

# Role of microRNAs as novel diagnostic biomarkers and potential therapeutic targets for hearing disorders (Review)

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Received July 22, 2022; Accepted August 31, 2022

DOI: 10.3892/ije.2022.12

**Abstract.** The ear is a complex structure consisting of the outer, middle and inner ear. For the proper function of the auditory system, the development of all these parts needs to be highly organized. Any alterations in this chain of events can lead to hearing loss or other impairments, such as dizziness, tinnitus and loss of balance. Of note, several external and internal elements, including noise exposure, ototoxic drugs, aging, as well as environmental or genetic factors, have been widely associated with hearing loss. Over the years, a growing body of evidence has indicated that microRNAs (miRNAs/miRs) play a critical role in ear development and auditory function, suggesting that an altered pattern of miRNA expression may be involved in the progression of these pathological conditions. On these bases, the present review article aimed to summarize the role of miRNAs in inner ear development and hearing disorders. Although further studies are warranted to provide more in-depth knowledge of the implicated mechanisms of action, according to the current data, miRNAs may be used as novel diagnostic biomarkers for the early detection of hearing loss. In addition, miRNAs and related signaling pathways may represent novel potential therapeutic targets for the treatment of hearing loss, ear inflammation and vestibular schwannoma.

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## 1. Introduction

The ear is the organ of the human body responsible for auditory function and maintaining balance. Anatomically, it consists of external and internal parts (outer, middle and inner ear), forming a complex structure. The auricle represents the visible part of the outer ear, whose function is to collect airborne sound waves and funnel them into the ear canal towards the eardrum (1). From the tympanic membrane, sound waves are transmitted to the oval window via an ossicular chain (malleus, incus and stapes) suspended within the air-filled middle ear cavity. The vibration of the three middle ear bones plays a key role in amplifying the auditory stimuli, which are conducted to a fluid-filled spiral structure, the cochlea (2,3). Within the cochlear structure of the inner ear, sound waves are transduced into electrochemical impulses by the auditory hair cells of the organ of Corti that in turn send these signals to the brain via the acoustic nerves (4,5). For a proper function of the auditory system, the development of all the parts must be parallel and in strict coordination from the outer to the inner ear. Any alterations in this composite structure can lead to ear defects and hearing loss (6).

Apart from hearing loss, other pathological conditions can affect the ear, including dizziness, tinnitus, loss of balance and inflammatory processes. These disorders occur in a large percentage of the population worldwide and may have a multi-factorial etiology (7,8). As reported in the literature, aging and environmental factors (noise exposure, chemical and physical factors) appear to be involved in the onset of hearing-related disorders (9-11). Moreover, even epigenetic alterations due to DNA methylation and histone modifications may play critical roles in hearing impairments and ear cancer (12).

In this context, microRNAs (miRNAs/miRs) have recently attracted the attention of the scientific community for their

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**Key words:** auditory system, microRNAs, inner ear development, hair cells, cochlea, hearing loss, ear infection, otitis, ototoxic drug, vestibular schwannoma

key role in gene regulation and expression, as well as for the interaction with various environmental factors able to modulate their expression levels (13-15). miRNAs are a wide class of small non-coding single-stranded RNAs, containing ~18-24 nucleotides, identified both in animals and plants (16,17). These highly conserved RNAs present a complex biogenesis that occurs not only in the nucleus, but also in the cytoplasm (Fig. 1). At the nuclear level, RNA polymerase II (RNA Pol II) transcribes the majority of the miRNAs from the non-coding region of DNA to obtain primary miRNAs (pri-miRNAs), while some miRNAs are transcribed by another polymerase (RNA Pol III) (18). Specifically, pri-miRNAs have a stem-loop structure and are characterized by a 5'-terminal 7-methyl-guanosine cap (m<sup>7</sup>G) and a 3'-terminal poly(A) tail (19). Within the nucleus, ribonuclease III Drosha modifies pri-miRNAs through a cleavage in association with DiGeorge syndrome critical region gene 8 (DGCR8) protein, to generate precursor-miRNAs (pre-miRNAs) (20). Subsequently, pre-miRNAs are transported to the cytoplasm via exportin-5, another ribonuclease III that acts synergistically with ras-related nuclear protein-guanosine triphosphate (Ran-GTP), recognizing the 3'-terminal of pre-miRNAs (21,22). In the cytoplasm, pre-miRNAs are cleaved by ribonuclease III Dicer with the aid of transactivation response rna binding protein and interferon-inducible double-stranded RNA-dependent activator proteins to form a mature double-stranded miRNA, indicated as miRNA-miRNA\* duplex (23). Finally, following the division of the miRNA-miRNA\* duplex, the miRNA strand is incorporated into the RNA-induced silencing complex (RISC), while the miRNA\* strand is degraded. Of note, the RISC is characterized by several proteins involved in the mRNA silencing process, such as Argonaute (Ago) proteins (24,25). In particular, the miRNA strand associated with the RISC binds to its complementary sequence in the 3'-untranslated region of the target mRNA, either promoting the degradation of mRNA or inhibiting protein translation (26,27).

Various studies have demonstrated that miRNA dysregulation is associated with a variety of pathological conditions, including tumors, neurological disorders and other acute and chronic diseases (28-31). Of note, some studies have also demonstrated that a number of miRNAs are involved in inner ear development, differentiation and the survival of inner ear hair cells, suggesting that changes in their expression levels may play a critical role in the onset of hearing disorders (32-35). On these bases, the aim of the present review article was to report a summary of the studies that have been conducted over the past years in order to provide a better understanding of the role of miRNAs in inner ear development. In addition, the present review article focused on the potential association between altered miRNA expression and hearing loss, ear inflammatory processes, drug-induced ototoxicity and cancers affecting the auditory system, such as vestibular schwannoma.

