Reliability of Serological Prestin Levels in Humans and its Relation to Otoacoustic Emissions, a Functional Measure of Outer Hair Cells

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Objectives: Serological biomarkers, common to many areas of medicine, have the potential to inform on the health of the human body and to give early warning of risk of compromised function or illness before symptoms are experienced. Serological measurement of prestin, a motor protein uniquely produced and expressed in outer hair cells, has recently been identified as a potential biomarker to inform on the health of the cochlea. Before any test can be introduced into the clinical toolkit, the reproducibility of the measurement when repeated in the same subject must be considered. The primary objective of this study is to outline the test-retest reliability estimates and normative ranges for serological prestin in healthy young adults with normal hearing. In addition, we examine the relation between serum prestin levels and otoacoustic emissions (OAEs) to compare this OHC-specific protein to the most common measure of OHC function currently used in hearing assessments.

Design: We measured prestin levels serologically from circulating blood in 34 young adults (18 to 24 years old) with clinically normal pure-tone audiometric averages at five different timepoints up to six months apart (average intervals between measurements ranged from <1 week to 7 weeks apart). To guide future studies of clinical populations, we present the standard error of the measurement, reference normative values, and multiple measures of reliability. Additionally, we measured transient evoked OAEs at the same five timepoints and used correlation coefficients to examine the relation between OAEs and prestin levels (pg/mL).

Results: Serum prestin levels demonstrated good to excellent reliability between and across the five different time points, with correlation coefficients and intraclass correlations >0.8. Across sessions, the average serum prestin level was 250.20 pg/mL, with a standard error of measurement of 7.28 pg/mL. Moreover, positive correlations (generally weak to moderate) were found between prestin levels and OAE magnitudes and signal-to-noise ratios.

Conclusions: Findings characterize serum prestin in healthy young adults with normal hearing and provide initial normative data that may be critical to interpreting results from individuals with sensorineural hearing loss. Our results demonstrate reliability of serum prestin levels in a sample of normal-hearing young adults across five test sessions up to 6 months apart, paving the way for testing larger samples to more accurately estimate test-retest standards for clinical protocols, including those involving serial monitoring. The positive correlations between serum prestin and OAE levels, although weak to moderate, reinforce that the source of serum prestin is likely the outer hair cells in the inner ear, but also that serum prestin and OAEs each may also index aspects of biologic function not common to the other.

Key words: Prestin, Serological biomarker, Inner ear, Otoacoustic emissions, Hearing loss.

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INTRODUCTION

At the current time, there are no clinically available blood-based biomarkers to inform on the health of the inner ear, comparable to serological markers that are commonly used to assess organ function in other domains of medicine (e.g., CA-125 for ovarian cancer, TSH for thyroid disorders). Biomarkers are powerful tools that can be used as a metric for disease or the functional state of an organism (Rüttiger et al. 2017). However, prestin, a motor protein uniquely expressed in the lateral membrane of the outer hair cells (OHCs) (Zheng et al. 2000), has recently come to the forefront as a potential biomarker to inform on the health of the cochlea (Parham 2015). Here, we provide normative ranges of serum prestin levels in a small sample of healthy young adults, evaluate the reliability of prestin by repeating measurements at five timepoints, and compare serum levels of this OHC-specific protein to another measure of OHC function (otoacoustic emissions—OAEs) that is routinely used in clinical hearing assessments.

OHCs are effector cells that augment the sensitivity and tuning of the cochlea and are particularly susceptible to the effects of aging and to injury from noise and ototoxins. The tuning and sensitivity functions of the cochlear amplifier are directly related to electromotility of the OHCs (Brownell et al. 1985; Zenner et al. 1985). A membrane protein, prestin, generates this electromotility, the physical change in length of the OHCs as a function of membrane polarization that occurs in the lateral plasma membrane of the OHCs (Zheng et al. 2000; for a thorough review of prestin, see He et al. 2014), with one study suggesting that there may be a gradient of prestin expression along the tonotopic axis in guinea pigs (Bai et al. 2010). Until recently, studies of this inner-ear protein were limited to animal models because of a lack of noninvasive measurement approaches. In animal models, a variety of invasive approaches have been adopted, including using real time polymerase chain reaction and Western blot to measure prestin expression directly from cochleae (e.g., Chen 2006; Xia et al. 2013). Moreover, genetic modification has been used to establish both that prestin is necessary for electromotility (Liberman et al. 2002) and that it plays a central role in cochlear tuning (Cheatham et al. 2004). Here, we are not using prestin to measure cases of genetic modification or prestin mutations (see Dallos et al. 2006), but rather, we are using it as an indirect marker of the integrity of OHC health.

We have previously proposed measuring prestin levels using less invasive methods (Parham 2015), with serological techniques where prestin is measured from circulating blood serum obtained via standard venipuncture approaches. Prestin levels in circulating blood may offer novel insight into OHC health, such as serving as a biomarker for the early detection of acquired

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sensorineural hearing loss (Parham 2015). A protein such as prestin is small enough (80 kDa) to cross the blood-labyrinthine barrier and enter blood circulation, allowing for prestin to be measured outside the cochlea via venipuncture from the superficial veins of the upper limb, as is common for other clinical biomarkers. Commercially available enzyme-linked immunosorbent assay (ELISA) kits offer extremely sensitive techniques for analyzing prestin from serum, allowing picogram quantities of prestin to be detected with such assays (Parham 2015). The ELISA technique is a useful starting point for measuring prestin serologically in humans for a number of reasons. First, it is commonly utilized in clinical medicine to quantify markers in the blood. Also, in addition to having been successfully utilized it in preclinical models of ototoxicity (Liba et al. 2017; Naples et al. 2018) and noise-induced hearing loss (Parham & Dhyrfjeld-Johnsen 2016; Parham et al. 2019), the ELISA technique has been embraced by other investigators who independently replicated and extended these findings in both preclinical (e.g., Doğan et al. 2018) and clinical (e.g., Sun et al. 2019) settings, thus facilitating comparisons across studies, which is crucial at this early phase of this nascent field.

Blood-based biomarkers offer a novel strategy in hearing diagnostics with the potential for widescale administration of hearing-related health diagnostics. A clear advantage for including serum prestin as a supplementary tool at the disposal of a clinician is that in the clinical setting, blood tests are routinely performed in patients both at regular primary care visits and for those receiving specialized care. For example, a patient undergoing treatment for cancer with cisplatin, an ototoxic chemotherapeutic cancer drug (Rybak et al. 2007), will regularly have their blood drawn for a variety of purposes. Current standards for audiometric surveillance of chemotherapeutic ototoxicity, being set apart by months, are not designed to detect cochlear injury at the earliest phases. The addition of a prestin blood test to the laboratory panel being monitored by the oncologist is simple and practical, and it would facilitate serial surveillance of cochlear health at shorter intervals of, for example, days.

