

# Western Blot Characterization of Human Serum Prestin, an Outer Hair Cell Biomarker

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**Hypothesis:** Western blot analysis of human prestin in the blood reveals multiple bands, rather than a single band.

**Background:** Previously, using the ELISA method, prestin was shown to be a good biomarker of outer hair cell (OHC) health and sensorineural hearing loss that could be measured in the blood. Recently, we found that a Western blot approach in an experimental model demonstrated three prestin bands providing greater insights into prestin in the blood and its origins. This approach has not yet been explored in humans.

**Methods:** Serum samples from 25 healthy human subjects were analyzed. An automated Western blot for each sample was generated, and bands were analyzed and compared with transient evoked otoacoustic emission levels (TEOAE).

**Results:** There were five bands at ~32, ~50, ~94, ~139, and ~171 kDa, respectively. Notably, the ~50-kDa band consistently

was the most prominent. When the subjects were divided based on TEOAE level, those with high emission levels had a significantly larger ~94-kDa band than those with low emission levels.

**Conclusions:** Western blot characterization of OHC biomarker prestin in humans shows that the band closest to the previously estimated molecular weight of prestin (81 kDa) is related to a functional measure of OHCs. This finding increases confidence in the value of serum prestin as a biomarker. The Western blot method appears to offer higher-resolution information on serum prestin. Future work will be carried out under pathological conditions to inform on the application of this quantitative method in clinical settings.

**Key Words:** Biomarker—Prestin—Sensorineural hearing loss—Transient otoacoustic emissions—Western blot.

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## INTRODUCTION

WHO estimates that half a billion people worldwide have disabling hearing loss (1). Twenty-three percent of Americans older than 12 years have hearing loss in at least one ear (2). Early diagnosis of hearing loss is essential to delaying progression, yet it is only currently measurable by audiometric testing (3). Our group has proposed using hearing health biomarkers through blood analysis based on inner ear proteins in circulation. Prestin, a motor protein found on outer hair cells (OHCs) in the inner ear, has been proposed as one such potential hearing biomarker (3,4). Critical for sensitive hearing, prestin takes different conformations as a part of cochlear amplification (5). Disruption of OHCs occurs by ototoxins, noise exposure, and aging

(6). Blood levels of prestin have been shown to be affected by these variables (7,8).

The effect of loud noise exposure on serum prestin levels has been shown in both experimental models and humans (7–12). A highlight of this work was that prestin levels were negatively correlated with average daily noise exposure in healthy humans (8,10,11). Quantified via the enzyme-linked immunosorbent assay (ELISA) technique, there is a statistically significant negative correlation between serum prestin levels and noise exposure. Those with louder environments had lower levels of prestin, but audiometric measures were unable to distinguish between those with low and high noise exposure levels (8). The hypothesis is that in response to more noise, the expression of prestin is decreased to reduce amplification (8). Our group has previously published human ELISA data suggesting the relative stability of prestin levels in blood over a 6-month period indicating its potential utility as a biomarker (7).

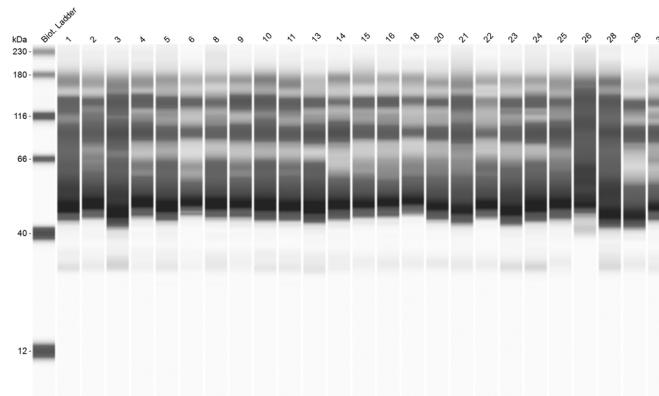
Prestin levels were shown to rise with ototoxin exposure, including cisplatin and cyclodextrin (13–16). We previously relied on the ELISA method for qualification. In our latest work with cyclodextrin, to gain more insight into prestin in the blood, we used the Western blot method after ototoxin cyclodextrin dosing in guinea pigs and found three prominent bands at ~58, ~83, and ~134 kDa (17). In

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**FIG. 1.** The composite image illustrating the high-contrast automated Western blots of all 25 subjects and the loading control (i.e., biotene ladder). Bands at about ~50, ~94, and ~139 kDa were most intense and consistently present.

particular, a prestin band of about 134 kDa was amplified after ototoxin treatment in guinea pigs (17). We believe that this 134-kDa band is a dimer of prestin representative of a prestin burst (17). Recently, we demonstrated that the 134-kDa band/prestin burst is sensitive to OHC injury even when the injury does not result in OHC loss (18). Identifying the species of prestin is essential to interpreting levels of circulating prestin and may improve our understanding of the mechanisms of injury in the cochlea.

