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## Age-related declines to serum prestin levels in humans

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### ABSTRACT

Measurement of the motor protein prestin offers a novel approach to assessing outer hair cell (OHC) status using serological techniques. Motivated by our prior work showing reduced serum prestin levels in healthy young adults at-risk for noise damage, the current study examined serum prestin levels, measured from circulating blood, across the age span from 18 to 82 years old. Results suggest that serum prestin levels negatively correlate with age, with young adults having higher levels of circulating serum in the blood than older adults. Group-level analyses showed minimal differences in prestin levels between 18 and 29, 30-39, and 40-49 year olds, but significant reductions in the 50+ years-old age group compared to the three younger groups, even though all groups significantly differed from each other in audiometric thresholds and distortion product otoacoustic emissions signal-to-noise ratio. Serum prestin levels declined with increasing levels of hearing loss, with a statistically significant relationship emerging between prestin and low-frequency hearing thresholds (0.25-2 kHz) but a weaker non-significant relationship for high-frequency hearing thresholds (3-8 kHz). This differential pattern between low- and high- frequency thresholds is consistent with the basal-to-apical progression of OHC loss with age. Findings support the idea that serum prestin is the product of residual OHCs in the less age-affected apical regions. Moreover, when entered in a regression model with audiometric thresholds, age was a stronger predictor than pure tone hearing threshold level for predicting serum prestin levels.

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## 1. Introduction

Serological (blood-based) biomarkers have recently come to the forefront as having the potential to inform on the health of the inner ear. Various cochlear proteins have been considered as possible biomarkers (Mulry and Parham, 2020). One of these proteins is *prestin*, a motor protein found in the lateral membrane of the cochlear outer hair cells (OHCs) (He et al., 2014) that is measurable in human blood samples taken via standard venipuncture techniques (Hana and Bawi, 2018; Iliadou et al., 2021; Parker et al., 2021, 2022; Sun et al., 2020). Since first being identified as a cochlear protein in 2000 (Zheng et al., 2000), a sizeable literature has emerged on prestin's role in cochlear amplification (Bai et al., 2019; Belyantseva et al., 2000; Chen, 2006; Lamas et al., 2015; Liberman et al., 2002; Xia et al., 2013; Yu et al., 2006). Serological measurement of prestin is more recent development: A hypothesis for prestin as a serum biomarker of OHC

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damage was first put forth in 2015 (Parham, 2015), followed by proof-of-concept studies in animal models (Parham and Dyhrfjeld-Johnsen, 2016; Parham et al., 2019). Motivating this line of work was the vision that such a marker could be combined with a panel of other serological measurements in humans and ordered during yearly medical examinations by a primary care physician. Such a technique may be particularly useful for individuals reluctant to get their hearing tested by an audiologist as they age (Rawool, 2018). The literature on serum prestin levels in humans is still in its early stages but has started growing more recently, with recent studies examining its relation with otoacoustic emissions (OAEs) (Parker et al., 2021), noise exposure (Hana and Bawi, 2018; Parker et al., 2022), ototoxicity (Jalali et al., 2021; Solis-Angeles et al., 2021), idiopathic sensorineural hearing loss (Sun et al., 2020), and Meniere's disease and vestibular migraine (Naples et al., 2022). The current study targets age-related changes to serum prestin levels, by measuring the protein in blood samples taken from young, middle-aged, and older adults.

By definition, "aging" is "a progressive decline or loss of tissue and organ function over time due to the gradual accumulation of deleterious biological changes" (Wang and Puel, 2020). With re-





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spect to hearing, aging is associated with presbycusis, a progressive loss of hearing characterized by hearing thresholds shifts (bilateral hearing loss above 2 kHz (Cheslock and De Jesus, 2021), increased difficulty hearing speech in noise (Wong et al., 2010; Zekveld et al., 2011), and trouble localizing sounds (Gates and Mills, 2005; Kim and Chung, 2013). Presbycusis is a complex, multifactorial phenomenon affecting both the peripheral and central hearing systems, with the origins of age-related cochlear hearing declines encompassing the loss of OHCs, the denervation of IHCs, declines in vascular function that affect the endocochlear potential, and a combination thereof (Gates et al., 1989; Gates and Mills, 2005; Schulte and Schmiedt, 1992). The current work specifically targets age-related loss of OHCs, one of the most common causes of presbycusis (Perez and Bao, 2011), using serum prestin levels in circulation as a novel tool for measuring the integrity of OHC function. Distortion product OAEs (DPOAEs) are included in the test battery as a second measure of OHC function (Lonsbury-Martin and Martin, 1990; Ueberfuhr et al., 2016). Prestin is proposed to be released into circulation after the phagocytosis of damaged OHCs (Abrashkin et al., 2006; Bird et al., 2010; Parham, 2015) and also naturally by intact OHCs as part of the homeostatic regulation process associated with protein turnover (see Harasztosi et al. (2021) for recent work on the recycling of OHC proteins).

Prestin is not the first cochlear protein to be examined under the lens of aging. Otolin-1, a cochlear protein that is also measurable in blood circulation and is of interest in patients with benign paroxysmal positional vertigo (BPPV) because of its specific expression in the vestibular components of the inner ear, has been found to have age-related increase in its blood levels. One study (Tabtabai et al., 2017) found that otolin-1 levels were significantly higher in patients older than 65 years, compared to those who were younger (22-64 years). However, BBPV, unlike age-related changes to hearing, is not associated with cell death but rather demineralization in the inner ear. Age has also been found to be a predictor of serological measures outside of the auditory system, such as in studies of ovarian cancer (Pauler et al., 2001) and chronic obstructive pulmonary disease (Lomas et al., 2008). Collectively, this suggests that such biomarkers may be susceptible to effects of aging on the human body, regardless of their anatomical origin.