## 2. Regulatory role of miRNAs in inner ear development

The inner ear is a labyrinthine structure composed of three semicircular canals, the utricle that connects these three canals and the saccule. In addition, it is characterized by the cochlea, a fluid-filled spiral structure able to convert sounds into hearing through the organ of Corti (Fig. 2) (36,37). The development

of the inner ear is a complex process that requires several steps, beginning from the transformation of the embryonic ectoderm to the formation of functionally mature structures. This process also includes an accurate histological organization of supporting cells and mechanosensory hair cells, which transduce hearing impulses to the neurons (38,39).

As widely described in the literature, a multitude of genes and transcription factors, including atonal BHLH transcription factor 1, neurogenin 1, neuronal differentiation 1, fibroblast growth factor, Hedgehog, SRY-box transcription factor 2 (Sox2) and eyes absent 1, as well as the Notch and Wnt signaling pathways, are responsible for this coordinated transition from precursor cells to high differentiated cell types (40-43). However, several studies have highlighted that inner ear development is not exclusively regulated by proteins, indicating that miRNAs may also play a key role in the development and function of the auditory system.

For example, Jiang *et al* (44) conducted an *in vitro* study on mouse neural stem cells to evaluate the expression levels of miRNAs during neuronal differentiation. Notably, since miR-124 exhibited the highest increase in expression among the considered miRNAs (six-fold compared to baseline conditions), the research group transfected the inner ear neural stem cells with RNA oligoribonucleotides (non-specific miRNA, miR-124 mimics and miR-124 inhibitor). Notably, the differentiation of neural cells was reduced by the knock-down of miR-124, whereas the overexpression of miR-124 resulted in a significant increase. In addition, the expression levels of receptor kinase B (TrkB), a receptor involved in the regulation of neurogenesis and survival of neurons, and cell division control protein 42 homolog (Cdc42), a GTPase, which controls the neurite extension of spiral ganglion neurons, were also investigated (44). Notably, Jiang *et al* (44) found that the overexpression of miR-124 enhanced the levels of both these factors, suggesting that this miRNA may regulate neural differentiation by regulating TrkB and Cdc42.

Similarly, Du *et al* (45) assessed the spatial expression of miRNAs during inner ear development using mouse embryos. In brief, miR-183 family members (miR-183/96/182) and miR-15a were more expressed in the sensory epithelium, while miR-194 was predominantly detected in the spiral ganglion. Since miR-194 exhibited a dynamic expression during inner ear development, they focused on this miRNA to investigate its function. Specifically, the transfection of suspended cells from the spiral ganglion highlighted that the overexpression of miR-194 affected neuron morphology, including a disorganized somatodendritic cytoskeleton (45). In addition, to further elucidate the mechanisms of action, the research group evaluated the expression of Ras homolog B (RhoB), a member of the Rho GTPase family functionally connected to microtubule associated protein 1A and actin regulatory proteins. Of note, miR-194 overexpression induced a significant reduction in RhoB expression, while the protein expression levels were enhanced following miRNA knockdown. Overall, these results suggest that miR-194 may be crucial to the morphogenesis of spiral ganglion neurons by targeting RhoB (45).

Subsequently, the physiological functions of the miR-183/96/182 cluster have also been investigated. For this purpose, Geng *et al* (46) used knockout mice in which the clustered gene had been inactivated. As demonstrated by the

