CHRONIC HPA AXIS RESPONSE TO STRESS
IN TEMPOROMANDIBULAR DISORDER

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partial fulfillment of the requirements for the degree of Master of Science in Dental
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ABSTRACT

CYNTHIA ANN LAMBERT: Chronic Hypothalamic Pituitary Adrenal Axis Response to Stress in Temporomandibular Disorder
(Under the direction of Anne E. Sanders)

Perceived stress is associated with temporomandibular disorder (TMD). We hypothesized that cortisol concentration, a biomarker of hypothalamic-pituitary-adrenal (HPA) axis function, was elevated in TMD cases relative to controls, and that perceived stress was positively correlated with cortisol concentration. In this case control study, TMD case status was determined by TMD Research Diagnostic Criteria. Adult participants (n=116) aged 18-59 years were recruited from within a 50-mile radius of the UNC at Chapel Hill. Cases (n=45) and controls (n=71) completed the 14-item Perceived Stress Scale using a reference interval of the past three months. Approximately 100 strands of hair were cut from the posterior vertex segment of their scalp and the most proximal portion was analyzed for cortisol concentration. TMD cases perceived higher stress than controls. Hair cortisol concentration was lower in TMD cases than controls. A weak negative relationship was determined between perceived stress and hair cortisol concentration.
ACKNOWLEDGEMENTS

Completing this thesis required the cooperation and support of many special people. I would like to thank Dr. Anne Sanders for her wisdom, time, efforts, and commitment with my thesis research; Dr. Gary Slade for his knowledge and expertise in research and writing; Rebecca Wilder for her endless support, encouragement, positive words and always believing in me to achieve this goal of thesis completion. Your guidance has been extremely valuable and I will always be thankful and grateful for your support.

To my best friend, Stan, I would never have been able to achieve this endeavor had it not been for you. Your constant and continual love, encouragement, positive comments, and belief in me are more valuable than words and I cannot express them adequately.

To my wonderful daughter, April, grandson Draven, and granddaughter Lillyana, you light up my life in more ways than you will ever know. I will always encourage you that life is worth living and your goals are possible - no matter your age. To all my brothers and sisters and their families, who believe in me and encourage me to excel in all that I do - I love you all!

To the many people at UNC who have helped me with this thesis and have interacted with me these last two years, you have made my dream more beautiful because you are part of the journey – You are my friends!
To Qun, you are a great friend. You and your prayers are always there to see me through the challenges. Viviana, your presence in these past two years and your help are appreciated and valued. I wish you the best in dental school.

To my main supporter, Jesus Christ, for without Him I am nothing. He reminds me daily that I can do all things because He strengthens me. His Holy Word, states in 2 Timothy 1:7 “For God has not given us a spirit of fear, but of power and of love and of a sound mind.”
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<tr>
<td>CRH</td>
<td>Corticotrophin Releasing Hormone</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic Pituitary Adrenal</td>
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<td>IRB</td>
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<td>OPPERA</td>
<td>Orofacial Pain Prospective Evaluation and Risk Assessment</td>
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<td>Perceived Stress Scale</td>
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INTRODUCTION

One of the most fundamental physiological responses to stress is activation of the hypothalamic-pituitary-adrenocortical (HPA) axis. The end product of HPA axis activation is stimulation of the adrenal cortex to increase secretion of the glucocorticoid cortisol. While protective in the short term, sustained activation of this hormonal response system is theorized to lead to tissue damage and subsequent dysregulation of biological systems.¹ Since the 1960’s investigators have measured cortisol levels in blood, saliva or urine to understand how stress increases vulnerability to disease.
REVIEW OF LITERATURE

Psychosocial Stress

Well before the role of HPA axis was theorized, stress was recognized to contribute to acute necrotizing ulcerative gingivitis—so-called trench-mouth—among First World War soldiers. Today stress has salience to oral health research because it is implicated in the pathogenesis of several dental conditions that have relevance to dental hygienist clinical practice. Heightened levels of stress are associated with oral mucosal lesions such as oral lichen planus, and recurrent aphthous stomatitis. Among middle-aged adults, those with greater perceived stress were less likely to have retained 20 teeth—the minimum number required for adequate function. Psychosocial stress is believed to increase susceptibility to gingival infection and depress immune responsiveness to periodontal pathogens. A cross-sectional study of 1426 adults, found that financial strain was associated with greater clinical attachment loss and alveolar bone loss.

