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Original Article

Circulating Immune Complexes and Heat Shock Protein 70 in the Sera of Patients with Sudden Sensorineural Hearing Loss

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OBJECTIVES: The aim of this paper is to analyze and assess the usefulness of heat shock protein 70 (Hsp70) and circulating immune complexes (CIC) in patients with sudden sensorineural hearing loss (SSHL) in a tertiary care hospital in a research university (2014-2017).

MATERIALS and METHODS: Patients were interviewed about the history of diseases; underwent an ear, nose, and throat (ENT) examination; a hearing test; and were analyzed for the presence of CIC and Hsp70 protein. A simple dot blot method was designed for the purpose of identification of Hsp70 bound to CIC and free Hsp70.

RESULTS: In 59.4% of patients with idiopathic SSHL, elevated levels of immune complexes were observed. Compared with healthy subjects, a significant difference was noted (p=0.00016). Positive reactions to the presence of free Hsp70 protein were noted in the sera of 48.4% of patients. In the control group, free Hsp70 was observed in 8% of patients (p=0.0000034). Hsp70 bound to CIC was detected in the sera of 62.5% of patients; and in healthy cases, in 12% (p<0.0001).

CONCLUSION: In the sera of persons with SSHL, there are elevated levels of CIC and Hsp70. After the application of the innovative method for determining the occurrence of Hsp70 bound to CIC, it was stated that its presence is greater than that of free Hsp70, commonly detected by means of the Western-blot method. The dot blot method applied in the present study increases the Hsp70 identification and by the same token increases the probability of detection of autoimmunological background of SSHL.

KEYWORDS: Sudden sensorineural hearing loss, heat shock protein 70, circulating immune complexes

INTRODUCTION

Sudden sensorineural hearing loss (SSHL) is frequently classified as "idiopathic" since the causative factor responsible for its onset is not identified in most cases. SSHL is defined as a loss of at least 30 dB in three contiguous frequencies over a period of 3 days or less [1]. The incidence of SSHL is five to 20 per 100,000 [2-4]. The true incidence of SSHL may be higher than these estimates because affected individuals who recover quickly do not present for medical care [5, 6]. Although individuals of all ages can be affected, the peak incidence is between the fifth and sixth decade of life. SSHL has an equal incidence in men and women. The incidence rate per 100,000 people in the Taiwanese population is 8.85 for men and 7.79 for women [1, 3, 5, 7]. Nearly all cases are unilateral; less than 2% of patients have bilateral involvement, and typically bilateral involvement is sequential [5]. The SSHL etiology and pathogenesis are unknown, but the proposed primary causes include viral infection, vascular disease, and autoimmunity [1]. SSHL is a complex multifactorial disease that includes genetic factors, such as prothrombin G20210A, factor V Leiden G1691A, and methylene-tetrahydrofolate reductase C677T [8]. In the majority of cases, the etiology of SSHL is unknown. It suggests that it may originate from autoimmune reaction in the inner ear [9-16]. The blood sera of people with hearing impairments indicate the presence of many specific antibodies to auto-antigens of the inner

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ear. It can be assumed that the antigen with a molecular weight of 68 KD, which was identified as the heat shock protein 70 (Hsp70), has the greatest diagnostic value [17-21]. According to the references, this marker was determined in approximately 59% of patients with autoimmune impairment of the inner ear [19]. In the available literature about the free Hsp70 occurrence in the serum, it was determined by qualitative and quantitative methods (enzyme-linked immunosorbent assay). Numerous publications indicated that the level of Hsp70 correlated with the occurrence of SSHL [22-24]. These identifications of Hsp70 were made in these studies by means of the Western-blot method. This method required the implementation of quite a time-consuming electrophoretic procedure, combined at a later stage with the transfer of the separated proteins onto nitrocellulose and immunochemical identification using specific monoclonal antibodies.

There is a need to develop a simple and rapid method for determining the presence of this marker. Hsp70 is an intracellular cryptic protein. It is emphasized that Hsp70 in inflammation may appear on the cell surface, it may dissociate into the intercellular space, and, as an antigen, it may stimulate the immunological mechanisms [25].

In the chronic form of the inner ear inflammation, continuous stimulation of the immune system with this antigen may lead to specific mobilization of the humoral mechanisms, in response to cell necrosis.