With respect to prestin, cochlear expression of prestin has been shown to be significantly reduced in older vs. younger mice (Jeng et al., 2020; Zhang et al., 2022) and rats (Chen et al., 2009). However, a relationship between serum prestin levels and age has never been tested in humans before and cannot be assumed without rigorous testing. Cochlear function, including OHC function, is known to change throughout the human lifespan from early gestation to geriatrics (Abdala and Dhar, 2012; Eggermont and Moore, 2012). While traditional methods of OHC counts cannot be conducted in live humans, a study in human cadavers suggest a continuous decrease in OHCs counts in humans starting from birth, particularly in the basal turn of the cochlea (Kusunoki et al., 2004). To examine OHC changes with aging, Kusunoki et al. (2004) studied the temporal bones (removed at autopsy) of 24 humans aged 1 day to 86 years and found that the infant group (0-2 years) had a significantly higher OHC count than the older groups (9-18 years, 25-59 years, and 64-86 years old), and their two middle groups a higher count than their oldest group of cadaver temporal bones. Thus, given that hair cells are fully mature at birth and they do not regenerate, hair cells show only declining aging patterns and not maturation (Chardin and Romand, 1995; Forge et al., 1998; Roberson and Rubel, 1994). The accumulating effects of noise exposure and a higher potential exposure to some ototoxic drugs may also contribute to OHC death and dysfunction in aging adults to compound the age effect. Jeng et al. (2020), Keithley (2020), and Liu et al. (2022) have recently discussed the biophysical, morphological, and molecular changes to the OHCs and the cochlea with age. Measuring prestin through the blood may provide insight into these changes and processes.

The current study examines the relationship between serum prestin levels and aging by measuring levels in adult participants aged 18-82 years old in self-reported good health and no history of diagnosed hearing loss, amplification use, or other significant hearing or neurological impairments. We have previously proposed that OHC loss may lower circulating prestin levels once the damaged cells are cleared from the body (Parham et al., 2019; Parker et al., 2022). Therefore, declining OHC counts with age are predicted to manifest as lower levels of circulating prestin in older compared to younger populations, and if there is a continuous decrease in OHC across the lifespan then a negative correlation between the two metrics is predicted. Additionally, we examined age-related changes to hearing thresholds, as well as DPOAEs and speech perception in noise and used a regression model to evaluate the contribution of age and hearing thresholds as predictors of our population's circulating prestin levels.

## 2. Materials and methods

### 2.1. Participants and exclusionary criteria

Seventy-two adults (18-82 years old, mean=39.98 years, 50 female) participated in this study. Most participants were undergraduate or graduate students, faculty, or staff at the University of Connecticut, or members of the broader community in Connecticut. Recruitment ads were placed in the UConn Student and Employee Daily Digests, a daily email listserv informing the UConn community about campus activities, including opportunities to participate in research studies. Respondents to the ads were screened via email correspondences for no known history of hearing loss, hearing aid amplification or use, chronic ear infections, ear surgery, seizures or neuropathy (e.g. multiple sclerosis (Furst and Levine, 2015), or past or current head trauma that resulted in limiting activity for more than one day (e.g. concussion (Turgeon et al., 2011)). All participants passed an otoscopic exam and a DPOAE screening (Madsen Alpha OAE Hearing Screener, Otometrics, Inc.). The DPOAE screener was conducted by measuring emissions to 2.5, 3, 3.5, 4, 5, and 6 kHz f2 frequencies in decreasing order (L1/L2 = 65/55 dB SPL). Participants were required to pass four out of the six frequencies with a 6 dB SPL signal-to-noise ratio (SNR), and the screener automatically terminated once four F2 frequencies were passed. One participant was excluded from data analvsis after not passing the DPOAE screening (n = 71 remaining). Data from the DPOAE screening was used only for screening purposes and was not investigated further for age-related effects. Air conduction audiograms were then conducted from 0.25 to 8 kHz via a Grason-Stadler GSI-561 clinical audiometer for the right and left ears separately. Hearing thresholds were not used as an exclusionary criterion due to the wide range of ages included in the study. Additionally, a questionnaire was administered about general health, tinnitus, and current loud occupational or recreational activities over the last year. When asked "In general, would you say your health is poor, good or excellent", no participants reported "poor" health (24% reported "excellent", 58% "very good", 14% "good", and 4% "fair"). Three participants over the age of 70 noted a "significant medical history", citing orthopedic injuries and diabetes, a stroke, and cancer (in remission) and kidney stones. One participant in their 40 s noted childhood thyroid cancer. All other participants noted no significant medical history. All participants tested in 2021 were screened for COVID-19 diagnosis or symptoms, including body temperature measured by UConn Health clinic staff, before entering the lab space or phlebotomy station.

Participants tested in 2018–2019 also had their body temperature taken to screen for factors that might affect peripheral and central hearing.

