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Chronic psychosocial stressors and salivary biomarkers in emerging adults

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Summary

We investigated whole saliva as a source of biomarkers to distinguish individuals who have, and who have not, been chronically exposed to severe and threatening life difficulties. We evaluated RNA and DNA metrics, expression of 37 candidate genes, and cortisol release in response to the Trier Social Stress Test, as well as clinical characteristics, from 48 individuals stratified on chronic exposure to psychosocial stressors within the last year as measured by the Life Events and Difficulties Schedule. Candidate genes were selected based on their differential gene expression ratio in circulating monocytes from a published genome-wide analysis of adults experiencing different levels of exposure to a chronic stressor.

In univariate analyses, we observed significantly decreased RNA integrity (RIN) score ($P = 0.04$), and reduced expression of glucocorticoid receptor-regulated genes (Ps < 0.05) in whole saliva RNA from individuals exposed to chronic stressors, as compared to those with no exposure. In those exposed, we observed significantly decreased BMI ($P < 0.001$), increased ever-smoking and increased lifetime alcohol abuse or dependence (P 0.03), and a reduction of cortisol release. In *post hoc* multivariate analyses including clinical and biospecimen-derived variables, we consistently observed significantly decreased expression of *IL8* (Ps < 0.05) in individuals exposed, with no significant association to RIN score. Alcohol use disorders, tobacco use, a reduced acute stress response and decreased salivary *IL8* gene expression characterize emerging adults chronically exposed to severe and threatening psychosocial stressors.

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Conflict of interest

All authors state that there are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence, or be perceived to influence, their work.

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1. Introduction

The stress response engages the hypothalamic pituitary adrenal axis and the sympathetic nervous system, activating or suppressing genes both centrally and in the periphery through the release of hormones at each level of the axis (Chrousos and Gold, 1998). These systems are normally under negative feedback control to respond appropriately to acute risks (flight-or-fight response), but upon chronic stimulation, these biological systems become dysregulated, leading to altered hormonal, cytokine, and metabolic states (McEwen, 2000). Exposure to chronic stressors (social and environmental toxicants) contributes to allostatic load and to disease, including behavioral, metabolic and inflammatory disorders (Cohen et al., 2007; Koob and Kreek, 2007; Miller, 2008; Epel, 2009; Rappaport and Smith, 2010). Identification of biomarkers of exposure to chronic stressors will not only facilitate identification and characterization of individuals suffering from the effects of exposure to chronic stressors, but will also elucidate the mechanisms mediating the etiologic link between chronic stressor exposure and chronic disease.

A biomarker of exposure to chronic psychosocial stressors in leukocyte RNA from adults in multiple distinct studies (Cole et al., 2007; Miller et al., 2008, 2009) is the gene expression signature of reduced glucocorticoid receptor (GR) regulated gene expression and increased nuclear factor kappa B (NF-κB) regulated gene expression. This signature was identified using differential gene expression analyses on DNA microarrays (Schena et al., 1995), gene ontology (GO) analyses (Ashburner et al., 2000) and transcription factor binding motif (TFBM) analyses (Cole et al., 2005). An advantage of utilizing the genomic technologies represented by the work of Cole (Cole, 2010) is the opportunity to identify functional biological elements and to leverage biomedical knowledge to hypothesize mechanisms at the pathway (Cole et al., 2007; Miller et al., 2008, 2009) or gene (Cole et al., 2010) level.

Our objective in this study was to evaluate how emerging adults with high or low levels of exposure to severe life events and difficulties differ in relevant clinical characteristics, salivary analyte metrics, and salivary gene expression. Emerging adulthood (ages 18–25) is a prime transitional period marked by departure from the childhood home and a decline in institutional structure and support (Arnett, 2000), potentially exposing the individual to both chronic and episodic stressors. Since substance use often peaks during emerging adulthood (Schulenberg et al., 2005), and the onset of substance abuse and dependence typically occurs at this time (Compton et al., 2005; Kessler et al., 2005), we included alcohol use disorders and tobacco use in our analyses. We analyzed behaviors and a demographic variable that might influence oral health and disease. We analyzed saliva metrics and several components of whole saliva (human DNA and RNA, bacterial DNA, and cortisol). As a specific biological hypothesis, we analyzed gene expression to observe whether the expression signature of differential exposure to a chronic stressor identified in monocytes derived from whole blood samples in adults was also present in whole saliva from emerging adults differentially exposed to chronic stressors. We chose to study whole saliva samples, as we seek to understand the potential of analyzing saliva collected in community and clinical trial settings for translation toward social genomics, health surveillance and analysis of treatment response applications. Saliva has obvious utility as a diagnostic tissue due to its ease of collection and ongoing efforts to identify salivary biomarkers of both oral cavity and non-oral cavity disease (Kaufman and Lamster, 2002; Streckfus and Bigler, 2002; Gao et al., 2009; Lee et al., 2009; Luther et al., 2010; Zhang et al., 2010).

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2. Methods

2.1. Oregon Youth Substance Use Project (OYSUP) participants

The study cohort of 48 individuals was chosen based on data from the Life Events and Difficulties Schedule (LEDS) obtained at age 21–23 from 123 participants within two grade-based cohorts from the OYSUP, a 15-year ongoing longitudinal study of approximately 1000 participants examining the etiology of substance use in Oregon youth (Andrews et al., 2003). OYSUP began in the 1997–1998 school year with students in five grade cohorts in the first through fifth grade, recruited from a single school district in a working class community in Western Oregon. Using a stratified random sample, parents of 2127 students in 15 elementary schools were sent a letter followed by a phone call describing the project and soliciting participation. We obtained parental consent for 1075 students (50.7%) to participate in assessments for the first four years of the study. An average of 215 students per grade (1st through 5th) participated in the study in the first year with an even distribution by gender (50.3% female, $N = 538$). Participants were comparable to elementary students in the district on race/ethnicity and participation in the free-lunch program (Andrews et al., 2003). However, they had significantly higher scores (albeit a small absolute difference) on academic achievement tests in both reading and math. Students in the study were comparable to students in Oregon on 30-day prevalence of use of all substances in the 6th grade (DHS, State of Oregon, 2000), with the exception of inhalants, where the prevalence of inhalant use was slightly higher in Oregon than in the OYSUP sample.

