Title

Salivary cytokines as a minimally-invasive measure of immune functioning in young children: correlates of individual differences and sensitivity to laboratory stress.

Journal

Developmental Psychobiology, 57(2)

Authors

DiPietro, Janet
Bandeen-Roche, Karen
Johnson, Sara
et al.

Publication Date

2015-03-01

DOI

10.1002/dev.21271

Peer reviewed

Published in final edited form as:

Dev Psychobiol. 2015 March ; 57(2): 153–167. doi:10.1002/dev.21271.

Salivary Cytokines as a Minimally-Invasive Measure of Immune Functioning in Young Children: Correlates of Individual Differences and Sensitivity to Laboratory Stress

Jenna L. Riis[1], **Douglas A. Granger**[1,2,3], **Janet A. DiPietro**[1], **Karen Bandeen-Roche**[4], and **Sara B. Johnson**[1,2,5]

[1]Department of Population, Family and Reproductive Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, jriis1@jhu.edu

[2]Institute for Interdisciplinary Salivary, Bioscience Research Arizona State University, Tempe, AZ

[3]Department of Acute and Chronic Care, Johns Hopkins School of Nursing, Baltimore, MD

[4]Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

[5]Department of Pediatrics, Johns Hopkins School of Medicine, Baltimore, MD

Abstract

There is growing interest in minimally-invasive measures of environmentally-responsive biological systems in developmental science. Contributing to that endeavor, this study explores the intercorrelations, correlates, and task-sensitivity of proinflammatory salivary cytokines in childhood. Saliva was sampled from 125 healthy five-year old children (49% male) across a series of cognitive and emotional challenge laboratory tasks. Samples were assayed for cytokines (IL-1β, IL-6, IL-8, TNFα), and markers of hypothalamic–pituitary– adrenal (HPA) and autonomic nervous system (ANS) activation (salivary cortisol and alpha-amylase [sAA]). Cytokines were positively intercorrelated and task-sensitivity varied. Except IL-8, cytokines were elevated in children with oral health issues and tobacco smoke exposure. Among boys, cytokines were positively related to sAA and negatively related to cortisol. The findings suggest that in healthy children, salivary cytokine levels reflect compartmentalized oral immune activity. Associations between ANS and HPA activity and cytokines in saliva may present opportunities for minimally-invasive methods to explore neuroendocrine-immune interactions during development.

Keywords

saliva; cytokines; salivary alpha-amylase; cortisol; children; acute stress

Correspondence to: Sara B. Johnson, 200 N Wolfe St, Room 2017, Baltimore, 21287, MD.

Conflict of interest: In the interest of full disclosure, we note that DAG is founder and Chief Scientific and Strategy Advisor at Salimetrics LLC (Carlsbad, CA), and this relationship is managed by the policies of the committees on conflict of interest at the Johns Hopkins University School of Medicine and the Office of Research Integrity and Adherence at Arizona State University.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

INTRODUCTION

There is growing interest among researchers and health care professionals in the calibration and functioning of the neuroendocrine–immune (NEI) network and its role in child health and development (Granger, Granger, & Granger, 2006; Johnson, Riley, Granger, & Riis, 2013; ThyagaRajan & Priyanka, 2012). Of particular importance is the impact of the early environment in shaping the development and regulation of the central nervous system (CNS) and the immune system (Johnson et al., 2013). Understanding the influence of early-life adversity and stress on NEI functioning is foundational to understanding and addressing the development and progression of inflammatory-related diseases in later life (e.g., Shonkoff, 2012).

Key to expanding NEI research in children is the development of minimally-invasive biomeasures that are valid, reliable, and feasible for use in a young population. Saliva collection is a minimally-invasive, low-cost, and socially acceptable method of obtaining biomeasure data (Granger & Johnson, 2013). Technical advances now allow us to measure in oral fluids many of the intracellular regulatory molecules that facilitate communication and coordination between the immune system (cytokines), hypothalamic–pituitary–adrenal axis (HPA; cortisol), autonomic nervous system (ANS; alpha-amylase), and behavior (see Granger et al., 2012 for review).

Despite increasing interest in the NEI network in early child development and the demand for minimally-invasive biomeasures, little is known about the nature and meaning of individual differences in salivary cytokines in young children. To our knowledge, only two studies have examined salivary cytokines in pre-pubertal youth, and these studies only examined interleukin-6 (El-Sheikh, Buckhalt, Granger, Erath, & Acebo, 2007; Keller, El-Sheikh, Vaughn, & Granger, 2010). Fundamental questions regarding salivary cytokine detection rates, variation, intercorrelations, stress-sensitivity, and correlations with measures of ANS and HPA activity, as well as associations with health and demographic factors in early childhood remain unanswered. In the present study, we begin to address several knowledge gaps by analyzing cytokines and key markers of the activity of the NEI network in saliva samples collected across a series of challenging laboratory tasks designed to elicit negative emotions and coping behaviors in healthy young children.

Conceptual Issues

Like the CNS, the immune system is tasked with adapting the body’s internal environment to meet the demands and challenges of the external environment. This adaptive calibration of the CNS and immune system is important for both within-system and across-system functioning and regulation. Environmental sensitivity is particularly important during early childhood when both systems are undergoing developmental change (Coe & Lubach, 2003; Miller, Chen, & Zhou, 2007). Communication between the CNS and immune system is essential for establishing homeostasis and allostasis (McEwen, 1998; Johnson et al., 2013). A set of shared chemical messengers (i.e., cytokines, hormones) and direct cell-to-cell interactions allow for the integration and reciprocal regulation of the CNS and immune system.

Cytokines are the primary intercellular protein messengers of the immune system. In addition to regulating immune processes, cytokines facilitate communication between the immune system and the rest of the body, and they are critical for development, growth, and healing. Cells throughout the body and CNS produce and release cytokines. Secretion of cytokines often involves new protein synthesis, however, some cytokines are produced and stored in intracellular granules for rapid release. Cytokines have multiple target cells and mechanisms of action; their effects are often redundant or synergistic. There are several classes of cytokines. Within the interleukin subclass, interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8), and tumor necrosis factor alpha (TNFα), are the four critical signaling molecules involved in initiating and maintaining inflammation (Clough & Roth, 1998; Owen, Punt, & Stranford, 2012).

These cytokines also coordinate and regulate communication within the NEI network, and their levels are influenced by stress-related activity in the ANS and HPA axis (see Irwin & Cole, 2011 for review). For instance, activation of the ANS in response to acute stress is associated with the release of noradrenaline and a subsequent increase in inflammatory cytokines (Doan, Melvold, Viselli, & Waltenbaugh, 2008; Irwin & Cole, 2011). Rising levels of inflammatory cytokines stimulate the HPA axis to release cortisol which then inhibits inflammatory cytokine production (e.g., Irwin & Cole, 2011). This negative feedback loop between the immune system and the CNS (e.g., Irwin & Cole, 2011; Bilbo & Schwarz, 2012) is critical to regulating inflammation and maintaining health. Dysregulation of this feedback loop can result in chronic inflammation and excessive cytokine secretion which has been associated with “sickness behaviors” (e.g., depressed mood and decreased appetite), and autoimmune and neurologic diseases (Dantzer, O’Connor, Freund, Johnson, & Kelley, 2008; Thomson & Lotze, 2003). On the other hand, insufficient immune functioning increases susceptibility to disease (see Granger et al., 2006 for review).

The Present Study

To our knowledge, this is the first systematic exploration of the nature and correlates of salivary cytokines in early childhood. We examined cytokine intercorrela-tions and associations with demographic and health factors, including measures of systemic and oral health, for salivary IL-1β, IL-6, IL-8, and TNFα in five-year old children. To investigate relations between cytokines and measures of ANS and HPA activity within the oral cavity, we also examined associations between cytokines and salivary alpha-amylase (sAA) and cortisol. We tested three hypotheses. In addition to these hypotheses, we explored changes in cytokine levels across a series of cognitive and emotional challenge tasks without a priori hypotheses regarding the direction and magnitude of cytokine change.

Hypothesis 1. Salivary Cytokine Activity is Positively Intercorrelated Across a Series of Cognitive and Emotional Challenge Tasks

The salivary cytokines examined (IL-1β, IL-6, IL-8, TNFα) are proinflammatory and share regulatory mechanisms (Elenkov, 2008). Findings from studies examining salivary cytokines in older youth suggest a coordinated immune response in the oral cavity and strong inter-cytokine correlations (Riis et al., 2013). It is hypothesized that the inter-cytokine associations in young children are similar to those observed in older youth. Although no

studies were identified that examined proinflammatory cytokine intercorrelations after acute stress, the cytokines examined have common regulatory mechanisms mediated by the activation of the ANS and HPA axis (Elenkov, 2008). Therefore, cytokine levels post-stress are hypothesized to be related to the ANS and HPA axis stress response and highly intercorrelated.

