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Salivary cytokines in healthy adolescent girls:

Intercorrelations, stability, and associations with serum cytokines, age and pubertal stage

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Abstract

To our knowledge this is the first study to rigorously explore salivary cytokines in youth. Cytokines in saliva and serum were analyzed across three annual assessments in healthy adolescent girls (*N*=114, 11-17 years at enrollment). We assayed saliva for GM-CSF, IFNγ, IL-1ß, IL-2, IL-6, IL-8, IL-10, IL-12p70, TNFα, adiponectin, and cotinine. Results revealed: (1) cytokine levels, except IFNγ and IL-10, were detectable in saliva, and salivary cytokine levels, except IL-8 and IL-1ß, were lower than serum levels; (2) salivary cytokine levels were lower in older girls and positively associated with adiponectin; (3) compared to serum cytokines, the correlations between salivary cytokines were higher, but salivary cytokines were less stable across years; and (4) only IL-1ß in one assessment year showed a significant serum-saliva association. Variation in basal salivary cytokines in healthy adolescent girls largely reflects compartmentalized activity of the oral mucosal immune system, rather than systemic cytokine activity.

Key words: saliva, serum, cytokines, immune markers, adolescent

Introduction

More so than any other time in the history of developmental science, the direction of child and adolescent research is being influenced by theoretical models that emphasize the confluence of biological, behavioral, social, and contextual forces in child development and health. Our understanding of the mechanisms linking the central and peripheral nervous and immune systems have evolved significantly, leading researchers to consider the nature and implications of effects of these systems on behavior, immunity, illness susceptibility, and negative health outcomes. However, generally speaking, how these relationships affect child and adolescent health and development has received only scant empirical attention (e.g., Granger, Kivlighan, Blair, El–Sheikh, Mize, Lisonbee, & Schwartz, 2006).

Technical advances now enable minimally invasive measurement (in saliva) of many of the intracellular regulatory molecules (i.e., cytokines) responsible for linking communications between the immune system and the central nervous system (CNS), hypothalamic-pituitary-adrenal (HPA) axis, and behavior (Granger, Fortunato, Beltzer,Virag, Bright, & Out, 2012). To date, with a few exceptions (El-Sheikh, Buckhalt, Granger, Erath, & Acebo, 2007; Keller, El-Sheikh, Vaughn, & Granger, 2010) the depth of our knowledge regarding the nature and meaning of individual differences in salivary cytokine levels in youth is extraordinarily shallow. Basic questions related to observed variation, intercorrelations between cytokines, stability over time, and the correlates and concomitants of salivary cytokine measurements in youth remain unanswered. In the present study, we begin to address several knowledge gaps by analyzing cytokines reflecting various components of intercellular and hormonal signaling within the neuro-endocrine-immune (NEI) network in saliva samples, which were collected across three years of annual assessments in a multi-cohort prospective study of healthy adolescent girls.

Conceptual Issues

The Neuro-endocrine-immune (NEI) Network. The history of science linking behavior and psychological states to changes in immune function is well documented (see Ader, 1996, 2000 for reviews). The first era spans the turn of the prior century through the 1950s. During this period, the field of immunology expanded our understanding of lymphoid cell and tissue function, antibody specificity and activity, and most other basic features of the immune system. Since the CNS lacks a lymphatic system to drain its tissues and capture potential antigens, it was assumed the CNS was protected from infiltration of many soluble substances (e.g., cytokines) and the inward migration of lymphoid cells by the blood-brain barrier. Correspondingly, it was widely held that the CNS operated relatively isolated from and independent of the immune system. Evidence supporting the veridicality of an immune-CNS link was not reported until the 1970-80s when anatomical pathways were discovered that physiochemically linked the CNS and lymphoid systems. Based on these findings, it was established that the HPA axis and autonomic nervous system (ANS) were able to communicate with and alter the function of lymphoid cells by releasing factors and hormones secreted into general circulation (see Ader, 2000 for review). These discoveries established the scientific legitimacy of studying interrelationships among the brain, behavior, and immunity.

Before the 1960-70s, it was assumed that cellular communication within the immune system depended largely on cell-to-cell contact. However, a series of landmark studies (e.g., Granger & Williams, 1968; Hessinger, Daynes, & Granger, 1973) revealed that lymphoid cells secreted soluble chemical messengers to initiate, maintain, and regulate cellular immune responses. Later it was discovered that these soluble signals (see Maier & Watkins, 1998; Smith, 1992; Vilcek, 1998) also had “hormonal” effects on nonlymphoid cells (e.g., Besedovsky, del

Rey, Sorkin, Da Prada, Burri, & Honegger, 1983; Besedovsky & del Rey, 1989). In recognition of their functional diversity, this group of signaling molecules was designated *cytokines* (see below for more detail) in the 1980s.

