TNF- α , IL-6 and IL-10. TNF- α increases inflammation and is significantly elevated in the ear mucosa in tympanosclerosis. IL-6, secreted by macrophages and epithelial cells, affects osteoclast differentiation and bone remodeling. Its expression occurs very early in the development of tympanosclerosis, and its high levels can induce calcification and fibrosis processes. IL-10 is also elevated, but its function seems to depend on its balance with IL-6.

The protein OPN, secreted mainly by macrophages, links inflammatory processes to calcification. It shows high affinity for hydroxyapatite and is present in calcium deposits in the tympanic membrane.

The TGF- β 1 level is significantly elevated in tympanosclerosis and stimulates fibrosis and collagen deposition. An increase in its expression correlates with the severity of the disease and leads to hyalinization and degeneration of the mucosa. In addition, it affects the expression of MMP-9, a type IV collagen-degrading enzyme, which promotes extracellular matrix remodeling and fibrosis. It is worth mentioning that modifications such as DNA methylation and histone acetylation may influence the expression of MMP-9 via affecting the accessibility of the MMP gene promoter regions to transcription factors [164,165]; however, such modifications have not yet been studied in relation to tympanosclerosis. In addition, a study reported that tranilast—an anti-allergic drug—inhibited the expression of MMP-9 [166]; therefore, future studies could be designed to investigate the effects of anti-allergic agents on the expression of MMP-9 with a view to potentially developing new therapeutic strategies. *COL2A1* (type II collagen) and *COL4A1* (type IV collagen) expressions are significantly increased, and are responsible for excessive fibrosis, while *COL1A1* is downregulated (see Figure 5). The expression of type II collagen (*COL2A1*) in chondrocytes is tightly regulated by epigenetic mechanisms (including DNA methylation), which may also be the case in tympanosclerosis [167].

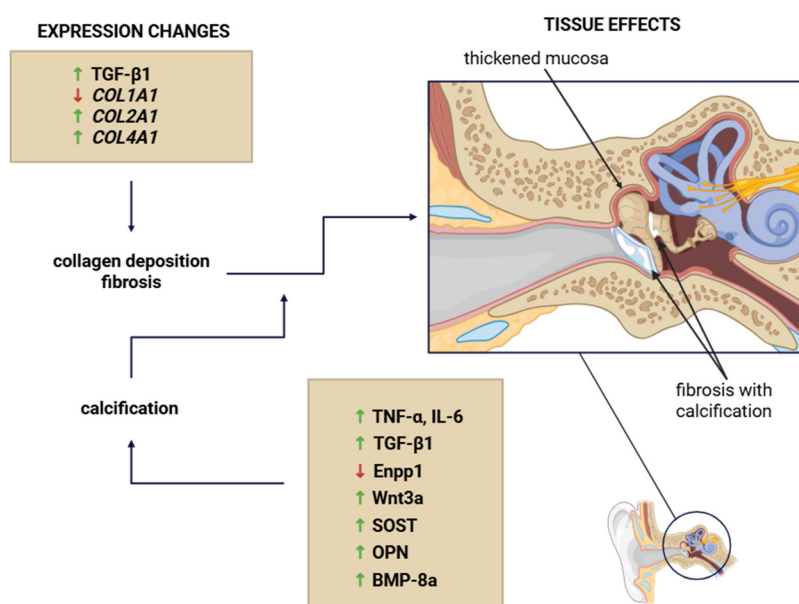


Figure 5. Increased levels of TGF- β 1, COL2A1, and COL4A1 and decreased levels of COL1A1 result in fibrosis, leading to the thickening of the middle ear mucosa. Upregulation of genes involved in calcium metabolism induces calcification, visible in plaques on the tympanic membrane and/or middle ear cavity (TGF- β 1—Transforming Growth Factor β 1; COL1A1—Collagen Type I Alpha 1 Chain; COL2A1—Collagen Type II Alpha 1 Chain; COL4A1—Collagen Type IV Alpha 1 Chain; TNF- α —Tumor Necrosis Factor α ; IL-6—Interleukin 6, *Enpp1*—Ectonucleotide Pyrophosphatase/Phosphodiesterase 1; Wnt3a—Wnt3a protein; SOST—Sclerostin; OPN—Osteopontin; BMP-8a—Bone Morphogenetic Protein 8a). Created in BioRender. Mazurek, M. (2025) <https://BioRender.com/fipieq4>.