Hypothesis 2. Salivary Cytokine Activity is Positively Associated With Oral Health Issues and Unrelated to Demographic and Systemic Health Factors

The immune system is highly compartmentalized. Salivary cytokines are produced within the oral cavity to respond to local immunological threats (Gröschl, 2009). Not surprisingly, previous studies show consistent positive associations between oral health problems and salivary cytokine levels in older youth and adults (Gornowicz et al., 2012; Gümüş, Nizam, Lappin, & Buduneli, 2013; Gursoy et al., 2009; Heikkinen et al., 2010; Nibali, Fedele, D'Aiuto, & Donos, 2012; Nishida et al., 2006; Rathnayake et al., 2013). Systemic health and demographic factors, however, are largely unrelated to levels of salivary cytokines (Nishanian, Aziz, Chung, Detels, & Fahey, 1998; Riis et al., 2013; Sjögren, Leanderson, Kristenson, & Ernerudh, 2006; Williamson, Munro, Pickler, Grap, & Elswick, 2012). It is hypothesized that cytokine levels in the saliva of healthy young children will follow the same patterns as observed in older youth and adults.

Hypothesis 3. Salivary Cytokines Are Positively Associated With ANS Activity and Negatively Associated With HPA Activity

The immune system and the ANS and HPA axis are reciprocally regulated (Irwin & Cole, 2011). Although not widely studied, two investigations reported negative associations between IL-6 and HPA activity in the oral cavity in adults (Izawa, Sugaya, et al., 2013b; Moons, Eisenberger, & Taylor, 2010). Izawa, Sugaya, et al. (2013) also found that salivary IL-6 was positively associated with ANS activity in adults participating in an acute stress task.

Numerous studies reveal that salivary cortisol and sAA provide important information regarding HPA and ANS activation in response to stress (Granger, Kivlighan, El-Sheikh, Gordis, & Stroud, 2007; Hellhammer, Wüst, & Kudielka, 2009; Vining & McGinley, 1987; Nater & Rohleder, 2009). The mechanisms underlying cytokine stress-reactivity in saliva are not well understood, and reactivity may reflect both immune system as well as ANS and HPA processes. Coordination between the psychobiology of stress and salivary cytokines could open up new opportunities for developmental studies linking brain, behavior, immunity, and health.

Cytokine Change in Response to Challenge

While changes in cytokine levels across the study visit may be partially driven by CNS activity in response to the challenge tasks, little is known about the nature and magnitude of stress-related change in salivary cytokines. Limited previous research suggests that salivary cytokines may be associated with negative emotional states. Three studies found increases in oral IL-6 in adults after social stressor tasks (Chiang, Eisenberger, Seeman, & Taylor, 2012; Izawa, Sugaya, et al., 2013b; Slavich, Way, Eisenberger, & Taylor, 2010). Higher levels of

salivary IL-6 are also associated with negative emotions and mental states (Sjo¨gren et al., 2006; Keller et al., 2010; Moons et al., 2010). In addition, salivary IL-1b has been associated with the activation of brain areas involved in emotional processing in adults (O'Connor, Irwin, & Wellisch, 2009). Little research, however, has focused on salivary cytokine associations with cognitive stress. There is also limited research in children. A small study of 8–9 year olds found an increase in salivary IL-6 in girls and a decrease in IL-6 in boys after two stressor tasks (one cognitive and one emotional; El-Sheikh et al., 2007). Given the limited information regarding the nature of acute cognitive and emotional stress-related change in salivary cytokines in young children, the changes in cytokine concentrations across the study visit were explored without specific a priori hypotheses.

MATERIALS AND METHODS

Participants

Participants were recruited from a pool of mothers who participated in a fetal development study conducted from 2006 to 2007 and from the local community in Baltimore, Maryland. Mother-child pairs were recruited from March 2011 to July 2013. Enrollment in the fetal development study in 2006–2007 was limited to relatively low-risk non-smoking women with healthy, singleton pregnancies (additional eligibility criteria are described in DiPietro et al., 2010). Mothers from the fetal development study were self-referred and mostly well-educated, married, and white (DiPietro et al., 2010). To increase the diversity of the sample, mother-child pairs were also recruited from Baltimore City through community postings and fliers. To be enrolled in the current study, children had to be five years old, and mothers and children had to be fluent in English. Children with significant health conditions or developmental disabilities that impaired cognitive, motor, or regulatory functioning were excluded. Community enrollment accounted for 64% of the sample.

Data were collected from 151 mother-child dyads. Of these pairs, three were unable to complete the study protocol, and 23 children had insufficient salivary data. The current investigation analyzed data from the 125 children with complete saliva data for all samples. Children with (n = 125) and without (n = 23) complete saliva data were similar with respect to age, sex, recent health, current sickness, oral health issues, and body mass index (BMI). Approximately half the sample was male, about a third was white, and the majority was in good health (Table 1). On the day of the study, about 19% were sick (e.g., runny nose or cough), and approximately 22% used an over-the-counter or prescription medication in the two days prior (Table 1). Also, a considerable proportion of the sample had oral health issues (including bleeding gums and very loose teeth), or were exposed to tobacco smoke (Table 1).

Procedures

The Johns Hopkins Bloomberg School of Public Health Institutional Review Board approved the study protocol. Mothers provided written informed consent. Figure 1 shows the study protocol for children. The 90-min study visit began with a free play session for the mother and child participants to allow the child to acclimate to the study room. After free play, mothers completed a survey, and children participated in a series of behavioral and

neuropsychological assessments. Children completed the Peabody Picture Vocabulary Test-Fourth Edition (Dunn & Dunn, 2007) and two cognitive challenge tasks that tested inhibitory control (the Silly Sounds Game and the Pig Game; Willoughby, Blair, Wirth, & Greenberg, 2010; Willoughby, Wirth, & Blair, 2012). Children also participated in three age-appropriate emotional challenge tasks: the Disappointing Gift Game, the Not Sharing Game, and Mischel's Delay of Gratification Task (Mischel & Mischel, 1983) (described in detail below). Four saliva samples were collected from children throughout the study visit (two samples before the emotional challenge tasks and two after these tasks). The majority (88%) of study appointments were conducted in the afternoon, and the elapsed time from child waking to study appointment was not associated with age, sex or health factors (except overweight and obese children were awake for longer when the appointment began compared to underweight and children of a healthy weight [$t(123) = -3.95$, $p < .01$]).

Emotional Challenge Tasks—The Disappointing Gift Game assessed the child's response to disappointment (Cole, Zahn-Waxler, & Smith, 1994). During Part 1 of this game, the child ranked six potential gifts (including undesirable items [e.g., a sock]) from favorite to least favorite. Part 2 of this game occurred approximately 15 min later when the child was given his/her least favorite gift. After receiving the gift, two study evaluators asked the child a series of questions that required the child to confront his/her disappointment, as well as the evaluators' authority (e.g., "Did you get a prize? Is this the prize you wanted?"). This task has been shown to challenge behavioral control and to elicit negative emotions (e.g., anger) in young children (Cole, Martin, & Dennis, 2004; Cole et al., 1994; Garrett-Peters & Fox, 2007; Jahromi & Stifter, 2008; Kieras, Tobin, Graziano, & Rothbart, 2005).

The Not Sharing Game is an emotional stressor and test of emotional regulation (LabTAB of Texas at Arlington, 2012; Spinrad et al., 2009). In this game, an evaluator unevenly distributed candy between herself and the child. Throughout this task, the evaluator asked the child a series of questions to elicit his/her feelings about the game and about the evaluator's behavior. This task has been shown to elicit negative emotions (e.g., frustration) and to increase salivary cortisol in young children (Spinrad et al., 2009).

The Delay of Gratification Task is an emotional stressor and test of coping abilities (Mischel & Mischel, 1983; LabTAB of Texas at Arlington, 2012). The child was left alone in the study room with either a marshmallow or a pretzel. The child was told that the evaluator must briefly leave the room, and he/she was asked to not eat the treat while the evaluator was away. The child was told that if he/ she waited for the evaluator to return on her own (i.e., without ringing a bell that would summon the evaluator), he/she would be receive two treats. The child was left alone with the treat for 8 min or until he/she rang the bell. This task has been shown to elicit negative emotions and correlate with inhibitory control measures in young children (Gagne, Hulle, Aksan, Essex, & Goldsmith, 2011; Mischel & Mischel, 1983; Jahromi & Stifter, 2008).