For researchers interested in the interactions between the brain, behavior, and immunity, this set of discoveries completed a critically important communication loop. That is, cells of the lymphoid, CNS, and endocrine systems use the same hormones, neurotransmitters, and other critical effector molecules to send and receive signals among one another. Thus, the central nervous, endocrine, and immune systems constitute an interactive information network, with each node capable of affecting and being affected by the activity of the others (Ader, 2000; Black, 1995; Cotman, Brinton, Glaburda, McEwen, & Schneider, 1987).

Decoding the syntax of these communications has enabled theorists to attribute new features to the immune system's functional repertoire. Blalock (e.g., 1994, 1997) proposed that the immune system recognizes unique environmental stimuli (e.g., viruses, bacteria, microbes, toxins) that are essentially undetectable by our classic sensory systems. Just as is true with our other sensory inputs, when the immune system recognizes such stimuli, the encounter is converted into a cascade of biochemical messages. We now know that those messages are responsible for inducing changes in the host's metabolic, thermogenic, and behavioral states in response to infection. Characterizing the immune system as a "sixth sense" (Blalock, 1994; 1997) highlights the biological plausibility that communications between the immune system and the brain represent a largely unexplored pathway through which environmental factors (non-self antigens) shape and reshape the structure and function of the biological systems underlying behavior.

Primer on Cytokines for Developmental Scientists. Cytokines are protein molecules that act as intercellular messengers which range in size from 8 to 30 kiloDaltons (kDa) and induce their effects by binding to high-affinity receptors on target cell membranes. Monocytes and macrophages are especially efficient cytokine producers. Most cytokine secretion involves *de novo* protein synthesis, a process that takes hours. Cytokines play important roles in maintaining health and are the causative agents in disease. In addition to regulating immune and inflammatory responses, the biologic consequences of cytokine signaling are critical for development, growth, healing, and maintenance of just about every tissue and organ of the body.

Cytokines can be grouped into families according to their structure, common receptor usage, and whether they have predominantly anti-inflammatory, proinflammatory, chemotactic, or growth-promoting functions. Within the immune system, in general, interleukins (e.g., IL-1ß, IL-2, IL-6, IL-8, IL-10, IL-12p70) are molecules that have a functional activity involving leukocytes; colony-stimulating and growth factors (e.g., GM-CSF) promote cell proliferation of hematopoeitic and somatic cells; tumor necrosis factors (e.g., TNFα) initiate inflammation, orchestrate the development and maintenance of lymphoid organs, and regulate lymphocyte homeostasis and proliferation; interferons (e.g., IFNγ) act to interfere with viral replication; and chemokines are chemotactic agents that recruit leukocytes to sites of inflammation and orchestrate lymphoid architecture and leukocyte trafficking (Clough & Roth, 1998). Cytokines are extremely potent, and their presence even at very low pg/ml levels can induce substantial cellular consequences. A complex set of mechanisms exists to prevent cytokines from doing damage to the host. Inadequate production of cytokines will result in an insufficient cellular immune response and a potentially negative outcome related to pathogenesis of the microbe or virus. On the other hand, excessive cytokine secretion has been linked to "sickness behaviors"

(see subsequent discussion), autoimmune disorders (e.g., arthritis), neurological disease (e.g., multiple sclerosis), toxic shock, and death (see reviews by Goldsby, 2003; Thomson & Lotze, 2003).

Peripherally Activated Cytokines Communicate with the CNS, HPA, and ANS. The most well-characterized pathway through which peripheral immunological stimuli signal changes in the brain begins with the macrophage. When activated, macrophages secrete a cascade of cytokines. The cytokine products released in response to immune stimulation, not the direct effect of the immunological stimulus (e.g., bacterial or viral pathogenesis), are particularly important for mediating many of the resulting changes in the brain (Dantzer, Bluthe, & Goodall, 1993; Dinarello, 1984a, 1984b). Molecular, cellular, and *in vivo* evidence suggests that macrophage-derived interleukin-1 (IL-1ß) has the most systemic and hormonal-type effects of all the cytokines (Smith, 1992), with widespread consequences for the brain (Weiss, Quan, & Sundar, 1994). Peripheral administration of IL-1ß stimulates corticotropin-releasing factor (CRF) from hypothalamic neurons (Harbuz & Lightman, 1992; Sapolsky, Rivier, Yamamoto, Plotsky, & Vale, 1987; Woolski, Smith, Meyer, Fuller, & Blalock, 1985), resulting in increased circulating levels of ACTH and corticosterone (Dunn, 1988, 1990). IL-6 and TNFα function similarly to IL-1ß in that they also stimulate the HPA axis during inflammatory stress (Smith, 1992).

Cytokines have a variety of effects on cerebral neurotransmission (see Dunn & Wang, 1995; Dunn, Wang, & Ando, 1999). IL-1ß stimulates cerebral norepinephrine (NE) metabolism. Together with IL-6 and TNFα, IL-1ß also stimulates indolamine metabolism of tryptophan and decreases the concentration of serotonin. Cytokine-stimulated indolamines also affect the synthesis of quinolinic (QUIN) and kynurenic acid. In the brain, QUIN is an agonist of

excitatory amino acid receptors, including the N-methyl-d-aspartate (NMDA) receptor. Prolonged activation of these excitatory amino acid receptors in the hippocampus, basal ganglia, and cerebral cortex may damage the nerve cells. In addition to CNS effects of peripheral cytokines, brain cells can release cytokines, and communication between the CNS and immune system is mediated via a direct connection by the vagus nerve (Heyes, 1992; Heyes, Brew, Saito, Quearry, Price, Lee, Bhalla, Der, & Markey, 1992; Heyes, Quearry, & Markey, 1989; Heyes, Saito, et al., 1992; Saito, Markey, & Heyes, 1992).