Recent studies support the use of serum prestin as a biomarker of hearing loss. Parham and Dyhrfjeld-Johnsen (2016) published a proof-of-concept animal study measuring prestin levels following noise exposure in rats that resulted in OHC loss in the basal portion of the cochlea, a permanent reduction of distortion product OAE (DPOAE) magnitudes, and elevated auditory brainstem response (ABR) thresholds. In this study, serum prestin levels were measured just once. This measurement occurred 2 weeks after exposure and it showed that prestin was detectable serologically in both the noise-exposed and the control groups of rats, but the noise-exposed animals demonstrated significantly lower levels in comparison to the controls, consistent with a reduction in prestin production from OHC loss. Follow-up work in rats focused on the time course of change in serological prestin levels in the immediate aftermath of a traumatic noise event (Parham et al. 2019). Blood samples measured six times throughout 14 days showed prestin levels that initially spiked from baseline when measured four hours after exposure to 120 dB SPL noise. This initial spike was followed by a gradual decline back to baseline ~24-hours postexposure and subsequently to below baseline by the 72-hours postexposure measurement. Histological findings showed strong decreases in hair cell count in the basal region of the cochlea, as well as DPOAE and ABR values consistent with a permanent hearing loss. At the end of two weeks, prestin levels were 20% below baseline, showing statistical significance when compared with baseline. A group exposed to 110 dB SPL noise, who experienced only a temporary threshold shift in hearing, also showed a small steady drop from baseline, but the change was not significant. Moreover, hair cell loss was significantly less than in the 120 dB SPL noise group, and their DPOAE levels and ABR thresholds largely recovered. Collectively, these studies show reproducible reductions in circulating prestin levels in rats where there was permanent noise-induced OHC damage.

Animal models of ototoxicity have also given insight into the timeline over which prestin is released into circulation following drug administration, as also studied by Parham and colleagues. Liba et al. (2017) and Naples et al. (2018) measured prestin levels in the blood after the administration of low-dose cisplatin in mice and guinea pigs. Prestin levels first increased in both animal models before lowering back to or below baseline levels 14 days after treatment.

These serological studies of prestin in animal models have paved the way for measuring serum prestin levels in humans, and a small literature has recently emerged focusing on clinical populations. Hana and Bawi (2018) found that serum prestin levels were significantly elevated in their noise-induced hearing loss (NIHL) group (n = 300, 35 to 45 years old) right after noise exposure relative to an age and sex matched control group (n = 200, 36 to 44 years old). Prestin levels were measured again in the NIHL group one month after treatment. (The treatment, including the duration of treatment itself, was not described.) At posttreatment, prestin levels remained elevated compared to the controls; however, the group showed a 55% drop from their own original levels when compared with pretreatment, suggesting that while prestin levels spike immediately after a traumatic noise event, they may eventually stabilize and return to near baseline levels. However, Hani and Bawi did not repeat prestin measurements in their control group, leaving open the possibility that the pre-to-posttreatment change in serum prestin levels in the NIHL group was not merely due to the treatment or circulating prestin being filtered out of the body but was instead an artifact of low test-retest reliability of serum prestin. This, and the lack of published normative data, serves to motivate the current study's examination of test-retest reliability in serum prestin levels in healthy adults with normal-hearing thresholds and OAEs.

Another recent human study measured prestin levels serologically in humans with idiopathic sudden sensorineural hearing loss (ISSHL). Sun et al. (2019) measured the serum protein in an ISSHL group (n = 14, 31 to 72 years old) between two and seven days after the onset of loss and found that prestin was detectable in blood samples from both their hearing loss patients and healthy controls (n = 24, 33 to 76 years old). (The "idiopathic" nature of ISSHL makes it difficult to determine the exact nature of the hearing loss and its relation to the OHCs, although all patients were treated with the same drug therapy strategy.) However, concentration of serum prestin was significantly higher in ISSHL compared with controls, although in those who responded to treatment, prestin levels eventually decreased from their initial test levels at retest. While these results, too, may support a temporal pattern where circulating prestin levels spike in the immediate aftermath of a trauma (e.g., dangerous sound levels, sudden hearing loss) followed by

a gradual stabilization as the protein is filtered from the body, they also suggest the need for more data on test-retest reliability of the measure to determine if the changes in levels can indeed be attributed to a response to treatment, or simply due to a lack of repeatability of measurement in humans. To the best of our knowledge, Hana and Bawi (2018) and Sun et al. (2019) are the only studies to have published prestin levels in humans to date. Tovi et al. (2019) assayed for prestin autoantibodies through the blood in an ISSHL population but was not a direct study of serum prestin levels. Other studies have used blood samples to study rare cases of genetic manipulations of prestin in human populations (Toth et al. 2007). Our interest in serum measurement of inner ear function is comparatively broader than the study of rare conditions—we envision it having potentially wider scale clinical application in assessing the integrity of OHC function.

The recent development of techniques to serologically measure inner-ear proteins means that there are still many unknowns and much to be explored before the serum biomarker can be realistically considered for inclusion in the clinical toolkit. Previous research on serum prestin focused on hearing loss, yet little is known about serum prestin levels from healthy human adults. The goal of our current study, therefore, was to study normal variation in circulating levels of prestin in ears that do not show any indication of clinical hearing loss. We evaluated the test-retest reliability of circulating prestin levels in healthy college students with clinically normal pure-tone audiometric thresholds and OAEs and provide normative values that will be valuable for future work on the serological marker. To be comprehensive, measurements were taken at five separate test sessions spaced throughout an academic term.

Although normative ranges and test-retest data do not currently exist for healthy adults, previous work has shown that prestin was detectable in the serum of healthy controls. What explains the presence of prestin in such cases? In the healthy ear, prestin, and other inner-ear proteins, are continuously recycled as part of the homeostatic regulation of OHC function (Parham 2015; see Morimoto & Cuervo 2009 for a discussion on protein homeostasis). Homeostatic regulation of cochlear function leads to the hypothesis that serum prestin levels should be stable in the absence of a change in cochlear function.

Reference values and test-retest reliability estimates are needed to establish the clinical utility of serological otological biomarkers such as prestin. By comparison, test-retest reliability has been heavily studied in other common audiological tests such as OAEs (e.g., Franklin et al. 1992; Marshall & Heller 1996; Ng & Mcpherson 2005; Wagner et al. 2008; Stuart et al. 2009; Reavis et al. 2015) and the ABR (e.g. Edwards et al. 1982; Oyler et al. 1991; Song et al. 2011). Furthermore, all clinical tests of auditory function have reference values—a range of cutoff values that differentiates "normal" from "abnormal" function (e.g., hearing thresholds ranging from 26 to 91+ dB HL in young adults are classified as a hearing loss from mild to profound; Clark 1981). Even small variances from normative ranges can potentially be meaningful. Variances, both inter- and intrasubject need to be accounted for and well-understood to determine if they are clinically significant.