Health and Demographic Data—An evaluator measured child height and weight. Children were classified as underweight or healthy weight versus overweight or obese based on the Centers for Disease Control and Prevention age- and sex-specific guidelines (Centers

for Disease Control and Prevention, 2001, 2011). Maternal smoking, child medication use in the last 48 hr, and child oral health issues, race/ ethnicity, and recent health were based on maternal report. Dummy variables were created for the child's use of any medication, and use of allergy or asthma medication. Current child oral health issues, based on mother-reported cuts or sores in the mouth, very loose/recently lost teeth, bleeding gums while brushing, or untreated cavities, were coded as yes/no. Child race/ethnicity was reported by mothers and data were dichotomized (white versus non-white), because of low percentages of Asian/Pacific Islanders and Native Americans/ Alaskan Natives in the sample (Table 1). Child current illness and recent health were assessed with the questions "Is your child currently feeling sick or ill? (e.g., runny nose, fever, cough, aching, etc.)" (yes/no), and "Compared to others his or her age, would you say your child's health in the last two days (48 hr) has been: (responses: excellent; very good; good; fair; poor)." Responses were dichotomized into excellent/ good health versus fair/poor health.

Timing Data—To assess the impact of circadian patterns on cytokine concentrations, a time since waking variable was created using maternal report of child's wake time and the study visit start time. A task timing variable was also created that indexed the elapsed time between Part 2 of the Disappointing Gift Game and each saliva sample. The task timing variable was used to adjust for the differential timing of emotional challenge tasks across children. Figure 1 shows the timing of the tasks and samples; the timing of the saliva samples was unrelated to age, sex, and health variables.

Salivary Analyte Data

Following Granger, Kivlighan, Fortunato, et al. (2007b), whole unstimulated saliva was collected by passive drool into a 2 mL cyrogenic vial for 3 min or until 0.75 mL of fluid was collected. Saliva samples were stored at −20°C until assayed. Saliva samples were assayed following Granger, Kivlighan, El-Sheikh, Gordis, and Stroud (2007a) for sAA by kinetic reaction assay. Salivary alpha-amylase results were determined in U/mL. For sAA, intra-assay variation computed for the mean of 30 replicate tests was less than 7.5%, and inter-assay variation computed for the mean of average duplicates for 16 separate runs was less than 6%. Cortisol was analyzed in duplicate using a commercially available enzyme immunoassay without modification to the manufacturer's protocol (Salimetrics, Carlsbad, CA) with a range of sensitivity from .007 to 3.0 μg/dL. Intra- and inter-assay coefficients of variation for cortisol were less than 5 and 10%, respectively.

Salivary cytokines were measured following Riis and colleagues (2013) using a 4-plex 96-well format electro-chemiluminescence immunoassay manufactured by Meso Scale Discovery (MSD, Gaithersburg, MD). Each well of each 96-well plate was coated with capture-antibodies to IL-1β, IL-6, IL-8, and TNFα. Detection antibodies were coupled to SULFOTAG™ labels that emit light when electrochemically stimulated via carbon-coated electrodes in the bottom each microwell. The 4-plex Multi-Spot Array assay was run following the manufacturer's recommended protocol without modification. Cytokine concentrations were determined with MSD Discovery Workbench Software (v. 3.0.17) using curve fit models (4-PL with a weighting function option of $1/y^2$). Lower limits of detection (LLD) and intra-assay coefficients of variation were as follows: IL-1β (0.06 pg/mL, 4.4%),

IL-6 (0.09 pg/mL, 4.8%), IL-8 (0.05 pg/mL, 2.1%), and TNFα (0.11 pg/mL, 6.6%). The detection rates for the four salivary cytokines ranged from 95% to 100% in the 500 saliva samples (125 children with four samples each). Only three saliva samples for IL-6 and 25 samples for TNFα had concentrations below the assays' lower limit of detection.

For all analytes, concentrations below the assay's LLD were estimated using the curve model for the plate. All biomeasure data distributions were very skewed (range across the four samples [skew, kurtosis] for IL-1β: 3.07–6.23, 14.68–51.50; IL-6: 3.40–6.47, 15.04–52.87; IL-8: 2.57–4.77, 10.60 – 30.98; TNFα: 4.16–8.90, 24.29–88.87; sAA: 1.96–4.13, 7.95–29.52; cortisol: 2.26–12.00, 4.37–10.97).

Statistical Analyses

Salivary analyte data were log-transformed and Winsorized to improve the normality of the distributions (Tabachnik & Fidell, 2001). Winsorization was performed by analyte and saliva sample separately. During Winsorization, ≤1% of each cytokine's concentrations, 2% of sAA concentrations, and 4% of cortisol concentrations were changed. Individual trajectories across saliva samples were maintained for all analytes during the Winsorization process, so Winsorization thresholds varied slightly by analyte; cytokine and sAA values were brought within three standard deviations of the mean, and cortisol values were brought within 3.7 standard deviations.

Winsorization and transformation improved the distribution of the analytes (range across the four samples [skew, kurtosis] for IL-1β: 0.28–0.48, 2.77–3.36; IL-6: 0.35–0.63, 2.88–3.34; IL-8: 0.27–0.63, 2.70–3.94; TNFα: −0.20–0.44, 3.42–3.85; sAA: −0.61–0.33, 3.28–4.56; cortisol: −1.81–1.03, 4.72–6.55). No consistent associations between flow rate and raw sAA and cytokine concentrations were found, so, following recommendations by Granger and colleagues (2012), data were not adjusted for salivary flow rate. We first explored the raw data, then log-transformed and Winsorized data were used in all statistical analyses.

T-tests and Pearson correlations were used to examine differences in task timing (i.e., elapsed time from Part 2 of the Disappointing Gift Game to saliva samples) by age, sex, and health factors. Paired *t*-tests examined changes in sAA and cortisol across samples and differences between cytokine concentrations at each sample. To test our first hypothesis, intercorrelations among cytokines at each sample were examined using Pearson correlations and a principal components analysis. For each set of *t*-tests and Pearson correlations, tests of statistical significance were two-sided with an alpha of .05, and Bonferroni-corrected alpha levels (.05/ number of tests) were also examined.

To test our second hypothesis, multilevel mixed models examined associations between cytokines and demographic and health factors. Each cytokine was modeled separately as an outcome with robust variance. Models included a random intercept and an autoregressive (AR-1) residual error matrix to account for the correlation of sample concentrations within subjects and correlated errors across samples. The association between each cytokine and each demographic and health factor was examined separately in models adjusted for the task timing variable. Variables associated with cytokines at $p \leq .1$ were selected as covariates in

fully adjusted models for each cytokine. Backwards stepwise regression with a retention threshold of $p < .05$ was used to create parsimonious adjusted models for each cytokine.

Furthermore, because we were interested in exploring the extent and nature of task-related change in cytokine levels, we examined the nonlinearity of cytokine trajectories across the study visit using a spline knot. With no a priori hypotheses about the timing of task-related change in cytokine levels, we tested the appropriateness of a knot placed at sample 2 or 3 in both null and parsimonious adjusted models. Spline models used a parameterization describing the intercept, the slope over the first samples (sample 1–2 or sample 1–3), and the slope over the last samples (sample 2–4 or sample 3–4), rather than the slope over the first samples and the deviation of slope over the first versus last samples. Differences between slope terms were examined using post-estimation tests, and significant differences suggested a change in cytokine slope beginning at the spline knot. For each cytokine, likelihood-ratio tests were used to select the most appropriate model (spline model with knot at sample 2, spline model with knot at sample 3, or linear model).

To test our third hypothesis, sAA and cortisol were added separately as independent variables to the final model for each cytokine. Based on literature that suggests that NEI relations may vary by sex, we also examined the interaction between sex and sAA or cortisol on each cytokine (Prather et al., 2009; Rohleder, Schommer, Hellhammer, Engel, & Kirschbaum, 2001).

Missing data were less than 2.5% for all variables except child age. Child age in months was missing for 8.8% of the sample (although all children were five years old). Missing data for age, BMI, sample and task timing, time since waking, and survey data were imputed. Evaluator notes were used to impute 16% of missing data, and the remaining missing data were imputed using mean values. Sensitivity analyses performed on complete case data demonstrated that this imputation did not substantively impact the findings (estimates and *p*-values). Statistical analyses were conducted using Stata 12 (Stata Corp LP, College Station, TX).

RESULTS

Salivary Cytokine Concentrations

The descriptive statistics for raw concentrations of each cytokine by saliva sample are shown in Table 2. Overall, IL-1β, and IL-8 were present at much higher concentrations than IL-6 and TNFα. For each saliva sample, levels of IL-8 were the highest, followed by IL-1β, IL-6, and TNFα ($t[123] = 47.42–74.45$, $p < .008$ for all). Analyte levels were quite stable across the study protocol with significant positive correlations across samples for each analyte at the Bonferroni-corrected alpha level (IL-1β *r*'s[123] = .74–.88; IL-6 *r*'s[123] = .81–.92; IL-8 *r*'s[123] = .61–.81; TNFα *r*'s-[123] = .67–.86; sAA *r*'s[123] = .80–.91; cortisol *r*'s-[123] = .59–.84, $p < .008$ for all).