Temporomandibular Joint Disorders

Perhaps strongest evidence for a putative role of stress in oral disorders comes from studies of the onset, severity and chronicity of temporomandibular disorders (TMD). TMD is the most common form of chronic orofacial pain affecting 5% of the United States population. Sanders et. al. demonstrated a strong dose-dependent relationship between severity of perceived stress and odds of examiner-determined TMD. Baseline findings from the Orofacial Pain Prospective Evaluation and Risk Assessment (OPPERA) prospective
cohort study investigating risk factors for TMD found that compared with controls, TMD cases reported higher levels of psychosocial symptoms, affective distress, somatic awareness, and pain catastrophizing. Longitudinal research that followed healthy adults with no prior history of TMD found that those with greater perceived stress were more likely to experience first-onset TMD than adults with less perceived stress.

**Cortisol Measurement**

It is perhaps surprising that cortisol measurement does not feature more prominently in oral health research as a biomarker of stress. New protocols for salivary cortisol collection offer advantages over blood and urine sampling protocols in terms of cost and simplicity. Yet major difficulties remain in obtaining valid and reliable measurements of cortisol in observational studies. Firstly, cortisol secretion follows a robust 24-hour rhythm, peaking around 8:00 with a nadir between 20:00 and 24:00. Overlying this daily pattern is a series of 8-10 pulses. Such variation means that exact timing of specimen collection is critical if cortisol concentrations are to be meaningfully compared, and multiple measures per subject are often required. The United States National Longitudinal Study of Adolescent Health study recently reported its decision to drop salivary cortisol measurement from its protocol because responses and protocol adherence were inadequate.

A second limitation of cortisol measurement in blood, saliva and urine is that each of these fluids provides a very limited temporal window of cortisol activity. Levels of cortisol in blood and saliva reflect average hormone levels in the past one hour while cortisol in urine captures a slightly longer interval of up to 24 hours. None of these is able to measure chronic stress exposure which is thought to pose a greater threat to health than the short-term physiologic responses to acute stress.
Cortisol in Human Scalp Hair

An important breakthrough was the development of an assay to measure endogenous concentrations of cortisol in human scalp hair\textsuperscript{18} permitting a reliable measurement of the stress response over a prolonged period, i.e. chronic stress exposure. \textsuperscript{19} Cortisol is thought to be incorporated into hair through diffusion from body secretion of sweat and sebum during formation of the hair shaft. \textsuperscript{20} Since hair grows at a precise rate of 0.35 mm per day, equivalent to one centimeter (cm) per month, \textsuperscript{21} hair length is an accurate index of exposure to stress over time. Thus hair cortisol promises a new, simple and non-invasive way in epidemiologic research to examine the role of stress.

To clarify the role of stress in TMD, the first aim of this study was to confirm the well-documented association between perceived stress and TMD. Once established, the second aim was to determine the relationship between hair cortisol concentration and TMD status. The third aim was to examine the correlation between perceived stress and hair cortisol concentration. The authors tested the hypotheses that both perceived and biologic measures of stress were elevated among TMD cases and that perceived stress was positively correlated with hair cortisol concentration.
INTRODUCTION AND LITERATURE REVIEW

One of the most fundamental physiological responses to stress is activation of the hypothalamic-pituitary-adrenocortical (HPA) axis. The end product of HPA axis activation is stimulation of the adrenal cortex to increase secretion of the glucocorticoid cortisol. While protective in the short term, sustained activation of this hormonal response system is theorized to lead to tissue damage and subsequent dysregulation of biological systems. Since the 1960’s investigators have measured cortisol levels in blood, saliva or urine to understand how stress increases vulnerability to disease.

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An important breakthrough was the development of an assay to measure endogenous concentrations of cortisol in human scalp hair\textsuperscript{18} permitting a reliable measurement of the stress response over a prolonged period, i.e. chronic stress exposure.\textsuperscript{19} Cortisol is thought be incorporated into hair through diffusion from body secretion of sweat and sebum during formation of the hair shaft.\textsuperscript{20} Since hair grows at a precise rate of 0.35 mm per day, equivalent to one centimeter (cm) per month,\textsuperscript{21} hair length is an accurate index of exposure to stress over time. Thus hair cortisol promises a new, simple and non-invasive way in epidemiologic research to examine the role of stress.

