



Proteomics studies in inner ear disorders: pathophysiology and biomarkers

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EXPERT
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Although proteomics has been exploited in a wide range of diseases for identification of biomarkers and pathophysiological mechanisms, there are still biomedical disciplines such as otology where proteomics platforms are underused due to technical challenges and/or complex features of the disease. Thus, in the past few years, healthcare and scientific agencies have advocated the development and adoption of proteomic technologies in otological research. However, few studies have been conducted and limited literature is available in this area. Here, we present the state of the art of proteomics in otology, discussing the substantial evidence from recent experimental models and clinical studies in inner-ear conditions. We also delineate a series of critical issues including minute size of the inner ear, delicacy and poor accessibility of tissue that researchers face while undertaking otology proteomics research. Furthermore, we provide perspective to enhance the impact and lead to the clinical implementation of these proteomics-based strategies.

KEYWORDS: biomarkers • hearing loss • inner ear • proteome • proteomics

Several pathological conditions of the inner ear cochlea-vestibular system including sudden hearing loss (SHL), vestibular neuronitis, labyrinthitis or autoimmune inner ear disease have no clearly defined pathophysiology. Although treatments for many of these conditions have been validated by rigorous studies and are overall well-agreed upon, they are still based on empirical experience and at best, offer theoretical explanations as to the cellular pathology. Similar to their pathophysiology, inner ear diseases are challenging in terms of diagnosis and are mostly referred to as idiopathic. The diagnostic challenge pertaining to these conditions is mainly due to the lack of a direct and definite method of inner ear assessment and the absence of specific biomarkers for inner ear diseases that can hint at disease origin, severity and progression [1]. These hurdles represent a major challenge despite the continual refinement of clinical vestibular exam using new technologies (head impulse testing and vestibular-evoked myogenic potentials) and the introduction of three-dimensional MRI and diffusion-weighted imaging [2].

Among the common conditions, idiopathic sudden sensorineural hearing loss (ISSHL) is one common disorder of the above-mentioned pathologies, with an estimated annual incidence of 5–20 per 100,000. This number is even considered an underestimate because patients recovering ‘relatively’ quickly may not present to medical attention [3]. Despite a plethora of studies suggesting several etiologic agents (infective, vascular and immune activation), ISSHL still lacks a clear established etiology [4–6]. This multifactorial origin may explain the high variability in terms of prognosis and treatment regimens of ISSHL patients [1,7–9]. Thus, identifying specific biomarker(s) to be used in early diagnosis is of paramount importance in guiding the diagnostic/treatment options in inner ear pathologies, besides providing new insight into the underlying pathophysiology. In this review, we aim at evaluating recent updates of proteomic applications related to inner ear pathology and physiology in terms of significance, challenges, current achievements and future implications.

Proteomics methodologies & approaches

The need for better classification, diagnosis and prognosis of complex diseases, as well as more targeted, and potentially more effective treatments calls upon an active quest for biomarker discovery using available technology such as genomics, proteomics and metabolomics coupled to systems biology tools. Currently, global approaches for SHL biomarker discovery are scarce; for instance, a simple PubMed search using the term '(Sudden Hearing Loss) AND biomarker' reveals only 39 hits compared with the term '(Traumatic Brain injury) AND biomarker' that gave 2065 hits or '(Macular Degeneration) AND biomarker' that gave 695 hits (PubMed search; December 2014). Even if these numbers do not reflect value or reproducibility of data, they still emphasize a lower general interest in biomarker discovery in SHL compared with other diseases.

Among the different available high-throughput methodologies, proteomics represents a promising field of biomarker discovery, especially in the post-genomics era [10–12]. Proteomics can better reflect on the dynamic cellular changes and responses than genomics and is still more complex and informative than metabolomics [3,13]. Noting the importance of proteomics, the National Institutes of Health (NIH) announced, in 2003, a new program entitled 'Proteomics in Auditory and Developmental Disease Processes' for the review of proteomic approaches and their application in otology [14]. In this section, we cover an overview of the different methodologies in proteomics that are relevant to applications in inner ear pathology.

Human proteomics & mass spectrometry approaches

The proteome represents the full complement of protein encoded by one's genome [15], and proteomics is 'the high-throughput study of the proteome, including protein quantification, protein–protein interactions, post-translational modifications and protein function' [16,17]. Proteomics, thus, aims at characterizing the information flow within the cell and the organism through pathways and networks [18].

Proteomics can be applied in a global approach studying the entire protein complement in tissue or biofluid samples for unbiased discovery of protein expression or abundance patterns [19]. Alternatively, a hypothesis-driven 'targeted' approach studies a subset of cellular proteome with specific properties based on insight from previous literature. An example of the latter is the study of calcium-binding proteins in specific locations of the inner ear [20]. However, limitations of inner ear proteomics, in particular, necessitate the use of hybrid paradigms [19].

Mass spectrometry (MS) is at the core of modern proteomics together with multidimensional separation techniques such as 2D-DIGE (2D differential gel electrophoresis) and liquid chromatography (LC) [21]. These techniques are used for protein profiling in which investigators assay protein abundance and expression levels. With the advances in proteomics techniques, novel and highly sensitive protein detection methods appeared in otology including quantification techniques, protein microarray chips and nanoproteomics [22–25]. Multiple MS add-on

labeling techniques have been used in otology to enable protein quantification such as isotope coded affinity tag, iTRAQ (isobaric tagging for relative and absolute quantification) and SILAC (stable isotope labeling of amino acids in cell culture) (for review see [22]). Alternative to MS, protein microarray chips are made of hundreds to thousands of reagents that can interact specifically for proteins and allow for detection of large numbers of proteins with high sensitivity [26]. Increased sensitivity of protein detection can also be achieved by the incorporation of nanotechnology into the traditional separation techniques and MS, an example of which is the use of nano-based high-resolution LC for protein separation [23] (for review see [27]). The use of highly sensitive approaches, protein microarrays and nanoproteomics tools, is limited in otology because the entire field of otology proteomics is still in its infancy.