ANS and HPA Activty

Alpha-amylase and cortisol concentrations changed across the study visit. Alpha-amylase was stable from sample 1 to sample 3, and concentrations increased after sample 3 (mean

sample 3 concentration = 114.02 U/mL, mean sample 4 concentration = 125.47 U/mL, sample 3 vs. sample 4: $t[124] = -3.00$, $p < .01$). In contrast, cortisol concentrations declined from sample 1 to 3 and then remained stable overall for the rest of the visit (mean sample 1 concentration = 0.09 μg/dL, mean sample 2 concentration = 0.08 μg/dL, sample 1 vs. sample 2: $t[124] = 2.46$, $p < .05$; mean sample 3 concentration = 0.07 μg/dL, sample 2 vs. sample 3: $t[124] = 2.76$, $p < .01$). For both analytes, differences across the study visit were significant at the Bonfer-roni-corrected significance level (corrected $\alpha = .017$). Using a 10% threshold for meaningful change (Granger et al., 2012), 44% of children showed a meaningful increase in cortisol after sample 3 and 40% showed a decrease. Most children (60%) exhibited a meaningful increase in sAA after sample 2 and only 18% exhibited a decrease.

Hypothesis 1: Cyokine Intercorrelations

Table 3 shows the intercorrelations between cytokines at each saliva sample. All cytokines were significantly and positively correlated with each other at each sample (r's[123] = .54–.77, $p < .008$ for all; see Table 3). Principal components analysis suggested that a single component explained 72–77% of the variance in cytokine concentrations at each sample. Intercorrelations among the cytokines at each sample remained significant after accounting for multiple comparisons (corrrected $\alpha = .008$).

Model Construction for Hypotheses 2 and 3

Multilevel mixed models were used to address hypotheses 2 and 3. A separate model was constructed for each cytokine using backward stepwise regression of demographic and health covariates. The nonlinearity of change across the study visit was also tested for each cytokine during the model-building process by evaluating the appropriateness of a spline knot placed at sample 2 or sample 3. Final models included the health and demographic covariates listed in Tables 4–7. Likelihood-ratio tests indicated that the change in IL-1β, IL-8, and TNFα was not linear across the study visit. The final models for IL-1β and IL-8 had spline knots at sample 3 indicating a significantly steeper decline in cytokine levels after sample 3 (Tables 4 and 6). The final model for TNFα had a significant spline knot at sample 2; TNFα declined from sample 1 to sample 2 and then remained stable for the rest of the study visit (Table 7). Results from spline models and likelihood-ratio tests indicated that the change in IL-6 across the study visit was linear, so IL-6 was modeled linearly in the final model. There was no significant change in IL-6 across the study visit (Table 5).

Hypothesis 2: Association Between Salivary Cytokines and Demographic and Health Factors

The results from the final model for each cytokine are shown in Tables 4–7. IL-1β and IL-6 were higher in children with current oral health issues compared to those without oral health issues (Tables 4 and 5, respectively). TNFα was higher in children with smoking mothers than those with non-smoking mothers (Table 7). IL-6 was also positively associated with time since waking and age (Table 5). IL-8 was the only cytokine not related to any demographic or health variable.

Hypothesis 3: Association Between Salivary Cytokines and ANS and HPA Activity

To examine the association between cytokine levels and ANS and HPA activity, sAA and cortisol were added separately as independent variables in final models for each cytokine (results shown in Supplemental Tables S1–S4). Alpha-amylase was positively associated with IL-1β (β = 0.16, z = 2.16, p < .05) and TNFα (although this association was only marginal, β = .16, z=1.82, p = .07). No significant relations were found between sAA and IL-6 and IL-8. Cortisol was not associated with any cytokine.

To examine sex differences in the relations between ANS or HPA activity and cytokine levels, we tested the significance of sex by sAA or sex by cortisol interactions in each final cytokine model. The interaction between sAA and sex was significant for IL-1β, IL-8, and TNFα (IL-1β: β = −.40, z = −2.82, p < .01; IL-8: β = −.30, z = −2.51, p < .05; TNFα: β = −.49, z = −2.62, p < .01), and it was marginally significant for IL-6 (β= −.44, z = −1.96, p = .05). These interactions indicated significant positive relations between cytokines and sAA among boys (effect of sAA on IL-1β: β=.43, z = 4.39, p < .001; IL-6: β = .46, z = 2.68, p < .01; IL-8: β = .30, z = 3.53, p < .001; TNFα: β=.49, z = 3.45, p < .01), and less positive relations among girls. The interaction between cortisol and sex was also significant for every cytokine (IL-1β: β = .25, z = 2.69, p < .01; IL-6 β = 0.33, z = 2.46, p < .05; IL-8: β = .25, z = 3.50, p< .001; TNFα: β = 30, z = 2.69, p < .01). These interactions indicated significant inverse relations between cytokines and cortisol among boys (effect of cortisol on IL-1β: β = −.17, z = −3.24, p < .01; IL-6: β = −.25, z = −2.81, p < .01; IL-8: β = −0.20, z = −3.68, p < .001; TNFα: β = −.26, z = −2.76, p < .01), and weaker inverse relations between cytokines and cortisol among girls.

To better understand the role of sex in moderating cytokine-CNS relations, we examined the effects of sAA and cortisol separately in sex-stratified final models for each cytokine. The results from stratified models are shown in Supplemental Tables S5–S8. Among boys, sAA was positively associated with each cytokine (IL-1β: β = .43, z = 4.62, p < .001; IL-6: β = .53, z = 3.87, p < .001; IL-8: β=.32, z = 3.66, p < .001; TNFα: β = .46, z = 3.33, p < .01), and cortisol was negatively associated with each cytokine (IL-1β: β = −.17, z = −4.28, p < .001; IL-6: β = −.27, z = −4.74, p < .001; IL-8: β = −.21, z = −5.16, p < .001; TNFα: β = −.26, z = −4.34, p < .001). Among girls, there were no significant associations between cytokines and sAA or cortisol (Supplemental Tables S5–S8).

Stratified analyses also revealed sex-specific cytokine trajectories across the study visit. The pattern of change in IL-1β and IL-8 observed in the whole sample was also seen among boys and girls separately; however, the decreases in slopes at sample 3 were only significant among boys (difference between slopes from sample 1–3 and 3–4 for IL-1β with sAA: β = −.54, z = −3.68, p < .001, with cortisol: β = −0.53, z = −3.63, p < .001; for IL-8 with sAA: β = −.36, z = −2.11, p < .05, with cortisol: β = −.35, z = −2.18, p < .05; Supplemental Tables S5 and S7). Also, IL-6 declined across the study visit in boys, but was stable across the visit for girls (for boys, IL-6 slope with sAA: β = −.16, z = −2.65, p < .01, with cortisol: β = −.15, z = −2.56, p < .05; Supplemental Table S6). The pattern of change in TNFα observed in the whole sample was also seen among boys and girls separately; however, the change in slope at sample 2 was only significant in girls (difference between slopes from sample 1–2 and 2–

4 with sAA: β=.42, $z = 2.19$, $p < .05$, with cortisol: β=.43, $z = 2.22$, $p < .05$; Supplemental Table S8).

DISCUSSION

The findings provide new information regarding salivary cytokine detectability, concentrations, task-sensitivity, and associations with demographic and health factors, as well as with measures of ANS and HPA activation in five-year old children. IL-1β, IL-6, IL-8, and TNFα all had high rates of detection and strong across-sample correlations suggesting that cytokine measurements were reliable and relatively stable within-individual. The findings also provide information about the constitution of saliva in young children. Consistent with findings from studies of adolescents and adults (Gursoy et al., 2009; Rathnayake et al., 2013; Riis et al., 2013; Suh, Kim, & Kho, 2009), levels of IL-1b and IL-8 were higher than levels of IL-6 and TNFα in this sample. Relative levels of cytokines were consistent across the saliva samples with IL-8 present at the highest concentrations, followed by IL-1β, IL-6, and TNFα. IL-1β and IL-8 are important for the migration and activation of neutrophils, which are the most prevalent phagocytic immune cell in the mouth (Bickel, 1993; Raeste, 1972; Shaftel et al., 2007). Despite varying concentrations, the cytokines were significantly positively intercorrelated at each saliva sampling time point. These findings support our first hypothesis; they add to findings from studies in older participants and suggest strongly coordinated immune processes within the oral cavity (Byrne et al., 2013; Riis et al., 2013).