2.2. Life Events and Difficulties Schedule

The phenotype for this study was defined using a contextually based assessment of participants' episodic and chronic life events and difficulties, The Life Events and Difficulties Schedule (LEDS) (Brown and Harris, 1978). The LEDS, adapted from the native British version for use on a sample of American emerging adults, was chosen to capture a multitude of developmental challenges encountered by the current sample—entering or struggling with college, problems finding a job, having children, moving out of their childhood homes, developing and maintaining romantic and platonic relationships, and achieving financial self-sufficiency. This semi-structured interview identifies acute life events (impacting the individual for up to four weeks) and chronic difficulties (ongoing problems lasting longer than four weeks) in 10 life domains [finances, work, education, housing, health, reproduction, crime/legal, and relationships (partner and others)]. Importantly, the biographical circumstances and context in which the events and difficulties were embedded were elicited from the participant and used to rate both immediate and long-term severity or threat. Using manuals providing thousands of case examples, raters who were blind to the interviewee's subjective responses assign standardized ratings to events and difficulties from cases presented by the interviewer. The LEDS was administered in the morning prior to a lunch break.

2.3. Assessment of alcohol use disorders, tobacco use, oral health behaviors and chronic medical conditions

We assessed the prevalence of alcohol use disorders and tobacco use in the sample to identify potential confounders of the relationship between stressors and differential gene expression. Lifetime smoking status was defined by an affirmative to the question, "Have you ever smoked at least 100 cigarettes in your lifetime?". Current smoking status was defined by an indication of at least one cigarette in answer to the question, "During the past seven days, how many cigarettes did you smoke on a typical day?". Alcohol abuse or dependence diagnoses were based on the structured clinical interview for DSM-IV Axis 1 disorders, the SCID-I (First et al., 2002). A lifetime diagnosis was defined as the presence of abuse and/or dependence at any time during the participant's life, and a current diagnosis

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was defined based on abuse and/or dependence on alcohol present during the last six months. We assessed the use of chewing tobacco using the question: "How many times have you used chewing tobacco or snuff in the last month?", and categorized it as yes if they had chewed tobacco within the last month. We assessed the frequency of dental care by asking whether the last dental visit was more than one year ago. Chronic medical condition status was defined by the answer to the question: "Do you have any chronic medical conditions such as asthma, depression, or Attention Deficit Disorder? (yes/no) If yes, what?". Chronic medical conditions were coded as a "yes" if they reflected a current condition, and "no" if the condition occurred long ago, *e.g.*, having asthma as a child.

2.4. Participant selection

We selected 48 individuals from the first 123 individuals in cohorts (years) 4 and 5 of OYSUP available for evaluation for differential gene expression analysis by qPCR in an attempt to replicate selected candidate genes from the gene expression signature of exposure to a chronic stressor identified by Miller et al. (2008) (Table 1). Among these 123 individuals, 24 (20%) individuals experienced the lowest level of exposure to stressors—no difficulties or only ones with low threat (*i.e.*, no moderate or severe chronic stressors) and no events or only low threat ones (*i.e.*, no moderate or severe episodic stressful events) in the six months before the interview. These individuals (17 males, 7 females) were chosen to comprise the low exposure to chronic stressor (or "low stressor exposure" sample). 31 of 123 (25%) experienced the highest exposure to chronic stressors—an ongoing ($N = 26$, 21%) or recent (within the last four months, $N = 5$, 4%) difficulty rated high in threat. To comprise a "high stressor exposure" sample which was matched for gender to those with low stressor exposure as closely as possible, we chose all available males ($N = 16$) and randomly selected 8 of 15 available females from those who had experienced the highest level of exposure to stressors.

2.5. Trier Social Stress Test (TSST) and cortisol AUCg estimation

The TSST is a standardized laboratory procedure to assess cortisol reactivity to a moderate psychosocial stressor (Kirschbaum et al., 1993b). Participants give a speech about their qualifications for a hypothetical job to two unknown "scientists" rating their behavior, and then they attempt a mental arithmetic task. Since salivary cortisol can be affected by tobacco, food or drink, the TSST was administered at least 2 h after lunch and after a 20 min observed rest period. In order to control for the diurnal variation of cortisol, TSSTs were scheduled between 15:00 h and 19:00 h. In order to control for the effect of estrogen on cortisol, all women were scheduled during the luteal phase of their cycle (days 15–26). The TSST is preceded by two salivary cortisol collections—one occurring following the rest period but preceding introduction to the committee, and another immediately preceding the speech and math tasks. Four collections occur after the TSST—once immediately after the TSST, twice more at 10 min intervals, and another 30 min following the previous sample. Salivary cortisol was assayed by immunoassay with mean intra and inter-assay coefficients of variation of 3.5% and of 5.1% (Salimetrics, State College, PA). We assayed cortisol using two samples per individual, with $r = 0.9986$ and $P < 0.001$. To assess cortisol reactivity, we used the measure of Area Under the Curve with respect to Ground (AUCg) (Pruessner et al., 2003), representing total cortisol secretion across time.

2.6. Gene selection

Candidate test genes were chosen based on transcripts that were observed to be differentially expressed in monocytes from 11 familial caregivers of brain-cancer patients and 10 control subjects (Miller et al., 2008), and based on the number of GR or NF-κB transcription factor binding motifs (TFBM) in the proximal promoter. We included a single candidate test gene based on biological criteria. We selected candidate reference genes for qPCR analysis of

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salivary RNA based on the reference genes used by Vandesompele et al. (2002) and Noutsias et al. (2008). Reference genes were selected based on their *M* values, a measure of gene expression stability (Vandesompele et al., 2002). For each candidate reference gene, the pairwise variation with all other candidate reference genes was calculated as the standard deviation of the logarithmically transformed expression ratios. A reference gene should have a gene stability measure below 0.5 in homogenous sample sets or below 1 in heterogeneous sample sets (Hellemans et al., 2007). Stepwise exclusion of the least stable gene resulted in an ordered gene list ranked by stability. The criteria used to select genes and the list of candidate test and reference genes selected for qPCR analysis can be found in Supplementary File.