Ctsb, present in the effusion fluid, contributes to sustaining the inflammation process and activation of apoptosis by caspases.

Decreased expression of *Enpp1* results in increased levels of phosphate and FGF23, which promotes mucosal mineralization and proliferation. *Rpl38*, whose expression affects phosphate levels, also plays a role in ectopic mineralization.

In addition, the expression of PAI-1 increases during the early phase of inflammation, as it participates in inflammatory cell migration.

BMP-8a and other BMPs present increased activity, promoting osteogenesis. The iNOS enzyme is responsible for the production of NO, which inhibits osteoclasts and promotes bone remodeling, and its expression is elevated in tympanosclerosis. Oxidative stress, caused by ROS and insufficient activity of antioxidant enzymes, leads to damage of the middle ear mucosa, collagen degradation and initiation of inflammatory processes, fibrosis and calcification, causing tympanosclerotic lesions to develop. High expression of iNOS increases the production of nitric oxide (NO), which has a protective effect in the initial phase by inhibiting osteoclasts, but in the long term, it intensifies oxidative stress and tissue remodeling. Increased iNOS expression is also observed in macrophage-like cells in tissues with developed tympanosclerosis. In addition, increased expression of IL-6, a cytokine linked to NO production and bone remodeling processes, may exacerbate inflammation and induce further iNOS activity.

The results of these studies shed light on the pathomechanism of tympanosclerosis, as they highlight how changes in the expression of the studied inflammatory components contribute to the progression of tympanosclerotic lesions. The formation of characteristic plaques, as these components are involved in process pathways, such as inflammation, fibrosis, sclerosis and calcification, which function correlates with the clinical picture of tympanosclerosis. These changes, when studied in more detail, could potentially serve as markers for detecting the risk or early development of tympanosclerosis in patients in whom we suspect the possibility of developing the disease, as the expression profile of the factors at the early onset or in tympanosclerosis differs significantly from that in normal inflammation, which does not present any signs of sclerosis. However, additional studies would be needed to definitively verify these results. A summary of the reported changes in expression of genes and proteins in tympanosclerosis in human populations is presented in Table 2 (see Table 2). Moreover, it may be important to identify the epigenetic processes behind these changes. Histone tail modifications and DNA methylation are very important epigenetic processes that regulate gene expression during inflammation [168]. Epigenetic modifications of the genes encoding pro- and anti-inflammatory molecules and their regulation by environmental signals play an important role in chromatin remodeling and transcriptional activation and are key to achieving an appropriate inflammatory response [169]. Recent studies indicate that epigenetic modifications play an important role in the pathomechanism and progression of alterations associated with the turnover of ECM proteins in cartilage and cartilage damage. Genome-wide association studies have shown that IL-1 β , IL-6, and TNF- α , which were epigenetically modified, were involved in

the abovementioned pathological process, contributing to the development of osteoarthritis [170]. We suspect that epigenetic changes in key factors also occur in the case of tympanosclerosis and may constitute another significant factor influencing differences in the development or progression of the disease among individuals. However, the current state of knowledge does not allow us to confirm this.

Table 2. A summary of changes in expression and protein levels between tympanosclerosis and healthy human study groups.