This leads to creation of immunological complexes containing Hsp70 proteins. In such a situation, a group of patients in which Hsp70 can only occur in a bound form CIC-Hsp70, undetectable by the classic Western-blot method, is likely to exist.

An innovative character of the dot blot, which was used in the present study, is in the fact that in the SSHL diagnostics, in addition to free Hsp70, the Hsp70 bound to circulating immune complexes (CIC) is also searched for.

In the literature on the subject, so far, this simple and inexpensive method of Hsp70 indication has not been used, that is, the composition of CIC has not been analyzed for the presence of Hsp70. Taking into account still unexplored etiology of SSHL, detection of a factor indicating an autoimmune process (Hsp70 is a remedial protein) would allow for a better choice of the treatment method.

MATERIALS and METHODS

The study involved a total of 64 patients aged 22 to 67 years (mean age 53.1±12 years) treated for SSHL. The control group included 50 people who were considered to be healthy, aged 24 to 65 years (mean age 48.1±13 years). The age and sex characteristics were not significantly different between the two groups. Information on demographic characteristics was collected. Informed consent was obtained from all participants, and the study followed the guidelines of the Declaration of Helsinki.

All the patients received no treatment for hearing loss or impairment before taking the hearing test. The exclusion criteria were the hearing loss caused by acoustic neuroma, central lesions, Meniere's disease, multiple sclerosis, drug-induced and noise-induced hearing losses, or hearing losses due to prior ear surgery. Audiometric tests, including pure tone audiometry, tympanometry, stapedius reflexes, auditory brainstem-evoked responses, and computer tomography or magnetic resonance imaging (to exclude acoustic neuroma) were performed in the SSHL patients. They were in the acute phase of the disease and fulfilled the following criteria for inclusion: (1) stable, unilateral SSHL of at least 30 dB for three subsequent one-octave steps in frequency occurring in less than 72 hours; (2) an interval shorter than 7 days since the onset of hearing loss; (3) the absence of retrocochlear disease as confirmed by temporal bone computed tomography or magnetic resonance imaging; (4) average hearing levels in the unaffected ear at less than 30 dB; and (5) the absence of diabetes and hypertension. There were diagnostic criteria of SSHL that corresponded to the literature [1, 26]. The SSHL etiology was in each case considered to be idiopathic.

The severity of hearing loss was evaluated with the average hearing level at five frequencies (250, 500, 1000, 2000, and 4000 Hz) on the pure tone audiogram performed for the first time after the sudden hearing loss was diagnosed. The improvement in hearing was assessed 3 months after the start of treatment according to the criteria proposed by Nomura [26]. Each patient was interviewed about the history of diseases, underwent an ENT assessment, a hearing test with pure tone audiometry, speech audiometry, tympanometry, supra-threshold audiometry (SISI, Peyser and Lüscher-Zwisłocki), tympanometry, otoacoustic emissions (products of distortion-DPOAE), assessment of short-latency auditory-evoked potentials (BERA), and analysis for the presence of CIC and Hsp70 protein in the serum. Venous blood samples were collected from each patient, centrifuged to obtain serum samples, and stored at -70°C until assayed. The serum samples were analyzed not later than 72 hours after obtaining them (mean, 42.2 hours). For further comparative analysis, the results from all patients were taken into account.

Healthy volunteers without a history of hearing loss or any ear disorders were enrolled as controls.

Hearing examination

Tone audiometry with suprathreshold tests was carried out on the Clinical Audiometer AC 40 (Interacoustics); hearing by air was evaluated at frequencies from 125Hz to 8000Hz, and via bone from 125Hz to 4000Hz. Speech audiometry was performed on the same apparatus, using Zakrzewski's monosyllabic test. Tympanometry, with the assessment of the susceptibility of the eardrum and stapedius reflexes, was performed using the AA 222 camera (Interacoustics). Evoked otoacoustic emissions were studied at frequencies ranging from 125Hz to 8000Hz as products of distortion on the Capella (Madsen) apparatus. Auditory Evoked Potentials of short latency were recorded with the Centor-C apparatus (Racia-Alvar), using as the stimulation intensity a crack from 100 to 0 dB SHL. On the basis of this battery of audiological tests, the degree of hearing loss and the sensorineural hearing loss were confirmed [27].