2.7. Saliva sample collection

We collected saliva for analysis of salivary RNA from OYSUP participants in the afternoon at least 2 h after the participant had eaten lunch and following the 20 min relaxation period. Saliva biospecimens were collected using commercially available kits (Oragene®DNA Self-Collection Kit, OG-500, and Oragene RNA for Expression Analysis Self-Collection Kit, RE-100, DNA Genotek, Ontario, Canada) using the manufacturer's protocol.

2.8. RNA extraction and metrics

DNA was extracted from saliva samples as previously described using Oragene® purifier and ethanol precipitation (Nishita et al., 2009) using the manufacturer's protocol for manual purification of DNA from 4.0 mL, PD-PR-015 Issue 3.1 instead of 2.0 mL. DNA was quantified by spectrophotometry using a Nanodrop ND-1000 (ThermoScientific, Wilmington, DE), by QuantiT™ PicoGreen®dsDNA Reagent (Invitrogen™, Carlsbad, CA), and qPCR (see Section 2.11). RNA was extracted from saliva according to the manufacturer's protocol and as described in Supplementary File. RNA was quantified by spectrophotometry using a Nanodrop ND-1000 and by Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA). A RNA Integrity Number (RIN score) was generated for each sample (Schroeder et al., 2006).

2.9. Reverse transcription and pre-amplification

RNA samples were standardized to the lowest common concentration (30 ng/μL), as determined by spectrophotometry, using RNase-free water. As a positive control for downstream applications, all 48 RNA samples, each standardized to 30 ng/ μL, were pooled to generate a "pooled saliva" sample. Stratagene QPCR Human Reference Total RNA (Agilent Technologies, La Jolla, CA), derived from pooled cancer cell line RNA, was standardized to 100 ng/μl and used as a positive control sample. As described in Supplementary File, the reverse transcription reaction was performed with negative controls lacking reverse transcriptase (–RT) and with no template (NTC), and was followed by a multiplex pre-amplification reaction.

2.10. qPCR protocol

Microfluidic arrays that enable testing of 48 cDNA samples against 48 Applied Biosystems Taqman® gene expression assays (a total of 2304 reactions) were used to analyze gene expression. A total of six arrays were used, each with 48 assays interrogating nine reference genes and 39 test genes on eight individual RNA samples (four high stressor/four low stressor exposure), as well as a five-point standard curve, commercial reference RNA sample, –RT pooled saliva, and NTC for each gene expression assay, each done in triplicate. A serial dilution curve made from pooled saliva cDNA was included on each array to normalize C_t values across arrays and perform a standard curve analysis. More details on the

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qPCR protocol and the BioMark™ system (Fluidigm, South San Francisco, CA) can be found in Supplementary File.

The output from each array was analyzed in the Fluidigm Real Time PCR Analysis software, as described in Supplementary File. Descriptive statistics and a calibration curve were then generated to represent data from across all six arrays. This included the mean and standard deviation of the triplicate C_t values calculated for each point on the standard curve, as well as the slope, intercept, r^2, and efficiency for each assay calculated based on this curve (Supplementary Table 2).

2.11. qPCR of human and bacterial DNA

The human and bacterial DNA content of salivary DNA was quantified by two qPCR assays. Human nuclear DNA was quantified using the human-specific inter-*Alu* primers described by Walker et al. (2005). Bacterial DNA was quantified using the 16S rDNA primers described by Nadkarni et al. (2002), which have been found to accurately quantify a broad taxonomic mixture of bacteria in clinical samples (Horz et al., 2005). The human and bacterial quantification assays were each performed in triplicate on 1.5 ng total sample DNA, as determined by quantification by QuantiT™ Pico-Green® dsDNA assay, and on standards ranging from 16 to 0.004 ng of human genomic DNA (Clontech, Mountain View, CA) or *Escherichia coli* genomic DNA (Affymetrix, Sunnyvale, CA). Nominal DNA concentrations were based on PicoGreen quantification, except for the standards, for which the manufacturer's specifications were confirmed by NanoDrop spectrophotometry. The absolute mass of human and bacteria DNA was determined by a standard curve for each assay, and the ratio between the quantities was calculated. The median as a measure of central tendency of the replicate values was used to calculate standard curves, because this tended to produce more linear standard curves. The mean CV of the human and bacterial quantification assays were 0.12 and 0.28, respectively.

2.12. Instrument comparison

A validation of the data collected in the Fluidigm BioMark™ instrument was done by collecting qPCR data on four assays in the Applied Biosystems (Foster City, CA) instruments 7900HT and ViiA™ 7. Gene expression assays chosen for the validation interrogated two GR-regulated test genes exhibiting differential expression (*GALC* and *RAB27A*) and two reference genes with the lowest gene expression variance (*CDKN1B* and *YWHAZ*) based on analysis of the individual sample qPCR on the Fluidigm Biomark™ instrument. cDNA samples (12 each from the high and low stressor exposure strata) with varying RIN numbers were run on one 384 well plate on the ViiA™ 7 and the remaining 24 samples were run on the 7900HT instrument. All samples were run in triplicate. Both plates had a five point (1:4) standard curve using pooled pre-amplified saliva cDNA used in the individual qPCR performed on the Fluidigm Bio-Mark™ and a reference RNA dilution.

2.13. Ethics

The protocol for the current study (RC2DA28793), a subset of the OYSUP (RO1DA10767), was reviewed and approved by the Institutional Review Boards of Oregon Research Institute (ORI) and SRI International (SRI). Prior to the assessment, OYSUP participants were provided with a written informed consent form describing the questionnaire, Trier Social Stress Test, clinical interviews, and biospecimen collection protocols to be administered and that information derived from each of these protocols would be linked with other information collected in the OYSUP project. Each of the protocols, the data linkage, and the risks associated with the protocols, were described by OYSUP staff to each potential participant. The rights to withdraw from parts of the project or the entire project at any time were explained to each potential participant. Participants positively indicated in writing their

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desire to participate or not to participate in each of the protocols and signed the written informed consent.

2.14. Statistical methods

Descriptive, correlation, association and mediation analyses of clinical data, RNA metrics and gene expression assay data were performed using SAS v9.2 (Cary, NC) and StataSE 11.2 (College Station, TX). Unadjusted odds ratios and 95% CIs were calculated for demographic variables, alcohol use disorders, tobacco use and behaviors influencing oral health. Comparison of means using appropriate parametric (clinical variables, cortisol, RNA metrics and normalized gene expression measures) or non-parametric tests after log transformation (human and bacterial DNA measures) were performed for continuous variables. False discovery rates (FDRs) (Benjamini and Hochberg, 1995) were calculated from the probability values from the differential gene expression analyses. The primary contrast of interest was to compare the high stressor exposure stratum to the low stressor exposure stratum in a dichotomous case-control design.