This Tinnitus Handicap Inventory (McCombe et al., 2001; Newman et al., 1996) was incorporated into this study with the intention of using the data as part of other, ongoing work in the lab. As such, tinnitus is not considered here in our analysis, and all participants who answered positively to experiencing tinnitus were included in the current study analysis. Specifically, twenty-three percent of participants (n = 15) responded positively to experiencing tinnitus ("Do you experience ringing in your ears (tinnitus)?"), though 14 out of these 15 participants scored <16 (slight or no handicap) on the Tinnitus Handicap Inventory and one participant scored a 38 (mild handicap). (The maximum score on the Tinnitus Handicap Inventory is a 100, labeled as a catastrophic handicap.)

## 2.2. Experimental protocol overview

All experimental procedures were approved by the Institutional Review Board at the University of Connecticut, and participants provided their written informed consent prior to study enrollment. During their test session, participants gave a blood sample. They also completed a battery of other tests that included pure-tone audiometry conducted for standard frequencies (0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8 kHz), speech perception in noise testing utilizing QuickSIN (Etymotic, Inc.), and DPOAEs (Mimosa Acoustics' HearID software).

The majority of the testing occurred during the 2021 summer season, with each participant coming to the lab for one test session, lasting approximately one hour. A small subset of the participants (n = 8) was tested in the 2018–2019 academic year, with blood samples frozen until batch processing with all the new samples in 2021. This subset of participants was not run under the full protocol of DPOAEs or tinnitus questionnaire but did have audiograms and QuickSIN data collected. All participants were monetarily compensated at the end of the test session.

For the QuickSIN, lists 1, 2, 3, and 4 were administered binaurally via inserts at 75 dB HL with SNR loss being calculated for each list individually (25.5 - score = SNR loss), and then averaged for one composite score to be used in our analyses. QuickSIN scores were not included in the analysis if the participant was not a native English speaker or was already familiar with the test and sentence lists, which was the case with 4 participants. For full DPOAE testing (separate from the initial screening tool), a twotone stimulus was used with a fixed ratio of 1.2 for the f1 and f2 frequencies, where f1/f2 = 1.22. This ratio has been found to optimally evoke robust DPOAEs (Abdala, 1996). Intensity levels (L1 and L2) were 65 and 55 dB SPL. For each of the 17 two-tone stimuli, DPOAE amplitudes were determined using the 2f1-f2 distortion product. Results were plotted with respect to the f2 frequency (0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 8.7, 9, 10, 11, 12, 13, 14, and 16 kHz). DPOAEs were measured twice per ear for each of the 17 f2 frequencies. DPOAE SNR was calculated by subtracting the measured noise floor from the DPOAE amplitude (dB SPL) for each run. Amplitude and noise floor were both averaged, separately, across the four runs, and then SNR was calculated using these averaged values. In our prior work (Parker et al., 2021, 2022), transient evoked OAEs, rather than DPOAEs, were used using a 50 dB SPL chirp, band passed from 1 - 5 kHz. Using DPOAEs in the current work may provide more frequency-specific information.

#### 2.3. Blood draw procedures

Blood draws occurred using standard venipuncture practices by a certified phlebotomist at the UConn Health Medical Services location in Downtown Storrs. Blood draws and audiological testing occurred on the same day. The phlebotomist collected two 6.0 mL tubes of non-fasting blood samples from the median cubital vein, a superficial vein in the upper limb. Blood samples were left in their tubes, standing upright, for approximately 30 min at room temperature, before being transported back to our research facilities for further processing by the first author, who had undergone the necessary biosafety training. To separate the serum, the specimens were spun at 3000 G for 10 min. After spinning, serum was collected via pipette and frozen at -80 °C until time of assay. At the conclusion of the study, samples underwent final batch processing in September 2021.

Prestin levels were measured in the serum using the MBS167508 ELISA kit (human prestin; MyBioSource, San Diego, CA) as described in the manufacturer's instruction manual. Given the number of participants, and the fact that each participants' serum was analyzed in duplicate, this required using two ELISA kits. To avoid the risk for imbalanced cross-plate variance, for each participant, one sample was run on Plate #1, and the duplicate on Plate #2. The Pearson's correlation for the two plates (r = 0.956, p < 0.000) suggests high reliability regarding cross-plate variance. The technician was blind to participant ID, sex, and age. Results were averaged across the two ELISA plates by the technician for one single serum prestin level value per participant. For full details on the blood draw procedure, sample processing, and assay, please see our recently published work (Parker et al., 2021, 2022).

### 2.4. Statistical analysis

In this participant sample, serum prestin values ranged over several orders of magnitude. So, to meet the assumption of normal residuals, serum data are analyzed and plotted using a log scale, unless otherwise noted (W = 0.97, p = 0.147 using a Shapiro-Wilk Test of normality). Audiometric thresholds and DPOAE SNRs used in the analyses were averaged across ears and runs, with thresholds being collected once in each ear, and DPOAEs twice in each ear.