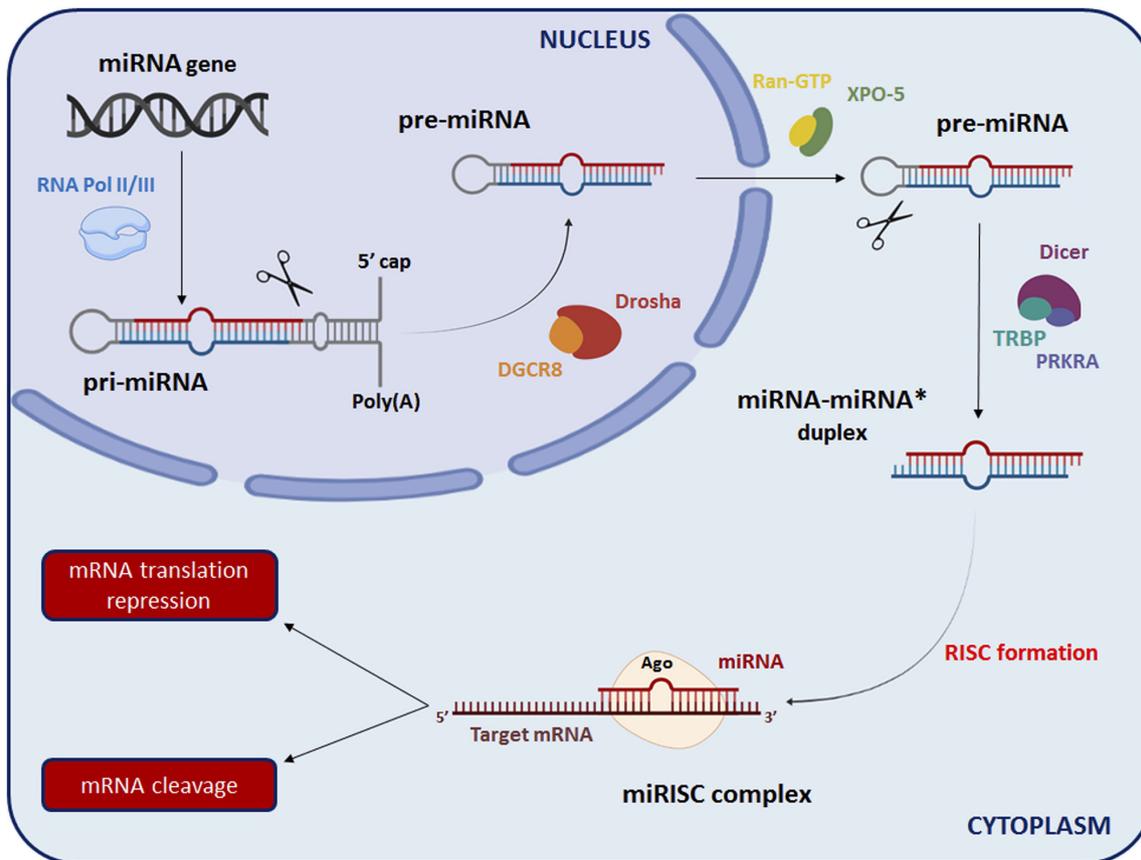


Figure 1. Schematic representation of miRNA biosynthesis. Ago, Argonaute proteins; DGCR8, DiGeorge syndrome critical region gene 8; miRNA, microRNA; pre-miRNA, precursor miRNA; PRKRA, Interferon-Inducible double-stranded RNA-dependent activator; pri-miRNA, primary miRNA; Ran-GTP, ras-related nuclear protein-guanosine triphosphate; RISC, RNA-induced silencing complex; RNA Pol II/III, RNA polymerase II/III; TRBP, transactivation response RNA binding protein; XPO-5, exportin-5.

auditory brain stem response test, the loss of function of the miR-183/96/182 cluster was strictly related to hearing loss in the knockout group compared to the wild-type group. At the same time, the cochlear hair cells of knockout mice exhibited severe morphological alterations, including abnormal apices, lack of kinocilia and immature stereocilia of equal length. Of note, due to severe defects that characterized the hair cells, the organ of Corti of mutant mice exhibited no functional mechano-electrical transduction. Moreover, the research group evaluated the expression of several predicted target genes (46). Specifically, chloride intracellular channel 5 protein, Radixin, ezrin, Rac family small GTPase 1, myosin 1C and Sox2 levels were upregulated in the cochlea of the knockout group compared to the controls. In summary, that study highlighted that the inhibition of the miR-183/96/182 cluster led to severe alterations in the morphology of hair cells and the function of the organ of Corti in mice, confirming the importance of miRNAs in the development of hearing functions (46).

### 3. Role of miRNAs in hearing loss

Hearing loss is a public health concern affecting a large percentage of the worldwide population and is more frequent in low- and middle-income countries than in industrialized ones (47). According to the World Health Organization (WHO), 466 million individuals (34 million children) are

currently affected by hearing loss, with a 50% increase in hearing disorders expected for the year 2050 compared to 2021 (~700 million individuals) (48).

It is possible to classify hearing loss in three clinical forms: Conductive, sensorineural and mixed. The conductive type is related to a disturbance in the transmission of sound waves from the outer to the inner ear, which leads to difficulties in speech comprehension and sound localization, as well as a negative effect on balance (49,50). Sensorineural hearing loss represents the most common clinical form among children and adults. It is due to the functional damage of the inner ear hair cells or the dysfunction of cochlear neural structures (51). As regards the mixed form, it occurs when both conductive and sensorineural features are present simultaneously (52).

Over the years, several factors have been implicated in the development of hearing loss, whose co-occurrence can result in severe impairments of the auditory system. Among the exogenous factors, noise exposure, smoking and chemotherapeutic drugs are the most well-known, while genetic mutations and aging represent the main involved endogenous factors (53-57). Of note, a number of recent studies have suggested that an altered regulation of genes mediated by miRNA expression may also contribute to hearing loss (58-61).

Xue *et al* (58) examined the potential association between miR-29b and the apoptosis of cochlear hair cells, one of the principal causes of age-related hearing loss. Through an