The effects of cytokines involve changes in neurotransmission in brain regions important for learning and memory, emotion regulation, and the psychobiology of the stress response (i.e., limbic system, hypothalamus) (e.g., Hart, 1988; Moldawer, Andersson, Gelin, & Lundholm, 1988; Opp, Orbal, & Kreuger, 1991; Sparado & Dunn, 1990; Avitsur & Yirmiya, 1999; Dantzer, 2001). Tyrell and colleagues (Smith, Tyrell, Al-Nakib, Coyle, Donovan, Higgings, & Willman, 1987; Smith, Tyrell, Coyle, & Higgins, 1988; Smith, Tyrell, Coyle, & Willman, 1987) extended these findings to humans by rigorously documenting the adverse effects of experimentally-induced respiratory virus infection and cytokine administration on psychomotor performance, mood, and memory.

Cytokines in Saliva

The immune system is organized into anatomically discrete compartments to respond to pathogens present in different body tissues (e.g., blood, mucosal surfaces, intestines, and airways). It is well known that variation in localized immune responses is largely confined to that particular compartment and relatively independent of the more general systemic level of immune activation (e.g., Kindt, Osborne, & Goldsby, 2006). Therefore, unlike many commonly investigated salivary analytes such as cortisol, the vast majority of variation in cytokines in oral

fluid may be produced locally in the oral cavity (Granger et al, 2006). To the extent this is true, the measurement of salivary cytokine levels may be of questionable value to the study of brain, behavior, and immunity. Consistent with this possibility, and in contrast to the strong serum-saliva associations for many endocrine markers, in studies with adults the serum-saliva correlations for immune markers has been shown to be small to modest (e.g., Nishanian, Aziz, Chung, Detels, & Fahey, 1998). Serum-saliva correlations for immune markers in children and adolescents, however, are unknown.

The Present Study

To our knowledge this is the first study to rigorously and systematically explore the nature, variation, and correlates of salivary cytokines in youth. Saliva samples were collected across three annual assessments in a multi-cohort prospective study of healthy girls aged 11 to 17 years at the first assessment. Samples were assayed for a representative panel of nine cytokines (GM-CSF, IFNγ, IL-1ß, IL-2, IL-6, IL-8, IL-10, IL-12p70, TNFα) reflecting various components of intercellular and hormonal signaling within the NEI network. We examine the amount of variation, intercorrelations, and stability over time in salivary cytokines. Given the expectation that salivary cytokine levels may reflect local-oral mucosal immune activation, we also examined associations between cytokines and an salivary adipokine (adiponectin) related to periodontal health (Iwayama, Yanagita, Mori, Sawada, Ozasa, Kubota, Miki, Kojima, Takedachi, Kitamura, Shimabukuro, Hashikawa, & Murakami., 2012) and controlled for smoking, a health behavior prevalent during adolescence and known to compromise oral health. Further, matching serum cytokine levels were employed to evaluate serum-saliva associations. In these analyses, we controlled for smoking and salivary adiponectin to test the hypothesis that controlling for oral

health improves serum-saliva associations for markers that are at least partly influenced by oral health.

Methods

Participants

This investigation analyzed data from a longitudinal study designed to examine the impact of depressive symptoms and substance use on reproductive and bone health across puberty in girls (Dorn, Pabst, Sontag, Kalkwarf, Hillman, & Susman 2011; Hillman, Dorn, & Huang, 2010). Using a cross-sequential design (Donaldson & Horn, 1992), a total of 262 girls were enrolled and grouped in four cohorts depending on their age at the first assessment. The first cohort consisted of girls aged 11 years (cohort 11), the girls in the second cohort were 13 years old (cohort 13), the third cohort included girls aged 15 (cohort 15) and the girls in the fourth cohort were 17 years old (cohort 17). Each cohort was assessed annually for three consecutive years. Enrollment took place between December 2004 and October 2007. Girls were recruited from a teen health clinic in a children's hospital in a Midwestern city and its surrounding community. Exclusion criteria for enrollment in the larger study included: 1) pregnancy or breast feeding within 6 months, 2) primary amenorrhea (> 16 years), 3) secondary amenorrhea (< 6 cycles/year; not due to hormone contraception), 4) body mass index (BMI) < 1st percentile or weight > 300 pounds, 5) medication/medical disorder influencing bone health (e.g., prednisone, seizure medications), and 6) psychological or cognitive disabilities impairing comprehension or compliance.