Gene/Factor	Value in Control Group (Healthy)	Value in Tympanosclerosis Group	p-Value	Correlation (R)	Data Type	Source
<i>SPP1</i>	– ^a	7-fold higher ^a	<0.05	0.82	Relative expression (mRNA/ACTB)	[120]
	– ^a	2-fold higher ± SD ^a	<0.05	N/A ^b	Relative expression (mRNA/ACTB)	[65]
	no significant changes ^c	2-fold higher ± SD ^c	<0.05	N/A ^b	Relative expression (mRNA/ACTB)	[65]
<i>COL2A1</i>	– ^a	4.9-fold higher ^a	<0.05	0.80	Relative expression (mRNA/ACTB)	[120]
	~5-fold higher ± SD ^c	~12-fold higher ± SD ^c	<0.05	N/A ^b	Relative expression (mRNA/ACTB)	[65]
<i>COL1A1</i>	– ^a	Lower ^a	<0.05	–0.82	Relative expression (mRNA/ACTB)	[120]
<i>COL4A1</i>	– ^a	Higher ^a	<0.05	0.48	Relative expression (mRNA/ACTB)	[120]
	no significant changes ^c	~2.5-fold higher ^c	<0.05	N/A ^b	Relative expression (mRNA/ACTB)	[65]
BMP-8a	20.0 ± 9.4% ^d	59.4 ± 15.2%	5.9 × 10 ^{–9} (<0.05)	N/A ^b	Percentage Area Fraction	[145]
IL-6	– ^a	~80% higher ± SD ^a	<0.05	N/A ^b	Protein release level	[65]
	8.15 ± SD pg/10 ⁵ fibroblast cells	11.4 ± SD pg/10 ⁵ fibroblast cells	<0.05	N/A ^b	Protein release level	[65]
	2-fold higher ± SD ^c	3-fold higher ± SD ^c	<0.05	N/A ^b	Protein release level	[65]
IL-10	– ^a	50% higher ^a	<0.05	N/A ^b	Protein release level	[65]
	– ^a	~3-fold lower ^a	<0.05	N/A ^b	Relative expression (mRNA/ACTB)	[65]
	~40% increase ^c	no significant changes ^c	<0.05	N/A ^b	Protein release level	[65]
MMP-9	– ^a	4–5-fold higher ^a	<0.05	N/A ^b	Protein release level	[65]
	1.1 ng/10 ⁵ ± SD fibroblast cells	3.6 ng/10 ⁵ ± SD fibroblast cells	<0.05	N/A ^b	Protein release level	[65]
	~3-fold higher ± SD ^c	~3-fold higher ± SD ^c	<0.05	N/A ^b	Protein release level	[65]
TGF-β	– ^a	Higher ± SD ^a	<0.05	N/A ^b	Protein release level	[65]
TNF-α	60% increase ± SD ^c	250% increase ± SD ^c	<0.05	N/A ^b	Protein release level	[65]

3.75 ± SD pg/10 ⁵ fibroblast cells	4.6 ± SD pg/10 ⁵ fibroblast cells	<0.05	N/A ^b	Protein release level	[65]
– ^a	3.87-fold higher ^a	<0.05	N/A ^b	Relative expression (mRNA/GADPH)	[25,26]
1.4 (median)	24.78 (median) ^e	<0.05	N/A ^b	Relative expression (mRNA/ACTB)	[27]

^a The control group is a baseline for assessing the fold change. No absolute values were reported. ^b N/A (not applicable/not available). ^c Factor expression levels in fibroblasts were measured after exposing the cells to activated human mast cells (HMC-1^{FceRI}). The results in the table are shown relative to the expression levels of inactive cells. ^d The control group consisted of patients with atherosclerosis, as the study compared gene expression between atherosclerosis and tympanosclerosis. ^e The mRNA level for the *TNF-α* gene was significantly higher in the group with advanced disease ($p = 0.0002$).

A thorough investigation of the processes behind the pathomechanism of tympanosclerosis may also enable the development of targeted drugs, potentially reducing the symptoms of the disease or even reversing the lesions. The current procedure of treatment of tympanosclerosis includes drainage of the middle ear, which significantly improves the prognosis by reducing the likelihood of advanced effects of tympanosclerosis, such as the fixation of the auditory ossicle chain. Dinç et al. (2019) have reported that the probability of a mobile ossicular chain or mobile stapes is higher when there is a clinical history of recent drainage [162,171]. However, if the fixation develops—especially fixation of the stapes—hearing loss will advance, and the only available procedure is surgical treatment: stapediolysis, stapedectomy, or stapedotomy [172], and this treatment aims to improve the patient's hearing. It is not, however, an immensely effective treatment, as the results are not always sufficient to restore hearing to a usable level [5,173]. A new, alternative treatment would certainly be of help when dealing with difficult-to-manage cases of tympanosclerosis. Detailed knowledge about involved factors and enzymes, about how they contribute to tympanosclerosis and how the changes in their expression occur, would be indispensable to developing such technology, and, therefore, should be acquired.

In addition to the epigenetic changes in expression, some studies have detected and reported mutations in patients with tympanosclerosis or animals with induced tympanosclerosis models—in particular, mutations in genes regulating immune response, oxidative stress and mineralization (see Table 3).

Table 3. Genetic variants associated with tympanosclerosis and their functional consequences.