2.15. *Post hoc* analyses of differential gene expression

We performed several additional analyses to evaluate the effect of clinical and biospecimen variables. In the first, we performed multivariate regression analysis of gene expression including chronic stressor exposure and RIN score on all genes (Supplementary Table 5). In the second, we performed multivariate regression analysis of gene expression including chronic stressor exposure, RIN score, BMI, cortisol and gender on all genes (Supplementary Table 6). In the third, we performed multivariate regression analysis of genes significantly associated with chronic stressor exposure, including smoking status, lifetime alcohol abuse or dependence and RIN score as covariates (data not shown). In the fourth, we performed multivariate regression analyses of clinical variables (BMI, lifetime alcohol abuse or dependence, smoking status, chewing tobacco, dental visits, chronic disease, and eligibility for a free lunch) and RIN score to evaluate the potential mediation of *IL8* association with exposure to chronic stressors by these variables (Supplementary Table 7). In the fifth, we calculated Cohen's *d* for association with the 37 candidate genes in this study using normalized data, and for the 37 candidate genes using averaged probe data from Miller et al. (2008), previously subjected to the Robust Multichip Averaging method (Irizarry et al., 2003), then calculated correlation statistics (Supplementary Table 8). In the sixth and last *post hoc* analysis, we interrogated the integrated 3′UTR adenylate—uridylate rich element (ARE) database (Halees et al., 2008) to identify ARE containing genes among our test genes and evaluated the distribution of ARE containing genes among differentially and non-differentially expressed genes using Fisher's exact test. We interrogated the same database to identify the prevalence of AREs in differentially expressed genes in previously published studies of genome-wide gene expression of response to chronic stressors (Cole et al., 2007; Miller et al., 2008, 2009), and compared the mean prevalence in these studies to the genome-wide average (Halees et al., 2008).

2.16. Power for gene expression analysis

Prior to the qPCR study, we performed a power analysis using data from an exon array-based genome-wide gene expression analysis of RNA from cell-free saliva that generated a salivary core transcriptome of 851 genes from 16 of 18 healthy individuals (Hu et al., 2008). Two of four reference genes and 26/38 test genes that we nominated for analysis were also available in this dataset (Gene Expression Omnibus (GEO) Accession GSE7760). We used the two sample *t*-test method of Wei et al. (2004), with the following parameters: variance component to be detected (the 90% least variable genes), the desired fold of change (1.5-fold), power to detect this change (80%), and type I error rate (two-tailed alpha equal to 0.01). The required sample size is 20 in each group with these parameters.

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2.17. Resource sharing

Data associated with this study has been deposited in GEO (Accession GSE27144). OYSUP data will become available through the National Addiction & HIV Research Database (http://www.icpsr.umich.edu/icpsrweb/NAHDAP/).

3. Results

3.1. Clinical differences between groups stratified by exposure to chronic stressors

We selected 48 participants from the first 123 individuals in Cohorts 4 and 5 of OYSUP with available saliva samples and LEDS data as the study sample (Table 1). At the time of the assessment, the 48 participants were on average 21.05 years of age (SD = 0.38), 31% female, and 94% self-identified white non-Hispanic race/ethnicity with no significant differences in age, gender or self-identified race/ethnicity between stressor exposure strata (Table 1).

We compared the stressor exposure strata for clinical variables likely to reflect or constitute significant stressors, including stressors that might specifically impact oral health (Table 1). Participants with chronic exposure to severe life events and difficulties were four times more likely to be an ever smoker, or to have a lifetime history of alcohol abuse or dependence (P 0.03) (Table 1). The high stressor exposure stratum had a significantly reduced BMI compared with the low stressor exposure stratum ($P < 0.001$). Although those in the high stressor exposure stratum were about twice as likely to be current smokers or to have a current diagnosis of alcohol abuse or dependence, neither current smoking nor a current diagnosis of alcohol abuse or dependence was significantly associated with exposure to chronic stressors (data not shown). Two variables that describe behaviors that could specifically impact on oral health (*i.e.*, whether participants chewed tobacco in the last month and whether the last dental visit was more than one year ago) were each about two-fold more common in the high stressor exposure stratum, but these did not reach statistical significance (Table 1). Similarly, whether participants had been eligible for a free lunch at baseline enrollment in the fifth grade or endorsed a chronic medical condition such as asthma, depression or Attention Deficit Disorder, was about twofold more common in the high stressor exposure stratum, but not statistically significant (Table 1). The univariate correlation matrix (Table 2) of these clinical variables reveals that the only significant ($P < 0.05$) correlation was that between smoking status and lifetime alcohol abuse or dependence.

There was no significant difference in cortisol reactivity (AUCg) to the Trier Social Stress Test between stressor exposure strata. Participants in the high stressor exposure stratum had a blunted cortisol response, with a mean value less than half that of the low stressor exposure stratum [mean (SD) 15.5 (7.5) and 34.9 (75.5), respectively, $P = 0.22$]. Moreover, there was much greater variability in the cortisol response in the low stressor exposure stratum. We estimated the amounts of human and bacterial DNA in salivary DNA using the Walker et al. and Nadkarni et al. assays and observed no significant differences in the Walker/Nadkarni ratio [mean (SE) −1.3 (10.1) and 2.2 (15.5), respectively, $P = 0.38$] or in the amounts of human [mean (SD) of log transformed median values, 3.3 (0.6) and 3.4 (0.6), $P = 0.78$] or bacterial [mean (SD) of log transformed median, −0.33 (2.2) and 0.07 (1.3), $P = 0.44$] DNA between high and low stressor exposure strata, respectively.