In addition to studying serum prestin levels over time, we were interested in how serum prestin levels relate to OAEs, given that both are presumed OHC measures. OAEs, currently the most specific measure of OHC function in the clinical assessment of human auditory function, are used routinely as a part of newborn hearing screenings and in audiology clinics to provide a quick, objective measure of OHC function (Kemp 1997). OAEs are low-level acoustic signals recorded from the ear canal arising in the cochlea. To be detectable in the ear canal, OHCs must be normal or near normal to create sufficient cochlear amplification for the emission to be back propagated through the middle ear and detected with a sensitive microphone in the ear canal. Cochlear amplification is powered by the OHC motility, arising from conformational changes in the motor protein prestin.

Electromotility of the OHCs is considered to be critical to detect OAEs in the ear canal (Cunningham 2011). It has been demonstrated that when the OHC electromotility is disrupted (e.g., from noise damage, ototoxic drugs, genetic mutation), OAEs are reduced or absent (e.g., Shehata et al. 1991). In addition to OHC electromotility, OAEs are also influenced by the integrity of the middle-ear space, and other aspects of the amplification mechanism including mechanotransduction and the endocochlear potential (Mills et al. 1993; Gillespie & Müller 2009). Thus, the connection between prestin, electromotility, and OAEs (Drexl et al. 2008) make prestin levels and OAEs sensible metrics of comparison. Work on animal models, where animals with permeant OHC loss show lower circulating levels of prestin, lead us to hypothesize that lower serum prestin levels in healthy adults may be associated with lower amplitude OAEs. However, the differences in the measurements, and different biological factors that influence each, may weaken the hypothesized positive relation. Our protocol utilized transient evoked OAEs (TEOAEs)—sounds emitted in response to a short acoustic stimulus, typically a click, tone burst, or chirp in our case.

MATERIALS AND METHODS

Participants

Thirty-four young adults (18 to 24 years old, mean = 20.26 years, 23 female), all undergraduate monolingual-English speaking students at the University of Connecticut, participated in this study. One participant left the study after two full test sessions due to scheduling issues; all others completed the longitudinal study in its entirety (n = 33). Recruitment ads were placed in the UConn Student Daily Digest, a daily email listserv informing students about campus activities, including opportunities to participate in research studies. Respondents to the ads were screened via a secured online questionnaire to rule out a history of chronic ear infections, ear surgery, hearing loss, hearing-aid amplification or use, seizures or neuropathy (e.g., multiple sclerosis), or past or current head trauma that resulted in limiting activity for more than 1 day (e.g., concussion). In the lab, all participants were confirmed to have clinically normal hearing bilaterally for the standard audiometric range (air conduction audiometric thresholds ≤20 dB HL for octave frequencies from 0.25 to 8 kHz) (Fig. 1) and to have passed an otoscopic exam, a distortion product OAE screener (Madsen Alpha OAE Hearing Screener, Otometrics, Inc.), and a middle-ear screener including tympanometry and acoustic reflexes (Tympstar Middle Ear Analyzer, Grason-Stadler, Inc). If any abnormalities were detected in the tympanometry or reflexes, testers were instructed to follow-up with bone conduction audiometry, although this was not necessary in any participants.

Additionally, the history screener asked about current participation in a music ensemble and engagement in loud occupational

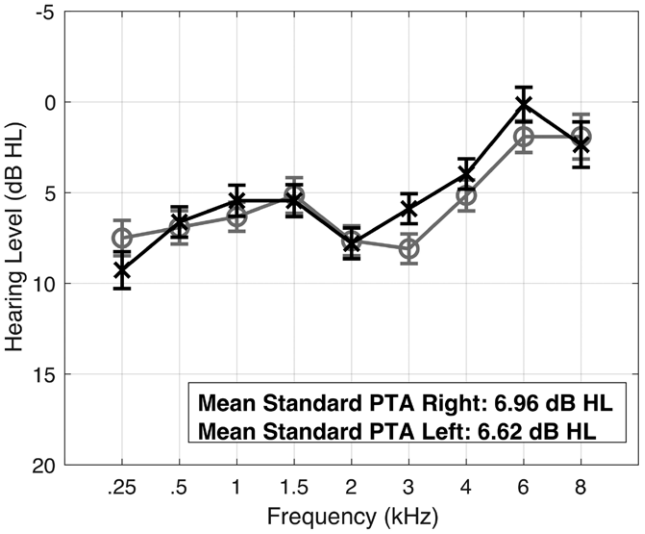

Fig. 1. Average hearing level thresholds across participants. To qualify for this study, participants were required to have thresholds <20 dB HL at all standard audiometric test frequencies (0.25–8 kHz). Error bars represent the standard error of the mean. PTA, pure-tone average across this frequency range.

or recreational activities. Data regarding prestin levels and OAEs will be discussed here, and analyses concerning musical training and noise exposure will be the subject of future analyses.

Experimental Protocol Overview

All experimental procedures were approved by the Institutional Research Board at the University of Connecticut, and participants provided their written informed consent before study enrollment. Testing occurred during the 2018 to 2019 academic year, with each participant coming to the laboratory for five test sessions (sessions 1, 2, 3, 4, and 5, not including first-day screening) spanning over three separate, nonconsecutive weeklong periods (rounds 1, 2, and 3) (Fig. 2). All testing occurred during an academic semester while classes were underway. Participants were monetarily compensated both after the completion of round 2 and round 3.

After confirming study eligibility, participants completed an 18- to 24-hour long "quiet period" before session 1. The purpose of the quiet period was to limit the possibility of the baseline hearing results being influenced by a temporary threshold shift that could have occurred from noise exposure the preceding day. The quiet period involved keeping exposure to noise to a minimum (e.g., no large social events, visiting loud bars or restaurants, music ensemble practice). We adopted this requirement from the US Department of Labor, Occupational Safety, and Health Administration. Compliance with the quiet period was confirmed by a personal noise dosimeter (ER-200DW8, Etymotic, Inc., Elk Grove Village, IL; overall noise dose <20% based on National Institute for Occupational Safety and Health criteria).

For each of the five sessions, blood samples and TEOAEs were obtained. At each session, participants also completed a battery of other tests that included pure-tone audiometry (conducted via a Grason-Stadler GSI-561 clinical audiometer for standard and extended high frequencies), speech perception in noise testing utilizing QuickSIN (Etymotic, Inc.) and a spatial release of masking task adapted from Jakien and Gallun (2018), medial olivocochlear reflexes (MOCR), and ABRs. For each round of testing, participants also engaged in one week of personal noise dosimetry, and completed questionnaires relating to noise exposure. The current study forms the framework for future analyses examining relations between serum prestin levels and these various other metrics of auditory function and noise exposure. The analysis has we present here two parts: first we examined the normative values and test-retest reliability of serum prestin in our data set, and second, we examined serum levels in relation to OAEs.