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METHODS

This study was approved by the UNC Biomedical Institutional Review Board. All participants gave written informed consent before their inclusion in the study.

Study Design

In this case control study, cases had examiner-diagnosed TMD. Controls were also examined and found not to have this condition.

Setting

During the period July 2010–October 2011, potential participants were recruited by advertisements placed in brochures, internet, radio, and newspapers within a 50 mile radius of the Center for Neurosensory Disorders at in the School of Dentistry at The University of North Carolina at Chapel Hill’s (UNC) in North Carolina USA.

Inclusion and Exclusion Criteria

Eligible participants were males and females aged between 18–60 years with scalp hair at least 3 cm in length. Respondents were first screened in a telephone interview to exclude those with conditions known to influence cortisol levels. Exclusionary criteria were diagnoses of any one of Cushing’s syndrome or Addison’s disease, diabetes, heart trouble or disease, hypertension that was not well controlled with medication, hyperthyroidism, major psychiatric disorder requiring hospitalization within the previous 6 months, chronic respiratory disease not controlled with medication, seizures, renal failure or dialysis. Also excluded were those who were pregnant, nursing, undergoing orthodontic treatment,
radiation or chemotherapy as well as persons with drug or alcohol abuse, trauma or surgery on the head, face or neck within the last six months. Persons having used corticosteroid treatment in the last 12 months (including cortisol containing creams and lotions, and nasal spray) were likewise excluded. Finally those having used permanent or semi-permanent hair color within three months were excluded since cortisol levels are lower in artificially colored hair.  

**TMD Case Classification**

A medical history was recorded for all screened participants prior to the clinical examination. Examinations were performed by six dental hygiene examiners trained in the examination protocol and calibrated for reliability and validity of their diagnostic decisions every six months. The standardized physical examination of the head and neck followed the research diagnostic criteria for temporomandibular disorders. In summary, TMD cases were people who reported a six-month history of pain in the temporomandibular structures, with at least 5 days of such pain in the month preceding the examination and where the examiner found at least three muscle groups in the temporomandibular region that were tender to palpation or jaw maneuver. Controls reported no history of orofacial pain within the preceding six months and no prior diagnosis for TMD. Additionally, their examination confirmed that they did not have TMD, arthralgia or myalgia.

**Hair Sampling**

A hair sample (~100 strands, ≥ 20 mg of hair) of at least 3 cm in length was collected by study personnel. The sample was cut using fine scissors from as close as possible to the scalp from the vertex posterior region. Intra-individual variation in cortisol content is less in this region (coefficient of variation = 15.6%), as compared to hair sampled from other than in
the posterior vertex, anterior vertex, nape, temporal and frontal regions (coefficient of variation = 30.5%). Because scalp hair grows one cm per month on average, analysis of three cm of hair most proximal to the scalp provides information about three months of systemic cortisol exposure. Hair samples were attached to a sheet of paper using Millipore (Billerica, MA, USA) tape, the scalp end was marked and the collection date and participant identification number were recorded. The paper was then enclosed in an envelope sealed with identification number and date on outside of envelope and stored at room temperature. Within six months of collection, samples were sent by mail to the laboratory at the University of Western Ontario, London, Ontario where cortisol levels for analyzed.

**Hair Sample Preparation and Quantification of Hair Cortisol**

In preparation for analysis, hair samples were measured and the length and color of the hair recorded. The most proximal 3 cm hair segment was cut, placed into a glass vial, labeled and weighed to ensure a minimal weight for analysis of 10-15 mg. Hair was then washed twice by immersing the segments in 3 ml of isopropanol, followed by a 3 minute incubated on a shaker at 0.11 g (100 rpm) at room temperature. Laboratory analysis was performed using a commercially available salivary cortisol enzyme immunoassay kit from Alpco Diagnostics (Salem, NH, USA). Details of the laboratory procedures are reported fully elsewhere.