Bioinformatics analysis & protein–protein interaction studies

Bioinformatics and systems biology analysis of proteomic data go hand in hand with the generation of high-throughput data. Current state-of-the-art bioinformatics tools include online ontologies and databases that allow for rapid data analysis. In addition, protein–protein interaction (PPI) databases and network analysis and visualization algorithms are available for mapping PPI *in situ*. This type of network analysis will allow investigators to determine major contributing proteins, that is, hubs, in the network of a process of interest. These hubs provide insight into underlying pathophysiology and are suggested as putative biomarkers and/or targets for therapy. In addition, online databases for genomics and proteomics enable the determination of cross-species orthologs of certain protein's binding partners to infer evolutionary conserved functions. In brief, systems biology tools are used to uncover new correlations and offer pathophysiological interpretations of the studied disease at a global level that could assimilate the multifactorial aspect of the disease (for review, see [28]).

In addition, several *in-vitro* and *in-vivo* approaches were used to detect PPI in otology. These include coimmunoprecipitation [29], surface plasmon resonance (SPR) analysis [30] and yeast two-hybrid screening (Y2H) [31]. Coimmunoprecipitation involves the isolation of immune complexes of binding partners using specific antibodies. This can be coupled to 2D-PAGE and MS analysis for a more robust identification and evaluation [32]. SPR is an optical-based procedure in which an analyte having the protein of interest is run over a metal film with an immobilized ligand. The binding of the protein to the ligand will cause a change in the refractive index of the film and can be thus detected. Y2H is a relatively recent technique for PPI in analysis and is currently exploited in otology [31]. The basic principle of Y2H procedure is that the protein of interest is coupled to the DNA-binding domain of a eukaryotic transcription activator, and unknown proteins are tagged to the activation domain so that only when two proteins interact, transcription will take place. Y2H technique was used to identify neuronal calcium sensor-1 binding proteins involved in

inner ear development, as well as to identify proteins interacting in hair cell mechanotransduction [33,34].

Available inner ear proteomic targets: from tissue to biofluids

Proteomics' analytical and quantification tools are suited for both tissue samples and biofluids. With regard to the otology, given the confined anatomy of the inner ear, the pathology is more accurately reflected in the protein profile of the perilymph and endolymph than in blood or cerebrospinal fluid (CSF), where the interpretation of the markers is hampered by many confounding factors, including dilution effect, variable blood-labyrinth barrier passage and clearance rates. Therefore, proteomics of the perilymph and endolymph biofluid samples would provide the optimal means to investigate the pathophysiology of inner ear diseases. Nonetheless, endolymph and perilymph are difficult to isolate and obtain, and only available in limiting and variable amounts. To compensate for the limited access to inner ear biofluids along with the minute samples obtained, several alternative sites like temporal bone biopsies with enrichment techniques have been used (discussed later). In addition, tissue samples of inner ear compartments can also be isolated from wild-type and genetically modified animals allowing for studying normal, abnormal and developmental physiology. However, due to the small size of the inner ear, pooling of multiple animals (up to 60 animals) may be needed in addition to the use of rigorous preparation and enrichment techniques [35,36]. Other more accessible targets of proteomics biomarker discovery such as blood and CSF are also investigated in attempt to discover systemic diagnostic biomarkers of inner ear diseases using samples from patients.

Applications of proteomics in otology

Current proteomic research has focused on both profiling the normal inner ear proteome and characterizing proteomic changes involved in inner ear pathology [37]. Examples of the animal models used in inner ear proteomics include guinea pigs, mice, chicken, zebrafish and chinchilla rodents, as well as bovine models [33,38–42]. Nevertheless, due to the complexity of inner ear structures and their specializations, the study of inner ear proteome has focused on assessing both location-specific subsets of the inner ear proteome and the entire inner ear proteome [19].

Early reports of protein deregulation in otology

Interest in inner ear biofluid composition dates back to early 1970, when preliminary qualitative studies explored the biochemical composition of inner ear biofluids in normal versus pathologic conditions. In the early work of H. Silverstein, fluid concentration of ions, glucose and proteins was evaluated and compared across different patient groups (Acoustic Neuroma, Ménière's Disease, and others) and controls [43]. Silverstein *et al.* devised a surgical procedure similar to stapedectomy called: 'diagnostic labyrinthotomy' as a method to obtain inner ear fluids for biochemical analysis. Proteins were collected from the

vestibular cistern in micropipets by capillary attraction and their quantity was assayed using *Folin phenol* reagent and spectrophotometry [43,44]. Results of this analysis showed marked elevation of perilymph proteins (>1000 mg/ml) in patients with small to large acoustic neuromas compared with controls [43]. Later, the same procedure was used by Makimoto *et al.* used diagnostic labyrinthotomy to demonstrate that the perilymph had a higher concentration of glucose and total protein compared to the endolymph [45]. Although these studies are more qualitative and far less informative than current proteomics approaches, they provided early evidence of putative involvement of protein dysregulation in inner pathologies and proposed potential diagnostic tests for some ontological conditions [44,46].