PART 1: SERUM PRESTIN NORMATIVE VALUES AND RELIABILITY

Blood Draw Procedures

Participants arrived at the laboratory in the morning each test day. To control for time of day, test sessions were limited to starting between the hours of 7:30 AM and 11:00 AM. In a small number of unavoidable circumstances, such as inclement weather or participant illness, test sessions occurred in the early afternoon, but before any significant noise events (e.g., band rehearsal) that day. Although the blood draws occurred in the morning, participants were not required to fast overnight. Blood draws always occurred before the administration of any other hearing tests. For the venipuncture, participants were escorted by a member of our research team to and from the UConn Health Medical Services location in Downtown Storrs, located 0.4 miles (approximately an 8-minute walk) from the laboratory. Venipuncture was performed by a certified phlebotomist who collected two 6.0 mL tubes of nonfasting blood samples (two red top tubes containing no anticoagulant or preservative) from the median cubital vein, a superficial vein in the upper limb. Blood samples were left in their tubes, standing upright, for approximately 30 minutes at room temperature, before being transported back to our research facilities for further processing by a member of our research team who had undergone the necessary biosafety training. In circumstances when samples could not be spun 30 minutes after collection, they were refrigerated up to 4 hours or placed on ice. Blood samples were transferred from the red top tubes

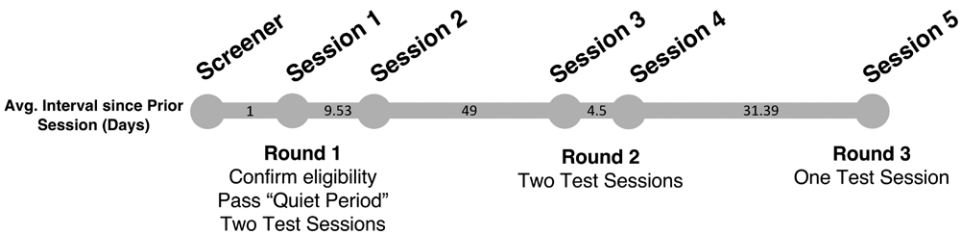

Fig. 2. Study timeline. Five sessions occurred over three rounds of testing over the span of an academic year. Numbers embedded in the timeline are the average interval since the prior session, in days.

to 1.7 mL microcentrifuge tubes by a trained research assistant before centrifuging. To separate the serum, the specimens were spun at 3,000 G for 10 minutes. After spinning, serum was collected via pipette and frozen at –80°C until time of assay. At the conclusion of the study, samples were transported over dry ice from the UConn's Storrs campus to the UConn Health campus in Farmington for final batch processing.

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. This kit was chosen for its wide detection range (10 to 3000 pg/mL)—particularly, its small low-end range—and its sensitivity to small changes (4.87 pg/mL) that are necessary for examining within-subject changes. A 1:5 dilution was prepared, and each serum sample was assayed in duplicate. The optical density in the wells of the ELISA microplate was measured at 450 nm using a Biotek ELx808 plate reader and data were compiled using the KCJunior software package (Bio-Tek Instruments, Inc., Winooski, VT). To avoid the risk for cross-plate variance, for each participant, the samples were processed in the same plate (e.g., All Participant ID no. 16's samples across all sessions were processed on Plate no.1, all Participant ID no. 26's samples across all sessions were processed on Plate no. 2), with the technician blind to participant ID and test date, as well as the fact that this was a repeated measures protocol.

Statistical Analyses

Descriptive statistics (e.g., mean, standard deviation, range, 95% confidence intervals of the mean) are reported for prestin levels at each session. Given that the prestin values (pg/mL) range over several orders of magnitude and to meet the assumption of normal residuals, the prestin data are plotted using a log scale, and subsequent statistics are performed on log-transformed values, unless otherwise noted (e.g., descriptive statistics tables and standard error of measurement). Raw data (i.e., not log transformed) are used for TEOAEs in all analyses and plots.

There is no consensus in the field of hearing science for the methods to evaluate reliability. Opinions on best approaches are varied, where not all are considered to be as equally valid at gauging reliability (McMillan 2014). Therefore, we take a multipronged approach to reporting measures of reliability:

1. Standard error of the measurement (SEM; Demorest & Walden 1984) was calculated across all five test sessions. SEM, calculated here using raw, not log-transformed, data, is used to index of the amount of test-retest variation due errors in measurement. It is expressed in the units of measurement and can be used to calculate the reference range for healthy persons. (See Reavis et al. 2015 for a meta-analysis on distortion product OAEs using SEM as an illustration of its application in hearing sciences). We calculated the SEM according to this formula:

$$\widehat{SEM} = s \cdot \sqrt{1 - ICC}$$

In this equation, s is the combined standard deviation of the five sessions and ICC is intraclass correlation coefficient across all sessions (see below). The 95% reference range for within-subject test-retest serum prestin shifts was calculated using:

$$\pm 1.96 \cdot \sqrt{2} \cdot \widehat{SEM}$$

Reference ranges are used in the literature to define the normal within-subject range (e.g., Reavis et al. 2015).
2. Pearson's correlations were conducted to examine the strength of the relation between test sessions. Pearson's correlations have been used to examine test-retest reliability in the field hearing sciences (e.g., Fournier & Hébert 2013; Ku et al. 2015).
3. Intraclass correlations (ICCs) with a two-way mixed model evaluating the absolute agreement were used to compare the repeatability of prestin levels across all five sessions. Similar to the Pearson correlation, the ICC can be used to estimate the magnitude of a relation between two test sessions, however, unlike Pearson correlations, it can also account for differences in the means across more than two sessions, such as this study, where there are five timepoints of measurement (Liu et al. 2016). Strong ICCs, between 0.75 and 0.9, and greater than 0.90, suggest "good" or "excellent" reliability, respectively (Koo & Li 2016). ICC has been used in the audiology and hearing science literature examining test-retest reliability of numerous tests auditory tests (e.g., Tremblay et al. 2003; He et al. 2013; Pronk et al. 2013; Bidelmen et al. 2018).
4. Linear-mixed effects modeling was conducted using a model that allowed us to handle missing or incomplete data and take into account the fact that the interval between sessions was not fixed. Restricted maximum likelihood estimations were conducted, and the mixed model included random intercepts to take the intersubject variability of baseline prestin levels into account. Time was treated as a continuous interval, measured in days since baseline (session 1). This method of coding time allows us to account for the variation in the test interval, both between and within subjects (see Fig. 2 for average intervals). We tested the null hypothesis that there is no relation between serum prestin level and test interval in the population. Similar mixed models have been used in existing hearing sciences literature (e.g., Bidelman et al. 2018).

Statistical analyses were run with MATLAB version 9.5 (The MathWorks, Inc., Natick, MA).