Association Between Salivary Cytokines and Demographic and Health Factors

We hypothesized that salivary cytokines are positively associated with factors related to oral health and unrelated to demographic and systemic health factors. This hypothesis was largely supported. Children with oral health issues had higher levels of IL-1β and IL-6, and children exposed to tobacco smoke had higher levels of TNFα. Previous studies have found elevated levels of salivary markers of inflammation in adults with gingivitis and periodontal disease (Gursoy et al., 2009; Nibali et al., 2012; Gümüş et al., 2013; Rathnayake et al., 2013), and in adolescents with dental caries (Gornowicz et al., 2012). Our findings extend these associations to young children who typically have low rates of clinically diagnosed periodontal conditions but may have high rates of loose or missing teeth and dental caries. Exposure to tobacco smoke is a risk factor for periodontal disease in adults (Yamamoto et al., 2005; Nishida et al., 2006) and adolescents (Heikkinen et al., 2010), and second-hand smoke exposure has been linked to elevated levels of salivary IL-1b in adults (Nishida et al., 2006). The current finding that maternal smoking is positively associated with inflammatory cytokine levels in children extends our understanding of the effects of tobacco smoke exposure on child health. Future studies should examine the utility of salivary cytokines as early indicators of inflammatory processes involved in oral and respiratory disease.

Unlike oral health factors, systemic health and demographic factors including sex, race, medication use, BMI, current sickness, and recent health were unrelated to salivary cytokine levels. Although the sample size was substantial, only a small group of children had fair or poor health. Therefore, the heterogeneity and size of the sample may not have been

sufficient to detect associations with recent health. The positive association observed between IL-6 and time since waking is consistent with findings of diurnal patterns of salivary IL-6 in adults with increasing concentrations beginning in the afternoon and peaking late at night (Izawa, Miki, Liu, & Ogawa, 2013). Despite the narrow age range of the sample, older children showed higher levels of IL-6. The two other studies of salivary cytokines in children (with age ranges from 8 to 12 years) found no age or puberty-related differences in IL-6 (El-Sheikh et al., 2007; Keller et al., 2010). In a study of adolescent girls, age and puberty were related to salivary cytokine levels, however there were inverse associations in this older sample (Riis et al., 2013). Early childhood is a period of complex neurologic and immunologic development (Johnson et al., 2013), therefore the positive associations between age and IL-6 found in the current study may represent developmental differences in neuroimmune processes specific to this developmental stage.

Overall, the finding that cytokines were largely unrelated to demographic and health characteristics, but were significantly elevated in children with oral health issues and those exposed to tobacco smoke supports the notion that salivary cytokines in young healthy children may not be representative of systemic inflammation, but rather reflect local immune processes of the oral cavity. Although no saliva samples in our study were visually contaminated with blood, future investigations should examine whether blood contamination may confound the relation between salivary cytokines and oral health issues among children (Granger et al., 2012). Also, future studies of cytokine serum-saliva correlations could help explain the cytokine associations observed in this study and provide direct evidence regarding the level of compartmentalization of oral immune processes. It's tempting to speculate that salivary cytokines may be more closely coordinated with serum levels during physiological states characterized as hyper-inflammatory (e.g., burns). Studies to address this possibility seem like a worthwhile next step.

Association Between Salivary Cytokines and ANS and HPA Activity

Our hypothesis that cytokine activity is positively associated with ANS and negatively associated with HPA activity was partially supported. To our surprise, these relations varied by sex. Among boys, cytokines were positively associated with sAA and negatively associated with cortisol. Among girls, however, there were no significant associations between ANS or HPA activity and any cytokine. Similar sex differences in NEI relations have been reported by studies of serum cytokines in adults (Dickerson, Gable, Irwin, Aziz, & Kemeny, 2009; Prather et al., 2009; Rohleder et al., 2001; Rohleder, Kudielka, Hellhammer, Wolf, & Kirschbaum, 2002). In these studies, men exhibited an increased sensitivity of inflammatory cytokines to the inhibitory effects of cortisol after a socioemotional stressor task, while women exhibited either no change or a decrease in sensitivity (Rohleder et al., 2001; Dickerson et al., 2009; Prather et al., 2009). These differences in cortisol sensitivity were associated with sex differences in cytokine levels across the stressor tasks with men showing a decrease in cytokines and women showing either no change or an increase (Prather et al., 2009; Rohleder et al., 2001). In the current study, similar sex-specific patterns of change were observed as boys exhibited declining cytokine trajectories and girl exhibited flatter trajectories across the visit.

To our knowledge, this is the first study to demonstrate these sex differences in NEI relations in children and using salivary markers of HPA and immune activity. Our findings suggest that sexual differentiation in CNS-immune relations are mediated, at least in part, by mechanisms independent of pubertal development; for example, intrauterine sex hormones may impact HPA axis development and regulation (Kajantie & Phillips, 2006), and sex differences in maternal–fetal relations, placental function, and neuroendocrine–neuroimmune interactions may have lasting effects on NEI relations (Bale, 2009). Our findings may help explain those of El-Sheikh and colleagues (2007) who found stress-related increases in salivary IL-6 in girls and decreases in boys. Although El-Sheikh and colleagues (2007) did not measure sAA or cortisol, the current findings suggest that sex-related changes in IL-6 may have been influenced by relations with ANS or HPA activity. Our findings also extend sex-specific NEI relations beyond the HPA axis to include measures of ANS activation and suggest that similar sex differences may exist in HPA-and ANS-immune relations. While additional research is needed to examine whether NEI relations in saliva reflect systemic NEI functioning, evidence of relations between CNS and immune activity in the oral cavity that mirror those found in serum demonstrates great potential for advancing the field of developmental psychoneuroimmunology and addresses the fundamental question of whether minimally-invasive methods can be used to examine NEI functioning.

Salivary Cytokine Task-Sensitivity

We examined changes in cytokine levels across a series of cognitive and emotional challenge tasks without a priori hypotheses regarding the nature, timing, and magnitude of task-related change in cytokine concentrations. The expected diurnal pattern of proinflammatory cytokine levels is an important context for interpreting these findings. While little is known about salivary cytokines in particular, the diurnal pattern of proinflammatory cytokines is influenced by cortisol; therefore, diurnal processes should exert positive change in cytokine levels across the study visit (Izawa, Miki, et al., 2013a; Logan & Sarkar, 2012). The majority of the cytokines, however, declined across the visit.

Among boys, IL-1β, IL-6, and IL-8 declined across all four saliva samples, suggesting sensitivity to the study environment or tasks. Furthermore, IL-1β and IL-8 exhibited a marked decline in slope immediately after the emotional challenge tasks indicating potentially increased sensitivity to the socioemotional stressors and the CNS response elicited by these tasks. In contrast, TNFα declined across the cognitive tasks and remained stable across the emotional tasks, suggesting sensitivity to the cognitive tasks. Among girls, cytokine trajectories were flatter, which may indicate less-specific and reduced sensitivity to the tasks. Interestingly, despite a lack of association between cytokine and CNS activation in girls, cytokines did not show diurnal increases across the visit, but rather exhibited declining or stable trajectories. Further research is needed to understand the diurnal patterns of salivary cytokines and the mechanisms underlying task-specific cytokine change in young children.

The interpretation of cytokine trajectories is complicated by aspects of the study protocol, including the timing, order, and nature of the tasks, and the potential influence of

unmeasured factors. The time between study tasks and saliva samples depended on the speed at which the child completed the tasks, and therefore varied across children. Given these differences in elapsed time from task to sample and the lack of information regarding salivary cytokine reactivity and recovery times, concentration changes are considered a cumulative response to all tasks rather than a specific response to one task. It is also important to note that increases in salivary cytokines often require new protein synthesis, and the time between our tasks and saliva samples may not have been long enough to allow for the examination of task-related increases in cytokine concentrations.

Isolating cytokine task-specific change is also complicated by the order and types of tasks administered. While cognitive challenge tasks engage ANS activation (Granger, Kivlighan, El-Sheikh, et al. 2007a; Lundberg & Frankenhaesuser, 1980; Nater & Rohleder, 2009), tasks involving emotional and social evaluative threat are typically needed to initiate an HPA stress response (Gunnar, Talge, & Herrera, 2009). We used emotional challenge tasks shown to be effective stressors for young children (Cole et al., 1994; Spinrad et al., 2009; Gagne et al., 2011). These tasks are commonly used with this age group and include elements of social evaluative pressure. The emotional challenge tasks required the children to interact, and confront or disagree with, an adult authority figure (the research evaluator). The varied cortisol response observed in the current study is typical for studies of the HPA response in children (Gunnar et al., 2009), and the observed increases in sAA indicate ANS activation in response to the emotional tasks. This increases our confidence that the emotional challenge tasks employed were appropriate socioemotional stressors for this age group. The extent of stress associated with the cognitive challenge tasks, however, is unclear (as cortisol levels declined across these tasks and sAA levels remained stable). Any CNS or cytokine response to the cognitive tasks likely influenced the subsequent response to the emotional challenge tasks. Future research is needed to delineate cytokine resting trajectories and sensitivity to specific types of stress.