Out of a possible 70 participants who passed the screening requirements, three exceeded the ELISA kit range (> 3000 pg/mL), leading them to be dropped them from the analysis, leaving us with n = 67. (Interestingly, all three of those out of range of the ELISA kit ranged in age from 31 to 37.) The remaining sample set was checked for high-end outliers, using a criterion of > 3rd quartile + 1.5 \* interquartile range for determining high-end outliers (which translates to prestin levels  $\geq$  1040 pg/mL). By this criterion, 11 were excluded, of which seven were considered "extreme" outliers falling outside of the range of a 3rd quartile + 3\*interquartile range calculation. (The ages of the extreme outliers ranged from 21 to 52 years of age.) The large majority of our samples measured below 1000 pg/mL, similar to the values from the control group in another study examining serum prestin levels in humans (Sun et al., 2020) and our prior reports (Parker et al., 2021, 2022). No outliers were found on the low-end using a 1st quartile -1.5\*interquartile range calculation. Excluding outliers, n = 57 for all subsequent analyses.

A Pearson's correlation, motivated by prior research suggesting a continuous age-related decline of OHC count (Kusunoki et al., 2004), was conducted between prestin levels and age across the full group of participants. Follow-up analyses were conducted by grouping participants into four different age groups: 18–29 years old (n = 26), 30–39 years old (n = 7), 40–49 years old (n = 5), and 50+ years (n = 19). The correlation allowed us to examine our participant pool as a whole. Additionally, examining different age groups allowed us to observe changes to prestin levels and hearing function by decade by life (similar to Arvin et al. (2013)), and also separate the young adults, who are generally at low-risk for age-related impacts to hearing (the 18–29 years-old), from the middle-age adults, who are at comparatively more at-risk for ex-

#### Table 1

Descriptive Statistics for Participants and Serum Prestin Levels.

		18–29 Years	30–39 Years	40-49 Years	50+ Years	Overall
#	n *	26	7	5	19	57
	Mean Age (yrs)	24.12	35.07	42.63	59.55	39.38
	Male:Female	18:8	1:6	1:4	6:13	17:39
	Out of Range of Detection or Outlier	4	7	3	0	14
pg/ml	Mean	229.99	243.06	281.14	172.69	231.72
	Median	215.53	209.91	342.62	184.30	204.71
	SEM	15.18	16.85	55.16	12.73	10.34
	SD	75.90	41.27	123.34	56.91	77.41
	Range	282.21	105.12	273.77	198.19	371.14
	Minimum	102.49	171.10	164.08	66.71	66.71
	Maximum	384.70	276.22	437.85	228.83	437.85

\* Excluding outliers. SEM = Standard Error of the Mean. SD = Standard Deviation.

periencing the earliest stages of age-related changes (the 30–39) and 40-49 year old groups) (Bonfils et al., 1988), and from the older adults (50+ years), who are at the highest risk for presbycusis (Arvin et al., 2013). One-way ANOVAs were conducted between groups for their prestin levels, bilateral audiometric thresholds, and bilateral pure tone averages (PTAs) including low frequency (LF-PTA: average of thresholds at 0.25, 0.5, 1, and 2 kHz) and high frequency (HFPTA: average of thresholds at 3, 4, 6 and 8 kHz. ANOVAs were also conducted between groups for individual DPOAE f2s, and also an average of the DPOAE SNRs for frequencies between 1 and 4 kHz (used to get an estimate as close to the transient evoked OAE chirp stimulus used in our previous work (Parker et al., 2021, 2022) averaged across both ears. To ensure a reliable DPOAE signal was present, a DPOAE SNR of 3 dB or greater was required. Six subjects, whose DPOAE SNR was less than 3, were excluded from the DPOAE analyses only. QuickSIN scores were also analyzed (using an average score across the four lists) to confirm known aging patterns in this well-studied metric (Zendel and Alain, 2012). A multiple regression model was also used to examine age and hearing status as predictors of serum prestin levels, testing the hypothesis that age is a stronger predictor. Statistical analyses were run with MATLAB version 9.10 (The MathWorks, Inc., Natick, MA). P < 0.5 was used for statistical significance in all analyses, and all presented p-values are uncorrected.

Before conducting any analyses with serum or aging data, an independent samples *t*-test examined whether prestin levels differed between the participant's biological sexes in the remaining dataset of n = 57 (see Table 1 for distribution of the sexes by age group). Similar to findings in our previous papers on serum prestin in humans (Parker et al., 2021, 2022), no difference was found between males and females (t(27.09) = 0.368, p = 0.715) using a Welsh's *t*-test, which was employed due to unequal group sizes. Thus, both sexes were pooled together for subsequent analyses.

## 3. Results

Overall, across the 57 participants, average prestin levels ranged from 66.71 pg/mL to 437.85 pg/mL, with a mean of 231.72 pg/mL. (See Table 1 for the full set of descriptive statistics on prestin levels.) The first step in analyzing the relation between serum prestin levels (logged, to meet the assumptions of normal distribution) and age was to conduct a Pearson's correlation. Our analysis shows a significant negative correlation between average serum prestin levels (pg/mL) and age (years) (r = -0.350, p = 0.008) (Fig. 1, panels A and B), with evidence to suggest that serum prestin levels show a general pattern of decreasing with increasing age, and that we can reject the null hypothesis that prestin and age are not related.

To supplement the correlation analysis, we divided participants into four different age groups: (1) 18–29 years old (n = 26), (2) 30–39 years old (n = 7), (3) 40–49 years old (n = 5), and (4)

50+ years (n = 19). Descriptive statistics for each age group are given in Table 1, and a bar plot (panel C) and box plot (panel D) of the distribution of prestin levels for each group can be seen in Fig. 1. After confirming equal population variances using a Levene's test (F(3,52) = 1.421, p = 0.247), a one-way ANOVA was conducted between the four groups. In line with the significant correlation across our sample, the one-way ANOVA showed a significant difference between the four groups' serum prestin levels (F(3,52) = 3.165, p = 0.032). Moreover, independent samples t-tests show a significant differences between the 50+ group and each of the younger groups (18–29: t(43) = 2.259, p = 0.014; 30–39: t(25) = 1.400, p = 0.047; 40–49: t(23) = 2.440, p = 0.011) (Fig. 1, panel C), but no significant differences were found between any combination of the three youngest groups amongst themselves.