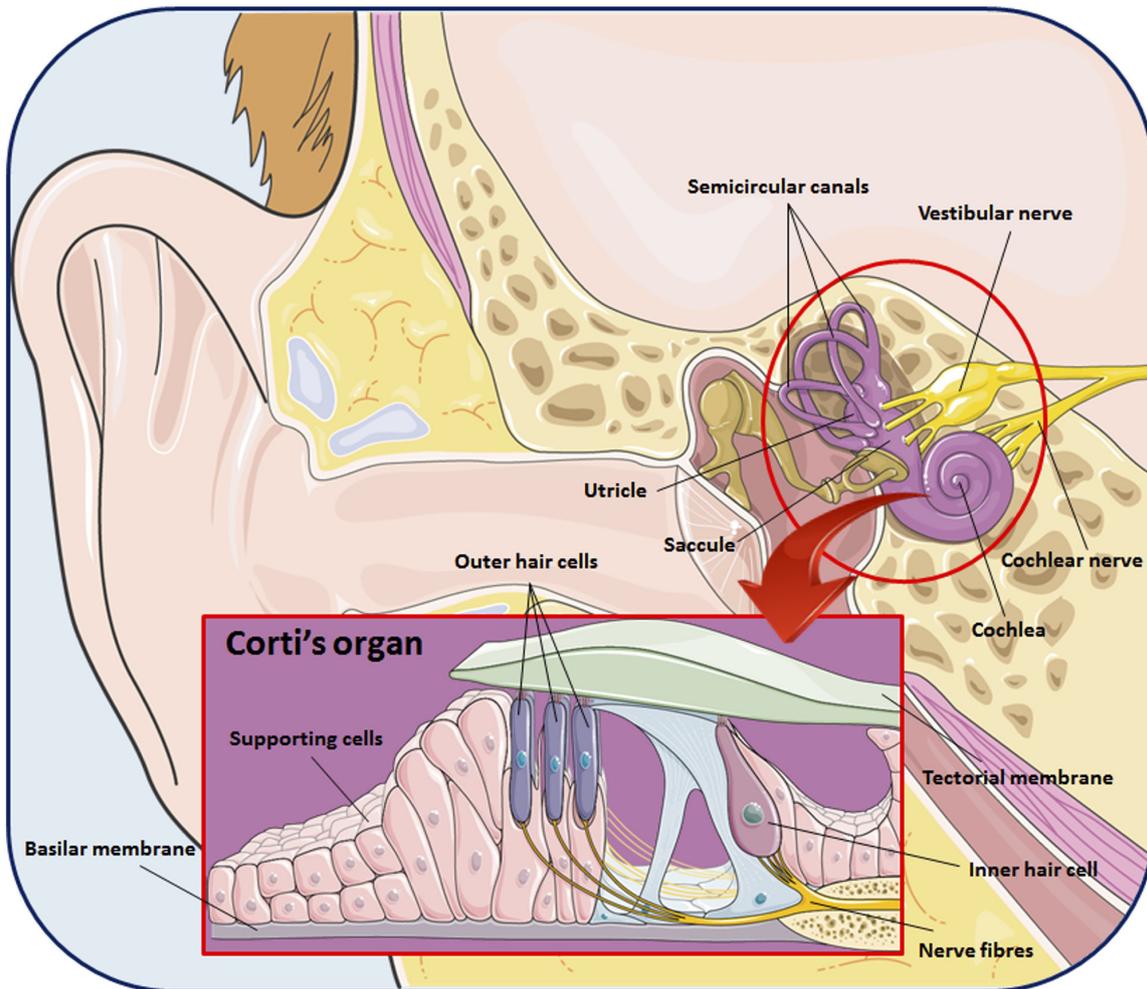


Figure 2. Anatomy of the inner ear.

*in vivo* study on a mouse model (C57BL/6), they observed that aged mice were characterized by a marked reduction in cochlear hair cells and higher miR-29b expression levels compared to the controls (young mice). At the same time, aged mice exhibited a significant decrease in Sirtuin 1 (SIRT1) and proliferator-activated receptor-gamma coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) expression levels, a deacetylase that regulates the intracellular oxidative stress and a coregulator involved in oxidative metabolism, respectively (58). The hypothesis that miR-29b may induce cell apoptosis and target SIRT1 and PGC-1 $\alpha$  was confirmed by the transfection of HEI-OC1 cells. Although further studies are required to better elucidate the mechanisms of action, the miR-29b/SIRT1/PGC-1 $\alpha$  signaling pathway may represent a novel potential target for the treatment of age-related hearing loss (58).

Similarly, the role of miR-34a in the pathogenesis of age-related hearing loss was evaluated in another study. Using C57BL/6 mice, Pang *et al* (59) noted that aging was strictly related not only to cochlear hair cell loss, but also to the upregulation of miR-34a expression. Moreover, an altered autophagic flux was observed in the cochlea of aged mice compared to young mice, as demonstrated by the variation in the levels of autophagy markers LC3-II and p62. To further investigate the mechanisms of action, the authors transfected

the HEI-OC1 mouse auditory cell line. Of note, the overexpression of miR-34a reduced autophagy-related protein 9A (ATG9A) expression, while the knockout of miR-34a exerted the opposite effect. The obtained data highlight the involvement of miRNAs in the progression of age-related hearing loss, indicating that the downregulation of ATG9A by miR-34a may play a crucial role in the impairment of the autophagic flux (59).

Of note, Li *et al* (60) focused on miRNA serum levels in subjects with occupational noise-induced hearing loss and healthy controls. Among the considered miRNAs, hsa-miR-4652-3p expression was downregulated in subjects with hearing issues compared to the controls, while the levels of hsa-miR-3162-5p, hsa-miR-4484 and hsa-miR-1229-5p were upregulated. However, only the hsa-miR-1229-5p serum levels were significantly increased. These researchers then conducted an *in vitro* study (293T cells) to validate the predicted targets of miR-1229-5p. Notably, the overexpression of miR-1229-5p markedly reduced mitogen-activated protein kinase 1 (MAPK1) levels (60). Taken together, these results indicate that miR-1229-5p may represent a novel diagnostic biomarker for noise exposure-related hearing loss. In addition, since MAPK1 has been described as a key molecule in regulating human genetic deafness, miR-1229-5p may be actively

Table I. miRNA expression in hearing loss.

Disease model	Study design/sample type	Up/downregulated miRNAs	Targets	(Refs.)
Age-related hearing loss	<i>In vivo</i> (mouse cochlea) <i>In vitro</i> (HEI-OC1 cells)	miR-29b (↑)	SIRT1, PGC-1 $\alpha$	(58)
Age-related hearing loss	<i>In vivo</i> (mouse cochlea) <i>In vitro</i> (HEI-OC1 cells)	miR-34a (↑)	ATG9A	(59)
Noise-induced hearing loss	Cohort (blood serum) <i>In vitro</i> (HEK293T cells)	miR-128-5p (↑)	MAPK1	(60)
Sudden sensorineural hearing loss	Cohort (blood serum)	miR-375-3p, miR-195-5p, miR-128-3p, miR-30a-3p, miR-140-3p, miR-590-5p, miR-132-3p, miR-186-5p (differentially expressed)	PI3K/AKT, MAPKs	(61)

Upward arrows indicate upregulation. ATG9A, autophagy-related protein 9A; MAPK1, mitogen-activated protein kinase 1; MAPKs, mitogen-activated protein kinases; PGC-1 $\alpha$ , proliferator-activated receptor-gamma coactivator 1 $\alpha$ ; PI3K/AKT, phosphoinositide 3-kinase/protein kinase B; SIRT1, Sirtuin 1.

involved in the progression of hearing disorders by inhibiting MAPK1 expression (60).