To our knowledge, this study represents the first examination of cross-system relations between salivary cytokines and measures of ANS and HPA activation. While this provides novel information regarding the coordinated response to stress in the oral cavity, it is important to recognize the potential influence of other unmeasured salivary analytes when interpreting cytokine task-sensitivity. Trueba et al. (2012) found that adults exhibit an increase in many anti-inflammatory salivary proteins after acute socioemotional stress. Additional research on the stress-related release of other anti-inflammatory proteins, the time course of their release, and the sensitivity of salivary cytokines to these proteins is important to illuminating the task-sensitivity of salivary cytokines and their associations with measures of ANS and HPA activity.

CONCLUSION

These results provide unique information regarding the concentrations of salivary cytokines in young children and the interplay of these immunologic analytes with markers of ANS and HPA activity across a series of challenge tasks. Confidence in our findings is bolstered by the tight controls afforded by laboratory-based sampling and repeated measures within individuals. Our findings suggest that oral immune processes are highly coordinated.

Consistent associations between some cytokines and oral health variables suggest that local oral immune processes are distinct from the systemic immune system. The NEI relations observed in this study were similar to systemic NEI relations. Therefore, levels of some salivary cytokines may provide insight into child oral health, and salivary cytokines may be used with ANS and CNS biomarkers to provide information regarding neuroendocrineimmune functioning. These findings present exciting opportunities for studying mechanisms linking brain, immunity and behavior, as well as the calibration of across-systems connections during early development and the impact this has on later-life health and well-being.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Contract grant sponsor: NIH/NIDA

Contract grant number: KO1DA027229-01

We are grateful to Jessica Bayer, Tracey Hand, Jessica Parker, and Jessica Acevedo at Johns Hopkins and Arizona State University for technical support with salivary assays.

REFERENCES

Arlington, U.; of, T. Laboratory Temperment Assessment Battery. 2012. Retrieved from http://www.uta.edu/faculty/jgagne/labtab/labtab_page.htm.

Bale TL. Neuroendocrine and immune influences on the CNS: it's a matter of sex. Neuron. 2009; 64(1):13–16. [PubMed: 19840541]

Bickel M. The role of IL-8 in inflammation and mechanisms of regulation. Journal of Periodontology. 1993; 64:456–460. [PubMed: 8315568]

Bilbo SD, Schwarz JM. The immune system and developmental programming of brain and behavior. Frontiers in Neuroendocrinology. 2012

Byrne M, O'Brien-Simpson N, Reynolds E, Walsh K, Laughton K, Waloszek J, Allen N. Acute phase protein and cytokine levels in serum and saliva: a comparison of detectable levels and correlations in a depressed and healthy adolescent sample. Brain, Behavior, and Immunity. 2013; 34:164–175.

Centers for Disease Control and Prevention. 2001. Data Table of BMI-for-age Charts Retrieved from http://www.cdc.gov/growthcharts/html_charts/bmiagerev.htm.

Centers for Disease Control and Prevention. 2011. About BMI for Children and Teens Retrieved from http://www.cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens _bmi.html.

Chiang JJ, Eisenberger NI, Seeman TE, Taylor SE. Negative and competitive social interactions are related to heightened proinflammatory cytokine activity. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109(6):1878–1882. [PubMed: 22308464]

Clough, N. Understanding immunology. St. Louis, Missouri: Mosby-Year Book, Inc; 1998.

Coe CL, Lubach GR. Critical periods of special health relevance for psychoneuroimmunology. Brain, Behavior, and Immunity. 2003; 17:3–12.

Cole PM, Martin SM, Dennis TA. Emotion regulation as a scientific construct: methodological challenges and directions for child development research. Child Development. 2004; 75(2):317–333. [PubMed: 15056186]

Cole PM, Zahn-Waxler C, Smith KD. Expressive control during a disappointment: variations related to preschoolers' behavior problems. Developmental Psychology. 1994; 30(6):835–846.

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature Reviews Neuroscience. 2008; 9(1):46–56. [PubMed: 18073775]

Dickerson SS, Gable SL, Irwin MR, Aziz N, Kemeny ME. Social-evaluative threat and proinflammatory cytokine regulation: an experimental laboratory investigation. Psychological Science. 2009; 20(10):1237–1244. [PubMed: 19754527]

DiPietro J, Kivlighan K, Costigan K, Rubin S, Shiffler D, Henderson J, Pillion J. Prenatal antecedents of newborn neurological maturation. Child Development. 2010; 81(1):115–130. [PubMed: 20331657]

Doan, T.; Melvold, R.; Viselli, S.; Waltenbaugh, C. Immunology. Harvey, R.; Champe, P., editors. Philadelphia, PA: Lippincott Willaims & Wilkins; 2008.

Dunn, LM.; Dunn, DM. Peabody Picture Vocabulary Test–Fourth Edition. Minneapolis, MN: Pearson Assessments; 2007.

Elenkov IJ. Neurohormonal-cytokine interactions: implications for inflammation, common human diseases and well-being. Neurochemistry International. 2008; 52(1–2):40–51. [PubMed: 17716784]

El-Sheikh M, Buckhalt JA, Granger DA, Erath SA, Acebo C. The association between children's sleep disruption and salivary interleukin-6. Journal of Sleep Research. 2007; 16(2):188–197. [PubMed: 17542949]

Gagne JR, Hulle CAV, Aksan Essex MJ, Goldsmith HH. Deriving childhood temperament measures from emotion-eliciting behavioral episodes: scale construction and initial validation. Psychological Assessment. 2011; 23(2):337–353. [PubMed: 21480723]

Garrett-Peters P, Fox N. Cross-cultural differences in children's emotional reactions to a disappointing situation. International Journal of Behavioral Development. 2007; 31(2):161–169.

Gornowicz A, Bielawska A, Bielawski K, Grabowska SZ, Wo´jcicka A, Zalewska M, Maciorkowska E. Pro-in ammatory cytokines in saliva of adolescents with dental caries disease. 2012; 19(4):711–716.

Granger DA, Fortunato CK, Beltzer EK, Virag M, Bright MA, Out D. Focus on methodology: salivary bioscience and research on adolescence: an integrated perspective. Journal of Adolescence. 2012; 35(4):1081–1095. [PubMed: 22401843]

Granger, DA.; Granger, GA.; Granger, SW. Immunology and developmental psychopathology. In: Cicchetti, D.; Cohen, D., editors. Developmental Neuroscience. Vol. 2. New York, NY: John Wiley and Sons; 2006. p. 677-709.

Granger, DA.; Johnson, S. Salivary biomarkers. In: Gellman, MD.; Turner, RJ., editors. Encyclopedia of behavioral medicine. New York, NY: Springer New York; 2013. p. 1697-1705.

Granger DA, Kivlighan KT, El-Sheikh M, Gordis EB, Stroud LR. Salivary alpha-amylase in biobehavioral research: recent developments and applications. Annals of the New York Academy of Sciences. 2007a; 1098(1):122–144. [PubMed: 17332070]

Granger DA, Kivlighan KT, Fortunato C, Harmon AG, Hibel LC, Schwartz EB, Whembolua G. Integration of salivary biomarkers into developmental and behaviorally-oriented research: problems and solutions for collecting specimens. Physiology & Behavior. 2007b; 92(4):583–590. [PubMed: 17572453]

Gröschl M. The physiological role of hormones in saliva. BioEssays. 2009; 31(8):843–852. [PubMed: 19554609]

Gümüş P, Nizam N, Lappin DF, Buduneli N. Saliva and serum levels of B-cell activating factors and tumor necrosis factor-alpha in periodontitis patients. Journal of Periodontology. 2013; 0:1–14.

Gunnar MR, Talge NM, Herrera A. Stressor paradigms in developmental studies: what does and does not work to produce mean increases in salivary cortisol. Psychoneuroendocrinology. 2009; 34(7): 953–967. [PubMed: 19321267]

Gursoy UK, Könönen E, Uitto V-J, Pussinen PJ, Hyvärinen K, Suominen-Taipale L, Knuuttila M. Salivary interleukin-1beta concentration and the presence of multiple pathogens in periodontitis. Journal of Clinical Periodontology. 2009; 36(11):922–927. [PubMed: 19799718]

Haggerty, RJRK.; Pless, IB. Child health and the community. New York, NY: John Wiley and Sons; 1975.

Heikkinen AM, Sorsa T, Pitkäniemi J, Tervahartiala T, Kari K, Broms U, Meurman JH. Smoking affects diagnostic salivary periodontal disease biomarker levels in adolescents. Journal of Periodontology. 2010; 81(9):1299–1307. [PubMed: 20450405]

Hellhammer DH, Wüst S, Kudielka BM. Salivary cortisol as a biomarker in stress research. Psychoneuroendocrinology. 2009; 34(2):163–171. [PubMed: 19095358]

Irwin MR, Cole SW. Reciprocal regulation of the neural and innate immune systems. Nature Reviews. Immunology. 2011; 11(9):625–632.