Annual assessments of salivary data were analyzed for a subset of girls selected from the larger study who had complete salivary and serum cytokine data (complete data available for 113 girls at year 1, 114 girls at year 2, and 107 girls at year 3). The mean age at the first assessment

was 11.35 years (SD = 0.27) for cohort 11, 13.52 (SD = 0.42) for cohort 13, 15.59 (SD =0.30) for cohort 15, and 17.66 (SD = 0.25) for cohort 17. The majority of the sample was Caucasian (78% for cohort 11, 64% for cohort 13, 58% for cohort 15, 68% for cohort 17). Socioeconomic status, measured with Hollingshead criteria, averaged 44.33 (SD =11.78) for cohort 11, 42.68 (SD = 12.80) for cohort 13, 32.21 (SD = 14.33) for cohort 15, and 39.92 (SD = 12.18) for cohort 17. The study was approved by the Institutional Review Board of the children's hospital. Written parental consent was obtained, and each adolescent provided assent.

Procedure

Data were collected in a Clinical Translational Research Center (CTRC). Lab visits were conducted between 11:30 A.M. and 12:30 P.M. and consisted of a physical examination, anthropometry, blood and saliva sampling, psychosocial questionnaires and interviews, and bone densitometry. Girls were instructed to fast for the two hours preceding their visit to the lab. If a participant was menarcheal researchers attempted to reschedule her visit to day 5-9 of her menstrual cycle. Lab visits were also rescheduled if girls reported an infection or acute illness. Saliva samples were collected using Salivettes (Sarsted, NC). For more details regarding the procedures, see Dorn and colleagues (2011).

Measures

Puberty. Pubertal maturation was determined by physical examination carried out by trained clinicians using visualization and breast palpation and inspection of pubic hair and was categorized by Tanner criteria (Marshall & Tanner, 1969).

Smoking. Smoking behavior was first determined by questionnaire asking participants to describe their current smoking behavior (i.e., if she had never smoked, tried smoking, used to smoke but stopped, or was currently smoking) (Mayhew, Flay, & Mott, 2000). In addition,

tobacco smoke exposure was tested by assaying the saliva samples for cotinine (a primary metabolite of nicotine) using a commercially available enzyme immunoassay without modification to the manufacturers protocol (Salimetrics, State College, PA). The test used 20 µl of sample, had a lower limit of sensitivity of .05 ng/mL, a range of sensitivity from .05 to 200 ng/mL and an average intra-assay coefficient of variability (CV) of less than 8%. Salivary cotinine was recoded into groups based on reported cutoffs for smoking status (low/non-smoker= 0-15 ng/mL; smoker > 15 ng/mL) (Etzel, 1990; Jarvis, Tunstall-Pedoe, Feyerabend, Vesey, & Salooiee, 1987; Patrick, Cheadle, Thompson, Dieher, Koepsell, & Kinne, 1994). All adolescents with cotinine levels higher than 15 ng/mL and adolescents who reported that they smoked (irrespective of their cotinine levels) were categorized as "smokers". Adolescents with low cotinine levels (≤ 15 ng/mL) and who also reported that they did not smoke were categorized as "non-smokers". Approximately one quarter of the girls were smokers (year 1: 23% of girls were smokers (26/113); year 2: 22% smokers (25/114); year 3: 27% smokers (29/107)).

Determination of salivary cytokines. Salivary cytokines were measured using 96-well format multiplex electrochemiluminescence immunoassays manufactured by Meso Scale Discovery (MSD, Gaithersburg, MD). Each well of each 96-well plate is coated with capture-antibodies to GM-CSF, IFNγ, TNFα, IL-1ß, IL-2, IL-6, IL-8, IL-10 and IL-12p70. Detection antibodies are coupled to SULFOTAG^{TM} labels that emit light when electrochemically stimulated via carbon-coated electrodes in the bottom each microwell. The MSD 9-plex Multi-Spot Array assay was run following the manufacturers recommended protocol without modification. Cytokine concentrations (pg/mL) 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 (CV) were as

follows: GM-CSF (0.26 pg/mL, 14%), IFNγ (3.04 pg/mL, 17%), TNFα (0.58 pg/mL, 12%), IL-1ß (0.58 pg/mL, 6%), IL-2 (0.39 pg/mL, 13%), IL-6 (0.21 pg/mL, 12%) , IL-8 (0.15 pg/mL, 12%), IL-10 (1.01 pg/mL, 8%), and IL-12p70 (0.48 pg/ml, 14%).

Determination of salivary adiponectin. Adiponectin is an endocrine factor secreted by adipose tissue. Studies show adiponectin plays a role in inflammation and immune responses and has a potent beneficial function on the maintenance of the homeostasis of periodontal health (Iwayama, et. al, 2012; Kraus, Winter, Jepsen, Jager, Meyer, & Dreschner, 2012). We assayed adiponectin using an enzyme immunoassay designed for use with serum (Duoset, R&D Systems, MN) modified for use with saliva. Samples were assayed at a 1:8 dilution (35uL volume of saliva was diluted), test volume was 100ul, standard curve ranged from 62.5 to 2000 pg/mL, and inter-assay CVs were on average 4.56%. A subset of samples were re-assayed at 1:4 (34/334 samples) or 1:16 (5/334 samples) based on values returned at 1:8 that were below or above the standard curve.