Gene	Type of Mutation/Polymorphism	Study Model	Functional Consequence
<i>NOS2</i>	−277A>G polymorphism	Human	Elevated nitric oxide levels, oxidative stress, cellular damage, tissue remodeling
<i>CAT</i>	−21A>T promoter polymorphism	Human	Lower catalase activity, weakened antioxidant defence
<i>NAT2</i>	<i>NAT2</i> *6A allele (slow acetylator phenotype)	Human	Impaired detoxification and ROS handling, significant increase in tympanosclerosis risk
<i>TLR4</i>	Asp299Gly (A>G) polymorphism	Human	Reduced cytokine response, development of chronic inflammation, increase in tympanosclerosis risk
<i>Evi1</i>	A2288T transversion (missense mutation)	Mice	Reduced inhibition of TGF-β pathway, increased mucin production,

				increased effusion, increased fibrosis
<i>Enpp1</i>	p.V246D (T>A in exon 7; “ <i>asj</i> ” mutation)	Mice		Decreased pyrophosphate levels, ectopic mineralization, bone deposition
<i>Rpl38</i>	Tail-short mutation	Mice		Increased phosphate levels, increased mineralization
<i>TCR Vβ6</i>	T-cell receptor usage	Mice		Autoimmune response to type II collagen
<i>NBPF15/16</i>	1q21.1 microduplication	Human		Association with bilateral tympanosclerosis, developmental delay and facial dysmorphism

Mutation of *Evi1* (A2288T) in mouse models weakens its inhibitory effect on TGF-β, resulting in increased expression of TGF-β and MUC2, a protein that increases the viscosity of the effusion and impairs the mucociliary clearance. These alterations further intensify the effusive processes in the middle ear. Mutation of *Enpp1* (*asj* mutation, p.V246D) in a mouse model leads to pyrophosphate deficiency, promoting mineralization and calcification of middle ear tissues. Mutations in *Rpl38* also lead to impaired phosphate metabolism and ectopic mineralization.

Regarding mutations detected in humans, the *TLR4* Asp299Gly polymorphism disrupts the immune response by attenuating the expression of TNF-α, IL-6 and IL-10, leading to chronic inflammation and the transformation of macrophages into osteoclasts, contributing to the formation of calcium plaques. The *CAT* (−21A>T) polymorphism impairs antioxidant enzyme activity, exacerbating oxidative stress. Moreover, the *NOS2* polymorphism (−277A>G) correlates with a higher risk of tympanosclerosis. Mutations in *NAT2*6A* are associated with impaired detoxification and oxidative stress and increased disease risk. The polymorphism frequencies in the studied human populations are summarized in Table 4 (see Table 4).

Table 4. A summary of genetic polymorphism frequencies and their associations with increased tympanosclerosis risk between healthy and tympanosclerosis study populations.

Gene	Polymorphism	Healthy [n; %]	Tympano- sclerosis [n; %]	Total Study Popula- tion [n]		p Value	Odd Ratio	95% CI	Source
				Healthy	Tympano- sclerosis				
CAT	−21A>T, genotype TT	16; 8.5	16; 22.2			0.002	0.54	0.29–0.99	
	−277A>G, genotype GG	44; 23.4	25; 34.7	188	72				[37]
NOS2	−277A>G, genotype AA	30; 16.0	20; 27.8			0.003	0.42	0.22–0.8	
NAT2*6	Mutant	3; 3.0	11; 11.6	100	95	0.03	4.3	1.15–16.1	[41]
TLR4	Asp299Gly	6; 6.0	5; 10.0	100	50	<0.005	N/A *	N/A *	[58]

* N/A (not applicable/not available).

In addition, clinical observations identify the possible involvement of *NBPF15* and *NBPF16* genes in cases of tympanosclerosis associated with microdeletions/microduplications of the 1q21.2 region, although their exact role requires further study, as observed in a case study.

Gene mutations, such as those described in the reviewed studies, may be a risk factor for the development of tympanosclerosis. Upon closer examination of a larger group of people, these mutations could be used to test for tympanosclerosis risk in patients. Patients with detected risk could be given additional measures to prevent the development of tympanosclerosis.