Izawa S, Miki K, Liu X, Ogawa N. The diurnal patterns of salivary interleukin-6 and C-reactive protein in healthy young adults. Brain, Behavior, and Immunity. 2013a; 27(1):38–41.

Izawa S, Sugaya N, Kimura K, Ogawa N, Yamada KC, Shirotsuki K, Nomura S. An increase in salivary interleukin-6 level following acute psychosocial stress and its biological correlates in healthy young adults. Biological Psychology. 2013b; 94(2):249–254. [PubMed: 23831278]

Jahromi LB, Stifter CA. Individual differences in preschoolers' self-regulation and theory of mind. Merrill-Palmer Quarterly. 2008; 54(1):125–150.

Johnson SB, Riley AW, Granger DA, Riis J. The science of early life toxic stress for pediatric practice and advocacy. Pediatrics. 2013; 131(2):319–327. [PubMed: 23339224]

Jones HP. Psychoneuroimmunology. 2012; 934:77–87.

Kajantie E, Phillips DI. The effects of sex and hormonal status on the physiological response to acute psychosocial stress. Psychoneuroendocrinology. 2006; 31(2):151–178. [PubMed: 16139959]

Keller PS, El-Sheikh M, Vaughn B, Granger DA. Relations between mucosal immunity and children's mental health: the role of child sex. Physiology & Behavior. 2010; 101(5):705–712. [PubMed: 20732336]

Kieras JE, Tobin RM, Graziano WG, Rothbart MK. You can't always get what you want. Effortful control and children's response to undesirable gifts. Psychological Science. 2005; 16(5):391–396. [PubMed: 15869699]

Logan RW, Sarkar DK. Circadian nature of immune function. Molecular and Cellular Endocrinology. 2012; 349(1):82–90. [PubMed: 21784128]

Lundberg U, Frankenhaesuser M. Pituitary-adrenal and sympathetic-adrenal correlates of distress and effort. Journal of Psychosomatic Research. 1980; 24(997):125–130. [PubMed: 7441580]

McEwen BS. Stress, adaptation, and disease: allostasis and allostatic load. Annals of New York Academy of Sciences. 1998; 840(1):33–44.

Miller GE, Chen E, Zhou ES. If it goes up, must it come down? chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. Psychological Bulletin. 2007; 133(1):25–45. [PubMed: 17201569]

Mischel HN, Mischel W. The development of children's knowledge of self-control strategies. Child Development. 1983; 54:603–619.

Moons WG, Eisenberger NI, Taylor SE. Anger and fear responses to stress have different biological profiles. Brain, Behavior, and Immunity. 2010; 24(2):215–219.

Nater UM, Rohleder N. Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. Psychoneuroendocrinol-ogy. 2009; 34:486–496.

Nibali L, Fedele S, D'Aiuto F, Donos N. Interleukin-6 in oral diseases: a review. Oral Diseases. 2012; 18:236–243. [PubMed: 22050374]

Nishanian P, Aziz N, Chung J, Detels R, Fahey J. Oral fluids as an alternative to serum for measurement of immune activation. Clinical and Diagnostic Laboratory Immunology. 1998; 5(4): 507–512. [PubMed: 9665958]

Nishida N, Yamamoto Y, Tanaka M, Maeda K, Kataoka K, Nakayama K, Shizukuishi S. Association between passive smoking and salivary markers related to periodontitis. Journal of Clinical Periodontology. 2006; 33(10):717–723. [PubMed: 16889628]

O'Connor M-F, Irwin MR, Wellisch DK. When grief heats up: pro-inflammatory cytokines predict regional brain activation. NeuroImage. 2009; 47(3):891–896. [PubMed: 19481155]

Owen, J.; Punt, J.; Stranford, S. Kuby immunology. 7th ed.. New York, NY: W. H. Freeman; 2012.

Prather AA, Carroll JE, Fury JM, McDade KK, Ross D, Marsland AL. Gender differences in stimulated cytokine production following acute psychological stress. Brain Behavior and Immunity. 2009; 23(5):622–628.

Raeste A. The differential count of oral leukocytes. European Journal of Oral Sciences. 1972; 80(1): 63–67.

Rathnayake N, Akerman S, Klinge B, Lundegren N, Jansson H, Tryselius Y, Gustafsson A. Salivary biomarkers of oral health: a cross-sectional study. Journal of Clinical Periodontology. 2013; 40(2): 140–147. [PubMed: 23174014]

Riis JL, Out D, Dorn LD, Beal SJ, Denson L, Pabst S, Granger DA. Salivary cytokines in healthy adolescent girls: intercorrelations, stability, and associations with serum cytokines, age, and pubertal stage. Developmental Psychobiology. 2013

Rohleder N. Variability in stress system regulatory control of inflammation: a critical factor mediating health effects of stress. Expert Review of Endocrinology & Metabolism. 2011; 6(2):269–278.

Rohleder N, Kudielka BM, Hellhammer DH, Wolf JM, Kirschbaum C. Age and sex steroid-related changes in glucocorticoid sensitivity of pro-inflammatory cytokine production after psychosocial stress. Journal of Neuroimmunology. 2002; 126(1–2):69–77. http://www.ncbi.nlm.nih.gov/pubmed/12020958. [PubMed: 12020958]

Rohleder N, Schommer NC, Hellhammer DH, Engel R, Kirschbaum C. Sex differences in glucocorticoid sensitivity of proinflammatory cytokine production after psychosocial stress. Psychosomatic Medicine. 2001; 63(6):966–972. http://www.ncbi.nlm.nih.gov/pubmed/11719636. [PubMed: 11719636]

Shaftel S, Carlson T, Olschowka J, Kyrkanides S, Matousek S, O'Banion M. Chronic interleu-kin-1 expression in mouse brain leads to leukocyte infiltration and neutrophil-independent blood-brain barrier permeability without overt neurodegeneration. The Journal of Neuroscience. 2007; 27(35): 9301–9309. [PubMed: 17728444]

Shonkoff JP. Leveraging the biology of adversity to address the roots of disparities in health and development. Proc.Natl.Acad.Sci.U.S.A. 2012; 109(Supplement 2):17302–17307. [PubMed: 23045654]

Sjo¨gren E, Leanderson P, Kristenson M, Ernerudh J. Interleukin-6 levels in relation to psychosocial factors: studies on serum, saliva, and in vitro production by blood mononuclear cells. Brain. 2006; 20(3):270–278.

Slavich GM, Way BM, Eisenberger NI, Taylor SE. Neural sensitivity to social rejection is associated with inflammatory responses to social stress. Proceedings of the National Academy of Sciences USA. 2010; 107(33):14817–14822.

Spinrad TL, Eisenberg N, Granger DA, Eggum ND, Sallquist J, Haugen RGG, Hofer C. Individual differences in preschoolers' salivary cortisol and alpha-amylase reactivity: relations to temperament and maladjustment. Hormones and Behavior. 2009; 56(1):133–139. [PubMed: 19348808]

Suh K-I, Kim Y-K, Kho H-S. Salivary levels of IL-1beta, IL-6, IL-8, and TNF-alpha in patients with burning mouth syndrome. Archives of Oral Biology. 2009; 54(9):797–802. [PubMed: 19555923]

Tabachnik, BG.; Fidell, LS. Using multivariate analysis. Vol. 4. Boston, MA: Allyn and Bacon; 2001.

Thomson, A.; Lotze, M. The cytokine handbook. Vol. 4. San Diego, CA: Academic Press; 2003.

ThyagaRajan S, Priyanka HP. Bidirectional communication between the neuroendocrine system and the immune system: relevance to health and diseases. Annals of Neurosciences. 2012; 19(1):40–46. [PubMed: 25205962]

Trueba AF, Mizrachi D, Auchus RJ, Vogel PD, Ritz T. Effects of psychosocial stress on the pattern of salivary protein release. Physiology & Behavior. 2012; 105:841–849. [PubMed: 22056539]

Vining R, McGinley R. The measurement of hormones in saliva: possibilities and pitfalls. Journal of Steriod Biochemistry. 1987; 27(1–3):81–94.

Williamson S, Munro C, Pickler R, Grap MJ, Elswick RKJ. Comparison of biomarkers in blood and saliva in health adults. Nursing Research and Practice. 2012:1–4.

Willoughby MT, Blair CB, Wirth RJ, Greenberg M. The measurement of executive function at age 3 years: psychometric properties and criterion validity of a new battery of tasks. Psychological Assessment. 2010; 22(2):306–317. [PubMed: 20528058]

Willoughby MT, Wirth RJ, Blair CB. Executive function in early childhood: longitudinal measurement invariance and developmental change. Psychological Assessment. 2012; 24(2):418–431. [PubMed: 22023561]

Yamamoto Y, Nishida N, Tanaka M, Hayashi N, Matsuse R, Nakayama K, Shizukuishi S. Association between passive and active smoking evaluated by salivary cotinine and periodontitis. Journal of Clinical Periodontol-ogy. 2005; 32(10):1041–1046.