Serum cytokine determinations. As part of the larger study, the cytokine panel assayed in saliva (described above) had been previously measured in serum using the Luminex platform and Lincoplex™ methodology without modification to the manufacturers recommended protocol (Millipore, Billerica, MA). For the serum cytokines, sensitivities were: GM-CSF (0.46 pg/mL), TNFα (0.05 pg/mL), IL-1ß (0.06 pg/mL), IL-2 (0.16 pg/mL) IL-6 (0.10 pg/mL), IL-8 (0.11 pg/mL), IFNγ (0.29 pg/mL, IL-10 (0.15 pg/mL), and IL-12p70 (0.11 pg/mL) with intra-assay CVs ranging from 3.11- 5.86% (see Dorn, Hillman, Houts, Denson, & Pabst, submitted).

Analytic Strategy

A log transformation was applied to all salivary and serum cytokines in order to correct for the positively skewed distribution. Any remaining outliers were winsorized (Tabachnik &

Fidell, 2001); that is, all values higher than three standard deviations from the mean were replaced with the next highest value in the distribution. The main analyses were conducted using the transformed and winsorized variables. To simplify the presentation, the main analyses report findings from the first assessment year, and results for the second and third assessment years are described only if they differ from those of the first year.

Paired *t*-tests were conducted to test whether cytokine levels in saliva were significantly different as compared to serum levels. Developmental differences in salivary cytokine concentrations were examined within each assessment year by Pearson's correlations (to examine the relation with age) and independent *t*-tests (to examine the relation with pubertal stage). Since very few girls were in Tanner Breast Stages 1-4 (34% for assessment year 1, 22% for year 2, and 12% for year 3), we compared levels of salivary cytokines between girls in stages 1-4 and girls in stage 5. The intercorrelations between serum cytokines and between salivary cytokines were examined by calculating partial correlations (controlling for age) and by principal component analyses (PCA) with promax rotation. Stability in cytokine levels over time and the associations with adiponectin were also evaluated using partial correlations (controlling for age).

Finally, serum-saliva associations for each of the cytokines were examined by calculating partial correlations (controlling for age). Then, hierarchical regression analyses were conducted to test whether controlling for age, smoking status, and adiponectin improved the associations between salivary and serum levels of the cytokines within each assessment year. For each salivary cytokine, a regression analysis was performed with age, adiponectin and smoking status in the first step and the serum levels of the respective cytokine in the second step. Although the transformation and winsorizing improved the distributions of the salivary and serum cytokines substantially, there was still modest positive skewness for some of the variables. Therefore,

robust regression analyses were performed, which are designed to be not overly affected by violations of assumptions for least squares regression (e.g., normal distribution, absence of outliers, absence of heterogeneity).

Results

Descriptive statistics for salivary and serum cytokine levels

Descriptive statistics (means, standard deviations, minimum and maximum, medians and 25% and 75% percentiles) are presented in Table 1, separately for each salivary cytokine and assessment year, but averaged across age-cohort. Similarly, descriptive statistics for the serum cytokines can be found in Table 2. With the exception of IL-10 and IFNγ, salivary levels were above the lower limit of detection (LLD) in the majority of samples for each cytokine (percentage of values below LLD: 0 – 49%). Values below the LLD were replaced with zeros, so as to be consistent with previous reports on this sample (Dorn et al., 2011). Salivary IL-10 and IFNγ were excluded from further analyses, because across all assessment years and age-cohorts (a total of 334 samples), 80% and 64% of samples had levels below the LLD for IFNγ and IL-10 respectively.

Within each assessment year, paired *t*-tests were conducted to examine whether cytokine levels in saliva were significantly different than serum levels. For the first assessment year, results indicated that salivary levels were significantly lower than those in serum (p's $<.01$), except for levels of IL-1β and IL-8 which had salivary levels greater than serum levels (p's $<.01$). Results were similar for years 2 and 3 with the exception of year 3 concentrations of IL-8 ($p = .08$) and GM-CSF ($p = .13$) which were not significantly different in serum and saliva.

Within each assessment year, Pearson's correlations were calculated to examine developmental differences by age in salivary cytokines. With the exception of IL-1β at years 1

and 2 and IL-6 at year 2, older girls had lower levels of all salivary cytokines at years 1 and 2 (for year 1: r [111] = -.40 – -.24, p's <.02; for year 2: r [112] = -.34 – -.20, p's <.04). At year 3, however, there were no significant correlations between age and salivary cytokine levels.

