

## Online Supplementary Material

# **Assessing Systemic Stress in Otolaryngology: Methodology and Feasibility of Hair and Salivary Cortisol Testing**

A.

1.

### Hair Collection Materials

Materials for each sample collection:

1 – pair of stylist’s thinning shears (reusable, after cleaned with alcohol pad)

1 – sheet of aluminum foil (30 x 30 cm)

2 – parting hair clips (reusable, after cleaned with alcohol pad)

1 – alcohol prep pad

1 – parting comb (disposed of after single use)

1 – strip of painter’s tape (approximately 5 cm in length)

1 – felt-tipped marker (to label foil after sample collection)

1 – letter-sized envelope (if patient has short [ $<3$  cm in length] hair)

2.

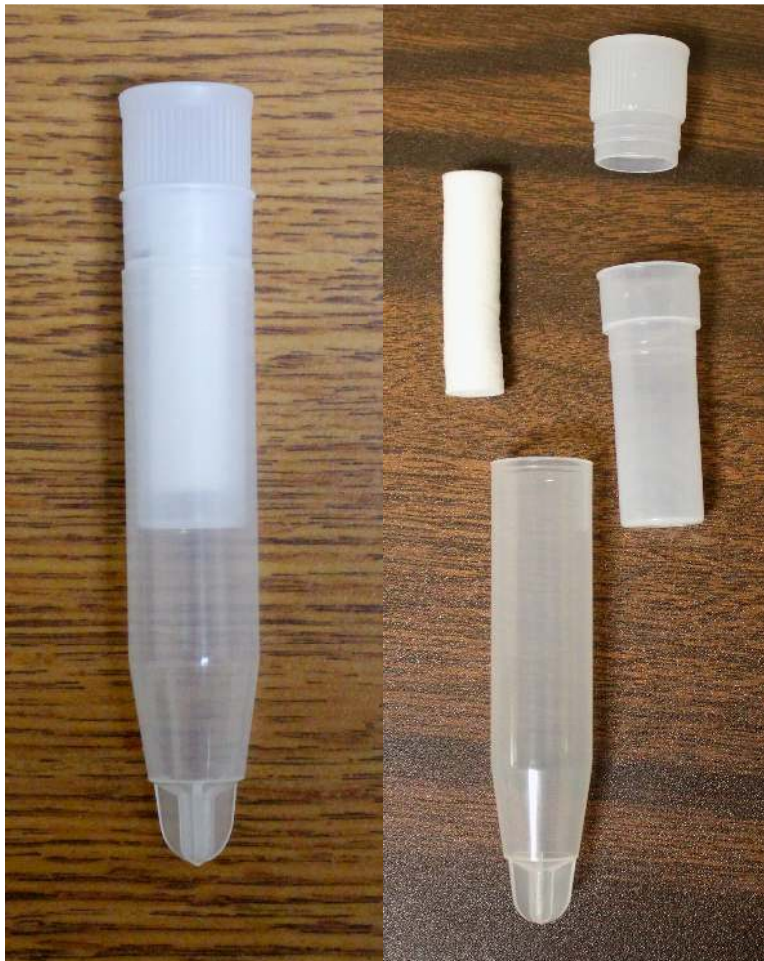
### Saliva Collection Materials

Materials for each prepared saliva collection packet:

1 – #4 (16.5 x 36.8 cm) bubble mailer (holds all materials) with prepaid postage

- 2 – quart-size plastic zipper bags (each holds one day’s collection materials)
- 8 – cotton swab Salivette tubes (Shown in **Figure A1**)
- 2 – small (golf-type) pencils
- 2 – timesheets for recording collection times (Appendix D1)
- 1 – set of saliva ID labels (Appendix D2)
- 2 – “Home Saliva Sample Collection Instructions” sheet (Appendix D3)
- 1 – “Cortisol Testing Patient Information” sheet (Appendix D4)