A study reported that bacterial otitis media caused by *Pseudomonas aeruginosa* can lead to tympanosclerosis [174]. More precisely, exotoxins produced by this bacterium can contribute to the development of tympanosclerosis. Although they are not endogenous changes in gene expression in cells of middle ear tissues, exotoxins are associated with calcification and, thus, can initiate processes leading to tympanosclerosis. It should be noted that although *P. aeruginosa* is a rare cause of acute otitis media, it is frequently detected in chronic suppurative otitis media, especially in cases with persistent tympanic membrane perforation and biofilm formation, with some studies reporting it as the predominant pathogen in such cases [175].

The limitation of this review is the insufficient number of large-sample studies in human populations, which makes it difficult to confidently assess the relationships between the studied factors and tympanosclerosis, and creates a risk of publication bias and selective reporting. A risk-of-bias assessment of human studies has shown that most studies did not identify potential confounding factors or discuss strategies to address them, thereby increasing the risk of confounding bias and limiting the reliability of the results.

Another limitation is that a substantial part of the included studies was conducted on animal models, which does not allow for a direct transfer of the results to humans, but requires additional research using human test subjects. After assessing the risk of bias in the reviewed animal studies, we discovered that many articles did not mention the animal allocation sequence generation and application, animal allocation concealment, description of baseline characteristics of the animals, random animal housing, and personnel blinding in the methods section. The inability to verify whether these bias precautions were applied is a significant limitation and may result in difficulties translating the results to clinical studies.

We currently do not have enough scientific evidence to reach conclusive results for this review, especially evidence acquired in studies using human tissues or studies on patients. It is certainly necessary to conduct longitudinal studies on a large, well-characterized cohort of patients with tympanosclerosis to assess causality between the molecular changes described in this review and the risk or progression of tympanosclerosis, and to differentiate it from co-occurrence. At the same time, in order to be able to conduct such clinical studies and achieve relevant results, we must reduce the translational gap between animal and clinical studies by ensuring the rigorous design, conduction and reporting of animal studies according to the current standards, as well as analysis of animal models for relevance to the planned clinical study [176,177]. The use of specific tools to facilitate and standardize validation and comparison of animal disease models to reduce the translational gap has been proposed [176].

The limitations of applied review processes include restriction to English-only studies and the inability to retrieve articles. Although we attempted to avoid reporting bias by conducting a comprehensive literature search across multiple databases and screening reference lists, we did not employ additional strategies aimed at limiting publication bias, such as contacting authors for unpublished data or searching trial registries. Therefore, we cannot exclude the possibility of publication bias or selective outcome reporting, especially in small-sample studies. The currently available evidence is heterogeneous, and a reliable sensitivity analysis would not be possible. However, such analyses would be essential in future studies, given the reported limitations, once sufficient data become available.

Identifying the genetic factors involved in the pathogenesis of tympanosclerosis is crucial for understanding the underlying mechanisms of the disease. Such data can help with the identification of risk factors for the disease in patients, selecting the most effective therapies, and, potentially, with developing targeted molecular treatments that not only inhibit the disease process but also potentially reverse the pathologies, which could be implemented in difficult cases. However, the current level of knowledge does not allow for developing such innovations, and further studies, particularly those involving human samples, need to be conducted to confirm the findings, solidify the current evidence and broaden our knowledge.

5. Conclusions

The reviewed studies indicate that persistent inflammation is a key factor in the progression of tympanosclerosis, characterized by elevated expression of TNF- α , IL-6, and IL-10. TNF- α sustains chronic inflammation, IL-6 promotes iNOS activation, osteoclastogenesis, calcification and fibrosis, while IL-10 modulates this response in balance with IL-6. Early inflammatory progression is further supported by PAI-1, which enhances neutrophil migration and inhibits tPA/uPA activity, thereby favoring chronic inflammation. Oxidative stress contributes through upregulation of iNOS and increased NO production, which initially inhibits osteoclasts but eventually induces oxidative damage and tissue remodeling. Polymorphisms in antioxidant and detoxification genes—including *CAT* (-21A>T), *NOS2* (-277A>G), and *NAT2* (*NAT2**6A)—impair antioxidant defenses and increase susceptibility to oxidative damage in tympanosclerosis. Fibrosis and extracellular matrix remodeling are mediated by elevated TGF- β 1, altered collagen composition (increased *COL2A1* and *COL4A1*, decreased *COL1A1*), and enhanced MMP-9 activity. Dysregulation is further exacerbated by *Evi1* mutations, which reduce inhibition of TGF- β signaling. Calcification and osteogenesis involve activation of the Wnt/ β -catenin pathway, increased expression of SOST, and osteogenic factors, such as BMP-8a. Additionally, alterations in phosphate metabolism caused by *Enpp1* mutations and modified *Rpl38* expression promote ectopic mineralization. These findings are derived from heterogeneous studies, predominantly in animal models, and require confirmation in rigorously designed human studies.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app16094420/s1>, Supplementary File S1: Review protocol; Supplementary File S2: PRISMA Checklist; Supplementary File S3: PRISMA-A; Supplementary File S4: List of excluded publications; Supplementary File S5: Risk of bias assessment; Supplementary File S6: Data extraction form S6.