Though our analyses would suggest a relation between serum prestin levels and age, it is important to consider that age is likely not the sole, nor perhaps the primary predictor of prestin levels. With that, we collected pure tone audiometric thresholds and DPOAEs. Table 2 shows age group differences for each frequency for audiometric thresholds including low and high frequency PTA (top) and DPOAE SNRs (bottom), and each's Pearson's correlation with serum prestin levels. All nine audiometric threshold frequencies, plus the PTAs, showed significant differences between age groups at the p < 0.001 level, but only three frequencies and one PTA calculation significantly correlated with prestin (0.25, 1.5, and 4 kHz, and LFPTA). However, all correlations were negative, suggesting that higher (worse) thresholds pair with lower prestin levels). Both LFPTA (r = 0.700, p < 0.001) and HFPTA (r = 0.777, p < 0.001) 0.001) correlated significantly with age, but only LFPTA correlated significantly with prestin (r = -0.295, p < 0.042). DPOAE results are less straightforward. Eleven out of the 17 DPOAE frequencies (plus the 1-4 kHz averaged value) show significant differences between age groups at the p < 0.05 level, and one more at the p <0.001 level using DPOAE SNRs. Five frequencies show no significant difference, many of these frequencies fall in the 8-12 kHz range where standing wave nulls are observed in the DPOAE dataset (Miller et al., 2018; Siegel, 1994). Four frequencies (0.75, 1, 3, and 4 kHz) plus the average metric show a correlation with serum prestin levels at the p < 0.05 level, and the correlation between serum prestin levels and DPOAE SNR are in the positive direction for all but one F2 frequency (10 kHz-non-significant and also corresponding to the frequency range where standing nulls are observed). The DPOAE average also correlated significantly with age (r = -0.636, p < 0.001).

Fig. 2 plots compares audiometric thresholds (panel A), DPOAE SNRs (panel B) and QuickSIN scores (panel C) for each group, to illustrate group differences. Unlike audiometric thresholds and DPOAES, QuickSIN scores do not show a significant difference between age groups (F(3,48) = 1.061, p = 0.375), but, generally, show worse (higher) scores with increasing age. Fig. 3 plots the relations



**Fig. 1.** Four plots showing the distribution of serum prestin levels across ages, and by age group. Scatter plots between serum prestin levels and age, with a color map used to plot low (A) and high (B) frequency pure tone average (dB HL). Different marker shapes are used to plot females (diamond) versus males (circle). A bar plot (C) shows the mean prestin level (pg/mL) by age group and indicates significant differences between the three youngest groups and the 50+ group (\* = significant at p < 0.5; non-significant differences amongst the three youngest groups are not shown), and a box plot (D) maps the distribution with individual data points scattered on top and the thick black line representing the median.

between the hearing status measures (HFPTA, LFPTA, DPOAE SNR average, and QuickSIN SNR loss) and age (top) and serum prestin levels (bottom). All four measures show a significant correlation with age, suggesting that each metric worsens as individual grows older, but only LFPTA (r=-0.295, p=0.042) and the DPOAE average (r=0.318, p=0.038) showed a significant relation with serum prestin levels (see Fig. 3 for all correlation and significance values). Therefore, we can reject the null hypothesis that LFPTA and DPOAEs do not show a significant relation to serum prestin levels, but must retain the null for HFPTA and QuickSIN. All audiograms, in concert with QuickSIN scores, were reviewed for indications of (undiagnosed) age-related hearing loss. A total of six participants showed a sloping high-frequency loss consistent sensory presbycusis. These participants ranged in age from 61 to 82.

With a better understanding of how hearing status differs between our four age groups, and how each measure correlates with serum prestin levels, we examined age and hearing thresholds as potential predictors of serum prestin levels in humans. To do this, we used multiple regression (Enter) with serum prestin levels as our dependent variable, and age and audiometric LFPTA (the PTA calculation that showed a significant correlation with serum prestin levels) as predictors (VIF = 1.993). In this model, R = 0.352, suggesting the overall model is a significant predictor of serum prestin levels (F(2,54) = 3.744, p = 0.030). Moreover,  $R^2 = 0.124$ , or, in other words, 12.4% of the proportion of variance in serum prestin levels explained by age and LFPTA. To better understand how each predictor contributed to the model, we examined the unstandardized beta coefficients for each, and found that while age significantly contributed to the model (B = -0.003, p = 0.040), LF-PTA did not (B = -0.003, p = 0.369) once accounting for age. Recall that log-transformed values are being used in the model for the prestin dependent variable when interpreting the beta coefficients.