PART 2: THE RELATION BETWEEN SERUM PRESTIN LEVELS AND OTOACOUSTIC EMISSIONS

OAE Procedures

For each of the five sessions, TEOAEs were collected on the same day as the blood samples. TEOAEs were measured in the right ear using HearID software (Mimosa Acoustics). When TEOAEs could not be obtained in the right ear due to probe fit or calibration difficulty, the left ear was used. A 50 dB SPL 1 to 5 kHz bandpass chirp stimulus was presented through an ER10C probe tip insert (Etymotic, Inc.) using a preset protocol (TE50_B2000_N60) within the HearID software that controlled the stimulus delivery, recording, and analysis process. The chirp increased logarithmically in frequency over time. The stimulus and protocol are identical to that described in Marshall et al. (2014) but with minor modifications to the bandpass filtering of the response (see Lapsley Miller et al. 2004; Mimosa Acoustics 2007). The stimulus was calibrated regularly using a Brüel & Kjær 2250 class 1 sound level meter with a 2-cc coupler. As part of this protocol, MOCRs were also collected using a

TABLE 1. Prestin levels—descriptive statistics (using raw data)

		Round 1		Round 2		Round 3
		Session 1	Session 2	Session 3	Session 4	Session 5
	n	25	28	27	29	28
	Days since baseline (avg)	–	9.53	80.49	84.94	116.55
	Out of range of detection	4	4	5	4	5
	Hemolytic	5	2	1	0	0
pg/mL	Mean	289.48	252.84	248.30	227.05	338.32
	STD	403.27	371.31	320.37	297.91	258.44
	Range	1761.07	1575.37	1357.93	1270.76	1084.64
	Minimum	41.06	19.30	17.92	11.76	48.21
	Maximum	1802.13	1594.67	1375.85	1282.52	1131.85
	Confidence intervals (95%)	117.61	122.25	133.31	121.74	143.51
		513.82	499.38	450.84	428.82	404.91

contralateral noise paradigm, but for the present study, we focus only on the TEOAEs recorded without contralateral noise.

Four blocks of OAEs were collected, and from each block, the TEOAE magnitude (dB SPL), OAE noise floor level (dB SPL), and OAE signal-to-noise ratio (SNR, dB) were calculated automatically by the software. Then, for each dependent measure, the values from the four runs were later averaged offline. The SNRs (dB) were calculated by subtracting the OAE noise level from the OAE magnitude. Details on how the OAE magnitude and noise floor were calculated to appear below.

For each block, a stimulus ensemble of four chirps was presented up to 500 times in nonlinear mode. In nonlinear mode, every *xth* chirp's polarity is inverted and occurs at a greater in intensity than preceding chirp, to minimize stimulation artifact and middle ear-components for better isolation of OHC function (Kemp et al. 1986; Berlin et al. 1993). In our case, every fourth chirp was inverted and presented at +9.5 dB above the preceding three. Each chirp in the ensemble had a duration of 10.5 ms, and a new chirp was presented every 32.5 ms. Data collection stopped after reaching 500 repetitions of the stimulus ensemble if the stopping criteria were reached. Hard stopping criteria were set for a minimum of 615 accepted repetitions [signal-to-noise ratio (SNR) ≥6 dB] or a maximum of 500 rejected repetitions (SNR <6 dB), whichever occurred first. The minimum level of the OAE magnitude was set to be 0 dB SPL and the maximum level of the noise floor to be –6 dB SPL, thus yielding an SNR of at least 6 dB SPL. Responses to the individual chirps were averaged over a 14-ms time window, which began 2 ms after the end of the chirp to limit the effects of stimulus ringing and which had a 2.5 ms onset and offset amplitude ramp. The recordings were bandpass filtered from 1000 and 5000 Hz with a 3-dB roll-off, creating an effective bandwidth of the response of 721 to 5075 Hz. The TEOAE magnitude was analyzed in the frequency spectrum by summing the power in all bins over the effective bandwidth. The noise floor was calculated by taking the difference between TEOAEs magnitudes from successive stimulus presentations.

Statistical Analyses

Pearson's correlations were conducted to measure the relation between prestin and OAEs.

RESULTS

Part 1: Serum Prestin Normative Values and Reliability

An initial independent samples t-test compared males and females with respect to prestin levels. No difference was found ($t[148] = -0.08$, $p = 0.964$), and so the variable was dropped from subsequent analyses and data from males and females were pooled. Across the five sessions, the range of detectable serum prestin levels spanned from 11.76 to 1802.13 pg/mL (n = 137; 250.20 ± 28.30, mean ± SE mean) (Table 1). Figure 3 shows the distribution of prestin levels across sessions, and Figure 4 shows how each participant patterns from session to session. Confidence intervals of the mean (95%) generally ranged from the low-mid 100s (pg/mL) at the lower bound to the 400s to 500s (pg/mL) at the upper bound, with some variation from session to session (Table 1). ("Global" confidence intervals, calculated across five sessions, ranged from 194.77 pg/mL at the lower bound to 305.64 pg/mL at the upper bound.) Moreover, we did not find that serum prestin levels showed significant correlations with low, standard, high, or extended high frequency pure-tone averages (Huh et al. 2018) (Table 2).

Out of a possible 167 serum samples (33 participants at five sessions each, plus one participant who dropped out of the study after only two sessions), prestin levels could not be measured from 30 samples. Hemolysis prevented accurate prestin measurements in eight of these samples. These hemolytic samples were discarded before ELISA processing, as pilot data showed hemolytic samples to provide inaccurate (erroneously high) prestin level measurements. In the other 22 cases where prestin could not be measured, this was because values were out of the range of detection for ELISA kit MBS167508. With a detection range of 10 to 3000 pg/mL, the ELISA kit cannot provide precise measurements of prestin levels that fall either below (n = 12 samples, six individual participants) or above (n = 10, two individual participants) that range. On the low end, six participants had nondetectable levels, but no single participant fell below the detection range across all five sessions. Conversely, on the high end, the 10 samples came from only two participants whose levels exceeded the kit range across all five sessions. Outside these two participants, the next highest serum prestin level is 1802.13 pg/mL, with the large majority of samples measuring below 1000 pg/mL. The range of prestin observed here is generally in line with the values from the control group in another study (Sun et al. 2019).

We computed a "global" standard error of the measurement (SEM) for serum prestin levels across all five test sessions. Global SEM was 7.28 pg/mL. This calculation used listwise deletion for those with missing data. Additionally, we calculated corresponding 95% reference range resulting in ±20.18 pg/mL.