**Perceived Stress**

Perceived stress was measured using the psychometrically-validated and widely used 14-item Perceived Stress Scale (PSS). Summary scores from this instrument and its shorter 10-item subset are shown in previous studies by our group to be positively associated with TMD. The PSS was developed to evaluate the theoretical construct of stress proposed
by Lazarus and Folkman\textsuperscript{27} that a stimulus is stressful when perceived as both threatening and exceeding one’s coping resources. The PSS takes into account these appraisals by measuring the degree to which respondents consider their lives to be unpredictable, uncontrollable and overloaded.\textsuperscript{25} In each question, respondents were asked to indicate how often they felt or thought a certain way. The conventional one-month reference interval was extended in this study to three months. This was considered to better represent exposure to chronic stress than the one month interval, without being so long that recall bias would limit the interpretation of findings. Responses were recorded on a five-point ordinal scale coded: never=0; almost never=1; sometimes=2; fairly often=3; very often=4. In computing a summary score, positively worded items were reverse coded, consistent with recommended scoring methods.\textsuperscript{25}

**Covariates**

Covariates were sex, age in years, race, ethnicity, educational attainment, annual household income and cigarette smoking status. This information was obtained by questionnaire at the time of the physical examination.

**Statistical Analysis**

Participants with hair cortisol concentrations >1500 ng/g were excluded from analysis on the basis of possible contamination due to use of creams or ointments containing hydrocortisone.\textsuperscript{28} Initial exploration using histograms and qnorm diagnostic plots showed that PSS scores were normally distributed, and cortisol concentrations were skewed towards higher values. Therefore log\textsubscript{10} transformed cortisol values were modeled when the continuous values were analyzed. To account for the potential effect of confounding, analyses were repeated after stratifying on TMD case status.
The Pearson’s product moment correlation coefficient was used to determine the strength and direction of the relationship between PSS scores and cortisol concentration. A scatter plot was fitted to graphical depict this relationship. Fisher’s exact test was used to compare dichotomous variables and the independent samples t-test (two-sided) compared differences in mean log_{10} cortisol concentration between TMD cases and controls.
RESULTS

Data were analyzed for 45 TMD cases and 71 controls after omitting three subjects whose cortisol concentrations exceeded 1500 ng/g. The age of participants ranged from 18 to 59 years (mean=29.9 years) and the sample was predominantly female (80.2%) and white race (84.2%).

TMD cases and controls did not differ on the basis of sociodemographic characteristics or smoking status (Table 1). However compared with controls, TMD cases perceived significantly higher levels of stress in their daily lives \( P < 0.001 \) (Figure 1A and Table 2).

Perceptions of stress and levels of hair cortisol did not differ significantly between participants on the basis of age, sex, race, smoking or socioeconomic status (Table 2). Despite perceiving higher levels of stress, cortisol concentrations were significantly lower in TMD cases than in controls \( P < 0.001 \).

Examination of the cloud of observations on the scatter plot revealed a weak, negative relationship but statistically significant relationship between perceived stress and cortisol concentration (Figure 2) \( r = -0.188, P = 0.044 \). When examined in separate strata of case status, the relationship was negative in each stratum, but failed to reach statistical significance for cases \( r = -0.111, P = 0.169 \) and controls \( r = -0.082, P = 0.498 \).

Examination of the stratum-specific odds ratios and their confidence intervals suggested that
the relationship between perceived stress and hair cortisol concentration was similar in TMD cases and controls.
DISCUSSION

Key Findings

In this study, TMD cases perceived significantly more stress than controls over the preceding three months, confirming a well-established relationship between psychosocial stress and TMD. Our expectation that higher stress perception in cases would correspond with elevated cortisol production was not supported. In fact, cortisol production was significantly lower in cases than controls. Among all subjects combined, perceived stress and cortisol concentration were significantly and negatively related, albeit in a weak relationship. When examined in stratum-specific analyses, perceived stress and cortisol concentration were negatively associated for both cases and controls, but non-significantly. In summary, individuals with higher perceived stress had lower hair cortisol concentration, and this effect was more pronounced among cases than controls.