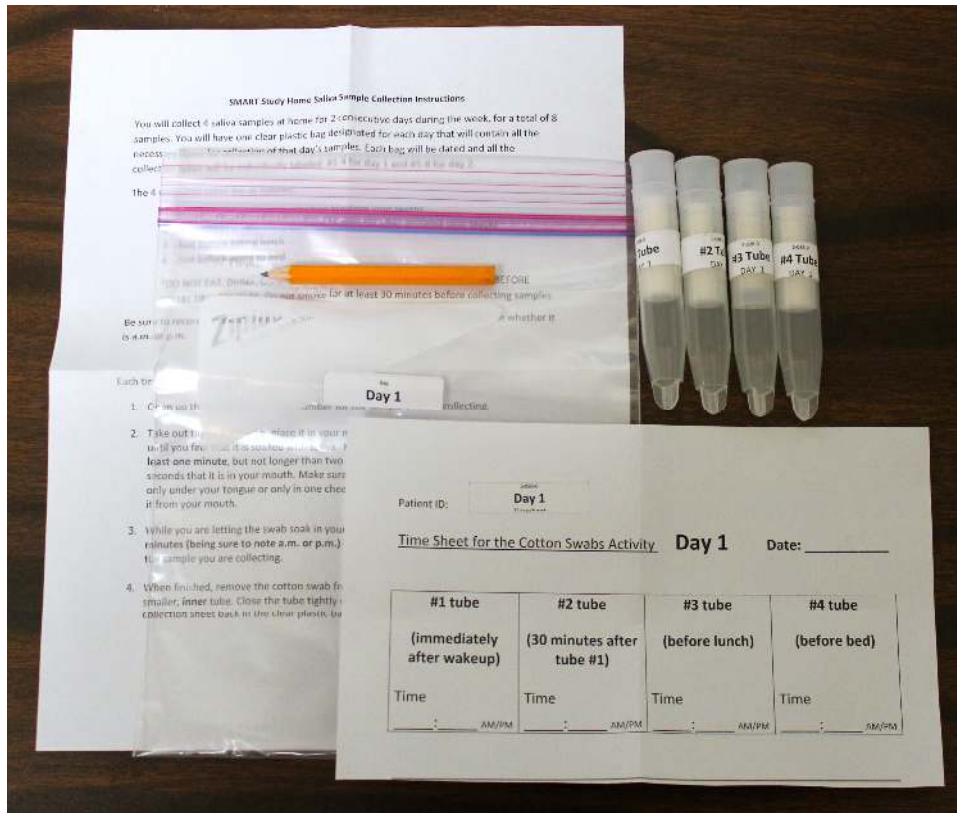
Figure A1. Complete Salivette tube (left) and individual components (right)



How to prepare packets:

- Label the tubes, timesheets, and plastic bags with appropriate saliva ID labels
- place tubes 1-4 with the timesheet for day 1 into the plastic zipper bag for day 1, along with one small pencil and a “Home Saliva Sample Collection Instructions” sheet; this completes day 1 materials. A completed day 1 pack is shown in **Figure A2**.
- place tubes 5-8 with the timesheet for day 2 into the plastic zipper bag for day 2, along with one small pencil and a “Home Saliva Sample Collection Instructions” sheet; this completes day 2 materials.
- Place both bags and the “Cortisol Testing Patient Information” sheet into the prepaid bubble mailer; this completes one home collection packet.

Figure A2. Day 1 materials for home saliva sample collection



B.

1.

Hair Sample Collection Procedure

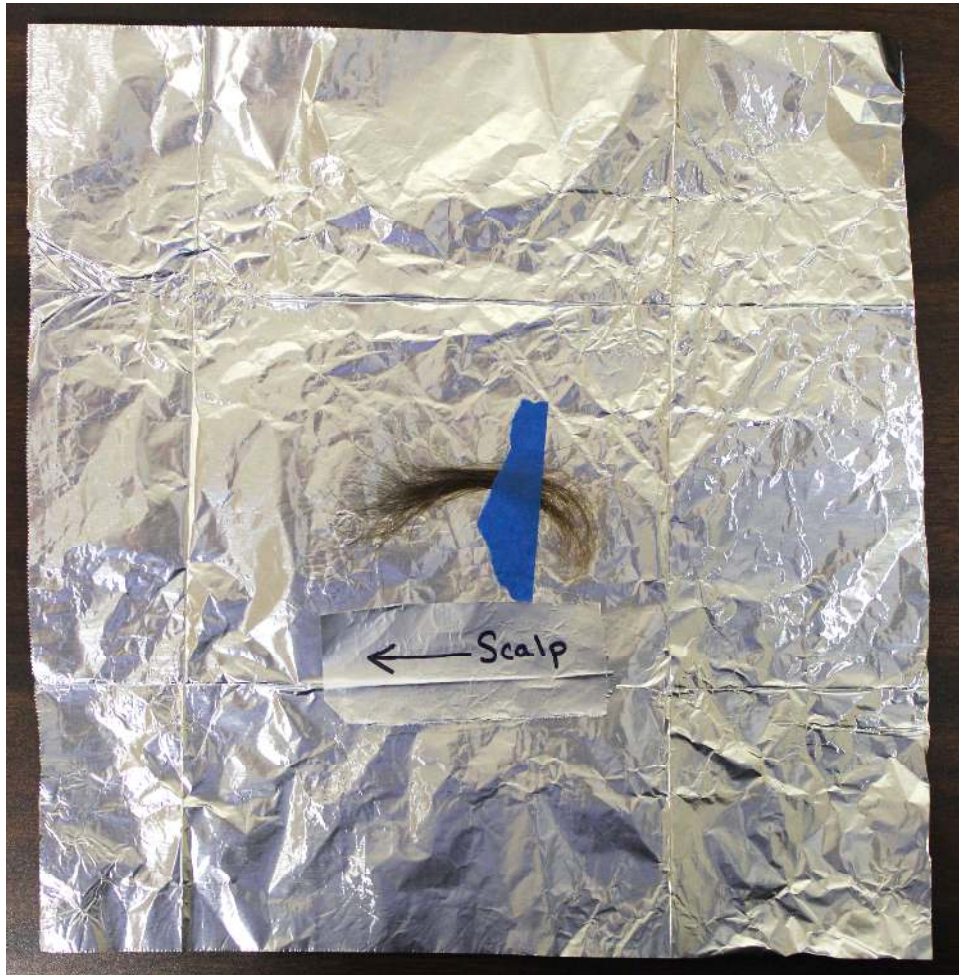
One of two procedures is followed according to the length of the hair. For participants with hair >3 cm in length (long hair) at the posterior vertex, a parting comb is used to isolate a strip of hair approximately 5 mm in width (cephalocaudal) and 3-5 cm in length (mediolateral), as shown in **Figure B1**.