Assay of CIC by the PEG test

Each serum sample (2 mL) was diluted in 2 ml of 7% PEG-6000 in borate buffer (0.1 M, pH 8.4). The samples were incubated at 4°C for 24 h and centrifuged at 15,000 g for 30 min at 4°C. The precipitate was washed with 3.5% PEG-6000 in borate buffer, suspended in 2 mL of 0.1 M NaOH, and incubated at 25°C for 30 min. The optical density was estimated at 280 nm on a spectrophotometer (0.1 optical density

unit was read as 0.07 g/L of CIC protein). The results were considered positive when the optical density (OD) value was greater than 0.130, based on the value of 0.112±0.018 OD of healthy men reported in our earlier publications [28].

Isolation of CIC

A serum sample (0.5 mL) from each patient was mixed with 0.5 ml borate buffer (0.1 M, pH 8.4) and 1 mL of 7% PEG in borate buffer and incubated for 24 h at 4°C. The precipitate was resuspended in 0.5 mL of solution for dissociation.

Dissociation of CIC

The identification of Hsp70 antigens was preceded by the dissociation of immune complexes. To expose the antigenic determinants, 2-mercaptoethanol was used to cut the sulfide bridges in the hinge regions of the immunoglobulins. The CIC samples were diluted in a dissociation buffer and applied to nitrocellulose filters [29].

Detection of Hsp70 with dot blot immunochemical analysis

The Hsp70 antigens were identified by a dot blot analysis on nitrocellulose (NC) filters. Mouse monoclonal antibody to mAbHsp70 (no. Sc-59571 Santa Cruz Biotechnology, Inc. USA) was used as the primary antibody. Alkaline phosphatase-conjugated polyclonal rabbit anti-mouse immunoglobulin (Dako, catalog number D0314) was used as the secondary antibody. A BCIP/NBT Alkaline Phosphatase Substrate Kit IV was used to visualize the results (Vector Laboratories, catalog number SK-5400). Appearance of color in the place of plotting of the probe under examination constituted a positive result for the Hsp70 protein presence. Figure 1 presents a sample result of determination of the free Hsp and immune-complex-bound Hsp using dot blot.

The research protocol was accepted by the Ethics Committee of Nicolaus Copernicus University Collegium Medicum in Bydgoszcz no KB 498/2014 (characteristics and etiology of progressive and sudden hearing loss), and a written informed consent was obtained from all participants.

Statistical Analysis

Data sorting and initial calculations were performed, and figures were drawn using Office 2010 (Microsoft Corp., Seattle, WA). These were assessed using the STATISTICA data analysis software system