Comparison with Previous Studies

Our study is not the first to find an inverse or null association between perceived stress and hair cortisol concentration. A study that administered the Perceived Stress Scale with a three-month reference interval to university students reported a weak negative correlation with hair cortisol content ($r = -0.061$, $P = 0.025$). Another study comparing long-term unemployed individuals with people in stable employment, found that while the unemployed reported higher Perceived Stress Scale scores, hair cortisol concentration was not associated with perceived stress. Likewise Perceived Stress scores and hair cortisol
concentration were not associated among patients attending a cardiac rehabilitation program. Elsewhere, a study comparing adults with severe chronic pain with healthy controls found a weak positive correlation between Perceived Stress Scale scores and hair cortisol that failed to reach statistical significance ($r = 0.24$, $P = 0.08$, Spearman). Similarly, the correlation between Perceived Stress Scale scores and hair cortisol concentration was weakly positive but not did not reach statistical significance ($r = 0.2$, $P = 0.06$) for subjects in case control study where cases were patients with adrenal insufficiency were on hydrocortisone replacement therapy. These findings differ from another conducted with pregnant women that reported a positive relationship between Perceived Stress Scale scores and hair cortisol concentration.

Few epidemiologic studies have measured hair cortisol in disorders in stress-related disorders. In these few studies, divergent findings report that cortisol is elevated in some disorders while lower in others. A pilot study compared hair cortisol concentration in severe chronic pain patients recruited from a chronic pain clinic who had received opioid treatment for at least one year ($n=15$), with pain-free control group recruited from the community ($n=39$). Perceived stress and cortisol levels were both higher in the opioid-treated chronic pain group with cortisol being almost elevated two-fold in the pain group (83.1 [33.0–204.9] pg/mg) relative to controls (46.1 [27.2–199.9] pg/mg).

Consistent with findings from the severe chronic pain study, a study of men hospitalized following acute myocardial infarction found significantly higher median hair cortisol levels in these men over the three months preceding the event (295.3 ng/g [105.4–809.3]) than had hospitalized men admitted for other conditions (224.9 ng/g [76.58–949.9]). By contrast to these two studies, in a case control study in which cases had
generalized anxiety disorder, hair cortisol concentrations were 50–60% lower in cases than in healthy age- and sex-matched controls; a result that contradicted earlier research using short term measures of cortisol.\textsuperscript{35}

A study that might shed light on these differential patterns examined hair cortisol levels in female adolescents at multiple time points following the 2008 Wenchuan earthquake in China.\textsuperscript{36} Subjects were classified into one of three groups: those who experienced the earthquake and developed post-traumatic stress disorder (PTSD); those who experienced the earthquake and did not develop PTSD; and a group of non-PTSD controls from a different region that was unaffected by the earthquake. Hair segments corresponding to time before and several occasions after the earthquake were compared for cortisol concentration in all three groups. Hair cortisol concentrations were similar in all groups before the earthquake suggesting no difference in HPA axis activity at baseline. In the first two months following the earthquake, cortisol levels were significantly higher in both groups exposed to the earthquake compared with the control group. Then, at 2 to 4 months after the earthquake, and again at 5 to 7 months after the earthquake, the non-PTSD group exposed to the earthquake had significantly higher cortisol concentration than both the exposed PTSD group and the control group. The authors interpreted this as a blunted HPA response in the PTSD group.\textsuperscript{36} The important finding was the change in cortisol secretion over time in the PTSD group from elevated initially, relative to controls, to suppressed.

**Possible Mechanisms and Explanations**

The noteworthy finding of the study of stress-responsive physiology to the earthquake is that timing since onset of chronic stress is important. It is possible that chronic stress elicits both an increased and a decreased production in cortisol, at different stages following onset.
of stress. In fact, this explanation was a major finding of a meta-analysis of 107 studies published between 1950–2005 that examined the relationship between chronic stress and HPA axis activity. The meta-analysis concluded that exposure to chronic stress initially activates the HPA axis producing elevated secretion of cortisol. Then, over time HPA activity subsides and cortisol secretion rebounds to below normal levels. The rebound may be a consequence of a cumulative stress burden. This is consistent with the concept of allostatic load that posits that overuse of systems designed to manage transient stress leads to impairment of the HPA function including a decrease in responsiveness to novel stressors and disturbance in the regulation of the key mediators.

Applied to the present study then, it is possible that prolonged or repeated perceptions of stress reported by TMD cases lead to blunted HPA activity and deficient cortisol signaling. In support of this idea are findings from a study of working women where high scores on the Perceived Stress Scale were associated with an 11% attenuation in diurnal variation of salivary cortisol characterized as a pronounced reduction in cortisol awakening response.