Figure B1. Hair sample to be collected for a participant with long (>3 cm) hair



Two cuts with thinning shears are then performed to remove the hair as close to the scalp as possible. Thinning shears are used because they do not remove the entire strip of hair and help to avoid any risk of a bald spot at the sampling site. This hair is then carefully pulled away from the subject's scalp, being careful to maintain the orientation of the hair. The oriented hair is secured to a piece of an aluminum foil sheet (approximately 30 x 30 cm) using a thin strip of painter's tape, which has low adhesiveness and does not leave behind a sticky residue, which may hinder sample processing at the laboratory. The scalp end of the hair is labeled for reorientation later at the laboratory, as shown in **Figure B2**.

Figure B2. Post-collection hair preparation



The hair should be taped at least 3 cm from the proximal (scalp) end of the hair because this is the section of hair that will be used in the cortisol analysis.

For participants with hair <3 cm in length (short hair) at the posterior vertex, a parting comb is used to isolate a strip of hair approximately 5 mm in width (cephalocaudal) and 3-5 cm in length (mediolateral), in the same fashion as for long hair. An open envelope is then placed under the strip of hair to catch the sample as it is cut, as shown in **Figure B3**.

Figure B3. Hair sample to be collected for a participant with short (<3 cm) hair



Two cuts with the thinning shears will yield an amount of hair adequate for analysis. This hair is not oriented with regard to the scalp, so it is simply dumped from the envelope onto the 30 x 30 cm aluminum foil sheet, which is then folded to securely hold the sample. All samples are stored in a dry, secure area at the study-coordinating center.

2.

#### Saliva Collection Procedure

Saliva samples are collected by the subject at his or her home. At each of the three study visits, the subject is instructed on the procedure for home saliva collection

and is given a prepared packet with all the necessary materials, including a prepaid envelope for return of the saliva samples after collection (Appendix A2). Each sample is collected using a Salivette (Sarstedt AG & Co., Nümbrecht, Germany), which is a clear plastic tube with a smaller internal tube that holds a cotton swab. The subject places the cotton swab into her mouth for 1-2 minutes, moving it around until the swab is saturated. The cotton swab is then placed back into the internal tube of the Salivette. Participants are instructed to collect the samples on a weekday during which they would be following their typical routine in order to minimize the occurrence of abnormal stressors during the time of sample collection. The participants are instructed to not brush or floss their teeth, eat, drink, or use tobacco for the 30 minutes prior to sample collection to decrease the possibility of sample contamination. The exact time of each sample collection is recorded on a timesheet that is provided with the sample collection materials.

Following collection, the participant mails the samples to the study-coordinating center using the prepaid envelope provided. If there is any delay in the time from collection to shipment, the subjects are instructed to refrigerate the samples at home to preserve the saliva. At the coordinating center, all samples are stored in a dedicated, secure refrigerator at 4 degrees Celsius until being taken to the laboratory for analysis. Saliva samples for cortisol analysis are stable at room temperature for 7-14 days, one month or more at 4 degrees Celsius, and indefinitely at -20 degrees Celsius.<sup>1</sup>

C.