Proteomic characterization of the organ of Corti

Isolde Thalmann *et al.* were among the pioneers in using 2D gel electrophoresis (2D-GE) to study the human inner ear proteome [19]. In 1980, Thalmann *et al.* used 2D-GE to identify proteins specific to the inner ear that could be probable markers of organ-specific dysfunction. The group collected tissue of the organ of Corti from normal guinea pigs and identified, using 2D-GE, two organ of Corti proteins I and II (OCP-I and OCP-II) in addition to oncomodulin [38,47]. OCP-I and OCP-II proteins were believed to be present specifically in the organ of Corti; however, they were not associated with a specific function [19]. Oncomodulin was first recognized as calcium-binding protein 15. Later, calcium-binding protein 15 was identified to be a β -parvalbumin isoform of the mammalian inner ear and called oncomodulin [48].

In addition to the hair cells involved in sensory signal transduction, the organ of Corti also contains non-hair cells that include neurons, glia and nonsensory epithelium. Different population of cells in the organ of Corti can be separated using flow cytometry prior to proteomics analysis allows for comparison of hair cell versus non-hair cell proteomes. Indeed, Herget *et al.* compared the proteome of sensory hair cells compared with non-hair cells in the vestibular system and identified 64 proteins specific to hair cells compared with 103 proteins only present in non-hair cells [49]. Although the role of all these proteins in hair cells is not well understood, the findings of the study remain significant in terms of identifying a specific proteome of the inner ear sensory cells. This proteome will probably house the more relevant proteins to the physiology of hearing and pathology of idiopathic hearing loss. A remaining question of interest is whether populations of auditory and vestibular hair cells exhibit any difference in their protein profiles. Elkan-Miller *et al.* [36] investigated the difference of protein, mRNA and microRNA profiles between cochlear and vestibular epithelia isolated from C3H mice using both microarray analyses (for RNA) and mass spectrometric analysis of isobaric stable isotope-labeled peptides (iTRAQ) for proteins. They detected around 460 proteins of which 25% were differentially regulated between cochlear and vestibular epithelia. In addition, this study demonstrated the presence of PSIP1-P75, a protein previously unknown in the vestibular epithelium. This protein

involved in transcriptional regulation is a target of miR-135b, a micro-RNA that was found to be differentially expressed between the two epithelia.

Recently, studies on hair cell bundles have revealed key elements in hair cell mechanotransduction [41,50]. First, MS analysis of sensory hair bundles in mice revealed a previously unidentified antigen associated with kinocilial links of sensory hair bundles [41]. This tip antigen was found to be an avian ortholog of human protocadherin-15, which is a product of the gene locus for the deaf/blindness; Usher syndrome type 1F/DFNB23 [41]. In a follow-up study using a yeast-two hybrid screen, TMIE was identified as a protein that interacts with the mature form of protocadherin-15 that has a special cytoplasmic domain (CD2) [51]. TMIE was found to be a critical component of inner ear mechanotransduction through coupling the tip-link to the transduction channel. Along the same line and following the identification of cyclic nucleotide-gated channel α -3 transcripts in vestibular hair cells [52], Selvakumar *et al.* used several PPI techniques (Y2H and SPR) to identify interacting partners important in mechanotransduction function in the inner ear [34]. These include a stereociliary tip-link protein, cadherin-23 (CDH-23) along with myosin VIIa proteins. Furthermore, the prominent work done by Shin *et al.* in studying chick vestibular hair bundle using shotgun proteomics approach revealed the role of creatine kinase circuitry in the normal homeostasis of vestibular hair cells. Proteins of purified chicken vestibular bundles were found to be most abundant in proteins involved in ATP synthesis followed by cytoskeletal, calcium signaling and stress-related proteins. Their finding highlighted the essential role of rapid energy turnover in hair cell functions supported by the fact that creatine kinase knockout mice exhibit hearing loss [53]. In a subsequent study [54], the same group analyzed the proteomes of the chick inner ear epithelia and vestibular bundle. A total of 336 proteins were significantly enriched in vestibular bundles and were found to be involved in cytoskeleton structure and dynamics, energy metabolism, phospholipid synthesis and cell signaling. In addition, network analysis of the interaction network of bundle proteins revealed the presence of two important proteins RDX (radixin) and SLC9A3R2 (NHERF2) as hubs within the network and potential prominent mediators of bundle functions and interactions with other inner ear structures.

In an effort to incorporate high-sensitivity capture technique in the area of otology proteomics, Peng *et al.* used nanoscale LC and tandem mass spectrometry (nano-LC-MS/MS) to study the proteome of the organ of Corti in mice. This high-sensitivity technique identified 628 proteins from six replicates forming the largest proteomic dataset of the organ of Corti. Of the identified proteins, 11 proteins including cochlin, myosin VI and myosin IX were found to be associated with hearing loss or impairment [23]. Using a similar approach, Darville and Sokolowski conducted an in-depth proteomics analysis using nano-LC-MS/MS applied on enriched samples cochlear sensory epithelium of 30-day-old mice [55]. In total, the different techniques allowed for identification of 4620 protein IDs which

form the most exhaustive proteomic investigation of cochlear sensory epithelium to date. Several of these proteins have not been previously reported in the sensory epithelium including calumenin, caskin, Nell2, copine-6 and Nipsnap, which are calcium-binding proteins, as well as sideroflexin-3, amphiphysin 2 and paralemmin-1 that are membrane-bound partner proteins. This exhaustive proteome is now available for future research investigating key components and interacting pathways in inner ear proteome.

In summary, proteomic analysis of inner ear sensory epithelia has emphasized three major processes that appear to be crucial for normal hair cell physiology, namely energy metabolism, signal transduction and cell cytoskeleton. Furthermore, the used proteomics and protein interaction analyses allowed uncovering key and previously unrecognized mediators of mechanotransduction in hair cells.