**Strengths and Limitations**

Strengths of the study relate to the rigor of the measurement protocols. The quantification of hair cortisol was conducted in laboratories in the Department of Physiology and Pharmacology, University of Western Ontario; an internationally prominent center for hair cortisol research. The Research Diagnostic Criteria for TMD case classification are standardized criteria that reliably ascertain TMD case classification. The Perceived Stress Scale is widely used and has well established reliability and validity. Our findings are the first in the oral health literature to investigate hair cortisol as a systemic biomarker of long-
term exposure stress. While our results did not support our hypothesis, the findings serve to challenge an over-simplistic view of psychoneuroimmunology in TMD and other stress-related disorders.

There are several limitations to our study. Firstly our expectation of a strong correlation between perceived stress and hair cortisol concentration rests on an erroneous assumption that these factors are two measures of the same phenomenon. However, one is a cognitive appraisal of stress and the other is the physiologic response to stress. Secondly, since we do not have information on the duration of TMD in the cases, we were unable to determine whether chronic cases were more likely than recent-onset cases to have a lower cortisol concentration. Neither did we collect information on other variables that may influence cortisol such as alcohol use and body mass index.

**Implications for Dental Hygiene Practice**

Psychosocial stress contributes to the etiology of several disorders that dental hygienists evaluate in clinical practice. Patients may be unaware that their orofacial muscle or joint pain has dental relevance. Likewise the patient may not recognize that stress might be a contributing factor to their symptoms. Dental hygienists are well positioned to observe, discuss and evaluate potential TMD and its risk factors in the course of their intraoral and extraoral examinations. This is consistent with the American Dental Hygienists’ Association Standards for Clinical Dental Hygiene Practice\(^4\) that hygienists perform an individualized assessment that includes interpretation of symptoms and clinical signs while systematically taking account of the general health status, history and needs of the patient. In discussing the patient’s oral status the dental hygienist may inform the patient that stress is a common factor
in TMD since this may be taken into consideration in formulating a patient-centered and evidence-based treatment plan.
CONCLUSION

Measurement of hair cortisol in epidemiologic studies is still in its infancy and the mixed findings make interpretations difficult. Our understanding will be improved with prospective cohort studies that collect hair samples before and after first-onset of TMD.
ACKNOWLEDGEMENTS

This study was supported by the North Carolina Translational and Clinical Sciences Institute grant 10KR30904 and National Institutes of Health grants U01DE017018 and P01NS045685.
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<td>36 (80.0)</td>
<td>46 (64.8)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td><strong>Household income (USD)</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;$40,000</td>
<td>24 (53.3)</td>
<td>37 (52.1)</td>
<td>0.91</td>
<td>0.26, 3.19</td>
</tr>
<tr>
<td>$40,000-$100,000</td>
<td>14 (31.1)</td>
<td>23 (32.4)</td>
<td>0.85</td>
<td>0.23, 3.21</td>
</tr>
<tr>
<td>≥$100,000</td>
<td>5 (11.1)</td>
<td>7 (9.9)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>2 (4.4)</td>
<td>4 (5.6)</td>
<td></td>
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<tr>
<td><strong>Smoking status</strong></td>
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<td></td>
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</tr>
<tr>
<td>Current</td>
<td>3 (6.7)</td>
<td>5 (7.0)</td>
<td>0.96</td>
<td>0.22, 4.27</td>
</tr>
<tr>
<td>Former</td>
<td>7 (15.6)</td>
<td>10 (14.1)</td>
<td>1.12</td>
<td>0.39, 3.21</td>
</tr>
<tr>
<td>Never</td>
<td>35 (77.8)</td>
<td>56 (78.9)</td>
<td>Referent</td>
<td></td>
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Table 2: Distribution of mean Perceived Stress Scale scores and mean log_{10} hair cortisol concentration