Independent t-tests examining differences in salivary cytokine levels between early (Tanner Breast Stages 1-4 (year 1, n = 38; year 2, n = 25; year 3, n = 13)) and late (Tanner Breast Stage 5 (year 1, n =75; year 2, n = 88; year 3, n = 93)) pubertal development showed similar findings. Lower cytokine concentrations in saliva were associated with more mature pubertal stage for most cytokines in assessment years 1 and 2 (for year 1 (n = 113), p's <.01 for all cytokines except IL-12p70 which showed no association with pubertal stage (p = .09); for year 2 (n = 113), p's < .04 except for IL-6 and IL-1β which showed no association with pubertal stage, p = .32 and p = .12, respectively). In contrast, only IL-8 (p = .02) and GM-CSF (p <.01) were significantly related to pubertal stage at year 3 (n = 106), with more advanced puberty associated with lower levels of these cytokines in saliva.

Insert Tables 1 and 2 about here

Intercorrelations of cytokines

Salivary cytokines were strongly and positively intercorrelated (all p's < .01; Table 3) for the first assessment year, with partial correlations (controlling for age) ranging between .51 (df = 110, p < .01) and .93 (df = 110, p < .01). A PCA pointed to one underlying component, explaining 75% of the variance in saliva cytokine levels. Standardized component loadings ranged between .77 and .95. Results were similar for the second and third year: partial correlations ranged between .42 (df = 111, p <.01) and .92 (df = 111, p < .01) for the second year

and between .39 (df = 104, $p < .01$) and .87 (df = 104, $p < .01$) for the third assessment year. Similarly, the PCAs for the second and third years also pointed to one underlying component explaining 72% and 65% of the variance in salivary cytokines, respectively.

By contrast, for serum cytokines, most of the partial correlations were modest (see Table 3) at the first assessment year, ranging between .20 (df = 110, $p = .03$) and .89 (df = 110, $p < .01$). A PCA with promax rotation was performed and results pointed to two underlying components which explained in total 73% of the variance. The first component included the cytokines IL-12p70, IL-1β, IL-2, IL-6, and GM-CSF (standardized component loadings between .74 and .87), whereas the second component consisted of IL-8 and TNFα (standardized component loadings: .63 and .75, respectively). For the second and third assessment years, partial correlations were comparable, ranging between .10 (IL-12p70 and TNFα, df = 111, $p = .31$) and .82 (IL-1β and IL-2, df = 111, $p<.01$) for the second year and between -.11 (IL-8 and GM-CSF, df = 104, $p = .25$) and .69 (IL-2 and IL-6, df = 104, $p <.01$) for the third year. A PCA with promax rotation for year 2 concentrations revealed a similar component structure to year 1 cytokines; 69% of the variance was explained by two underlying components each made up of the same cytokines groupings as year 1 cytokines. A PCA with promax rotation for year 3 cytokines also pointed to 2 components explaining 73% of the variance. However, unlike assessment years 1 and 2, in year 3 IL-12p70, IL-2, IL-6, IL-8 and TNFα loaded on the first component (standardized component loadings between .64 and .89), and IL-1β and GM-CSF loaded on the second component (standardized component loadings: .75 and .78, respectively).

Insert Table 3 about here

Stability of individual differences in cytokines across assessment years

As can be seen in Table 4, salivary cytokines were modestly associated over time. On average, the size of the partial correlation (controlling for age) for the stability across a one-year period was .30 (assessment years 1 and 2; *r* [110] = .21 - .38, *p*'s < .05) and .25 (years 2 and 3; *r* [104] = .09 - .36, *p* = .34 - <.01). The coefficients for the stability across a two-year period averaged .34 (*r* [103] = .19 - .46, *p's* = .06 - <.01). No specific salivary cytokine was necessarily more stable across time than any other. By contrast, for serum cytokines the size of the coefficients between assessment years 1 and 2, years 2 and 3, and years 1 and 3 were, on average .61 (*r* [110] = .39 – .75, *p*'s < 01), .33 (*r* [104] = .10 - .54, *p* = .32 - <.01) and .40 (*r* [103] = .16 - .57, *p* = .09 - <.01), respectively. Noteworthy is the observation that individual differences in levels of IL-12p70, IL-1β, IL-2, and IL-6 were more stable over time when measured in serum than in saliva.

Insert Table 4 about here

Cytokines and salivary adiponectin

Table 5 presents correlation coefficients for the association between salivary adiponectin and each salivary and serum cytokine, controlling for age, at the first assessment year. For every salivary cytokine, there was a significant and strong positive association between cytokine level and adiponectin (*r* [110] = .46 - .77, *p*'s < .01). In striking contrast, there was no significant association between salivary adiponectin and any cytokine measured in serum (*r* [110] = -.08 – .00, *p's* = .38 – > 1.00). Results were similar for the second and third assessment years.