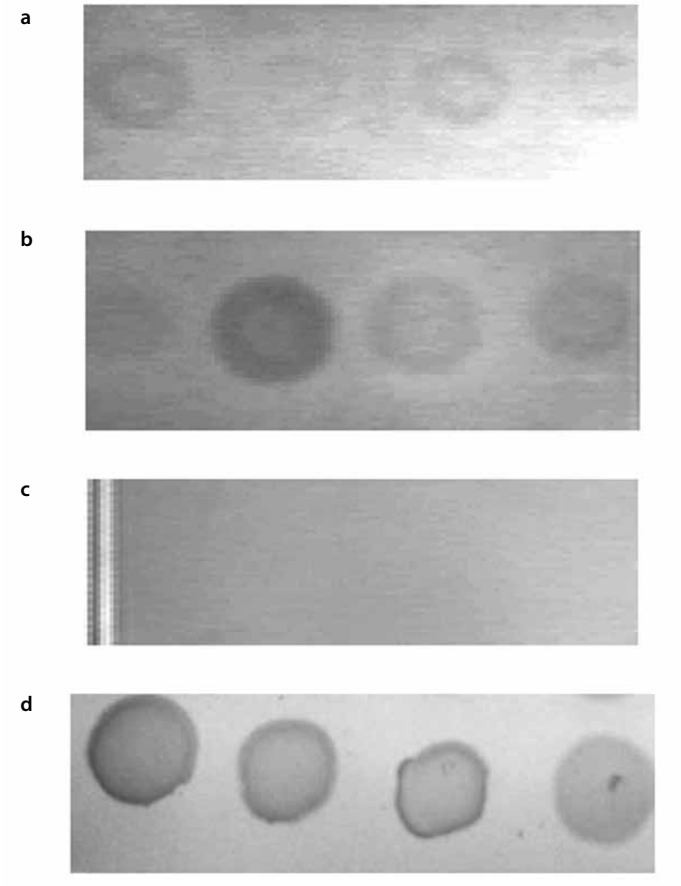

Figure 1. a-d. Dot blot immunochemical analysis for the presence of bound Hsp70 protein
in the circulating immune complex and free Hsp70 protein in the sera of patients with SSHL. a) Exemplary positive response to the presence of free Hsp70 protein in sera, b) Exemplary positive response to the presence of Hsp70 protein in circulating immune complexes, c) Exemplary negative control for the presence of Hsp70 protein, d) Exemplary positive control for the presence of Hsp70 protein.

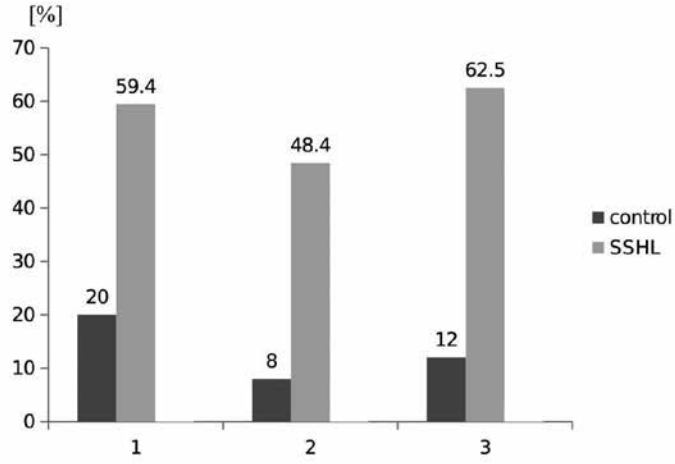

Figure 2. The presence of CIC and Hsp70 in the control and the SSHL group.
1) Elevated circulating immune complexes values in test sera
2) Presence of free Hsp70 protein in test sera
3) Presence of CIC-bound Hsp70 protein in test sera

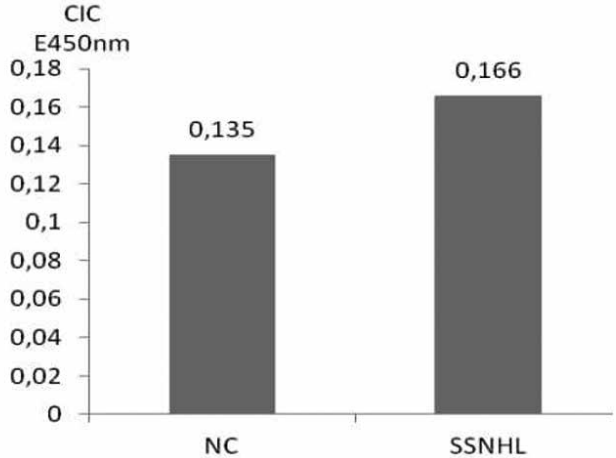

Figure 3. The mean values of serum circulating immune complexes in the patients with sudden sensorineural hearing loss and normal control.

(v. 10, StatSoft, Inc.; www.statsoft.com). The results are presented as the arithmetic means (x) and standard deviations (±SD). The $\chi 2$ non-parametric test for random distributions was used to search for significant differences between tested groups. Results at the $p<0.05$ level were considered to be statistically significant.

RESULTS

The SSHL was observed in all (n=64) patients. Twenty-six patients (41%) had a profound hearing loss, 20 patients (31%) had moderate hearing loss, and 18 patients (28%) had minor hearing loss. The right ear was affected in 27 patients (44.3%), and the left ear in 34 (55.7%). Thirty patients (47%) had a history of additional dizziness episodes. Tinnitus was observed in 49 patients (76%).

In the patients with SSHL, both elevated CIC values and positive reactions to the presence of Hsp70 were observed. Figure 2 summarizes the percentage of elevated CIC values, and positive responses to the presence of the Hsp70 protein in the form of free protein and the serum bound protein.