<table>
<thead>
<tr>
<th></th>
<th>Perceived Stress score</th>
<th></th>
<th>Log_{10} cortisol concentration</th>
<th></th>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>P-value</td>
<td>Mean</td>
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<tr>
<td>TMD status</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.69</td>
<td>7.24</td>
<td>0.001</td>
<td>2.38</td>
</tr>
<tr>
<td>Case</td>
<td>24.80</td>
<td>8.27</td>
<td></td>
<td>2.19</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>22.27</td>
<td>7.89</td>
<td>0.108</td>
<td>2.29</td>
</tr>
<tr>
<td>Male</td>
<td>19.26</td>
<td>8.25</td>
<td></td>
<td>2.34</td>
</tr>
<tr>
<td>Age group (years)</td>
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<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>21.02</td>
<td>6.13</td>
<td>0.723</td>
<td>2.31</td>
</tr>
<tr>
<td>25-34</td>
<td>22.41</td>
<td>8.60</td>
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<td>2.27</td>
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<tr>
<td>35-60</td>
<td>21.50</td>
<td>9.52</td>
<td></td>
<td>2.34</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>21.58</td>
<td>8.24</td>
<td>0.842</td>
<td>2.30</td>
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<tr>
<td>Not white</td>
<td>22.00</td>
<td>7.37</td>
<td></td>
<td>2.32</td>
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<tr>
<td>Educational attainment</td>
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<tr>
<td>≤High school graduation</td>
<td>20.62</td>
<td>7.25</td>
<td>0.364</td>
<td>2.37</td>
</tr>
<tr>
<td>Some college or higher</td>
<td>22.11</td>
<td>8.32</td>
<td></td>
<td>2.28</td>
</tr>
<tr>
<td>Household income (USD)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;$40,000</td>
<td>22.59</td>
<td>8.18</td>
<td>0.414</td>
<td>2.29</td>
</tr>
<tr>
<td>$40,000-$&lt;100,000</td>
<td>21.11</td>
<td>8.77</td>
<td></td>
<td>2.31</td>
</tr>
<tr>
<td>≥$100,000</td>
<td>19.50</td>
<td>5.87</td>
<td></td>
<td>2.29</td>
</tr>
<tr>
<td>Smoking status</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>23.38</td>
<td>6.44</td>
<td>0.729</td>
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</tr>
<tr>
<td>Former</td>
<td>20.65</td>
<td>10.05</td>
<td></td>
<td>2.40</td>
</tr>
<tr>
<td>Never</td>
<td>21.71</td>
<td>7.78</td>
<td></td>
<td>2.29</td>
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</tbody>
</table>
Fig 1A: Box and whisker plot of the distribution of Perceived Stress scores for TMD controls and TMD cases. The horizontal line within the box is the median value while the lower and upper hinges are the 25th percentile and 75th percentile, respectively. The ends of the whiskers represent the minimum and maximum values. A two-group mean comparison t-test indicates the mean value for controls (19.7, s.e. 0.0) is statistically significant from that of cases (24.8, s.e. 1.2), $P = 0.0007$. 
Fig 1B: Box and whisker plot of the distribution of log$_{10}$ cortisol concentration for TMD controls and TMD cases. The horizontal line within the box is the median value while the lower and upper hinges are the 25$^{th}$ percentile and 75$^{th}$ percentile, respectively. The ends of the whiskers represent the minimum and maximum values. A two-group mean comparison t-test indicates the mean value for controls (2.4, s.e. 0.03) is statistically significant from that of cases (2.2, s.e. 0.05), $P = 0.0002$. 
Fig 2: Scatter plot of the relationship between Perceived Stress score (X-axis) and $\log_{10}$ cortisol concentration (Y-axis) showing the fitted line and 95% confidence interval (n=116 observations). The Pearson correlation coefficient for this relationship is -0.188, $P = 0.044$. 
APPENDIX A

Figure 3: Perceived Stress Scale Questionnaire

In the LAST THREE MONTHS, how often have you:

<table>
<thead>
<tr>
<th>Question</th>
<th>Never</th>
<th>Almost never</th>
<th>Sometimes</th>
<th>Fairly often</th>
<th>Very often</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Been upset because of something that happened unexpectedly?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Felt that you were unable to control the important things in your life?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Felt nervous and &quot;stressed&quot;?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Dealt successfully with irritating life hassles?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Felt that you were effectively coping with important changes that were occurring in your life?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Felt confident about your ability to handle your personal problems?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Felt things were going your way?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Found that you could not cope with all the things you had to do?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Been able to control irritations in your life?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Felt that you were on top of things?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Been angered because of things that happened that were outside of your control?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. Found yourself thinking about things that you have to accomplish?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. Been able to control the way you spend your time?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. Felt difficulties were piled up so high that you could not overcome them?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4: Hair Sample from Vertex Posterior Portion of Head and Scalp