Characterization of other inner ear proteomes

Other than the sensory epithelium, different structural components of the inner ear were studied by proteomics approaches. Ikezono *et al.* studied bovine cochlear and vestibular membranous labyrinthine tissues proteomes in an attempt to understand the mechanism of hereditary hearing impairment with the proposed hereditary deafness gene, *Coch* [40]. *Coch* gene mutation is believed to be responsible for a hereditary form of hearing loss involving the locus DFNA9. *Coch* encodes the protein Cochlin whose function remains to be elucidated fully [56]. The mutant form of Cochlin is associated with deposition of acid polymucosaccharide in inner ear structures leading to neuronal degeneration [57]. *Coch* gene mutation was also associated with predisposition to Ménière's disease [58]. Ikezono used 2D-PAGE and identified 16 major spots having high homology with the *Coch* cDNA. Those spots were found to form 70% of the bovine inner ear proteins. Later, Robertson *et al.* compared mice with *Coch* (+/+) genotype to those with *Coch* (-/-) genotype using 2D-GE and MS. The protein profile revealed that cochlin was the most abundant cochlear protein and, thus, may be involved in hearing loss and vestibular dysfunction in humans [59]. This was further studied using human temporal bone samples isolated from normal subjects and those with DFNA9-subtype of inner dysfunction. Similar to mice data, results of protein profiling showed that cochlin was the most abundant protein, and its high expression levels and stability were believed to be the pathophysiological basis of the hearing loss and vestibular dysfunction occurring in DFNA9 [59]. This represents a major finding showing that cochlin protein is associated with hearing loss dysfunction.

In other studies, otoconial matrix proteins were evaluated and analyzed. Wang *et al.* isolated tissue from guinea pigs and studied otoconial proteome via 2D-PAGE. The group discovered otoconin-90 protein that was found to be a close homologue of phospholipase-A2 and was called phospholipase-A2 like protein [60]. Although it is still early to implement such experimental findings into direct clinical application, the study of the otoconial matrix proteins may

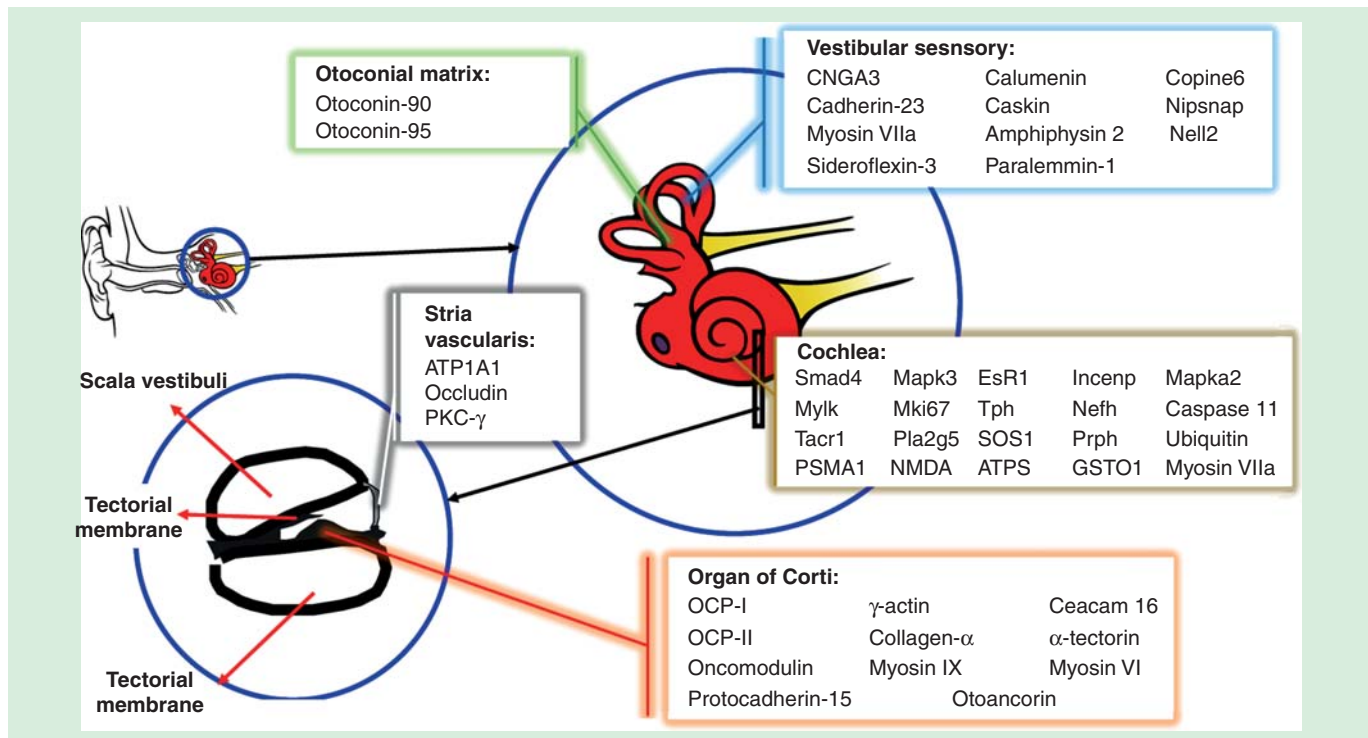


Figure 1. Prominent proteins expressed in different structures in the inner ear.