Recently, the potential association between miRNAs and sudden hearing loss was also investigated. In this regard, Nunez *et al* (61) conducted a prospective cohort study on a group of sudden sensorineural hearing loss patients and a control group consisting of normal hearing subjects. Briefly, the research group analyzed the circulating miRNA expression profiles observing that eight miRNAs (miR-375-3p, miR-195-5p, miR-128-3p, miR-30a-3p, miR-140-3p, miR-590-5p, miR-132-3p and miR-186-5p) were differentially expressed between the two groups examined. In addition, they noted that the genes involved in phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and MAPK signaling pathways represented the putative targets of the identified miRNAs. These findings confirm the involvement of miRNAs in hearing disorders, supporting their application as novel circulating biomarkers. However, further studies are warranted in order to provide a better understanding of the role of miRNAs in hearing loss (61).

A summary of miRNAs that have been investigated in hearing loss is presented in Table I.

#### 4. Involvement of miRNAs in ear infection and inflammatory processes

Ear infection represents one of the most common disorders affecting individuals of all ages. If left untreated, these infections can cause hearing loss and other auditory system complications, such as vestibular dysfunction. Depending on the triggering pathogen, ear infections can be classified into viral, bacterial and fungal (62).

Viruses frequently affect inner ear structures, inducing the development of inflammatory processes and enhancing the susceptibility to infection caused by other pathogens (63). Among viral infections, congenital cytomegalovirus infection is the leading cause of hearing loss in children (64). Other viruses, such as Rubella, Herpes simplex virus and Zika virus,

have been described as pathogens strictly related to hearing loss in newborns and adults (65-67).

As regards ear bacterial infections, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* and *Moraxella catarrhalis* are the most frequently involved pathogens (68-72). Of note, bacteria-induced meningitis has a cytotoxic effect on the cochlea and may lead to neural damage (73). In addition, even the middle ear can be affected by bacterial-induced inflammatory processes, of which otitis media with effusion and chronic suppurative otitis media are the most severe forms (74,75).

Compared to other ear infectious processes, fungi are usually involved in otitis externa, a cutis/subcutis inflammation of the external auditory canal. As reported in the literature, *Aspergillus* and *Candida* are the main causative genera of otomycosis (76,77). Of note, several factors can promote the progression of otitis externa, such as humidity, swimming and diving (78).

Over the past few years, the involvement of miRNAs and epigenetic modifications in the pro-inflammatory and anti-inflammatory processes have been investigated (79,80); however, the role of miRNAs in the pathogenesis of ear inflammation has not yet been clarified.

For example, Rudnicki *et al* (81) demonstrated that miR-224 plays a crucial role in inner ear inflammation, downregulating pentraxin 3 (Ptx3), a protein involved in the immune response, whose release is regulated by nuclear factor  $\kappa$ B (NF- $\kappa$ B). Briefly, the transfection of 293T and NIH3T3 cells revealed that the overexpression of miR-224 was strictly related to the decrease in Ptx3 levels (81). This inverse association was also evaluated through an *in vivo* experiment on a mouse model of inner ear inflammation (otitis interna). Notably, the injection of lipopolysaccharide into the scala tympani of mice induced an increase in miR-224 expression and Ptx3 mRNA levels, while Ptx3 protein levels exhibited no significant variations compared to the controls (81). As was reported in that study, since even the miR-224 promoter has a binding site for NF- $\kappa$ B, both miR-224 and Ptx3 may be

Table II. Altered miRNA expression during ear inflammation.

Disease model	Study design/sample type	Up/downregulated miRNAs	Targets	(Refs.)
Otitis interna	<i>In vivo</i> (mouse inner ear) <i>In vitro</i> (293T, NIH3T3 cells)	miR-224 (↑)	Ptx3	(81)
Otitis media with effusion and recurrent	Case-control (middle ear biopsies) <i>In vitro</i> (HMEECs)	miR-146a, miR-146b (↑)	TRAF6	(82)
Otitis media with effusion	Case-control (middle ear effusion and blood serum) <i>In vitro</i> (HMEECs)	miR-210 (↓)	HIF-1 $\alpha$	(83)

Upward and downward arrows indicate upregulation and downregulation, respectively. HMEECs, human middle ear epithelial cells; HIF-1 $\alpha$ , hypoxia-inducible factor-1  $\alpha$ ; Ptx3, pentraxin 3; TRAF6, TNF receptor-associated factor 6.

recruited during inflammation. Taken together, these findings suggest that miR-224 and Ptx3 form a feedback loop, where miR-224 downregulates Ptx3 reducing the inflammatory process (81).

Even miR-146 appears to be involved in ear inflammatory processes. In this regard, Samuels *et al* (82) conducted an *in vitro* study on human middle ear epithelial cells (HMEECs) to evaluate the expression of miR-146 following treatment with IL-1 $\beta$  or tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). Notably, miR-146a and miR-146b expression levels were upregulated by treatment with these pro-inflammatory cytokines (82). Moreover, they evaluated miR-146 expression in middle ear biopsies of patients with otitis media. Specifically, miR-146a was upregulated in patients with otitis media with effusion and recurrent otitis media, while miR-146b expression was significantly higher only in the recurrent group compared to the controls (82). At the same time, that research group noted that miR-146a and miR-146b expression was inversely related to TNF receptor-associated factor (TRAF6) levels, a member of the Toll-like receptor signaling pathway. In summary, that study highlighted that miR-146a and miR-146b may play a key role in the pathogenesis of otitis media by targeting TRAF6 (82).

Recently, the role of miR-210 in otitis media was also investigated. Specifically, Zhang *et al* (83) observed that miR-210 levels were significantly downregulated in middle ear effusion in the serum of patients affected by otitis media. On the contrary, the expression levels of pro-inflammatory cytokines, as well as nitric oxide and vascular endothelial growth factor (VEGF) were increased in the otitis group compared to the controls (83). These authors then conducted a functional assay to evaluate the potential regulatory role of the aforementioned miRNA. Of note, the transfection of HMEECs with miR-210 mimics led to a significant reduction in the expression levels of pro-inflammatory cytokines and cell apoptosis, whereas cell viability was increased. In addition, miR-210 overexpression was also related to the downregulation of hypoxia-inducible factor-1 $\alpha$ , a transcriptional factor involved in pro-inflammatory gene expression (83). Overall, the obtained results suggested that miR-210 overexpression may represent the starting point for a novel therapeutic approach with which to reduce ear inflammatory processes. However, further studies are required to better understand the mechanisms of action of this miRNA (83). Of note, inflammatory processes are not

only sustained by microbial infections. Indeed, it has been demonstrated that aging, occupational or environmental noise and drugs can induce ototoxic damage sustained by inflammatory processes (84-86). As regards drug-induced ototoxicity, an in-depth description is provided in the following chapter.