No study has yet explored species of prestin in humans. Defining prestin species in humans is essential to translating our experimental discoveries to the clinical realm. Here we describe our prestin findings in Western blot analyses of normal-hearing young adults. This effort will advance blood-based biomarkers for hearing health.

## MATERIALS AND METHODS

Our methods expanded upon the previous study of prestin carried out at our institution (8). Participants with clinically normal hearing bilaterally (ages 18–24 yr) were enrolled in the study. All subjects underwent dosimetry, and the average daily noise exposures (LAeq8h) were calculated (8). Blood samples were available at five different time points (8). Samples were collected in 6.0-ml tubes with no anticoagulant or preservative drawn by a certified phlebotomist from the median cubital vein (8). The samples were standing upright for 30 minutes at room temperature before being centrifuged for 10 minutes at 3000g (8). The serum was collected and is currently stored at  $-80^{\circ}\text{C}$ . The time point with the most specimens ( $n = 25$ ) available was selected. All subjects underwent audiometric testing including transient evoked otoacoustic emissions (TEOAEs).

### Automated Western Blot

Western blot was performed by Raybiotech, Inc. (Peachtree Corners, GA), using an automated Capillary Electrophoresis Immunoassay machine (ABBY; ProteinSimple Santa Clara, CA). In brief, serum samples were diluted  $20\times$  and mixed with a master mix (ProteinSimple) to a final concentration of  $1\times$  sample buffer,  $1\times$  fluorescent molecular weight marker, and 40 mM dithiothreitol (DTT) and

then heated at  $95^{\circ}\text{C}$  for 5 minutes. The samples, blocking reagent, wash buffer, primary antibody (SLC26A5 polyclonal Invitrogen PA5-42533), an HRP-conjugated secondary antibody, and chemiluminescent substrate were dispensed into designated wells in the manufacturer-provided microplate. After plate loading, the separation electrophoresis and immunodetection steps took place in the fully automated capillary system. Auto-Western analysis was performed at room temperature, and instrument default settings were used.

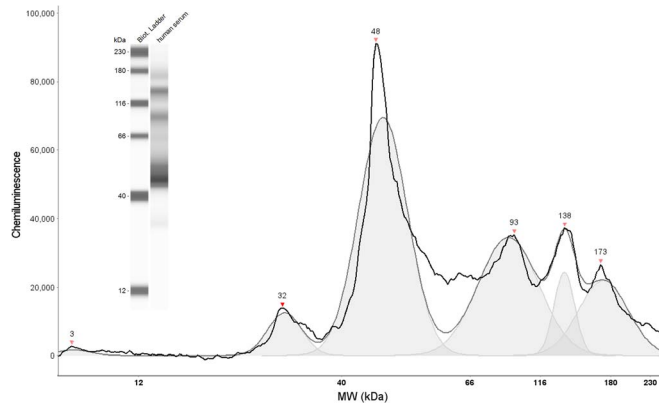
### Data Analysis

Automated Western blots were quantitatively analyzed by generating the corresponding electropherograms (see Fig. 2). In the electropherogram, bands are represented as peaks. We measured the height, the area under the peak, and the signal-to-noise ratio for each peak. Samples were divided into two groups based on subject TEOAE value, with 10 dB being established as the cutoff. Statistical analyses were carried out using SPSSv26. Independent-samples *t* test was run based on these groups for each peak height. Because of the OHC damage hypothesis, stating that prestin would increase with greater TEOAE levels, a two-sided *p* value was utilized (8). To determine the relationship of each electropherogram peak measure with other variables including TEOAE, LAeq8h, and previously collected ELISA-measured prestin levels, which were not normally distributed, Spearman rho correlation coefficient was examined.