We conducted Pearson's correlations to examine the relation between all combinations of sessions. Pearson's correlation

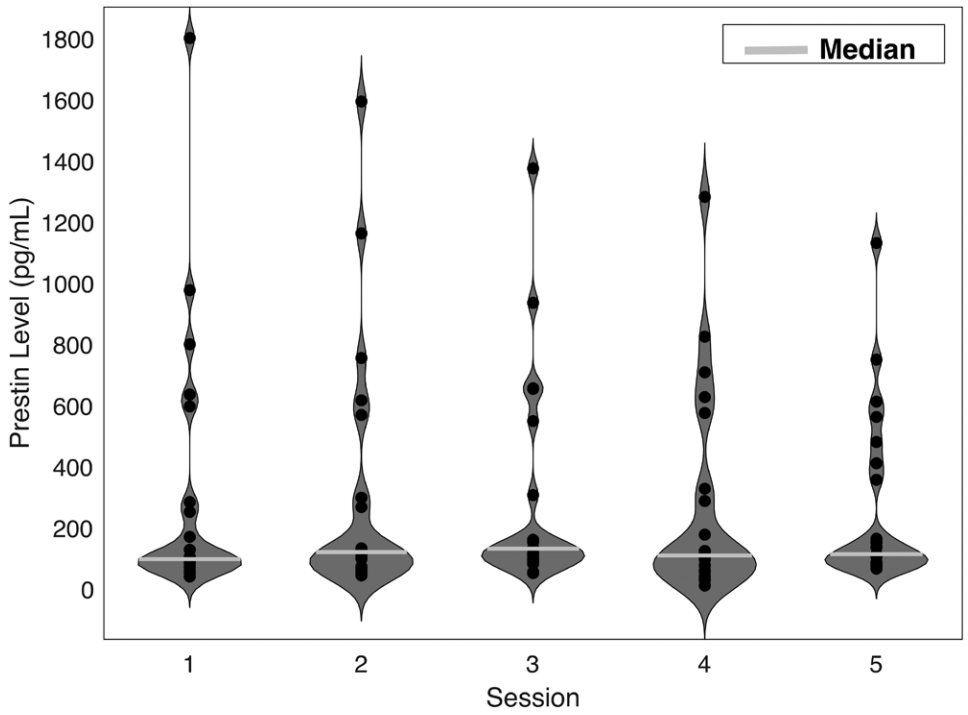

Fig. 3. Distribution of prestin levels by session. This plot uses raw (nonlogged) data to illustrate the range of values. The white horizontal bar represents the median prestin level of each session.

coefficients showed "good" positive relations ($r > 0.8, p < 0.0001$) across all pairwise comparisons (Fig. 5), and there were no discernable patterns of stronger correlations for more proximal time points (e.g., session 1 versus session 2) in comparison to more distal sessions (e.g., session 1 versus session 5). To further compare the reliability and agreement of prestin levels between time intervals, we measured "global" ICC across all five test sessions. Global ICC showed "excellent" reliability (ICC = 0.98). This ICC value was used in our SEM calculation.

We also tested a linear-mixed effects model because it has the capacity to handle the unbalanced nature of the dataset (i.e., missing data due to being out of the range of ELISA kit detection or data that were unanalyzable due to being hemolytic). Our model supported the null hypothesis by showing no appreciable difference between time intervals on prestin levels [$F(1,121.46) = 0.31, p = 0.582$].

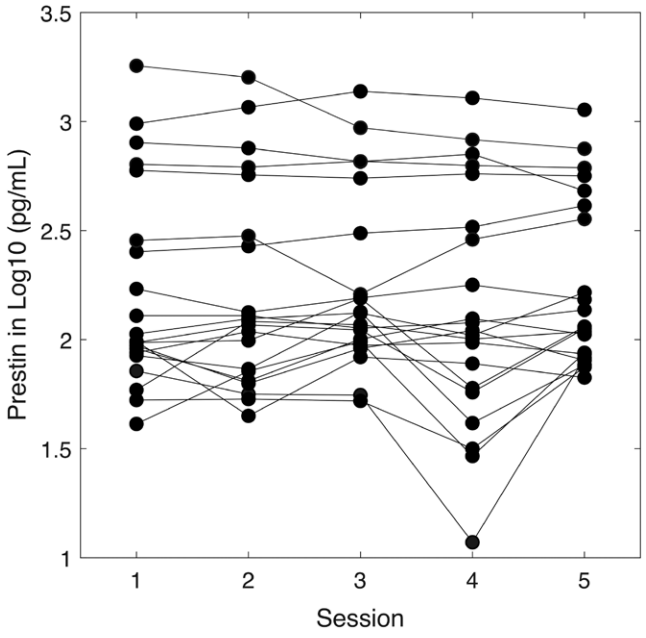

Fig. 4. Line plot of prestin levels across sessions by participant. Each line represents one participant. Only participants with detectable levels at all five sessions are plotted (n = 27). This plot uses a base-10 log scale.

Part 2: The Relation Between Serum Prestin Levels and Otoacoustic Emissions

TEOAE magnitudes, across participants and sessions, ranged from 2.38 to 18.92 dB SPL (n = 153; 10.03 ± 0.25) (Table 3). Out of a total possible 167 TEOAE data points, 14 are missing from the analysis due to equipment error. To examine the relation between TEOAEs and serological prestin levels, Pearson's correlations between prestin levels and TEOAE magnitudes were conducted for each session (Table 4). As hypothesized, the correlations between TEOAE magnitudes and prestin are all positive (higher prestin levels correlating with stronger OAEs and vice versa) but generally weak to moderate. When both OAE magnitudes and serum prestin levels are averaged for each participant across the five sessions and then correlated, the relation is overall stronger ($r = 0.47, p = 0.050$) than for any intrasession pairwise comparison.

Within the field of audiology, OAE screening protocols commonly use OAE SNR instead of magnitudes, motivating the next set of analyses. TEOAE SNRs, across participants and sessions, ranged from 6.30 to 20.75 dB (n = 153; 11.1 ± 0.24) (Table 3). Similar to TEOAE magnitudes, correlations with prestin are all positive, and the r values fall into the weak to moderate range (Table 4). Likewise, when OAE SNRs and serum prestin levels

TABLE 2. Bilateral pure-tone averages—descriptive statistics and correlations with average serum prestin levels

kHz		Low frequency (0.5, 1, 2)	Standard frequency (0.5, 1, 2, 4)	High frequency (3, 4, 6)	Extended high frequency (10, 12.5, 14, 16)
dB HL	**Mean**	6.79	6.23	4.19	1.45
	STD	3.41	3.14	3.22	7.80
	Range	12.50	12.50	15.00	41.25
	Minimum	0.85	–1.25	–3.33	–13.75
	Maximum	13.35	11.25	11.67	27.50
	r	0.05	0.05	0.19	0.21