In 38 patients (59.4%) out of 64 with SSHL, elevated levels of immune complexes were observed. In the control group, elevated levels of immune complexes were noted only in 10 subjects (20%). The differences were significant (p=0.00016).

Using the dot blot method to determine the prevalence of free Hsp and proteins bound in immune complex, the following results were stated. Positive reactions to the presence of free Hsp70 protein were observed in the sera of 31 patients (48.4%) with SSHL. In comparison to healthy cases, free Hsp70 was observed in 4 persons (8.0%). The differences between groups were significant (p=0.0000034). Hsp70 in the form of a serum-bound protein was noted in 40 patients (62.5%). In the control group, this bound protein was present in only 6 cases (12.0%). The differences were the most significant ($p<0.0001$) taking into consideration results of the immune complexes, free Hsp70 and Hsp70 in the form of a serum-bound protein.

The average serum CIC protein level was significantly higher in patients than in the control group: E450nm=0.135±0.015 in the control group and E450nm=0.166±0.041 in the SSHL group. The median values were E450nm=0.14 and E450nm=0.17 in the control group and in patients with SSHL, respectively. Figure 3 summarizes the results of studies related to the CIC levels in the test groups.

DISCUSSION

An analysis of the immune system disorders in diseases of the inner ear is complicated. The structure of the inner ear tissue is delicate and thus difficult to access during a physical examination. Immune responses within the inner ear structures are often a consequence of systemic mechanisms [9, 12]. An example may be changes in the inner ear found in Cogan's disease or systemic lupus erythematosus [16, 30]. The immune system of the inner ear is connected to the systemic immune system, the so-called mucosa-associated lymphatic system of the mucosa (MALT), in two ways: through the spiral modiolar vein and through the round window of the middle ear. The dominant way is the venous one. It seems that the inner ear is separated from the systemic circulation with the blood-brain barrier-perilymph. Experimental studies have shown that the inner ear is the place of immune processes anatomically connected with the endolymphatic duct and the endolymphatic sac. In the tissues and lymphatic vessels, there are cells and proteins responsible for non-specific and specific reactions of the immune system. The local action of the immune system of the inner ear is associated with systemic action [10, 12]. This is possible through the process of continuous circulation of immunologically competent lymphocytes between the endolymphatic sac and the MALT. Immune reactions in the environment of the inner ear play an important protective role. An excessive immune response may, for example, damage the structure of receptor organs in the cochlea (hearing loss), adversely affecting the vestibular tissue structures, which can lead to vestibular disorders. Circulatory disorders in the cochlea and related tissue hypoxia are considered to be one of the mechanisms that cause SSHL. Hypothetically, this may be related to the activity of the local and/or systemic immune response. Sources of such behavior are different, from the reaction associated with the operation of exogenous pathogens (viruses) to the reaction modulated by the phenomenon of autoimmunity. The etiology and pathogenesis of idiopathic SSHL are controversial. Serum markers, such as Hsp originating from the cochlea and correlating to the clinical characteristics and prognosis of SSHL, have not been well studied. The objective of this study was to determine the presence and relation of Hsp70 and SSHL. The clinical significance of serum Hsp70 in patients with SSHL were studied.

The autoimmune process that causes damage to the structures of the hearing organ and balance organ may initially involve the inner ear, as in the autoimmune inner ear disease.

Hsps, which are believed to protect cells by dissolving and refolding miscoded or denatured proteins, are induced by various forms of stress, including heat, ischemia, free radicals, and toxic agents [31]. Cells produce high levels of Hsps to protect themselves against these unfavorable conditions. A previous study showed that Hsp70 may be released from damaged cochlear cells into the blood during cochlear insult [32]. The paper attempted to identify the antigenic determinants of the Hsp70 protein in CIC through a simple method of dot blot in the sera of people with SSHL. An innovative analysis of the antigen composition of CIC for the presence of proteins connected with the pathogenesis of a given disease may be the basis for designing simple procedures of identification of a given protein, for example Hsp70, by means of the immunochromatographic strip test method. Such a test may serve as a fast confirmation of presence or absence of the antigen searched for.