Take the hair sample from the vertex posterior region of the scalp.
1. Frontal
2. Temporal
3. Vertex anterior
4. Vertex posterior
5. Nape
APPENDIX C
Figure 5: Photo Indicating Hair Sample Taken from Vertex Posterior Portion of Head and Scalp
APPENDIX D

Figure 6: Steps in Hair Cortisol Analysis:

Hair Cortisol Analysis
University of Western Ontario

Day 1: Cut, Weigh & Wash

On this day we segment the hair into appropriate segment lengths to reflect the period of interest that is being studied. In this study the last 3 months of cortisol production was studied, the most proximal 3cm of hair was segmented with a box-cutting knife. The knife was wiped with isopropanol in between sectioning hair samples to avoid possible contamination.

Hair samples were weighed on a scale that is precise to 0.01mg. The assay has been validated for hair samples weighing between 10-15mg of hair, so that amount of hair was placed in a scintillation vial.

A standard practice when analyzing hair samples is to wash the hair segments. The purpose of this is to remove external contaminants. Although cortisol is produced endogenously, hair may be contaminated with glucocorticoids from topical creams, and possibly even glucocorticoids excreted in the sweat. This wash is meant to ensure that the cortisol being quantified by the assay is strictly from endogenous secretions.

The wash consists of immersing each hair sample with 3 ml of isopropanol. Hair samples are then placed in an incubator and spun at 0.28g for 3 minutes at room temperature. The isopropanol is then decanted from each vial in sequence. Care is taken to ensure that every strand of hair remains in the vial. This process is repeated again and then the hair samples are allowed to dry for at least 5 hours in a fume hood. There are several compounds which cross-react with the assay and therefore the wash is important to remove external contaminants from the surface of the hair (e.g. transferred topical hydrocortisone cream).

Day 2: Extraction

One ml of methanol is added to each hair sample (in scintillation vial). Using a pair of surgical scissors, the hair is finely minced in the methanol until it is granular in appearance (approx. 3 minutes per sample). The purpose of the mincing is to increase the surface area and maximally expose the medulla of the hair shaft to the solvent, where cortisol is believed to be primarily stored. In between mincing the samples, the scissors are wiped clean with laboratory tissue. Once all of the samples have been minced, they are placed in an incubator and spun at 0.28g for 16 h 50°C. Sixteen hours has been shown to optimally dissolve the cortisol from the hair in the methanol.
*For each kit we also run a homogenized hair sample that we have established a reference hair cortisol concentration range for. This acts as our positive control (in addition to the controls provided by the ELISA kit) to assess consistency with previous kits.

**Day 3: Analysis**

The extraction is now complete, but the cortisol-containing methanol solution cannot be directly run on an ELISA, they must first be dissolved in a more stable solvent. To do this, the vials are removed from the incubator and the 1 ml of methanol (with dissolved hair contents) is pipetted into 5 ml test tubes. The methanol is then evaporated by placing the test tubes on a test tube hot plate set to 50°C and under a steady stream of nitrogen. Following evaporation, a white residue is left at the bottom of the test tubes. To reconstitute the cortisol solution 250μL of phosphate buffered saline is added (the 4 fold change in solvent volume in accounted for later when calculating the hair cortisol concentration).

This cortisol containing solution is then analyzed using an Alpco Salivary ELISA kit (competitive ELISA), running it just at if it were saliva. The only difference we have is that our lab uses a 100 RPM rotation speed rather than a 200 RPM rotation speed.

The microwell plate from the ELISA is read using a plate reader and the optical density of each well is determined and can be read on software called “softmax”. From this software, optical densities are obtained and then corrected (with the blank) in a spreadsheet. These corrected values are used to get an interpolated cortisol content (ng/ml) based off of the standard curve that is produced. This ng/ml concentration is then volume-corrected and corrected by the weight of hair that was used to produce a ng/g value.
REFERENCES