3.2. Saliva and RNA sample characteristics

Mean (SD) saliva volumes overall were 4.5 (0.6) mL, and 4.5 (0.5) and 4.6 (0.6) for high and low stressor exposure strata ($P = 0.81$), respectively. The median (range) of saliva volumes overall was 4.5 (3.5–6.0) mL, and for high and low stressor exposure strata was 4.5 (3.7–6.0) and 4.5 (3.5–6.0) mL, respectively. Summary statistics of RNA metrics are

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provided in Table 3. RNA metrics (yield, AUC or concentration) measured either by spectrophotometry (Nanodrop ND-1000) or electropherogram (Agilent 2100 BioAnalyzer) did not differ significantly between the stressor exposure strata. RNA quantity was more variable and the purity of the RNA (absorbance ratios) was lower in the high stressor exposure stratum, but not significantly so. RNA quality (RIN score and 28S/18S ratio) was lower in the high stressor exposure stratum, significantly so for the RIN score ($P = 0.04$). Similar results were observed when evaluating RNA integrity using individual components of the electropherogram (data not shown). In multivariate analyses exploring the relationship between significant clinical (Table 1) and salivary RNA (Table 3) variables, RIN score was not formally significantly associated with stressor exposure strata when controlling for ever smoking and lifetime diagnosis of alcohol abuse or dependence [$\beta = -0.29$, $P = 0.052$]. The associations between RIN score, chronic stressor exposure, and substance use remain a subject for further study in larger samples from OYSUP. Analyses that include biomarkers of substance use, *e.g.*, cotinine for tobacco smoking, would provide information on possible under-reporting and recent smoking status that might help characterize the relations between psychosocial stressors, substance use and salivary RNA quality.

3.3. Candidate gene TFBMs and gene expression assay performance

We selected genes from the lists of differentially expressed genes by expression ratio (Miller et al., 2008) and based on the number of TFBMs of interest for a total of 18 and 20 genes with previously observed increased and decreased expression, respectively (Supplementary Table 1). 30 of the 38 genes chosen had one or more GR or NF-κB TFBMs. 10 of 18 candidate genes with previously observed increased expression had one or more NF-κB TFBMs and none had GR TFBMs, while 19 of 20 genes with previously observed decreased expression had one or more GR TFBMs and one had a single NF-κB TFBM, respectively. Gene expression assay performance from aggregate standard curve analysis of data from triplicate standard curves on six arrays, *i.e.*, on 18 standard curves, for each of 47 assays (39 test assays and nine reference assays) is described in Supplementary Table 2 and accompanying text (results of analysis of the qPCR performed using the Fluidigm Biomark™). qPCR normalization was performed using the geometric mean of the four most stable reference expression assays (Supplementary Table 3).

3.4. qPCR results

Of the 39 target genes analyzed in our study, two gene expression assays were dropped from further analysis due to a high failure rate (the assay interrogating *CRP* that was added *post hoc* as a biological candidate failed completely, and 25/48 samples failed for the assay interrogating *HLA-DQB1*). We excluded a total of seven individual C_t values (one each from *CX3CR1*, *GNA15*, *NAIP*, *NDUFB7* and *WDR7* and two from *GADD45B*) with normalized Z scores >2.96 prior to gene expression analysis (Grubbs, 1969). After deletion of outliers, only the CSF1R_Hs00911250_m1 assay had a maximum C_t 30 (a mean, standard deviation, minimum and maximum of 24.32, 2.33, 20.14, and 30.94, respectively).

3.5. Differential gene expression

16 of 47 analyzed assays, and two of four selected reference assays, had amplification efficiencies $\leq$90%, which precluded the use of the comparative standard curve (CSC), also known as the $\Delta\Delta C_t$ (Livak and Schmittgen, 2001) method of analysis. Thus, data were analyzed using the relative standard curve (RSC) method (Khan-Malek and Wang, 2011). The RSC method transforms sample C_t values into nanogram (ng) amounts based upon the standard curve produced from the aggregation of data from across all six chips. The normalized signal is the ratio of the transformed ng in the target gene to the geometric mean of the transformed ng amounts of the four reference genes (Vandesompele et al., 2002).

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Of the 37 target gene expression assays analyzed, eight exhibited statistically significant (two-tailed *t* test $P < 0.05$) differential gene expression between the high and low stressor exposure strata (Table 4 and Supplementary Table 4). Of the gene expression assays with some evidence for differential gene expression, six (*GALC*, *IL8*, *NSF*, *RAB27A*, *SLC35A1* and *STX7*) were underexpressed, and two (*GADD45B* and *HSPA1B*) were overexpressed, in the high stressor exposure stratum relative to the low stressor exposure stratum, respectively. The *IL8* gene expression assay exhibited a FDR of 0.044; no other gene expression assay exhibits a FDR < 0.124.

3.6. *Post hoc* analyses

Multivariate analysis with chronic exposure to stressors and with RIN score identified a group of five and seven test genes significantly associated with chronic stressors and RIN score, respectively (Supplementary Table 5); there were no genes significantly associated with chronic stressors and with RIN score. Five genes lost significant association with chronic stressor exposure (*GALC*, *NSF*, *RAB27A*, *SLC35A1*, and *STX7*), and two genes became significantly associated with chronic stressor exposure (*C7orf68* and *GNA15*) when adding RIN score into the multivariate regression.

In the second *post hoc* analysis (Supplementary Table 6), only *IL8* retained significant association with chronic stressor exposure when analyzed together with other clinical variables and RIN score. There were significant differential gene expression associations with BMI (*SERPINB2*), smoking status (*GADD45B*, *GNA15*, *MAFF*, *RPA1*) and gender (*CD9*), but no significant associations with cortisol. The same seven genes were significantly associated with RIN score in this analysis as in the first *post hoc* analysis, suggesting that the gene associations observed with RIN score are not influenced by the clinical variables added to this analysis.

In the third *post hoc* analysis to evaluate the association of each of the significantly associated gene expression assays with stressor exposure adjusting for smoking status, lifetime alcohol abuse or dependence and RIN score, *IL8* was the only gene that retained statistical significance ($\beta = -0.48$, $P = 0.034$) (data not shown).

In the fourth *post hoc* analysis, we evaluated potential mediation of the association of *IL8* with chronic stressor exposure by the clinical variables in Table 1 and by RIN score; we did not observe significant mediation by any clinical variable or by RIN score (Supplementary Table 7).

In the fifth *post hoc* analysis (Supplementary Table 8), we observed non-significant correlations between the calculated effect size (Cohen *d*) of the gene associations with chronic stressor exposure in our study and the calculated effect size of the same genes in the dataset of Miller et al. (2008) (Pearson $r = 0.27$, $P = 0.06$, Spearman $r = 0.27$, $P = 0.10$).