Insert Tables 5 and 6 about here

Serum-saliva associations

No significant saliva-serum correlations were found for any cytokine in assessment year 1 (r [110] = -.11 - .11, *p's* = .24 - .92; Table 6), and similar results were found for years 2 and 3. Hierarchical regressions revealed that the predictors from the first step (age, adiponectin and smoking status) significantly predicted levels of salivary IL-12p70 (F [3, 109] = 19.30, p <.01), IL-1β (F [3, 109] = 61.12, p <.01), IL-2 (F [3, 109] = 51.78, p <.01), IL-6 (F [3, 109] = 19.56, p <.01), IL-8 (F [3, 109] = 74.03, p <.01), TNFα (F [3, 109] = 25.27, p <.01), and GM-CSF (F [3, 109] = 50.83, p <.01) in the first assessment year (Table 7). For all salivary cytokines, adiponectin was significantly associated with cytokine levels (t = 5.80 – 14.73, *p's* < .01). Older girls had significantly lower levels of IL-8, TNFα and GM-CSF (t = -3.43 – -2.35, *p's* < .03), and this association was marginally significant for IL-2 (t = -1.96, p = .05). Also, current smokers had lower levels of GM-CSF (t = -2.46, p =.02) and marginally lower levels of IL-6 (t = -1.87, p = .06). In contrast, for all cytokines, serum levels of the respective cytokine did not significantly predict salivary levels after controlling for age, adiponectin and smoking status in the first assessment year (t = -0.87 – 1.61, p's > .05).

Results were similar for the second and third assessment year. However, differences between years were seen in the relation between smoking status and salivary cytokine concentrations. IL-1β was higher in smokers than non-smokers in year 2 (t = 2.67, p <.01), and IL-1β, IL-2 and IL-6 (t = 2.24 - 3.17, p < .03) were higher in smokers than non-smokers in year

3. Also, in addition to the age effects seen at year 1, at year 2 IL-12p70 was negatively associated with age (t= -3.07, $p < .01$), and at year 3 the negative relation between IL-2 and age reached significance ($t = -2.22$, $p = .03$). Also, in year 3 serum concentrations of IL-1β were significantly positively associated with salivary concentrations of IL-1β ($t = 2.30$, $p = .02$) after adjusting for age, smoking status, and salivary adiponectin levels.

Insert Table 7 about here

Discussion

To the best of our knowledge, this is the first study to rigorously examine serum-saliva cytokines among healthy adolescents. The findings provide important information about salivary cytokines' measurement and concentrations, intercorrelations, stability, and associations with individual factors and other analytes, including serum-saliva correlations. *Measurement and concentrations:* The results reveal that levels of cytokines, except IFNγ and IL-10, were detectable in the majority of saliva samples, but that the distributions of salivary cytokines were non-normal in every case. The levels of all cytokines were lower in saliva than serum, except IL-8 and IL-1ß. Salivary levels for these two cytokines were more than 20-times higher than other salivary cytokines. *Intercorrelations:* The intercorrelations between cytokines in saliva were higher than in serum, and the factor structure in saliva was also distinct from that in serum with a single component explaining individual differences in the salivary cytokines. *Stability across time:* Cytokine levels were more stable across years when measured in serum than in saliva. *Cytokine associations with individual factors and other analytes:* Using adiponectin as a marker of oral health, there were strong associations between salivary adiponectin and salivary, but not

serum, cytokines. Some of the salivary cytokines were inversely associated with age and pubertal stage. *Cytokine serum-saliva association:* Surprisingly, only IL-1ß showed a significant serum-saliva correlation, and, although statistically significant, the magnitude of the association was negligible. Also, this finding was not consistent across assessment years and only revealed in hierarchical regressions after controlling for age, adiponectin, and smoking status. Overall the findings suggest that variation in basal salivary cytokine levels in healthy adolescents largely reflects compartmentalized local inflammatory activity of the oral mucosal immune system.

At the practical measurement level, for the majority of saliva samples, the cytokine values fell within the dynamic range of measurement afforded by the MSD multi-plex electrochemoluminescence immunoassay platform. For salivary IFNγ and IL-10, however, levels were below the detection limit (< 1.0 pg/mL) for the majority of the samples and consistently so across the assessment years. This observation highlights the possibility that it may not be worthwhile to include measures of IFNγ and IL-10 in future studies of salivary cytokines in healthy girls. Also, the distributions of all cytokines in saliva were non-normal. Therefore, the salivary cytokine data required intensive restructuring and transformation so as not to violate assumptions of normality in statistical analyses. The description of these atypical distributions has important implications for estimating statistical power when planning studies, how salivary cytokine data are prepared prior to data analyses (i.e., transformation and dealing with statistical outliers), as well as for the selection of appropriate parametric or non-parametric statistical techniques.

Serum levels of cytokines were higher than levels in saliva for all cytokines except IL-1ß and IL-8. Levels of IL-1ß and IL-8 were more than 20-25-times the levels of the other salivary cytokines. The literature on oral immunology suggests that IL-8 is a chemotactic factor that

influences the migration of neutrophils and increases their concentration (Bickel, 1993). Also, IL-1ß is important for the activation of neutrophils (Shaftel, Carlson, Olschowka, Kyrkanides, Matousek, & O'Banion, 2007; Struyf, Coillie, Paemen, Put, Lenaerts, Proost, Opdenakker, & Van Damme, 1998). Neutrophils are the most prevalent type of phagocyte in circulation and in whole saliva (Raeste, 1972). Their granules contain proteins, proteolytic enzymes, defensins, and myeloperoxidases that kill bacteria, break down bacterial proteins and cell walls, and stimulate phagocytosis of IgG-coated bacteria (see Kindt et al., 2006). The oral compartment is home to hundreds of species of bacteria, and the migration and activation of neutrophils plays a major role in promoting and maintaining oral health. The high levels of IL-8 and IL-1β observed in the current study likely reflect this important role of neutrophils in oral health.