Several studies discussed the relationship between Hsp70 and SSHL. Some studies revealed a positive association between Hsp70 and clinical outcomes [26, 33], but other studies did not [27]. Papers have been published on evaluating the usefulness of the Hsp70 protein and Hsp70 antibodies in diseases of the inner ear. Yoo et al. [18] studied a group of patients with SSHL and observed the incidence of Hsp70 protein in 51% of the patients. These studies used the complex method by Western-blot. Also, the presence of antibodies directed against the Hsp70 enzyme was analyzed using the immunoassay method. Bonaguri et al. [31] showed the presence of anti-Hsp70 in 52% of people with hearing loss, compared with the control group (4%). On the other hand, Zeitoun et al. [19] showed the presence of auto-antibodies in 31 of 63 patients, representing 59% of people with hearing loss.

These results suggest an association between hearing loss and the autoimmune inner ear disease [31]. Currently, it is believed that testing the presence of the Hsp70 protein and the level of anti-Hsp70 auto-antigen may be helpful in the serological diagnosis of autoimmune hearing loss. It is believed that these proteins are the only available diagnostic markers that identify the origin of autoimmune hearing loss [3, 30].

Our tests involving the group of 64 patients with the SSHL impairment showed a positive reactions to the presence of Hsp70 proteins in the sera of 48% of patients, which seems to be consistent with the results obtained by other authors. We also performed an analysis of the CIC occurrence in groups with SSHL. In 59% of the SSHL cases, these values were greater than normal. We analyzed the occurrence of the bound protein Hsp70 in CIC. Additionally, the results indicate that the protein may be present in the bound form only. With a large capacity of local immune mechanisms, it seems to be possible. The presence of the Hsp70 protein and elevated CIC values are often the result of recurrent respiratory infections of various origins [34]. As a further consequence, this may be related to the occurrence of tinnitus (76% of our patients reported tinnitus), hypersensitivity to sound, and hypoacusis in the patients. Modugno et al. [32] hypothesized the occurrence of dizziness as a result of the formation of immune complexes in the fluids of the inner ear, which would lead to impairment of the composition of endolymph and cause mechanical irritation of the inner ear receptors. Hypothetically, such mechanisms in the form of chronic processes could cause precipitation of immune complexes (exceeding the concentration of a CIC-saturated solution, or solubility) in the environment of the endolymph, which could further lead to phenomena promoting both hearing loss and even dizziness.

The clinical aspect of the presence of the Hsp70 protein may be associated with different efficacy of the SSHL treatment with steroids between individuals. The widely discussed mechanism of the glucocorticosteroid action in hearing loss is to be based primarily on their immunosuppressive and anti-inflammatory effects [35]. The Hsp70 protein is easily subjected to regulation with dexamethasone in experimental studies in mice [36]. Its varied expression in the investigated cases of hearing loss may determine the success of steroids and can become an indicator to take or refrain from such a therapy. This hypothesis may be confirmed by earlier observations on the correlation between the decrease in the level of antibodies against HSP70 in the serum and success of steroid treatment in cases of sudden idiopathic sensorineural hearing loss [37].

The present study does not include a comparative analysis of the dot blot method with the Western-blot, the reason being that the Western-blot is not suitable for the Hsp70 indication in immunological complexes.

This limitation presents a fundamental advantage of the dot blot method used by the present authors.

CONCLUSION

Idiopathic SSHL is a difficult problem, both from the diagnostic and treatment point of view. Treatment results still remain unsatisfactory. Introduction of the proposed method of the Hsp70 protein indication in CIC, which is fast and inexpensive, into hospital departments would allow for detection of autoimmune etiology of a disease and its direct customized treatment.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Nicolaus Copernicus University Collegium Medicum in Bydgoszcz (no: KB 498/2014).

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – R.G., K.P., G.P.; Design – R.G., K.P., W.K., H.K., G.P.; Supervision – H.K.; Resource – S.O., M.M., G.P.; Materials – G.P., K.P., R.G.; Data Collection and/or Processing –R.G., K.P., S.O.; Analysis and/or Interpretation – R.G., K.P., G.P.; Literature Search – S.O., M.M., R.G.; Writing – R.G., K.P., G.P.; Critical Reviews – W.K., H.K.

Conflict of Interest: The authors have no conflict of interest to declare.

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