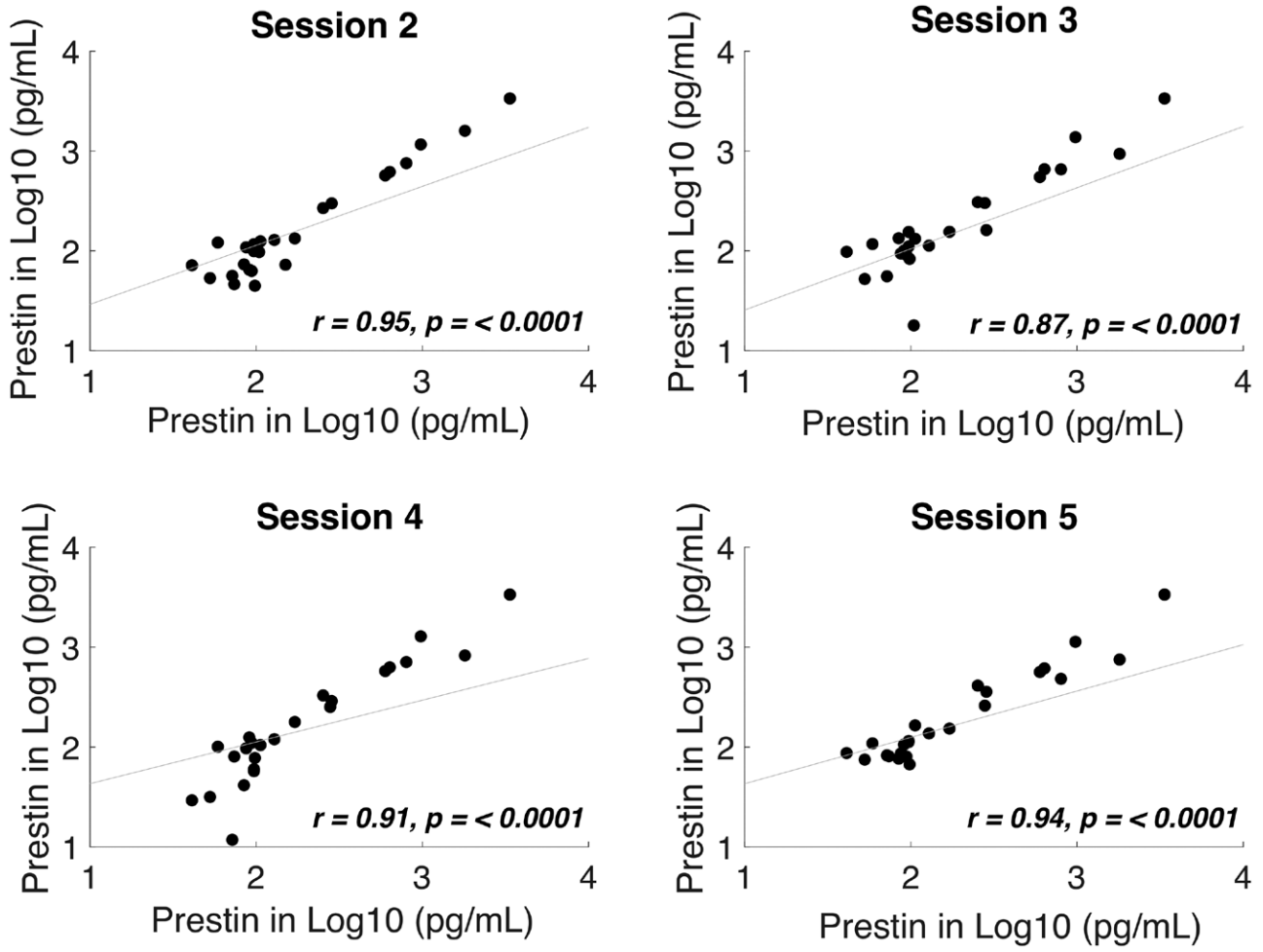

Fig. 5. Scatter plots of the relation between prestin levels (pg/mL) at session 1 and each of the other sessions. Pearson's correlations show a strong relation between prestin levels at all sessions ($r > 0.8$; $p < 0.0001$). These plots, and accompanying statistics, use a base-10 log scale. Session 1 is plotted on the x axis of each plot.

TABLE 3. OAE magnitude (dB SPL) and SNR (in dB) descriptive statistics

	Session 1		Session 2		Session 3		Session 4		Session 5	
n	31		28		30		33		31	
	OAE	SNR	OAE	SNR	OAE	SNR	OAE	SNR	OAE	SNR
Mean	10.47	11.51	10.85	11.68	9.85	10.86	9.48	10.8	9.58	10.73
SD	3.05	2.1	2.75	2.88	3.1	2.1	3.15	3	3.45	2.9
Range	13.77	14.2	11.61	11.9	13.36	11.34	14.42	11.33	15.25	11.51
Min.	5.15	6.55	6.21	6.61	4.21	6.39	2.94	6.3	2.38	6.32
Max.	18.92	20.75	17.81	18.51	17.57	17.73	17.36	17.64	17.63	17.83

OAE, otoacoustic emission; SNR, signal-to-noise ratio.

TABLE 4. Pearson's correlations between OAEs and prestin levels

	Session 1	Session 2	Session 3	Session 4	Session 5	Average
			Prestin			
OAE magnitude	0.12	0.43	0.19	0.15	0.31	0.47*
OAE SNR	0.16	0.43	0.25	0.20	0.37	0.50*

**Significant at the p = 0.05 level.*
OAE, otoacoustic emission; SNR, signal-to-noise ratio.

are averaged for each participant across the five sessions and then correlated, the relation is overall stronger (r = 0.50, $p = 0.044$).

DISCUSSION

The present study provides reference ranges and test-retest reliability statistics of circulating prestin levels measured serologically in healthy young adults with normal audiometric thresholds and present OAEs. The average prestin level across all five session was 250.20 pg/mL and 95% confidence intervals of the mean spanned from 194.77 to 305.64 pg/ mL. Pearson's correlations and ICCs show good to excellent relations, respectively, between sessions. Further, our linear-mixed effects model, which was able to handle missing data points, helped to confirm that levels are stable at retest by showing no effect of time interval. Across five time points, the SEM was calculated to be 7.28 pg/mL and the 95% within-subject reference range was found to be ±20.18 pg/mL. As well as providing reference values to guide future work, our findings suggest that serum prestin levels, although they vary between participants, are quite repeatable at the individual level when measurements occur within a span of six months. This study adds to the growing body of work that serves as proof-of-concept that this inner-ear protein can be measured safely and reliably in humans from blood serum.

In addition to reporting reliability statistics for prestin, we compared prestin levels to TEOAEs, another measure of OHC integrity. We hypothesized that TEOAEs and circulating prestin levels would show a positive relation given a common underlying connection to OHC integrity, but that the relations might be weak given that the two metrics differ vastly in their execution and measurement and are likely influenced by a different set of other physiological factors. OAEs are acoustic signals emitted by the cochlea that are detected from a microphone in the ear canal. OAEs depend on the OHC electromotility and other aspects of the cochlear amplifier but also the middle-ear status and acoustical conditions in the ear canal, while serological prestin levels are measuring quantities of the protein released into the circulating blood stream resulting from OHCs homeostatic regulation or damage. Overall, our results support the predicted relationship. When taking the average measurement across all five sessions, there is a statistically significant relationship between TEOAEs and serum prestin levels in the moderate range. Correlations performed for each individual session are comparatively weaker, potentially due to variable sample sizes and increased random error in this small dataset.