## Hair Analysis Procedure

At the analysis laboratory, each hair sample is cut to 3 cm and washed three times in isopropanol and dried as previously described.<sup>2</sup> After washing, drying, and weighing, the hair is ground using a ball mill (Retsch, Haan, Germany) within a 2-ml cryovial (Wheaton, Millville, NJ, USA), which holds the sample as well as a 3/16-inch (4.8 mm) stainless steel ball bearing. Cryovials are placed in specially milled aluminum cassettes holding three cryovials. The cassette containing the cryovial is immersed in liquid nitrogen to freeze the hair to facilitate grinding. Samples are ground for approximately 5 minutes. The powdered hair (5 – 10 mg) is extracted in the same cryovial in HPLC-grade methanol at room temperature for 24 hours with slow shaking. Following extraction, the cryovial is spun for 120 seconds in a microcentrifuge at 20,000 g, and the supernatant is removed, placed into a second microcentrifuge tube, and dried. These extracts are then reconstituted with assay buffer, and cortisol levels are determined using a commercial high-sensitivity enzyme immunoassay (EIA) kit (Salimetrics LLC, State College, PA, USA) according to the manufacturer's directions. The samples are read using an enzyme-linked immunosorbent assay (ELISA) microplate reader to determine the concentration of cortisol in each sample. A pooled control consisting of previously ground hair is extracted and run on each plate for determination of inter-assay coefficients of variation (CV).

D.

1.

Saliva Collection Timesheets

Patient ID:			
<u>Time Sheet for the Cotton Swabs Activity</u>		<b>Day 1</b>	Date: _____
<b>#1 tube</b>  (immediately after wakeup)	<b>#2 tube</b>  (30 minutes after tube #1)	<b>#3 tube</b>  (before lunch)	<b>#4 tube</b>  (before bed)
Time _____:____ AM/PM	Time _____:____ AM/PM	Time _____:____ AM/PM	Time _____:____ AM/PM
Patient ID:			
<u>Time Sheet for the Cotton Swabs Activity</u>		<b>Day 2</b>	Date: _____
<b>#5 tube</b>  (immediately after wakeup)	<b>#6 tube</b>  (30 minutes after tube #1)	<b>#7 tube</b>  (before lunch)	<b>#8 tube</b>  (before bed)
Time _____:____ AM/PM	Time _____:____ AM/PM	Time _____:____ AM/PM	Time _____:____ AM/PM

2.

Saliva ID Labels

S-XXX-V <b>#1 Tube</b> DAY 1	S-XXX-V <b>#2 Tube</b> DAY 1	S-XXX-V <b>#3 Tube</b> DAY 1	S-XXX-V <b>#4 Tube</b> DAY 1
S-XXX-V <b>#5 Tube</b> DAY 2	S-XXX-V <b>#6 Tube</b> DAY 2	S-XXX-V <b>#7 Tube</b> DAY 2	S-XXX-V <b>#8 Tube</b> DAY 2
S-XXX-V <b>Day 1</b> Timesheet	S-XXX-V <b>Day 2</b> Timesheet 1	S-XXX-V <b>Day 1</b> Materials Bag	S-XXX-V <b>Day 2</b> Materials Bag

Labeling scheme: “S-XXX-V,” where “S” denotes that this is a saliva sample, XXX denotes the participant’s 3-digit study number, and V denotes the number of the study visit.

3.

SMART Study Cortisol Testing Patient Information Sheet

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**SMART Study Cortisol Testing Patient Information Sheet**

**Why is this study being performed?**

This study is being performed to learn more about the effects of hearing loss on the body. We specifically would like to learn more about the relationship between hearing loss and stress. We will attempt to do this by measuring cortisol (stress hormone) levels before and after your hearing loss treatment to see how the cortisol levels change.

**What is cortisol?**

Cortisol, also known as hydrocortisone, is a steroid hormone released by your adrenal glands. This hormone is naturally secreted (released) in all of us throughout the day. Cortisol plays important roles throughout the body and has a variety of functions. It works to regulate our sleep-wake cycle, to manage blood sugar levels, to regulate the

responsiveness of the immune system, and to aid in metabolism of fats, proteins, and carbohydrates. Additionally, cortisol has also been called the “stress hormone” because it is released in response to stressful situations.

### **Why are we measuring cortisol?**

We are measuring cortisol in the SMART study because we suspect that hearing loss is stressful to the body and brain and may result in abnormally increased cortisol secretion. We also suspect that cortisol secretion will decrease to more normal levels following adequate treatment of hearing loss, either through the use of hearing aids or cochlear implantation. Knowing the effects of hearing loss and subsequent hearing loss treatment on cortisol secretion will give us more information on the effects of hearing loss on the body as a whole. In time, information gained from this study may help doctors treat hearing loss and other medical conditions more effectively.