ATPS: ATP-Synthase; BK channel: Large conductance $\text{Ca}^{(2+)}$ -activated $\text{K}^{(+)}$ channel; Ceacam16: Carcinoembryonic antigen-related cell adhesion molecule 16; CNGA3: Cyclic nucleotide-gated channel α -3; EsR1: Estrogen receptor1; GSTO1: Glutathione-S-transferase homologue 1; Incenp: Inner centromere protein; Mapka2: MAP Kinase activated protein kinase2; Mki67: Proliferating cell protein Ki67; Mylk: Myosin light chain kinase; Nefh: Neurofilament 200; NMDA: N-methyl-D-aspartate receptor; Pla2g5: Phospholipase A2 group V; Prph: Peripherin; PSMA1: Proteosome subunit alpha type 1; SOS1: Son of sevenless1; Tacr1: Substance P receptor; Tph: Tryptophan hydroxylase.

help understand the underlying pathogenesis behind benign paroxysmal positional vertigo and the degenerative changes of the otoconia [60]. Thalmann *et al.* in 2006 studied mouse otoconial proteins using 2D-GE and MS analysis identifying nine otoconial proteins including otoconin 90 and 95 that may be implicated in otoconial development and prevention of degenerative diseases of balance [61]. In summary, FIGURE 1 illustrates the major proteins reported in different structures of the inner ear.

Interestingly, Jamesdaniel *et al.* carried a major study to characterize the cochlear protein in three different strains of rats with normal hearing. These included Wistar, Sprague-Dawley and Fischer 344 rats. Broad spectrum antibody microarray was used, and 725 proteins were screened in the entire cochlea. More than 80% of the studied proteins were found in the different strains at different expression levels, and 16 proteins were found to be expressed with twofold increase compared with actin levels in all strains. This recent attempt to elucidate the inner ear proteome identified seven new proteins including MAP kinase-activated protein kinase 2, Smad4, tryptophan hydroxylase, son of sevenless1 protein, inner centromere protein, protein arginine methyl transferase6 and caspase11. The differential expression of these proteins across the different strains may be related to their differential susceptibility to ototoxicity, aging and noise [24].

In addition to efforts to map different components of the inner ear, Thomas *et al.* uncovered the presence of lipid-rafts in the membranes of hair cells and non-hair cells in the inner ear and used mass spectrometric analysis of detergent resistant membrane proteins to detect about 600 proteins present in those membrane domains. Among those proteins, there are key regulators of signal transduction in the inner ear such as the potassium channel KCNQ1 along with several gap junction proteins [62]. Bioinformatics analysis of isolated proteins identified the enriched cellular processes in the lipid raft proteome that include energetics, transport, ion homeostasis and cell contact.

Blood-labyrinth barrier integrity & cochlear interacting protein

In a recent study, Yang *et al.* used MS shotgun approach on mice cochlear tissue samples to assess blood-labyrinth barrier integrity [63]. The study identified more than 600 proteins of the stria vascularis capillaries in the mouse cochlea and provided the first database on protein components in the blood-labyrinth barrier [63]. The ion transporter ATP1A1 was also identified as the most abundant protein in the blood-labyrinth barrier, and its interaction with protein kinase C- γ and occludin proteins was found to be involved in maintaining the integrity of the blood-labyrinth barrier.

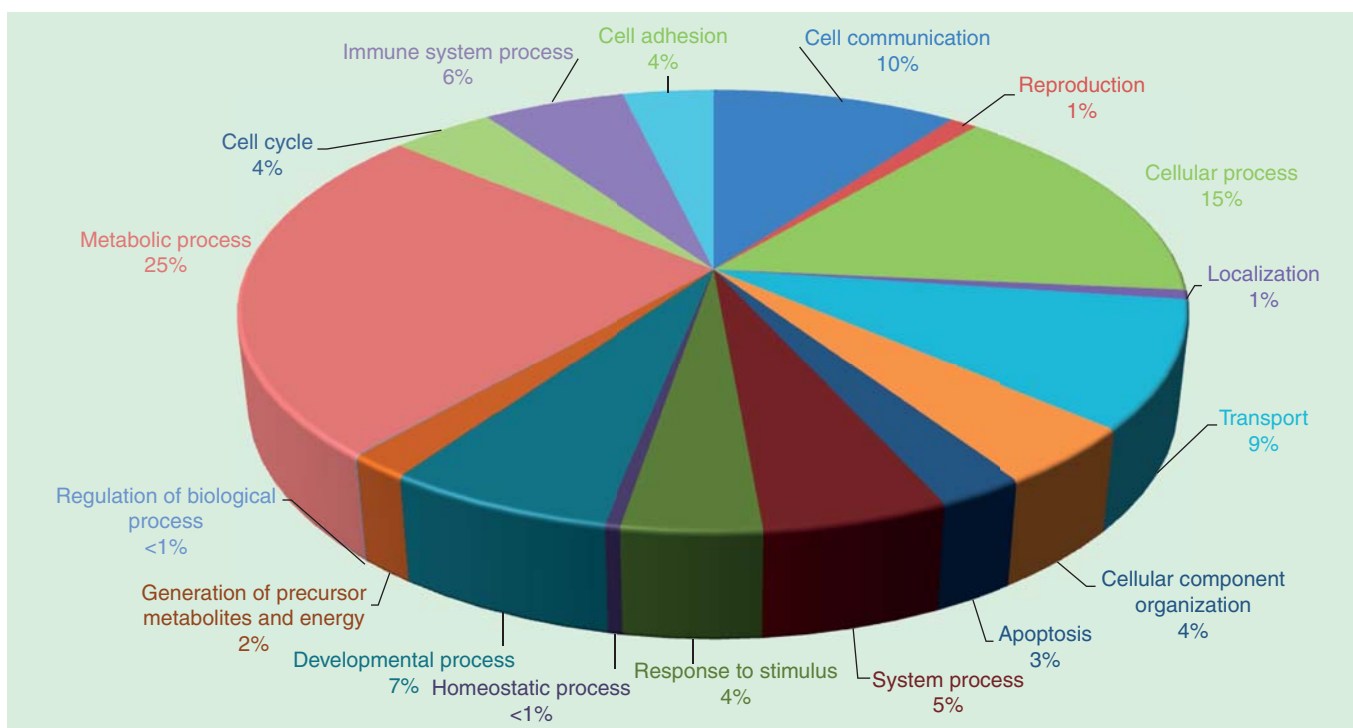


Figure 2. Pathway analysis of prominent proteins in the organ of Corti: proteins were pooled from the different reported studies and the analysis was performed via PANTHER-db [92].