Aging is recognized as a main risk factor for various pathologies. As already mentioned in the previous chapter, miRNAs and inflammation play a pivotal role in age-related hearing loss. In this context, the mechanisms through which aging is able to alter the expression levels of miR-34a, which is involved in the regulation of various processes, including inflammation and autophagy, have been previously demonstrated in mice (59).

Similarly, it has also been demonstrated that noise-related inflammation is responsible for miRNA dysregulation and, in turn, hearing loss. As widely described in the previous chapter, the dysregulation of different miRNAs has been associated with noise-induced hearing loss (Table I) (60). Among the miRNAs dysregulated due to environments with high levels of noise, miR-4652-3p, miR-3162-5p, miR-4484 and miR-1229-5p were recently identified and associated with the regulation of pathways notoriously involved in inflammatory processes, such as the MAPK pathway (60).

Table II summarizes the expression of miRNAs during ear inflammatory processes (Table II).

## 5. miRNAs and drug-induced ototoxicity

Ototoxicity is a pharmacological temporary or permanent adverse event that affects the auditory system, causing a dysfunction of the inner ear structures, particularly cochlear and vestibular tissues (87,88). Over the years, several studies have reported a wide spectrum of chemicals associated with ototoxicity through both direct and indirect mechanisms mediated by miRNAs. In this context, it has been demonstrated that chronic pesticide exposure is associated with the dysregulation of various miRNAs responsible for the regulation of key genes involved in inflammatory processes or cellular homeostasis, and in turn, with potential ototoxic effects (13,89-91). Apart from pesticides, drugs used for the treatment of various diseases may also exert ototoxic effects. Among platinum-based anticancer drugs, cisplatin, carboplatin and oxaliplatin are the most ototoxic (92,93). Moreover, aminoglycoside antibiotics (neomycin, gentamicin, kanamycin, streptomycin and

amikacin), loop diuretics and macrolide antibiotics have been also described for their ototoxic potential (94-96).

Typical symptoms of drug-induced ototoxicity are represented by hearing loss, tinnitus, dizziness and loss of balance. These hearing disorders can occur during or at the end of the treatment period with a gradual or sudden appearance (97). Although ototoxic drugs can compromise auditory function in all age groups, some studies have reported that children are exposed to a higher risk of impaired cognitive performance and language than adults (98-100). Of note, the incidence of ototoxicity and severity is dependent on several factors, including the selection of the therapeutic agent and its pharmacokinetic, the route of administration and dose, concomitant medications and genetic factors (101,102).

In this context, an increasing number of studies have focused on miRNAs and their potential involvement in drug-induced ototoxicity to discover novel early diagnostic biomarkers and otoprotective therapeutic approaches.

For example, Kim *et al* (103) investigated the role of miRNAs in neomycin-induced ototoxicity using zebrafish embryos. Specifically, miRNA expression levels were evaluated during inner ear hair cell regeneration following treatment with neomycin. Microarray analysis revealed that the miR-183 cluster (miR-183/-96/-182) was significantly upregulated at 12-24 h in the treatment group, while the levels returned to basal levels after 48 h. The reported data suggest that the overexpression of these miRNAs may be crucial in repairing cochlear hair cell damage (103). Moreover, the same research group noted that the knockdown of miR-183 partially affected the regeneration process of hair cells. Taken together, these results demonstrated that the miR-183 cluster exerts an otoprotective effect able to contrast aminoglycoside antibiotics-induced hair cell damage in zebrafish. This miRNA cluster may represent the starting point for the development of novel therapeutic approaches (103).

Circulating miRNA levels as novel diagnostic biomarkers of drug-induced ototoxicity have also been investigated. By using a mouse model of ototoxicity induced by the administration of kanamycin and furosemide, Lee *et al* (104) detected the serum levels of circulating miRNAs. Notably, they found that miR-205 expression was higher in the ototoxic group compared to the controls (four-fold increase until day 14). They then focused on the inner ear, observing a significant difference in miR-205 levels among the cochlear components (104). Notably, the organ of Corti exhibited a slight increase only in the first phase (day 5), while stria vascularis was characterized by a gradual increase in miR-205 expression until day 14. Therefore, it can be hypothesized that the increase in serum miR-205 levels may be due to extravasate via stria vascularis (104). Overall, the obtained results highlight that miR-205 expression is strictly related to antibiotics-induced ototoxicity in mice, suggesting its potential use as a novel diagnostic biomarker of drug-related hearing impairments (104).

Subsequently, Li *et al* (105) conducted an *in vivo* study on zebrafish embryos to evaluate the association between  $\beta$ -diketone antibiotic-induced ototoxicity and the regulatory role of miRNAs in hearing. Firstly, they noted a decreased response to acoustic stimuli in the treatment group compared to the controls. At the same time, treatment with  $\beta$ -diketone antibiotics also induced the downregulation of miR-96 and

miR-184 expression in zebrafish otoliths (105). Subsequently, to confirm the regulatory role of these miRNAs in hearing development, the research group evaluated the effects of their inhibition and overexpression. Of note, only the overexpression of miR-96 restored hair cells following exposure to  $\beta$ -diketone antibiotics, indicating that this miRNA plays a critical role in hearing development. On the other hand, miR-184 overexpression exerted no effect on hair cells, demonstrating that miR-184 was only involved in the development of otic vesicles. These findings on miR-96 and miR-184 may provide the theoretical bases for the development of novel intervention strategies against drug-induced ototoxicity (105).