In the sixth *post hoc* analysis, we identified nine test genes containing 3′ UTR AREs (*GALC*, *GBP1*, *HSPA1B*, *IL8*, *MAFF*, *RAB27A*, *SERPINB2*, *STX7*, and *THBS1*) and significant over-representation of ARE-containing genes among differentially expressed test genes (two-tailed Fisher $P = 0.002$). We observed a mean (SD) ARE prevalence of 0.23 (0.02) among differentially expressed genes in the studies of Cole and Miller (Cole et al., 2007; Miller et al., 2008, 2009).

Confirmation of differential *IL8* expression was performed on an ABI ViiA™ 7 instrument in 384-well format using non-preamplified cDNA from the same 48 saliva samples. Two ABI Taqman® Gene Expression assays were tested, Hs00174103_m1, as before, located in exons one and two of *IL8*, and another assay targeting *IL8*, Hs01553824_g1, located in

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exons three and four, with two reference gene expression assays, Hs00153277_m1 and Hs00237047_m1 (*CDKN1B* and *YWHAZ*, respectively). Samples were run in triplicate with 1 μl of unamplified cDNA. The *IL8* assay data from the two gene expression assays tested were highly correlated ($r = 0.992$) and, in univariate analyses, *IL8* exhibited significant differential gene expression as before (dif = −0.449, $t = -3.2$, $P = 0.0027$ and dif = −0.497, $t = -3.2$, $P = 0.0027$) analyses.

4. Discussion

To explore whether whole saliva might be used to identify biomarkers of exposure to chronic stressors, we evaluated clinical features, salivary analyte metrics, and expression of *a priori* selected candidate genes in whole saliva samples from 48 individuals stratified by exposure to chronic stressors. These 48 individuals were selected from an ongoing assessment of five grade-based cohorts of ~1000 participants (Andrews et al., 2003), specifically from a subset of 123 individuals from two grade-based cohorts that had been assessed for exposure to chronic stressors. Our univariate analyses identified RIN score, BMI, smoking status, lifetime alcohol abuse or dependence, and a group of GR-regulated genes significantly associated with exposure to chronic stressors. Association of RIN score with exposure to chronic stressors raises the possibility that some correlate of chronic stressors, *e.g.*, substance use, might be responsible for changes in RNA integrity. Indeed, when adjusted for smoking status and alcohol abuse or dependence, RIN score was no longer formally statistically significantly associated with exposure to chronic stressors. This association may reflect the effect of substance use on oral health, systematic health, or both health domains, perhaps through the number or activation of specific types of cells responding acutely or chronically to substance use that are more likely to degrade RNA. Multivariate analyses with clinical and biospecimen variables significantly associated with exposure to chronic stressors identified significant differential gene expression of *IL8* with exposure to chronic stressors. In multivariate analyses, *IL8* was found to be down regulated in the stratum exposed to high levels of severe life events and difficulties, *i.e.*, in the opposite direction to that previously observed in a study of monocyte gene expression in individuals differentially exposed to the chronic stress of caring for a dying relative (Miller et al., 2008). These results suggest that analysis of RNA from whole saliva has the potential to identify biomarkers of exposure to chronic severe stressors, but that further study of gene expression in different tissues from samples with different clinical characteristics will be necessary to characterize specific gene expression differences (see below).

The strengths of this study include the well-characterized longitudinal cohort, the extensive collection of clinical data and of biospecimens for analyses of cortisol, DNA and RNA, and the quality of the molecular analyses performed on the biospecimen analytes. This permitted exploration of clinical and molecular variables potentially relevant to stressor exposure and to behaviors related to general health and to oral health. We observed associations with health behaviors and clinical variables in the expected direction, *i.e.*, increased prevalence of variables associated with poorer general and oral health in the high stressor exposure stratum. Multiple quantitative assays interrogating different molecular components of saliva (cortisol, human and bacterial DNA, and human RNA) were performed. With respect to saliva characteristics *per se*, the strata did not differ in terms of volumes, RNA mass, or purity, although the high stressor exposure stratum exhibited significantly lower levels of RNA integrity using a sensitive electrophoretic metric. The gene expression assay data was of high quality, with low variation between replicate assays. Some of the gene expression assays resulted in statistically significant associations with chronic exposure to stressors, but the analyses of salivary cortisol and human and bacterial DNA did not reveal significant differences between strata. Multivariate analyses of gene expression with chronic stressor exposures with significantly associated biospecimen and clinical variables reduced the

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number of significantly associated genes, but consistently identified *IL8* as the most significantly associated gene in this small sample of candidates previously nominated in a genome-wide study of response to chronic stressor exposure. We observed some significant gene associations with clinical (*e.g.*, gender, smoking) and biospecimen (*i.e.*, RIN score) variables, though most associations were novel, *i.e.*, did not overlap with those genes observed to be significantly associated with exposure to chronic stressors in univariate analysis. This suggests that a reduction in power for future biomarker discovery is to be expected when adjusting for biospecimen and clinical variation within individuals with exposure to chronic stressors. We investigated the possibility that clinical variables or RIN score could be mediating the association of *IL8* gene expression exposure with chronic stressors but failed to observe evidence for mediation, although power to do so was limited. Notably, *IL8* expression was not associated with RIN score in three *post hoc* analyses.

The two strata are distinguished by significant differences in smoking status and lifetime alcohol abuse or dependence, which are significantly correlated with each other. The large reduction of cortisol release observed after the TSST in the high stressor stratum compared with the low stressor stratum was in the expected direction, given previous evidence for attenuated cortisol response in smokers (Kirschbaum et al., 1993a) and in individuals exposed to chronic stress (Heim et al., 2000). The significant association between exposure to chronic stressors and lifetime alcohol abuse or dependence diagnosis is consistent with evidence associating severity of psychosocial stressors and alcoholism in depressed adults (Zimmerman et al., 1985), life stress in adolescence with alcohol use (Colder and Chassin, 1993), and life events with problem drinking (Steinhausen et al., 2008). Tobacco smoke and other drugs of abuse are known to have profound acute and chronic effects on the human stress response, often contributing to the development of drug dependence (Koob and Kreek, 2007). Thus, in the present study, we observed the expected significant association between exposure to chronic stressors and substance use behaviors in a sample of emerging adults.