In a PPI assay using coimmunoprecipitation and 2D-PAGE-MS, Kathiresan investigated mouse cochlear interacting protein partners of large conductance Ca^{2+} -activated K^{+} (BK) channel involved in sensorineural excitation, signal transduction and metabolism. In total, 11 BK-binding partners were verified including annexin, apolipoprotein, calmodulin, hippocalcin and myelin P0 proteins. Furthermore, *in situ* PPI analysis revealed a network of BK partners that included 12 hubs and involved cellular metabolism, trafficking/scaffolding and mitochondrial function [64]. A more recent study by the same group used the same approach to identify BK-binding partners in chicken cochlea to elucidate evolutionary conserved function of this channel [65]. A total of 16 partners were identified this time, and a similar PPI database analysis was performed. Compared with mice, common hub proteins included N-methyl-D-aspartate receptor and ATP-synthase. Further analysis of orthologs across six species allowed identification of common partners involved in apoptosis, Ca^{2+} binding and trafficking. FIGURE 2 illustrates the different functional categories of the proteins reported in proteomics studies of the inner ear.

Proteomics investigation in inner ear development

Proteomics studies implicated several proteins involved in inner ear development using zebrafish models that allow for easy visualization of the ear along with the potential of developmental manipulation via mutation [33]. Petko *et al.* used Y2H (described previously) to identify proteins interacting with neuronal calcium sensor-1 that was shown to be involved in semicircular canal development. Y2H screening followed by

BLAST analysis to determine human orthologs of proteins of interest have led to the identification of the Ncs-1a/Pi4k β /Arf1 signaling pathway as a new pathway critical to vestibular apparatus development.

New insight on the development and elongation of utricle stereocilia was inferred from MS-based proteomic analysis of chicken utricle by Peng *et al.* [42]. Twifilin 2 protein was identified in postnatal mice as a utricle protein found at a higher concentration in short stereocilia compared with longer ones. *In vitro* analysis using twinfilin 2 expression in LLC/PK1-CL4 cells showed reduced microvilli elongation suggesting a role of twinfilin 2 in suppressing stereocilial elongation.

Proteomics applied to the study of inner ear pathology **Noise-induced changes**

Few proteomics investigation was conducted to study noise-induced inner ear protein changes [25,66]. Using broad-spectrum antibody microarray, Jamesdaniel *et al.* detected an upregulation of nuclear proteins E2F3 and WSTF in chinchilla rodents after noise exposure. Those proteins are downstream effectors of the p38/MAPK signaling pathway that support the critical role of this pathway in noise-induced hearing loss [25]. On the other hand, Yeo *et al.* used 2D-GE and MALDI-TOF-MS to analyze the differential expression of cochlear proteins in noise-treated and noise-free mice groups. About 286 spots were detected in the noise group including tyrosine kinase MEG2, angiopoietin-like 1, 70-kDa heat shock protein, etc. Although these proteins are not specific to the ear, they are believed to be involved in noise-induced hearing loss and may

provide insight into the underlying pathology and possible targets of treatment [66].

Hearing & vestibular dysfunction

Chance *et al.* studied changes in cochlear proteins in Ames Waltzer mouse model of deafness in Usher syndrome 1F using 2D-GE and MS [67]. Overall, 69 protein spots showed significant alterations, of which 20 spots corresponding to cochlin proteins were upregulated. These proteins were mapped into interaction networks using bioinformatics tools; results suggested that elevation in cochlin protein level may contribute to the degeneration of cochlear neuroepithelia in the USH1F model. In addition, hearing impairment and vestibular dysfunction were associated with *Clic5* recessive mutation. Mice carrying *Clic5*, an intracellular chloride channel protein, recessive mutation with absence of *Clic5* on western blot were found to have impaired hearing and vestibular dysfunction [68]. *Clic5* was found to be present at high levels in stereocilia of the chicken utricle with a 1:1 molar ratio with radixin, suggesting that the association between *Clic5* and radixin may help stabilize connections between the plasma membrane and the filamentous actin core in hair cell stereocilia. Identifying such connection is of essential importance for the understanding of hair bundle morphogenesis and maintenance [68].