Finally, a further illustration that PTA is not the predominant driving factor of serum prestin levels, we took a subset of our participants (those from out 50+ years age group, n = 19) and correlated serum prestin levels with age and with HFPTA and LFPTA. Correlations were stronger between serum prestin levels and age (r = -0.443, p = 0.050) than they were between serum prestin levels and HFPTA (r = -0.098, 0.682) or LFPTA (r = -0.101, p = 0.672), though none of the correlations were significant. Moreover, as another way to illustrate this, our participant with the lowest HFPTA (8.13 dB HL) did not have the highest serum prestin level measured in the current study (222.79 pg/mL), nor did the participant with the highest HFPTA (65.63 dB HL) have the lowest serum prestin levels (208.30 pg/mL). Similar results were found with LF-PTA, where our participant with the lowest LFPTA (13.75 dB HL) did not have the highest serum prestin level measured in the current study (190.78 pg/mL), nor did the participant with the highest LFPTA (36.25 dB HL) have the lowest serum prestin levels (170.32 pg/mL).

## 4. Discussion

In the current study, we examined the relation between serum prestin levels and age in human adults between the ages of 18 and 82 years with no history of a diagnosed hearing loss or amplification. In our participant sample, on average, hearing abilities measured by hearing status, DPOAEs, QuickSIN, and serum prestin levels—decline gradually with age. Our findings show a significant negative relation between age and prestin, where older individuals tend to have lower prestin levels. With respect to hearing thresh-

#### Table 2

Age-Related Differences in Audiometric Thresholds Distortion Product Otoacoustic Emissions, and their Correlations with Serum Prestin.

Audiometric Thresholds						
Frequency (kHz)	Age Group Differences	Correlation with Prestin	Frequency (kHz)	Age Group Differences	Correlation with Prestin	
0.25		r = -0.492,	3		r = -0.407,	
	F(3,53) = 12.20,	p < 0.001		F(3,53) = 10.79,	p = 0.006	
	$p < 0.001^{**}$			$p < 0.001^{**}$		
0.5		r = -0.219,	4		r = -0.346,	
	F(3,53) = 13.99,	p = 0.154		F(3,53) = 11.30,	$p = 0.021^*$	
	$p < 0.001^{**}$			$p < 0.001^{**}$		
1		r = -0.201,	6		r = -0.205,	
	F(3,53) = 13.19,	p = 0.191		F(3,53) = 14.25,	p = 0.183	
	$p < 0.001^{**}$			$p < 0.001^{**}$		
1.5		r = -0.372,	8		r = -0.240,	
	F(3,53) = 9.76,	$p = 0.003^*$		F(3,53) = 14.51,	p = 0.117	
	$p < 0.001^{**}$			$p < 0.001^{**}$		
2		r = -0.314,				
	F(3,53) = 6.78,	p = 0.038				
	$p < 0.001^{**}$					
LFPTA		r = -0.295,	HFPTA		r = -0.227,	
(0.25 – 2 kHz)	F(3,53) = 16.92,	$p = 0.042^*$	(3 – 8 kHz)	F(3,53) = 14.84,	p = 0.092	
	$p < 0.001^{**}$			$p < 0.001^{**}$		

Distortion Product Otoacoustic Emission Signal-to-Noise Ratios

F2 Frequency (kHz)	Age Group Differences	Correlation with Prestin	Frequency (kHz)	Age Group Differences	Correlation with Prestin
0.5		r = 0.044,	8.7		r = 0.050,
	F(3,40) = 3.24,	p = 0.775		F(3,40) = 0.49,	p = 0.749
	$p = 0.032^*$			p = 0.688	
0.75		r = 0.317,	9		r = 0.006,
	F(3,40) = 2.21,	$p = 0.036^*$		F(3,40) = 0.17,	p = 0.970
	p = 0.102			p = 0.914	
1		r = 0.368,	10		r = -0.002,
	F(3,40) = 3.94,	$p = 0.027^*$		F(3,40) = 2.11,	p = 0.991
	$p = 0.015^*$			p = 0.115	
1.5		r = 0.055,	11		r = 0.007,
	F(3,40) = 2.169,	p = 0.723		F(3,40) = 4.60,	p = 0.965
	$p = 0.035^*$		10	$p = 0.007^*$	
2		r = 0.232,	12		r = 0.028,
	F(3,40) = 3.87,	p = 0.130		F(3,40) = 2.86,	p = 0.857
2	$p = 0.016^*$	0.270	12	$p = 0.049^*$	. 0.100
3	F(2,40) 2,02	r = 0.370,	13	F(2.40) 4.7C1	r = 0.180,
	F(3,40) = 3.92,	$p = 0.030^{*}$		F(3,40) = 4.761,	p = 0.243
4	$p = 0.015^{\circ}$	- 0.248	14	$p = 0.006^{\circ}$	
4	F(2,40) = 4.06	n = 0.022*	14	F(2,40) = 7.02	n = 0.151,
	P(3,40) = 4.50	p = 0.032		$\Gamma(5,40) = 7.52,$	p = 0.329
6	p = 0.005	r = 0.049	16	p < 0.001	r = 0.002
0	F(3.40) - 4.54	n = 0.753	10	F(3.40) - 5.21	n = 0.052, n = 0.553
	n = 0.008	P = 0.755		$n = 0.004^*$	P = 0.555
8	p = 0.000	r = 0.003	1-4 kHz	p = 0.004	r = 0.318
0	F(3.40) - 1.51	n = 0.982	Ανσ	F(3.40) - 5.59	$n = 0.038^*$
	n = 0.228	p = 0.302		$n = 0.003^*$	P = 0.050

\* *p* < 0.5.