Recently, the role of miR-182 on the hearing loss induced by ototoxic drugs was also investigated. In this regard, Chen *et al* (106) demonstrated that miR-182 was able to reduce harmful effects on the auditory system induced by kanamycin and furosemide. In brief, pre-treatment with miR-182 attenuated the permanent threshold shift caused by the concomitant administration of kanamycin and furosemide in rats. At the same time, miR-182 overexpression significantly reduced hair cell death, protecting stereocilia and enhancing PI3K regulatory subunit p85 $\alpha$  levels (106). In summary, that study suggested that miR-182 played a key role in protecting the auditory function against drug-induced deafness in rats. However, further *in vivo* and *in vitro* studies are warranted in order to better clarify the mechanisms of action and the potential targets of this miRNA (106).

## 6. Altered miRNA expression in vestibular schwannoma

Vestibular schwannoma, also known as acoustic neuroma, is a slow-growing non-malignant tumor (average growth rate of 1-2 mm/year) originating from the Schwann cells of the vestibular nerve (107,108). This benign tumor, characterized by a mortality rate of <1%, has been classified into two typologies, sporadic and associated with neurofibromatosis type 2 (NF2) syndrome. Although vestibular schwannoma generally occurs unilaterally, it may also appear bilaterally when related to NF2 (5%) (109-111).

The overall incidence of vestibular schwannoma is 1.4 per 100,000 individuals per year and the majority of cases occur among middle-aged individuals of both sexes (112). Clinically, hearing loss and tinnitus are the typical symptoms, observed in 94% and 83% of patients, respectively. Other less frequent symptoms are represented by vertigo (20%), facial numbness (12%) and facial palsy (6%) (113,114).

Currently, the gold standard for the diagnosis of vestibular schwannoma is represented by magnetic resonance imaging, while surgical resection, fractionated radiotherapy and radiosurgery represent the available treatment options (115,116). However, it is necessary to identify novel therapeutic targets in order to develop less invasive intervention strategies able to improve the management of patient and hearing outcomes. Over the past decade, a growing body of evidence has demonstrated that several miRNAs may be involved in the progression of vestibular schwannoma, suggesting their potential value as novel drug targets.

For example, miR-21 has been found to be overexpressed in several tumor types, including vestibular schwannoma. In this context, Cioffi *et al* (117) observed that the miR-21 expression

Table III. Upregulated and downregulated miRNAs in vestibular schwannoma.

Study design/sample type	Up/downregulated miRNAs	Targets	(Refs.)
Case-control (tumor tissue vs. normal vestibular nerve tissue) <i>In vitro</i> (primary human VS cell cultures)	miR-21 (↑)	PTEN	(117)
Case-control (tumor tissue vs. normal peripheral nerve tissue) <i>In vitro</i> (HEI-193 cells)	miR-7 (↓)	EGFR, Pak1, Ack1	(118)
Case-control (tumor tissue vs. normal vestibular nerve tissue) <i>In vitro</i> (HEI-193 cells)	miR-1 (↓)	VEGFA	(120)
Case-control (tumor tissue vs. normal great auricular nerve tissue) <i>In vitro</i> (primary human VS cell cultures, 293T cells)	miR-205 (↓)	CDK14	(124)

Upward and downward arrows indicate upregulation and downregulation, respectively. Ack1, activated Cdc42-associated kinase 1; CDK14, cyclin-dependent kinase 14; EGFR, epidermal growth factor receptor; Pak1, p21-activated kinase 1; PTEN, phosphatase and tensin homolog; VEGFA, vascular endothelial growth factor A; VS, vestibular schwannoma.