The source of human DNA from saliva is thought to derive mostly from peripheral leukocytes transiting the mucosa (Endler et al., 1999), and an obvious corollary is that leukocyte numbers in saliva samples will vary depending upon the level of inflammation in both the peripheral circulation and in the oral cavity. This will complicate the interpretation of transcriptomic biomarkers due to different proportions of inflammatory cells in different individuals and over time in response to stressors and disease. Potential confounding of inflammation in the identification and validation of biomarkers is common to all biomarker studies involving diseases with an inflammatory component, *e.g.*, with cancer (Chechlinska et al., 2010). However, Cole et al. (2011) compared gene expression in a variety of leukocyte cell types, enabling the calculation of a cell type diagnosticity score for each gene, and a mean diagnosticity score for each group of differentially expressed genes for each cell type. This identified plasmacytoid dendritic cells, monocytes and B lymphocytes as the source of differentially expressed genes in individuals stratified by the chronic stressor of subjective loneliness (Cole et al., 2007). Other gene expression analysis methods are available that identify the tissue source of differential gene expression (Chaussabel et al., 2010; Shen-Orr et al., 2010). With large numbers of differentially expressed genes in a given experiment, which will require genome-wide gene expression data, it will be possible to identify the cell types contributing to differentially expressed genes in saliva.

Miller et al. (2008) observed significant over expression of *IL8* in monocytes from middle-aged adults with exposure to a chronic stressor. Interleukin-8 (IL-8, CXCL8) is a member of the chemotactic chemokine protein family that activates neutrophils and stimulates migration of these proinflammatory cells from blood into surrounding tissue. *IL8* expression is regulated by NF-κB activation, but also regulated by the GR (Tobler et al., 1992), where the GR acts to suppress NF-κB activation of transcription of NF-κB targets, including *IL8*,

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through competing with protein P-TEFb, responsible for promoting transcription elongation at ser276-phosphorylated and promoter-bound NF-κB (Beck et al., 2009). In a sample of 74 adult individuals with chronic periodontitis [mean (SE) age 50 (13) years] and 44 younger periodontally healthy individuals [mean (SE) age 31 (8) years], examination of 10 cytokines in cell-free saliva, including IL8, adjusting for age and smoking status, did not identify association between mean salivary cytokine levels and periodontal disease (Teles et al., 2009). Analysis of *IL8* cytokine and transcript levels in cell-free saliva in a sample of 32 patients [mean (SD) age 49.3 (7.5) years] with oral cavity and oropharyngeal squamous cell carcinoma (OSSC) and 32 controls [mean (SD) age 48.8 (5.7) years] demonstrated significant positive association with OSSC with high correlation between transcript and protein levels (St. John et al., 2004). These three examples suggest that the ability to detect a particular association with *IL8* transcript or IL8 protein may be related to the individuals' ages, the tissue sampled, and the health status and behaviors of the individuals examined. These clinical factors may influence the relative proportions of leukocyte subsets in a sample of saliva, or the gene expression behavior of oral salivary glands or epithelial cells. *E.g.*, the Gene Expression Atlas contains curated data on *IL8* gene expression from hundreds of experiments and disease states and many examples of each one of the three possible results (overexpressed, underexpressed, and not differentially expressed) represented (http://www.ebi.ac.uk/gxa/gene/ENSG00000169429?ef=disease_state). Examination of *IL8* differential expression in saliva from a novel sample of individuals exposed to chronic stressors to validate these results is a necessary future step. The correlation of effect sizes between our results and those in Miller et al. (2008) are nominally non-significant. Further tests of the hypothesis that whole saliva and venous monocyte transcriptomes may show similar responses to chronic stressors will require larger numbers of transcripts in samples matched for other clinical factors. The best comparison would be between the different tissues from the same individuals with transcripts measured using the same gene expression analysis platforms. Such analyses would identify and characterize differentially expressed genes shared or not shared between two different tissue types, and would contribute to a deeper understanding of the stress response.

AREs are 3′ UTR sequence motifs that regulate the stability of transcripts and are found in ~10% of transcribed genes in the human genome (Halees et al., 2008). AREs were first observed in cytokine-related genes and their function experimentally demonstrated 25 years ago (Caput et al., 1986; Shaw and Kamen, 1986). The status of immune cell regulation directly affects ARE-containing transcripts, *i.e.*, large number of ARE-containing transcripts are either transiently stimulated and then downregulated, or downregulated immediately after immune cell activation (Raghavan et al., 2004). Wong et al. have evaluated the complexity of the salivary transcriptome (Li et al., 2004), the integrity, sources and stability of RNA in saliva (Park et al., 2006), and the specific regulation of *IL8* by ARE binding proteins in saliva (Palanisamy et al., 2008). We identified that ARE motifs were over represented among differentially expressed genes in whole saliva in our univariate analyses. From these two results, we hypothesized that psychosocial stressors and the human stress response may influence the transcriptome through differences in GR-regulated gene transcription and in the levels of ARE binding proteins, leading to differences in transcription and stability of transcripts with these characteristics. We validated the latter part of this hypothesis by identifying a substantial increase in the prevalence of AREs in differentially expressed genes in the studies of Cole and Miller (Cole et al., 2007; Miller et al., 2008, 2009) over the prevalence in annotated genes. Because these prior results were based on analyses of venous blood leucocyte gene expression, the over-representation of ARE motif containing genes among differentially expressed genes in the human stress response should be observable in other tissues that contain leucocytes.