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Abbreviations

The following abbreviations are used in this manuscript:

TNF- α	Tumor Necrosis Factor alpha
IL-6	Interleukin 6
CAT	Catalase
iNOS	inducible Nitric Oxide Synthase
Wnt	Wingless-related integration site signaling pathway
TGF- β 1	Transforming Growth Factor beta 1
TLR4	Toll-Like Receptor 4
NOS2	Nitric Oxide Synthase 2
<i>Enpp1</i>	Ectonucleotide Pyrophosphatase/Phosphodiesterase 1
NAT2	N-acetyltransferase 2
TNF	Tumor Necrosis Factor
mRNA	messenger Ribonucleic Acid
ROS	Reactive Oxygen Species
NO	Nitric Oxide
VEGF	Vascular Endothelial Growth Factor
IL-1 β	Interleukin 1 beta
PAI-1	Plasminogen Activator Inhibitor Type 1
<i>Serpine1</i>	Serine (or cysteine) peptidase inhibitor, clade E, member 1
<i>Plat</i>	Plasminogen Activator, Tissue
<i>Plau</i>	Plasminogen Activator, Urokinase
NTHi	Non-typeable Haemophilus influenzae
IFN- γ	Interferon gamma
tPA	tissue-type Plasminogen Activator
uPA	urokinase-type Plasminogen Activator
CTSB	Cathepsin B
CTSK	Cathepsin K
CTSL	Cathepsin L
IL-10	Interleukin 10
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
SMAD2	SMAD Family Member 2
SMAD3	SMAD Family Member 3
SMAD4	SMAD Family Member 4
Col1	Collagen Type I
α -SMA	α -Smooth Muscle Actin
<i>Evi1</i>	Ecotropic Viral Integration Site 1
MUC2	Mucin 2
<i>Rpl38</i>	Ribosomal Protein L38
FGF23	Fibroblast Growth Factor 23
Ppi	Inorganic Pyrophosphate
LRP5/6	Low-Density Lipoprotein Receptor-Related Protein 5/6
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
TCF/LEF	T-cell Factor / Lymphoid Enhancer Factor
OPG	Osteoprotegerin
RANKL	Receptor Activator of Nuclear Factor κ B Ligand
RANK	Receptor Activator of Nuclear Factor κ B
SOST	Sclerostin
ERK1/2	Extracellular Signal-Regulated Kinases 1 and 2
MAPK	Mitogen-Activated Protein Kinase
OPN	Osteopontin
<i>SPP1</i>	Secreted Phosphoprotein 1

CaSR	Calcium-Sensing Receptor
DKK1	Dickkopf-related Protein 1
MMP	Matrix Metalloproteinase
MMP-9	Matrix Metalloproteinase 9
MMP-3	Matrix Metalloproteinase 3
MMP-12	Matrix Metalloproteinase 12
SARA	Smad Anchor for Receptor Activation
JNK1/2	c-Jun N-terminal kinases 1 and 2
BMP	Bone Morphogenetic Protein
BMP-8a	Bone Morphogenetic Protein 8a
COL1A1	Collagen Type I Alpha 1 Chain
COL2A1	Collagen Type II Alpha 1 Chain
COL4A1	Collagen Type IV Alpha 1 Chain
CII	Collagen Type II
TCR	T-cell Receptor
TCR V β	T-cell Receptor Variable beta chain
NBPF	Neuroblastoma Breakpoint Family
NBPF15	Neuroblastoma Breakpoint Family Member 15
NBPF16	Neuroblastoma Breakpoint Family Member 16
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses

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