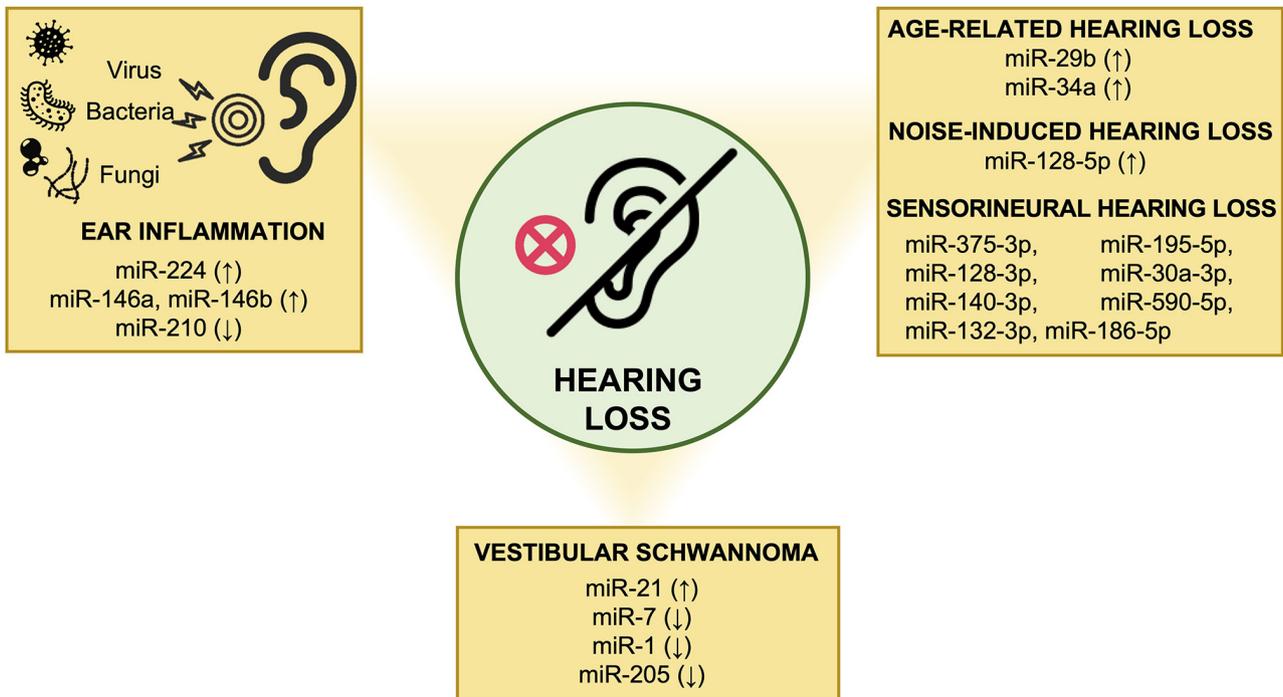


Figure 3. Schematic representation of the causes of alterations in miRNA expression associated with hearing loss. miRNA/miR, microRNA.

levels were higher in vestibular schwannoma samples than in the controls. Moreover, the aberrant expression of miR-21 was strictly related to low levels of phosphatase and tensin homolog (PTEN), a protein that acts on the PI3K/AKT pathway, inhibiting cell proliferation. The inverse association between miR-21 and PTEN levels was confirmed through a functional analysis. Specifically, the transfection of anti-miR-21 led to a significant reduction in cell proliferation due to miR-21 knock-down (117). In summary, these data underline that miR-21 overexpression is also involved in vestibular schwannoma formation and growth by targeting PTEN, which results in the hyperactivation of the PI3K/AKT pathway (117).

As previously reported by Saydam *et al* (118), another miRNA that could be involved in vestibular schwannoma is miR-7. In brief, they noted that several miRNAs were

deregulated in tumor samples compared to normal peripheral nerve tissues derived from fresh autopsies. They then investigated the functional significance of miR-7, the most notably downregulated miRNA in vestibular schwannoma samples. Specifically, the miR-7 levels were inversely related to epidermal growth factor receptor (EGFR) and p21-activated kinase 1 (Pak1) levels, both involved in cell division, survival and migration. In addition, Saydam *et al* (118) found that miR-7 overexpression also targeted activated Cdc42-associated kinase 1 (Ack1), a non-receptor tyrosine kinase whose activation has been found in several tumor types (prostate cancer, lung cancer, breast cancer, etc.) (119). These findings suggest that miR-7 could potentially act as a tumor suppressor in vestibular schwannoma formation and growth by inhibiting the EGFR, Pak1 and Ack1 signaling pathways (118).

Of note, Li *et al* (120) focused on miR-1, a small non-coding RNA downregulated in various types of cancer (oral cancer, colorectal cancer, lung cancer, etc.) (121-123). As was expected, the miR-1 expression levels were significantly decreased in vestibular schwannoma specimens compared to the controls (normal vestibular nerve). The hypothesis that miR-1 may play a suppressive role in this tumor was further confirmed by the transfection of the HEI-193 cell line. Specifically, the overexpression of miR-1 led to a significant reduction in cell proliferation, enhancing the apoptotic process, while the knockout of miR-1 exerted the opposite effect (120). At the same time, *in vitro* experiments revealed that transfection with miR-1 mimic was negatively related to VEGFA levels. Overall, the described results highlight that miR-1 may play a crucial role in the progression of vestibular schwannoma, providing a theoretical basis for the development of novel effective treatments against this pathological condition (120).

Recently, the potential inhibitory role of miR-205 in sporadic vestibular schwannoma was also investigated. Notably, Yin *et al* (124) found that miR-205 expression levels were lower in tumor tissues than in normal great auricular nerves (controls). Secondly, the authors transfected primary human vestibular schwannoma cell cultures and the 293T cell line with miR-205 mimic to evaluate the effects of miRNA overexpression on cell proliferation in vestibular schwannoma. Of note, the functional assay revealed that miR-205 overexpression resulted in a significant decrease in cell growth. Finally, Yin *et al* (124) concentrated on the mechanisms of action. Specifically, they observed that the *in vitro* overexpression of miR-205 significantly downregulated cyclin-dependent kinase 14 (CDK14) levels, whose high expression was positively related to cell growth. The obtained results demonstrate that miR-205 may play an inhibitory role against vestibular schwannoma progression by targeting CDK14. However, further studies are required to better clarify the effects of miR-205 expression in this tumor and its mechanisms of action (124).

The alterations of miRNAs involved in vestibular Schwannoma are summarized in Table III.

## 7. Conclusions and future perspectives

Hearing disorders affect an increasing number of individuals worldwide, particularly during childhood, leading to a significant reduction in the quality of life. Over the years, it has been widely demonstrated that miRNAs play a key role in the development and function of the auditory system. However, altered miRNA expression levels appear to be involved in the progression of hearing loss along with other factors, including aging, noise exposure, ototoxic drugs, environmental and genetic factors (Fig. 3). According to the studies described in the present review article, miRNAs may be used as novel diagnostic biomarkers for the early detection of hearing loss. At the same time, miRNAs and related signaling pathways may be considered the starting point for the development of novel therapeutic approaches against hearing loss, ear inflammation and vestibular schwannoma. Overall, although the current findings represent a promising avenue, further *in vitro* and *in vivo* studies are warranted in order to provide a better knowledge of the mechanisms of action of miRNAs.

## Acknowledgements

Not applicable.

## Funding

No funding was received.

## Availability of data and materials

Not applicable.

## Authors' contributions

LF and AL conceptualized the manuscript. AL, CMG, GG and SC wrote the original draft of the manuscript. LF, AL, DAS and SC provided critical revisions. AL, MS and CL prepared the tables, figures and critically analyzed the literature. All authors contributed to manuscript revision and have read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

LF is an Editor of the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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