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Future analyses will require additional clinical and molecular data to elucidate the effects of stressors on the salivary transcriptome. For example, the measurement of salivary cotinine (Zevin et al., 2000) would inform us objectively of recent tobacco use as well as the level of exposure to secondhand smoke (Benowitz et al., 2009), where tobacco use has been associated with similar changes in gene expression in nasal, buccal and bronchial tissues (Sridhar et al., 2008). The measurement of salivary alpha-amylase (RNA or protein) would inform us of the extent of sleep deprivation (Seugnet et al., 2006), which has been associated with increased cortisol levels in children (Raikkonen et al., 2010) and adults (Leproult and Van Cauter, 2010). Meta-genomic analysis would reveal the bacterial diversity of saliva samples (Nasidze et al., 2009), where family stressors and increased salivary cortisol have been associated with increased cariogenic bacteria load and dental caries (Boyce et al., 2010). The measurement of salivary metabolites might inform us of the presence of oral inflammation and disease through increased inflammatory mediators, bacterial amino acids and decreased levels of anti-oxidants, which have been associated with peridontitis in gingival crevicular fluid (Barnes et al., 2009). Proteomic analyses would enable validation of transcriptomic findings and potentially identify differentially expressed salivary proteins without identified salivary transcripts (Steiling et al., 2009). Future analyses of saliva samples will benefit from information from different molecular analysis technologies to distinguish the effects of psychosocial stressors, smoking, sleep deprivation and the presence of oral disease on salivary gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.psyneuen. 2011.11.010.

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Table 1

Clinical characteristics, by chronic exposure to severe life events and difficulties.

Stratum	Chronic exposure	No chronic exposure	P^a	Unadjusted OR (LCI, UCI)[b]
N (%)	24 (50)	24 (50)		
Age mean (SD) years	20.9 (0.33)	21.1 (0.41)	0.07	
BMI	26.1 (6.3)	28.7 (8.8)	<0.001	
Gender				
Male	16 (67)	17 (71)		
Female	8 (33)	7 (29)	0.76	0.82 (0.20, 3.3)
Self-identified race				
White	23 (96)	24 (100)		
Native American	1 (4)	0 (0)	>0.99	
Self-identified ethnicity				
Hispanic	2 (8)	1 (4)		
Non-Hispanic	22 (92)	23 (96)	>0.99	
Smoking				
Ever	13 (54)	5 (21)		
Never	11 (46)	19 (79)	0.02	4.5 (1.1, 20.1)
Lifetime alcohol abuse or dependence				
Yes	11 (46)	4 (17)		
No	13 (54)	20 (83)	0.03	4.2 (1.2, 15.4)
Chew tobacco, last month				
Yes	4 (17)	2 (8)		
No	20 (83)	22 (92)	0.67	
Last dental visit >1 year				
Yes	13 (54)	7 (29)		
No	11 (46)	17 (71)	0.08	2.9 (0.88, 9.26)
Chronic health condition				
Yes	10 (42)	5 (21)	0.11	2.7 (0.65, 12.3)
No	14 (58)	19 (79)		
Eligible for free lunch (baseline)				
Yes	13 (54)	8 (33)		
No	11 (46)	16 (66)	0.15	2.4 (0.75, 7.47)

[a] *t*-test, Pearson χ^2 or Fisher exact test P value.

[b] OR and Cornfield's 95% CI.

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Table 2

Correlation among clinical variables in 48 OYSUP participants.

	Stressor exposure level	BMI	Smoking	Alcohol	Chew tobacco	Dental visit	Free lunch	Chronic health condition
Stressor exposure level	1							
BMI	0.17	1						
Smoking	0.34	−0.13	1					
Alcohol	0.31	−0.12	0.59*	1				
Chew tobacco	0.21	−0.04	0.34	0.21	1			
Dental visit	0.25	0.04	0.31	0.25	0.29	1		
Free lunch	0.21	0.06	0.18	0.13	−0.04	0.19	1	
Chronic health condition	0.22	0.03	0.13	−0.16	−0.01	0.07	0.13	1

* $P < 0.05$.

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Table 3

Salivary RNA metrics, by chronic exposure to severe life events and difficulties.

Stress	NanoDrop ND-1000 metrics				Agilent 1200 Bioanalyzer metrics			
	Yield[b]	Conc.[c]	260/280[d]	260/230[e]	RIN[f]	AUC[g]	Conc.[h]	28S/18S[i]
High								
Mean	4.59	191.16	1.96	1.51	6.02	157.90	84.08	0.83
SD[a]	5.23	217.78	0.06	0.36	2.78	227.41	114.47	0.60
Median	2.44	101.56	1.99	1.55	7.0	53.15	34.0	1.0
Minimum	0.73	30.46	1.76	0.72	1.0	2.3	2.0	0.0
Maximum	19.69	820.23	2.02	2.03	9.6	823.1	412	2.2
Low								
Mean	4.26	177.48	1.99	1.63	7.50	145.01	83.50	1.28
SD	2.39	99.65	0.05	0.46	1.96	101.67	59.75	1.08
Median	3.79	158.03	2.00	1.76	7.9	119.60	66.50	1.0
Minimum	1.07	44.42	1.80	0.16	2.1	21.4	20	0.0
Maximum	9.88	411.82	2.05	2.06	9.8	451.3	230	6.1
P	0.781	0.781	0.097	0.248	0.039	0.802	0.983	0.086

[a]Standard deviation.

[b]Yield in µg.

[c]Conc. in ng/µL.

[d]260/280 = 260 nm/280 nm ratio.

[e]260/230 = 260 nm/230 nm ratio.

[f]RNA Integrity Number.

[g]Area Under the Curve.

[h]Conc. = ng/µL DNA concentration.

[i]28S/18S = 28S/18S RNA ratio.

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Table 4

Genes exhibiting significant differential expression in saliva by chronic exposure to severe life events and difficulties.

Gene	M^a (SD^b) [H^c]	M (SD) [L^d]	P^e	Ratio^f
GADD45B	1.10 (0.85)	0.64 (0.33)	0.023	1.72
GALC	0.52 (0.41)	0.88 (0.48)	0.018	0.58
HSPA1B	1.60 (1.59)	0.80 (0.44)	0.026	2.00
IL8	0.24 (0.24)	0.55 (0.36)	0.001	0.44
NSF	0.53 (0.27)	0.70 (0.23)	0.036	0.76
RAB27A	0.41 (0.24)	0.57 (0.21)	0.026	0.72
SLC35A1	0.56 (0.29)	0.77 (0.29)	0.034	0.72
STX7	0.41 (0.25)	0.57 (0.27)	0.041	0.72

[a] Mean normalized ratio ng target to geometric mean of ng reference genes.

[b] Standard deviation.

[c] H = chronic exposure to severe life events and difficulties.

[d] L = no chronic exposure to severe life events and difficulties.

[e] P from two-sided t-test of differential gene expression (H–L strata).

[f] Ratio of mean H/mean L.