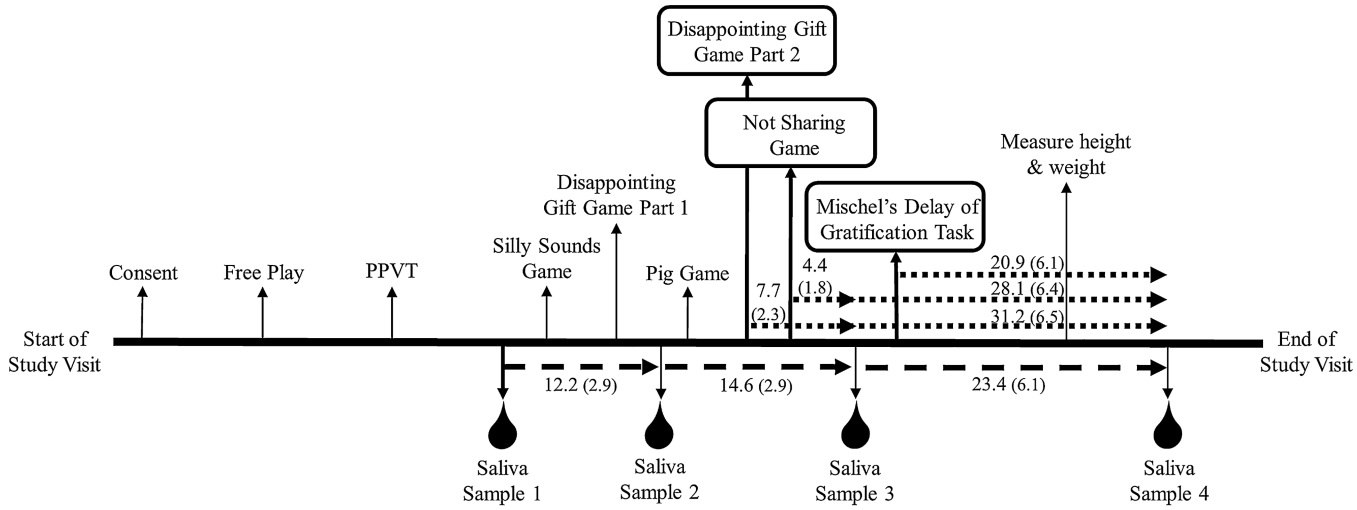

FIGURE 1.

Study protocol for children with elapsed time between saliva samples and emotional challenge tasks (n = 125).

Table 1

Demograpic and Health Characteristics of the Sample of 125 Five-Year Old Children

	Frequency	Percent (%)
Demographic Characteristics		
Age, mean years (SD)	5.45 (0.29)	
Male	61	49
Race		
White	42	34
African American	80	64
Asian/Pacific Islander	3	2
Health Characteristics		
Overweight/Obese[a]	44	35
Fair/Poor health in last 2 days	9	7
Currently sick with cold or fever[b]	24	19
Taken any medicine in last 2 days	28	22
Taken allergy/asthma medicine in last 2 days	20	16
Current oral health issues	14	11
Mother smokes	28	22

SD, standard deviation.

[a]Weight status (underweight or healthy weight vs. overweight or obese) assigned using the Centers for Disease Control and Prevention's percentile ranges by sex and age.

[b]Current sickness assessed with the question: "Is your child currently feeling sick or ill? (e.g., runny nose, fever, cough, aching, etc.)".

Table 2

Descriptive Statistics for Raw Salivary Cytokine Data in Five-Year Old Children Across a 90-minute Study Visit With Challenge Tasks (pg/mL; $n = 125$)[a]

	Sample 1	Sample 2	Sample 3	Sample 4
IL-1β				
Mean (SD)	212.12 (417.50)	182.62 (367.75)	136.77 (174.03)	90.68 (117.10)
Minimum- Maximum	13.54–3908.97	10.72–3401.80	9.58–1242.97	3.39–762.47
IL-6				
Mean (SD)	16.70 (34.50)	16.36 (39.70)	14.23 (39.42)	16.01 (45.13)
Minimum- Maximum	0.00–203.46	0.10–253.51	0.19–349.51	0.32–416.10
IL-8				
Mean (SD)	769.52 (772.13)	732.65 (998.32)	631.04 (593.65)	495.08 (712.39)
Minimum- Maximum	92.10–4660.69	48.13–7368.48	97.05–4306.81	61.58–5841.91
TNFα				
Mean (SD)	4.18 (8.55)	3.86 (13.90)	2.47 (3.55)	2.26 (3.85)
Minimum- Maximum	0.01–71.92	0.10–145.68	0.02–26.82	0.00–26.39

IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; TNFα, tumor necrosis factor alpha; SD, standard deviation.

Table 3

Correlations Between Salivary Cytokines at Each Saliva Sample (n = 125)

	IL-1β	IL-6	IL-8
Sample 1			
IL-1β			
IL-6	0.70*		
IL-8	0.69*	0.64*	
TNFα	0.73*	0.71*	0.62*
Sample 2			
IL-1β			
IL-6	0.70*		
IL-8	0.77*	0.64*	
TNFα	0.73*	0.70*	0.61*
Sample 3			
IL-1β			
IL-6	0.63*		
IL-8	0.67*	0.64*	
TNFα	0.62*	0.66*	0.54*
Sample 4			
IL-1β			
IL-6	0.72*		
IL-8	0.75*	0.71*	
TNFα	0.65*	0.71*	0.64*

IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; TNFα, tumor necrosis factor alpha.

* p <.008 (Bonferroni-corrected significance level).

Table 4

Results From Final Mixed Model of Log-Transformed IL-1β (n = 125)

	IL-1β		
Fixed Effects	**Parameter**	**SE**	***p***
Intercept	4.77	0.14	<.001
Task timing	0.00	0.01	.48
Sample 1–3	−0.13	0.04	<.001
Sample 3–4	−0.58[a]	0.15	<.001
Current oral health issues	0.59	0.18	<.01
Random effects	Estimate	SE	95% CI
Intercept			
Variance (Intercept)	0.68	0.12	0.49–0.96
Residual: AR(1)			
Rho	0.51	0.09	0.32–0.66
Within-subject variance	0.30	0.06	0.20–0.45

SE, robust standard error; CI, confidence interval.

[a]Difference between the slopes from sample 1–3 and 3–4: β = −0.45, z = − 3.36, p < .01.

Table 5

Results From Final Mixed Model of Log-Transformed IL-6 (n = 125)

	IL-6		
Fixed Effects	**Parameter**	**SE**	***p***
Intercept	1.41	0.13	<.001
Task timing	0.00	0.00	.29
Sample	−0.07	0.04	.11
Current oral health issues	1.03	0.39	<.01
Age	0.10	0.04	<.01
Time since waking	0.10	0.04	<.05
Random effects	Estimate	SE	95% CI
Intercept			
Variance (Intercept)	1.48	0.21	1.12–1.97
Residual: AR(1)			
Rho	0.36	0.09	0.16–0.53
Within-subject variance	0.39	0.08	0.26–0.60

SE, robust standard error; CI, confidence interval.

Table 6

Results From Final Mixed Model of Log-Transformed IL-8 (n = 125)

	IL-8		
Fixed Effects	**Parameter**	**SE**	***p***
Intercept	6.32	0.13	<.001
Task timing	0.00	0.01	.77
Sample 1–3	−0.06	0.03	.08
Sample 3–4	−0.34[a]	0.16	<.05
Random Effects	Estimate	SE	95% CI
Intercept			
Variance (Intercept)	0.41	0.07	0.30–0.58
Residual: AR(1)			
Rho	0.27	0.11	0.05–0.46
Within-subject variance	0.23	0.04	0.17–0.32

SE, robust standard error; CI, confidence interval.

[a] Difference between slopes from sample 1–3 and 3–4: β = −0.28, z = −1.97, p < .05.

Table 7

Results From Final Mixed Model of Log-Transformed TNFα (n = 125)

	TNFα		
Fixed Effects	**Parameter**	**SE**	***p***
Intercept	0.54	0.19	<.01
Task timing	−0.02	0.01	<.05
Sample 1–2	−0.23	0.06	<.001
Sample 2–4	0.10[a]	0.11	.36
Maternal smoking	0.52	0.22	<.05
Random effects	Estimate	SE	95% CI
Intercept			
Variance (Intercept)	0.85	0.13	0.62–1.16
Residual: AR(1)			
Rho	0.36	0.12	0.12–0.57
Within-subject variance	0.48	0.10	0.32–0.74

SE, robust standard error; CI, confidence interval.

[a]Difference between slopes from sample 1–2 and 2–4: β = 0.33, z = 2.66, p <.01.