\*\* p < 0.001.

olds, we also find that low-frequency hearing correlates significantly with serum prestin but high frequency, where age-related threshold changes are most evident, does not.

### 4.1. Prestin in an aging population informing our earlier work

Our group has led several previous studies, both in animal models (Parham and Dyhrfjeld-Johnsen, 2016; Parham et al., 2019) and in young adult humans (Parker et al., 2022), that examined the relation between serum prestin levels and noise exposure. In Parker et al. (2022), we found a significant negative relation between prestin levels and noise exposure in a group of young adults with normal audiograms, where higher prestin levels paired with lower levels of routine noise exposure—that is, people who lead quieter lives tended to have higher circulating levels of prestin. One theory was that this may be due to individuals with louder

lives experiencing hidden OHC loss/dysfunction that is not apparent from the audiogram. Though the current study was not designed to examine the participant's routine sound environment like our past study (Parker et al., 2022), the negative correlation between serum prestin levels and age, is consistent with known declines in OHC count with age, suggesting that the relations between prestin levels and noise exposure could likewise be attributed to OHC loss.

## 4.2. Age vs. hearing level—which is a stronger predictor of serum prestin levels?

It is important to examine whether the negative relation between serum prestin levels and age is indeed a function of age, or rather simply one of hearing threshold status, that is known to also increase with age (Huang and Tang, 2010). If prestin declines



Fig. 2. Subplots showing differences in audiometric thresholds (A), distortion product otoacoustic emissions (B) and QuickSIN scores (C) by age group. Error shading/bars represent the standard error of the mean.

with age, but hearing threshold status also declines with age, perhaps the decrease in prestin levels we've observed are more accurately predicted by thresholds than age. Fig. 1 (Panel A) shows the relation between prestin levels and age and uses a colormap to plot LFPTA (0.25 - 2 kHz), indicating that generally, LFPTA increases (i.e., worsens) with age. A similar pattern is observed for the HFPTA (3-8 kHz), although the degree of decline with age is greater (Fig. 1, Panel B).

Though we found that prestin declines with age, and audiometric thresholds, especially in the high frequency range, increase with age, we found a statistically significant relationship between the low frequency PTA (LFPTA) and serum prestin levels, but not the high frequency PTA (HFPTA). Table 2 shows that only two out of the nine measured thresholds at individual frequencies showed statistically significant relations with serum prestin, although all correlations between serum prestin and audiometric thresholds are negative in direction, suggesting that decreased levels of serum prestin with increased levels of age-related hearing loss. To examine the impact of age and hearing status individually, we performed multiple regression with serum prestin levels as the dependent variable and age and LFPTA as the predictors. While the overall model showed significance as a predictor of serum prestin levels (F(2,52) = 4.564, p = 0.015), only age contributed significantly to the model as a predictor (B = -0.003, p = 0.042). Our results suggest that prestin levels are more strongly voked to the

aging cochlea than they are to hearing status once the effect of age on hearing thresholds are considered.

The DPOAE and audiometric data support the conclusion that decreasing serum levels with age is related to OHC function. Specifically, the significant correlations between DPOAE SNR and LFPTA with serum prestin indicate that circulating prestin levels are dominated by protein output from OHCs in the healthier, less-age-affected apical region of the cochlea. If so, the relation between serum prestin and age, would further suggest that serum production decreases with age in these residual OHCs. Overall, our findings contribute to a changing interpretation of prestin in the blood by suggesting that serum levels are an indicator of the functional integrity of surviving OHCs.

Previously, we have reported that in the *acute* setting (at 24 hour after trauma), be it in the immediate aftermath of noise (Parham and Dyhrfjeld-Johnsen, 2016; Parham et al., 2019) or cisplatin exposure (Liba et al., 2017), there is a strong positive correlation between hearing loss and prestin (the higher the thresholds, the higher the serum prestin), which is consistent with the rapid spike in circulating prestin levels after OHC damage observed in our other work (Parham et al., 2019). However, here, we are working with participants, who have not undergone recent trauma or chemotherapy, and whose serum prestin levels therefore presumably reflect fewer hair cells producing prestin as the result of a gradual (not acute) age-related decrease in OHC. Thus, direct com-



**Fig. 3.** Scatter plots between four metrics of hearing status and age (top) and serum prestin levels (bottom). High Frequency Pure Tone Average (3-8 kHz, left), Low Frequency Pure Tone Average (0.25 - 2 kHz, 2nd from left), Distortion Product Otoacoustic Emissions Signal-to-Noise Ratio (average of F2 1–4 kHz) (2nd from right), and QuickSIN score (signal-to-noise ratio) (right). Age group is plotted by color. All participants (excluding outliers, n = 57) are plotted in the PTA plots. DPOAE plots include participants with DPOAEs >3 dB SNR from the second arm of the study only (n = 44), and QuickSIN plots include participants who were native English speakers with no prior exposure to the test (n = 53).

parisons between the acute and chronic types of hearing loss need to take into account when, in time, relative to the OHC loss, the blood assay is taken. Collectively, this suggests that in the immediate aftermath of trauma, prestin in circulation likely stems from recently traumatized OHCs, but when taken distal to a trauma or under conditions where OHC counts are expected to decline gradually, the dominant source of prestin in circulation is more likely intact OHCs.