## RESULTS

Figure 1 shows the automated Western blots of all 25 subjects. These blots revealed five bands at ~32, ~50, ~94, ~139, and ~171 kDa, respectively. Notably, the second band (~50 kDa) consistently was the most prominent. Bands 2, 3, and 4 were present in 100% of the samples, whereas bands 1 and 5 were the most variable and least intense.

A representative electropherogram, used for quantitative analysis, is shown in Figure 2. The heights of peaks at ~54, ~94, and ~139 kDa were negatively related to width ( $\rho = -0.175$ ;  $\rho = -0.62$ ,  $p < 0.001$ ;  $\rho = -0.15$ , respectively), but positively correlated with their area ( $\rho = 0.33$ ;  $\rho = 0.5$ ,  $p = 0.005$ ;  $\rho = 0.53$ ,  $p = 0.004$ , respectively)



**FIG. 2.** A sample electropherogram representation of the human prestin automated Western blot used for quantitative analyses. The corresponding automated Western blot and load control are shown in the inset. Multiple “peaks” corresponding to “bands” are defined demonstrating five isoforms of prestin. The solid black line represents raw tracing, whereas the smooth gray line was used for measuring peak height, and the curves represent the area under the curve.

and signal-to-noise ratios (and 0.58,  $p = 0.001$ ;  $\rho = 0.53$ ,  $p = 0.003$ ;  $\rho = 0.88$ ,  $p < 0.001$ , respectively). These results suggest that the electropherogram peak height, representing the intensity of the Western blot band, is a reasonable representative metric for further analyses.

When the subjects were divided based on TEOAE level, those with high emission levels had a more prominent ~94-kDa band than those with low emission levels (mean height difference, 3273.29;  $p = 0.021$ ) as demonstrated in Figure 3. A similar analysis of the other four bands based on TEOAE levels was not statistically significant.

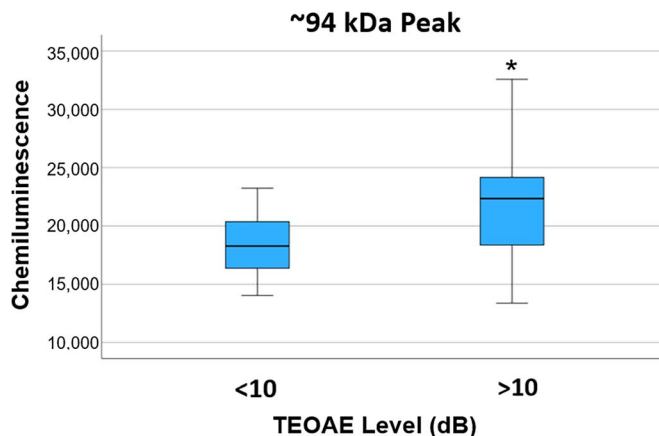
As we had previously reported (8), ELISA-measured prestin levels were negatively correlated with LAeq8h ( $\rho = -0.51$ ,  $p = 0.005$ ). There was no statistically significant correlation between various band/peak metrics of the three main peaks, total three-peak height, and average three-peak height with either ELISA-measured prestin levels or LAeq8h.

**DISCUSSION**

Here we have characterized, for the first time, isoforms of prestin from healthy human serum using automated

Western blot analysis. Five isoforms were present in our sample, with bands at ~32, ~50, ~94, ~139, and ~171 kDa, with the ~50-kDa band being the most prominent. Previously, we had reported three bands in guinea pigs (17). Those bands were at ~58, ~83, and ~134 kD, with the ~58-kDa band being the most prominent. Thus, the three guinea pig bands appear to be present in humans with comparable molecular weights. In humans, we found additional lower and higher bands at ~32 and ~171 kDa. What formulation of prestin each band actually corresponds to and how they are generated remains to be determined. The human ~139-kDa band is of particular interest because we demonstrated that in guinea pigs exposed to an ototoxin, the ~134 kD band is amplified (16). In other words, this particular isoform accounts for the rapid rise in blood prestin levels reported after ototoxic exposure in animal models (12, 13, 15).

Before undertaking the present study in humans, we investigated and compared two anti-prestin antibodies in guinea pigs (16,18). The two antibodies were the Invitrogen (PA5-42533) and the Santa Cruz (SC293212). Both antibodies produced multiple bands, but the Invitrogen antibody appeared to have better sensitivity and thus was adopted for our human investigations.