Drug-induced ototoxicity

Cisplatin, a common anticancer drug, was studied for its effect on cochlear proteins in rat. Rat cochlear organs were cultured in the presence or absence of cisplatin for 3 h and then subjected to 2-D DIGE and MS to compare protein profiles of cisplatin exposed and control cells. This study by Jamesdaniel *et al.* identified 17 downregulated proteins and 5 upregulated proteins after cisplatin treatment [69]. Similarly, Coling *et al.* used 2D-DIGE coupled to MALDI-TOF to analyze protein profile changes in P3 rat cochlea after treatment with cisplatin. Results showed significant change in the expression of 22 different proteins after cisplatin treatment and a less significant change of 7 other proteins [70]. To assess salicylate ototoxicity in mice model, Jung *et al.* injected mice with high dose of salicylate to induce ototoxicity and evaluated at 3 h or 3 days post injection. Proteomic analysis on cochlear tissue revealed 3 downregulated and 16 upregulated proteins at 3 h with none of these changes persisting to the 3 days time point. This suggested that the ototoxic effect of salicylate on cochlea is a transient one [71]. Further investigation of protein dysregulation after ototoxic drug insult may help study the mechanism of hair cell toxicity induced by cisplatin and provide possible targets for therapy. For instance, corticotrophin-releasing factor-2 (CRF2) was found to induce protective changes in organ of Corti cells. This effect was studied *in vitro* using organ of Corti cell line culture subjected to CRF2 post-aminoglycoside stress. Protein profiling by iTRAQ-LC-MS/MS approach detected 942 total proteins, of which 173 proteins were found to have more than 20% fold change in either directions after CRF2 treatment [50]. Those proteins were involved in mitochondrial homeostasis and modulation of nuclear function.

Vestibular compensation after labyrinthectomy

Vestibular compensation after labyrinthectomy involves several physiological mechanisms that occur to promote postural stability and assist in recovery of damage. Several studies investigated protein profile changes in ipsilateral vestibular compensation [72,73]. Studies in both rats and guinea pigs revealed protein dysregulation in vestibular nuclei after labyrinthectomy [72–74]. Dysregulated proteins were involved in axon guidance, coordinated upregulation of mitochondrial function, calcium homeostasis, cell signaling, cellular cytoskeleton, ATP biosynthesis and phosphate metabolism [73]. Although these studies report protein changes in different cellular processing during vestibular compensation, the actual role that these proteins play in the process is still not clear. Further investigation is thus needed to incorporate more data to understand specific underlying pathophysiological changes after labyrinthectomy.

Endolymph, perilymph & endolymphatic sac proteomes: implication for biomarker identification

Protein profiling of the perilymph is of importance to discover biomarkers for inner ear diseases such as perilymph fistula (PF). PF, an abnormal communication between the middle and inner ear, usually manifests as vertigo, tinnitus, aural fullness and sensorineural hearing loss [14]. Early attempts to identify quantitatively specific perilymph protein were carried by Arrer *et al.* using postmortem perilymph samples [75]. Arrer used comparative protein analysis between perilymph, CSF and blood to determine characteristic proteins of each of these body fluids. An initial observation was that the perilymph had more than 10-times less amount of albumin compared with the CSF [76]. Later, Thalmann's group (1991) used 2D-GE to study the perilymph, serum and CSF from guinea pigs and human subjects. The group identified a set of perilymph proteins that could serve as candidates biomarkers for PF [39]. Interestingly, certain proteins were expressed at higher levels in perilymph than in plasma such as PLS:33, whereas others such as UPS:2 were found to be comparable [77]. These findings indicated that the blood-perilymph barrier is not based on molecular weight differences, and that endolymph is derived from perilymph because its protein levels are uniformly five- to eight-times lower than the perilymph [77]. Following this study, the same group used amino acid sequencing to identify PLS:33 and PSL:29/30 protein IDs as apolipoproteins (apo) D and apo J proteins, respectively [78]. In addition, β 2-transferrin, not present in plasma, was found in perilymph at a concentration similar to that of CSF. Those proteins were evaluated as potential biomarkers for PF in the human subjects only when pure fistula samples are analyzed [78]. However, the use of better techniques for protein assessment found that previously detected proteins except for β 2-transferrin were not specific to the perilymph [78,79]. Similarly, Arrer *et al.* reported a possible utility of β 2-transferrin (β -trace protein) as a marker for CSF rhinorrhea [79]. One problem of β 2-transferrin is that it cannot be stained easily as antibodies against β 2-transferrin bind other transferrins [19]. Possible advances using more specialized

techniques like proteins chips may help assess the actual utility of β 2-transferrin as a marker for PF [79].

A comparison between CSF and perilymph protein content was carried by Swan *et al.* using LC-MS/MS coupled with iTRAQ labeling to study potential interactions with delivered drugs through implantable intra-cochlear drug delivery systems. Protein content in the perilymph was found to be three-times more concentrated than in the CSF and included majorly albumin, protease inhibitors, especially serpins (30%) and apolipoproteins (16%). Both serpins and apolipoproteins had high perilymph/CSF ratios. Relevant to the aim, albumin and high-molecular-weight kininogen proteins were found to participate in biofouling, processing of adsorption of microorganisms to surfaces. In addition, apo and albumin were reported to function as acidic and lipophilic drug reservoirs, whereas α -2-glycoprotein was found to bind basic drugs [80].

Human perilymph proteome of patients with vestibular schwannoma was compared by Lysaght *et al.* with that of patients undergoing cochlear implantation using LC-MS/MS. It was shown that 71 proteins were conserved in all samples, whereas 15 candidate biomarkers for vestibular schwannoma, associated with poor hearing, were identified. Among these, there are -crystallin and low-density lipoprotein-related protein 2 [1]. Nonetheless, no clinical utility of these markers is apparent owing to the low sample size used and the need for further validation [1].