# 4.3. How does the decline of prestin levels pattern with increasing age?

While our analysis shows a statistically significant, negative correlation between serum prestin levels and age, suggestive of a gradual, continuous decrease of prestin levels with age, the distribution of participant ages is a caveat to interpreting this analysis. This was one factor in motivating the division of the dataset into four age groups not only to examine group levels differences in prestin and hearing measures, but also to gather insight into the manner of declining prestin levels-whether it be continuous with age or show a sharp decline at a certain point. Mean prestin levels in our three youngest age groups were found to all fall within the 200-300 pg/mL range once extreme outliers are removed. Based on group means, prestin levels do not appear to follow a gradual decline pattern across the decades, but increase across the three youngest groups, though increases in means are relatively negligible within the span of 54 pg/mL and are not statistically significant. However, a sharp decrease in group mean is seen in the oldest age group. The 50+ years old group, while broad in age range, also shows the lowest median of the four groups, the smallest minimum serum prestin level value, the smallest maximum of any of the four groups, and the tightest standard error of the mean. Moreover, no outliers needed to be removed nor were any serum prestin levels too high to be read by the ELISA kit. No other group shows similarly consistent patterns.

One limitation of our study is the small number of participants, particularly when analyzed in groups, leading to an imbalance in group sizes. Because we were only able to test during hours that the phlebotomy clinic was open, we were limited to collecting blood samples on weekdays during "normal" work hours, potentially hindering the chance for a more robust sample of middleaged individuals. Future research may want to investigate patterns in middle age, as the majority of the "out of range" of the ELISA kit, or outlier, samples in our dataset were in our 30 s and 40 s groups. Another limitation that must be considered in any study that focuses on age-related changes to auditory measures is that it is near impossible to isolate age-related impacts to hearing from other environmental and health factors that accumulate with age Examples of confounding factors include noise exposure (Shone et al., 1991; Wu et al., 2021), intake of ototoxic mediations (Joo et al., 2018, 2020; Skalleberg et al., 2020) including aspirin consumption (Yu et al., 2008), increasing health concerns-particularly cardiac health (Friedland et al., 2009; Helzner et al., 2011) with new suggestions of the presence of prestin in the cardiovascular system (Zhang et al., 2021), loss of spiral ganglion neurons (Perez and Bao, 2011), and increased inflammation (He et al., 2020). Though our regression model helped us to examine age and LFPTA as predictors of serum prestin levels, age and hearing thresholds cannot be completely disentangled. Beyond that, presbycusis is heterogeneous and factors other than OHC can contribute to declines in hearing. For instance, we must consider that strial atrophy with consequent changes in the endocochlear potential can disrupt OHC function without OHC loss (Fettiplace, 2011; Jacob et al., 2011).

Moreover, because we did not collect personal sound level data on our participants as we did in our previous prestin studies in humans, we were not able to examine how environmental noise levels factor into our results. While we did administer the Noise Exposure Questionnaire (NEQ) (Johnson et al., 2017) on our participants, the NEQ asks solely about the last twelve months of noise exposure, and so it was deemed untrustworthy given the circumstances of unordinary decreases in recreational noise exposures (e.g. concert attendance, large gatherings, changes to hobbies and routines from occupations) throughout the early days of the COVID-19 pandemic. (Data was collected in Summer 2021.) This is a limitation given our previous finding of a relation between serum prestin levels and noise (Parker et al., 2022), and the known accumulation of noise exposure that comes with age. Our prior work has shown that noise exposure can predict prestin levels, at least in normal hearing young adults (Parker et al., 2022), suggesting that noise exposure may be one of the unaccounted factors in the current analysis. We also did not ask about the menopausal state of the female participants, and so the role of estrogen in modulating age-related changes to hearing cannot be assessed (Shuster et al., 2019). Another limitation is that we did not ask about how recently the participant had taken medications, such as aspirin or NSAIDs, that are known to affect cochlear function (Yu et al., 2008). Future studies on serum prestin levels in humans should focus on these various factors and expand to clinical populations with different etiologies of sensorineural hearing loss. Other clinical populations may be of interest for future studies, too. For example, with a recent study suggesting prestin as an amplifier for cardiac motor function (Zhang et al., 2021), studies with cardiac patients may also contribute valuable information to the growing literature on the mechanisms, utility, validity, and utility of serological measurements of prestin levels in humans.

## 5. Conclusion

In summary, we found that older individuals tended to have lower serum prestin levels circulating in their bloodstream than did younger participants. Prestin may potentially be able to provide information about cochlear function (or other non-cochlear factors) that standard audiological measures cannot, but also may be a useful tool as an adjunct to formal testing with an audiologist. Moreover, we discovered a significant relationship between serum prestin levels and DPOAE SNR and LFPTA throughout this age span. Serological measures of cochlear status continue to provide new information on the auditory system and insights about inner ear function and dysfunction. These results, along with a growing body of literature of measuring serum prestin levels in humans (Iliadou et al., 2021), suggest changes in serum prestin levels coincide with well-established changes in OHC function, and encourage the continuing study of the biomarker for its potential to improve hearing loss detection by extending the already ubiquitous use of blood-based of biomarkers in medical science.

## Data Availability

Data will be made available on request.

## **CRediT** authorship contribution statement

**Ashley Parker:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Visualization, Project administration, Funding acquisition. **Kourosh Parham:** Conceptualization, Methodology, Resources, Writing – review & editing. **Erika Skoe:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

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