The consistently positive association between TEOAEs and circulating prestin, where decreased TEOAEs pair with decreased prestin levels, may be explained by a common connection of the two metrics to OHC count. While direct methods of OHC counts utilized in animal model studies cannot be conducted in humans (beyond histopathologic microscopy methods), hearing loss that presents itself as reduced OAEs is often interpreted as being a consequence of OHC loss, and OAEs have long shown to reliably separate individuals with normal hearing from those with hearing loss across the lifespan (e.g., Probst et al. 1987; Harris 1990; Hussain et al. 1998; Harrison & Norton 1999; Norton et al. 2000). OHC loss is theorized to lead both to reduced OAEs and reduced production of prestin, which, in the long-term, is predicted to lead to decreased levels of prestin circulating in the blood stream, and therefore decreased levels of the protein detectable serologically. Supporting this theorized relation, lower serum prestin levels and lower OAEs were observed in animals following noise-induced damage to OHCs (Parham & Dyhrfjeld-Johnsen 2016). However, it should be remarked that in the current study, the relationship between OAEs and serum prestin levels was found in a sample of healthy young adults whose hearing thresholds and OAEs were not indicative of clinical definitions of noise-induced hearing loss. These findings warrant replication and further investigation in a larger and more diverse sample of healthy ears.

While our results suggest that serum prestin and OAEs, two putative metrics of OHC integrity, pattern together, the weak to moderate relationship is noteworthy. We speculate that, in addition to varying in their measurement and having different physiologic influences, that they could have differential sensitivity to OHC count. Circulating prestin is hypothesized to be sensitive to as little 1% OHC loss (Parham 2015), whereas OAEs may not be as sensitive to very small levels of OHC loss (a loss of about 10% of OHCs can produce a 2.5–4 dB decrease in OAE amplitudes (Hofstetter et al. 1997)). Furthermore, it has been argued that there may not be a clear-cut relationship between OAE level and OHC loss (Linss et al. 2005). Finally, compensatory changes have been observed for both OAEs (Wake et al. 1996) and prestin (Xia et al. 2013), which may further obscure their relationship. Further studies, including those with different etiologies of hearing loss, younger and older individuals, and frequency-specific OAEs, are necessary to draw stronger conclusions about the relation to levels of OHC function, to establish age-dependent reference values that distinguish normal from abnormal serological prestin levels, and to understand other factors that might influence prestin levels but not OAEs.

While the focus of this analysis was within-subject stability, our results do show a range of prestin levels in our participants, even in this population of young adults with normal-hearing thresholds. However, our participants primarily "stay in their lane"—that is, those who have high levels remain high throughout all five measurements points and those with low levels remain low. The participants on the low end are also the ones more likely to drop out of the range of detection of the ELISA kit at one or more points. The source(s) of the interindividual variation in prestin (and OAEs) in healthy young adults are not fully understood and warrant investigation, but we offer up the possibility that the gradient of prestin levels observed here may reflect normal variation in

the strength of the cochlear amplifier or subclinical levels of OHC loss in young adults with lower prestin levels. Between-subject differences will be a target for our future investigation to understand why some healthy adults have more or less prestin circulating in their blood.

Other limitations of our protocol should be noted. In addition to the limited sample size, missing data due to hemolysis or kit sensitivity reduced the number of participants for which a full set (all five sessions) of data was available for analysis. Measurements outside detection limits are called "censored" and can be modeled using "survival" or "accelerated failure time" models (Bernhardt et al. 2014). In the current work, our approach to handling censoring data (and hemolytic samples) was to discard these data points from our analysis. Our future work will involve advanced techniques in statistical modeling to best handle all of our missing data without bias. However, failure to measure levels is not an indication of bad data nor poor technique. We expect that some young adults might not have measurable levels, and failure to measure levels may be related to range of the ELISA kit. Additionally, because venipuncture was performed by a trained phlebotomist at a local clinic, and not by a member of our research team, our testing schedule was limited to hours that the clinic was open. This prevented testing from occurring at the earliest hours of the morning and on weekends, which may have influenced the demographic composition of the study sample.

Finally, it should be noted that prestin is not the only protein gaining traction as a potential biomarker of inner-ear function and health. Mulry and Parham (2020) cataloged several other proteins that may be candidate biomarkers of inner-ear health, for example, otoancorin (e.g., Zwaenepoel et al. 2002; Lukashkin et al. 2012), otogelin (e.g., Simmler et al. 2000; Schraders et al. 2012), cochlin (e.g., Ikezono et al. 2009; Calzada et al. 2012), and otolin-1 (e.g., Parham et al. 2014; Sacks and Parham 2015; Doğan et al. 2019). Our group has studied otolin-1 serologically in humans with benign paroxysmal positional vertigo (Parham et al. 2014; Sacks & Parham 2015). Otolin-1 is a protein that, like prestin, is specific to the inner-ear but is restricted to the support cells of the vestibular maculae, semicircular canal cristae, organ of Corti, and marginal cells of the stria vascularis (Deans et al. 2010). A serological measurement of otolin-1 could be a valuable tool in the diagnosis of benign paroxysmal positional vertigo, particularly in challenging cases such as subjective benign paroxysmal positional vertigo, multicanal or bilateral disease, or when diagnostic positional maneuvers prove difficult (Tabtabai et al. 2017). Thus, we envision a serological hearing screening protocol that involves multiple biomarkers.

Blood-based measures are not currently found in the clinical audiology or other health care practice specific to the inner-ear. However, if future studies reveal that they have greater sensitivity than methods of hearing assessment, or if they are found to be equally or less sensitive but can reach a wider population than current measures by being included in routine blood panels, serological measures could potentially have broad reaching implications. Serological measures could dramatically improve hearing loss detection and our understanding of inner ear pathophysiology, shape audiological and primary care practice and counseling services, and boost the quality of life and financial situations for individuals who seek preventative measures. Additionally, such a biomarker could prove valuable to ototoxic monitoring efforts and aid in the development of therapeutics that serve to protect OHCs and target regeneration of hair cells after injury by providing a means to track changes to the inner-ear. It must be emphasized though that we do not propose prestin as a substitute for audiometric evaluations such as OAEs. Rather, we propose prestin as a supplementary tool at the disposal of the clinician, which would then be followed by frequency-specific hearing assessments performed by an audiologist.

CONCLUSIONS

The OHC-specific protein prestin shows potential as a biomarker of inner-ear function through its reliability and relation to an existing metric of OHC function. Prestin levels can be measured in human serum, as obtained by a blood draw using phlebotomy techniques that are commonplace in other areas of medicine. Our results suggest that circulating levels show high test-retest reliability in normal-hearing young adults, as measured over five test sessions. Moreover, we show a positive weak-moderate relation between prestin levels and OAEs, the current clinical test most specific to the OHCs. While continued evaluation of serological prestin is warranted before clinical translation, a biomarker like prestin could hold clinical potential if incorporated into routine blood testing. This study contributes to the growing body of literature on serological prestin; collectively this literature shows promise that the era of such markers may be on the horizon.

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