### **How is cortisol measured in the SMART study?**

Cortisol can be measured in a variety of ways. In the SMART study, we are measuring cortisol levels by testing samples of saliva and hair. We chose these methods because they are the easiest for participants to do, while still being very accurate. Also, testing hair and saliva DOES NOT require any invasive or painful techniques.

### **If I choose to participate in the cortisol testing, what will be required of me?**

For the saliva testing, you will collect multiple samples at home. You will gently chew on a piece of cotton for about 1 minute until it is soaked and, then, place it in a storage container; this will give 1 sample. We will ask that you do this 4 times a day at specific times for 2 days, giving 8 samples total. You will get specific in-person instruction on how to do this and will be given an instructional sheet to take home with you.

For the hair testing, we will clip a few strands of hair from a non-obvious place on the back of your scalp. Specifically, we will collect about 50 strands of hair. The hair will be clipped by a well-trained member of the research team using a pair of stylist thinning shears, which allow us to cut some but not all of the hair from an area. The area from which the hair is removed is typically not visible once the hair is laid back into place.

### **What are the possible discomforts or risks?**

For the salivary testing, there is no risk to you. It only requires that you set aside 2-3 minutes, 4 times a day, for 2 days.

For the hair testing, discomforts that you might experience while in this study include slight uneasiness while having your hair cut by a research assistant. This will be minimized by ensuring adequate training for any person who would collect your hair sample. The impact of the hair collection is typically not noticeable. Otherwise, there are no risks beyond those experienced normally when your hair is trimmed or cut.

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4.

### SMART Study Home Saliva Sample Collection Instructions

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#### **SMART Study Home Saliva Sample Collection Instructions**

You will collect 4 saliva samples at home for 2 consecutive days during the week, for a total of 8 samples. You will have one clear plastic bag designated for each day that will contain all the necessary items for collection of that day's samples. Each bag will be dated and all the collection tubes will be individually labeled: #1-4 for day 1 and #5-8 for day 2.

The 4 collection times are as follows:

1. Immediately after waking (before brushing your teeth)
2. 30 minutes after waking (at least 15 minutes after brushing your teeth)
3. Just before eating lunch
4. Just before going to bed

**\*DO NOT EAT, DRINK, OR BRUSH YOUR TEETH FOR AT LEAST 15 MINUTES BEFORE COLLECTING SAMPLES.** Do not smoke for at least 30 minutes before collecting samples.

Be sure to record the **exact time** using the time that you take each sample and note whether it is a.m. or p.m.

Each time you need to collect a saliva sample you will do the following:

1. Open up the container with the number for the sample you are collecting.
2. Take out the cotton swab, place it in your mouth, and gently chew and move it around until you feel that it is soaked with saliva. Keep the cotton swab in your mouth for **at least one minute**, but not longer than two minutes. Do not chew it for the last 15 seconds that it is in your mouth. Make sure to move the swab around. Do not keep it only under your tongue or only in one cheek. The swab should be **WET** before removing it from your mouth.
3. While you are letting the swab soak in your mouth, write the **exact time in hours and minutes (being sure to note a.m. or p.m.)** on the time collection sheet in the space for the sample you are collecting.
4. When finished, remove the cotton swab from your mouth and place it back into the smaller, **inner** tube. Close the tube tightly with the plastic stopper. Put the tube and time collection sheet back in the clear plastic bag and close the bag securely.

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**Important things to remember:**

1. *Choose the tube with the right number!!*
2. *Write the exact time on the timesheet.*
3. *The timing of the first TWO samples for each day (right when you wake up and 30 minutes later) is VERY important and should be exact, using the same clock/watch.*
4. *Do not eat, drink, or brush your teeth 15 minutes before collecting the sample.*
5. *The cotton must be WET. Make sure you put the swab back in the tube.*

You will need to carry the zip lock bag with you during your day. After you collect all the samples, you can store the bag in your refrigerator until you can mail the samples. Samples should be mailed soon after all samples are collected (within a couple of days) using the pre-paid mailing package.

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**References**

1. Wood P. Salivary steroid assays - research or routine? *Ann Clin Biochem* 2009; 46:183-196.
2. D'Anna-Hernandez KL, Ross RG, Natvig CL, Laudenslager ML. Hair cortisol levels as a retrospective marker of hypothalamic-pituitary axis activity throughout pregnancy: comparison to salivary cortisol. *Physiol Behav* 2011; 104:348-353.