**FIG. 3.** Samples were categorized into two groups based on TEOAE level to compare the height of peak 3, ~94-kDa band. Group determination was based on TEOAE level of less or greater than 10 dB. A *t* test showed a statistically significant difference (N = 25,  $*p = 0.021$ ) indicating that those with TEOAE >10 dB had a more robust ~94-kDa peak/band.

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The human ~94-kDa band likely corresponds to the predicted molecular weight of 81.4 kDa for the full-length human prestin (19). Its prominence reflects constitutive expression and release of prestin in the cochlea resulting in a steady-state low level of prestin detection in blood. We believe that the difference in molecular weight is due to post-translational modification of prestin, specifically glycosylation. It has been demonstrated that prestin is N-glycosylated at asparagine residues in the second extracellular loop—N163 and N172 (20,21). We previously showed that deglycosylation with PNGase shifts the molecular weight of all bands larger than ~58 kDa by 10 kDa lower (18), placing our suspected ~94-kDa prestin band closer to 81 kDa with the 10-kDa correction applied.

The most prominent band in both human and guinea pig Westerns were at ~50 kDa, respectively. As we had previously noted, this band represents a challenge (16). As noted above, the ~94-kDa band may correspond to a prestin monomer. Similarly, the ~139- or ~171-kDa band may correspond to prestin dimers. The ~50-kDa variety is probably too small to represent an entire prestin molecule (16). This study and a previous guinea pig study (18) used the same prestin antibody; thus, the ~50-kDa band may represent a prestin fragment that contains the amino acid sequence targeted by the antibody. We have also demonstrated that this fragment is not N-glycosylated, and thus does not contain the asparagine residues in the second extracellular loop—N163 and N172 (18). The presence of this fragment at baseline suggests that a constitutive, controlled process is in place for breakdown and/or disposal (16). Consistency of findings between the two species adds strength to this hypothesis. The prominence of the ~50-kDa band in healthy-hearing humans highlights its importance. The significance of this band is further highlighted by our finding that this band decreases after exposure to an ototoxin, suggesting that there is a shift away from fragmentation under normal conditions toward dimeric release into the circulation under pathological conditions (16).

Our previous guinea pig Western blot investigations did not include any functional measures (16,18). The present study in human subjects integrated TEOAEs. Otoacoustic emissions represent a functional measure of the unique electromotile properties of OHCs bestowed by prestin (22). In the present study, we examined electropherogram peak height, corresponding to band prominence on the blot, in relation to TEOAE amplitude. Subjects who had higher TEOAE amplitudes were found to have higher heights in the ~94-kDa peak. Because this band is in close proximity of the estimated molecular weight of full-length prestin, this finding suggests that higher full-length prestin levels are associated with a more robust OHC function. This is consistent with experimental studies demonstrating decreased and increased otoacoustic emissions with decreased and increased prestin expression, respectively (23,24). This finding further supports serum prestin as a biomarker for cochlear health.

Although starting with younger healthy individuals without hearing loss establishes an important baseline of what isoforms of prestin are present in humans, a limitation of

this study is the lack of varying levels of hearing loss. Thus, further studies should seek to explore different types of sensorineural hearing loss and other conditions in relation to prestin isoforms. Examining particular isoforms may give a greater understanding of how prestin expression changes when induced by different states whether chronic, acute, noise, ototoxin, etc.

The importance of our Western blot approach to quantification of serum prestin levels is highlighted by the finding that prestin is expressed outside the inner ear (25), our finding that level changes are not dependent on OHC death (18), and that prestin is still detected in the blood when using ELISA quantification in prestin knockout mice (26). Our approach, as opposed to the ELISA quantification, is more granular and better positioned to potentially differentiate between different sources of prestin and hone in on the isoforms that are meaningful (e.g., ~94 versus ~50 kDa). Further exploration is needed to evaluate the utility of prestin as a biomarker. Here we present an investigation into prestin isoforms demonstrating five bands, where the band closest to the molecular weight of prestin relates to a measure of outer hair cell health. Continued exploration of the five bands should be pursued to understand their identity and if other correlations exist. In addition, prestin isoforms should be explored in pathologic conditions and in subjects with hearing loss. The use of prestin as a measure of hearing health at earlier stages than currently possible with audiometric measures would allow for intervention and potentially reduce the negative health impacts of hearing loss on millions of people.

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