Thalman *et al.* sampled endolymph from the endolymphatic sac and the cochlea of mice and guinea pigs and used 2DIGE-MS showing a high protein content in the endolymphatic sac that included glycosylated proteins such as α -antichymotrypsin, α 2-HS-glycoprotein (fetuin A), α -1 antitrypsin, transferrin and ApoD [61]. The protein profile of the cochlear endolymph showed 40-fold lower protein content, and unlike the endolymphatic sac, ApoD protein was not among the prominent proteins detected. Those aforementioned studies that undertook protein profiling of both the endolymph and the endolymphatic sac had contributed to a better understanding of normal inner ear fluid physiology and challenged the previous hypothesis that endolymphatic sac is a sink of the endolymph. Yet, the evolutionary and physiological benefit of having those different protein profiles in inner ear fluids remains to be discovered.

Search for serum biomarkers of inner ear diseases

To the best of knowledge, no specific studies have addressed biomarkers for ISSHL in CSF and blood. One study by Chiarella *et al.* reported the investigation of serum biomarkers of Meniere disease (MD). Plasma samples were collected from 16 patients with MD, and protein profiling was performed using 2D-GE and LC-MS/MS analysis. Results showed an overexpression of factor H and B, fibrinogen α and γ , β actin and pigment epithelium-derived factor proteins. Concurrently, there was an under expression of β -2 lipoprotein I, vitamin D binding protein and Apo lipoprotein I proteins compared with controls. The study concluded that protein profile might serve

as a diagnostic tool in MD. However, these findings are still far away from clinical implementation owing to the low sample size (only 16 patients) that hinders the ability to select for a disease-specific biomarker [81].

To date, serum identified markers in ISSHL include erythrocyte sedimentation rate, interleukin 6, C-reactive protein and a variety of autoimmune and prothrombotic markers [82–89]. These markers are nonspecific and yielded limited and inconsistent information regarding the diseases pathophysiology [90].

Expert commentary

Despite the surge in the number of studies investigating the inner ear tissue and biofluid proteomes, it appears that these findings are currently being used to understand the pathophysiology rather than as diagnostic biomarkers, as this field is still at its infancy [19]. The major limitations of otology proteomics research centers around the acquisition of inner ear samples and the sensitivity of the inner structure. In contrast to blood and even to CSF, a much higher risk resides in obtaining inner ear biofluids samples owing to the minute size of the delicate inner ear structures [14,19,37]. Thus, at this stage of inner ear proteome investigation, we are limited to infer information about the state of function or dysfunction of such sensory systems rather than indulging in the biomarker studies of inner ear pathologies. Similarly, the small volume of fluids in the inner ear necessitates much more sensitive techniques for the detection of extremely low amount of sample. To illustrate this point, the endolymphatic sac in guinea pigs holds 200 nL, whereas the endolymph and perilymph hold 4 and 30 μ L, respectively, which is not compatible for analysis with traditional proteomics tools, yet [19].

Other limitations include the difficulty of sample quality involving purity and preparation. Surgically obtained samples of the perilymph are commonly contaminated with traces of plasma and other exudates [1]. As suggested, a possible alternative to the *in vivo* studies would be the use of *in vitro* cell cultures; nevertheless, for the inner ear, this seems to be of limited utility because the hair cells of the organ of Corti are post mitotic [91]. To summarize, there are a number of limitations that have contributed to the slow advance in the inner ear proteomics requiring further optimization and better instrumentation.

Five-year view

The application of proteomics platforms in various areas of inner ear pathologies is still in its infancy compared with other disciplines such as in cancer research, neuroscience and psychiatric disorders. Owing to the anatomy of the inner ear, little can be done to decrease the invasiveness of inner ear fluid acquisition. The use of animal models is highly informative, especially when several species are used and online bioinformatics tools are used to determine possible human orthologs and protein interactions maps.

As we are being challenged by a number of obstacles in the specialized area of inner ear, it is anticipated that the field of inner ear proteomics will grow rapidly as more proteomics

tools and separation platforms are being introduced with better refined and advanced multidimensional separation systems (SILAC, Super SILAC, CAX, DIGE, etc.). Furthermore, we would expect the introduction of advanced bioinformatics predictive tools that go hand in hand with proteomics analysis and which can provide a more comprehensive, enhanced data mining output that would be more meaningful for clinicians and for clinical settings. Similarly, we envision the introduction of even more advanced clinical diagnostic assays (ELISA, flow cytometry or other high throughput assays), which will correlate the inner ear pathologies with the molecular and cellular perturbations. Despite the application of proteomics in the inner ear pathology is very promising, yet there are quite a

number of limitations. A more concerted and coordinated effort may allow for significant breakthroughs that can advance us from the exploratory phase of proteome profiling to the next step of biomarker identification with a direct application to the clinical settings.

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Key issues

- Proteomics techniques have recently been incorporated into the study of inner ear pathology and physiology.
- Inner ear proteomics used both biofluids and tissue biopsies of humans and animal models to detect biomarkers of inner ear pathologies.
- Previously uncharacterized proteins were detected in the inner ear hair cells and found to be key components of mechanotransduction pathways including TMIE-protocadherin 15 interactions.
- Proteomics studies on normal inner ear physiology uncovered prominent pathways in inner ear homeostasis that may be disrupted in different pathological conditions.
- Mass spectrometry-based protein profiling of inner ear development revealed the role of Twifilin 2 protein in stereocilial elongation during inner ear development.
- Pathology-associated changes in protein profile of the inner ear uncovered key processes in normal homeostasis and injury-related compensatory responses.
- Protein profiling of inner ear fluids suggested potential biomarkers of hearing loss etiologies; yet, the poor access to these fluids hinders clinical translation of findings.
- Despite current efforts, the only clinically available serum or cerebrospinal fluid biomarkers of inner ear pathology are acute-phase reactant proteins that are nonspecific and of poor utility.
- Future advancement in proteomics techniques in addition to separation and enrichment tools hold promise for ground-breaking findings in proteomic organization of the inner ear.

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