On average, the correlations between the various cytokines in saliva were higher than the intercorrelations among the same cytokines when measured in serum. This pattern was consistent across assessment year and was more pronounced for IL-12p70, IL-1ß, IL-2 and IL-6. The PCA findings confirm two principal components that explain the majority (~75%) of the variance in cytokines when measured in serum. By contrast, there was just a single component to the structure of the variation in cytokines when they are measured in saliva. Moreover, the intracorrelations for each cytokine over time were stronger for cytokines measured in serum than in saliva, and there was only negligible evidence of any serum-saliva correlation. Oral biologists consider the mouth the most complex microbial microenvironment in the body (Giannobile, Beikler, Kinney,Ramseier, Morelli, & Wong, 2009; Zhang, Henson, Camargo, & Wong, 2009). The oral mucosa immune system is exposed to a wide variety of non-self antigens including bacteria, fungi, virus, and toxins. Thus, in contrast to circulating blood, oral fluid is not sterile. It is tempting to speculate that because of compartmentalized host-microbe interactions salivary

cytokines reflect a variable environmental microbial influence in the oral compartment, compared to invariant host genetic influences on serum cytokines in these healthy adolescents in the sterile serum compartment. Collectively the findings of the study suggest that compartmentalized cytokine activity in the mouth of healthy individuals may be more pronounced and variable than in the systemic immune system.

All salivary cytokines were strongly associated with salivary adiponectin. Studies suggest that adiponectin may play a role in maintaining periodontal health (Iwayama et al., 2012). The fact that all salivary, but not serum, cytokine levels were correlated with adiponectin and that these correlations were very strong is particularly noteworthy. We interpret these finding as confirmatory evidence that salivary cytokine levels largely reflect the degree of local oral, rather than systemic, inflammation; a point crucial for developmental scientists.

There were no associations between serum and saliva levels of cytokines. It is especially important to note that this was largely true even after controlling for adiponectin and smoking status. The fact that only IL-1ß showed an association with serum should not go unnoticed. As noted above, serum levels of IL-1ß play a major role in communication within and across the NEI (Smith, 1992, Weiss et.al, 1994). It could be that in the case of systemic inflammation (e.g., sepsis, fever, infection), the serum-saliva association for IL-1ß would have been stronger and more consistent across assessment years. Interestingly, studies show that IL-1ß released by cells in the gingival crevicular gap (the space between the gum line and the tooth) during the course of periodontal disease moves into circulation and stimulates the liver to secrete C-reactive protein (CRP) (Megson, Fitzsimmons, Dhamapatri, & Bartold, 2010). At least one study suggests modest serum-saliva correlations for CRP (Out, Hall, Granger, Page, & Woods, 2012; see also Floriano, Christodoulides, Miller, Ebersole, Spertus, Rose, Kinane, Novak, Steinhubl, Acosta,

Mohanty, Dharshan, Yeh, Redding, Furmaga, & McDevitt, 2009; Punyadeera, Dimeski, Kostner, Beyerlein, & Cooper-White, 2011; Quellet-Morin, Danese, Williams, & Arseneault, 2011). It is possible that salivary IL-1β may play a role in communication across and within the NEI. The next generation of studies might explore these serum-saliva relationships in special circumstances and cases representing confirmed systemic and oral inflammatory disease, or both. For instance, in early childhood when the rate of oral health problems is so low, do levels of salivary cytokines more accurately reflect levels in the circulation? What about disease states where the levels of cytokines in blood are atypically high, is the serum-saliva association during these special physiological circumstances stronger? How do salivary cytokines respond to challenge: can similar stress-reactivity patterns be observed in salivary cytokines as for serum cytokines?

Interestingly, both age and pubertal stage were associated with salivary cytokine levels. Older and more pubertally mature girls had lower levels of most salivary cytokines, particularly in assessment years 1 and 2. These findings raise the possibility that inflammation in the oral mucosa is associated with hormonal changes associated with girls' pubertal transition. For instance, studies show the rate of inflammatory autoimmune diseases to be higher in females than males, and implicate differences in estradiol levels as a mechanism partially responsible for this robust gender difference (Fairweather, Frisancho-Kiss, & Rose, 2008). Unfortunately, pubertal relations with salivary cytokine levels could not be fully explored in this sample, because the range in pubertal stage was very restricted, particularly at assessment year 3 (i.e., most girls were in advanced pubertal stages). Studies also suggest that the rate of oral inflammation increases substantially as children transition through puberty (Guncu, Tozum, & Caglayan, 2005; Oh, Eber, & Wang, 2002). The lack of age and puberty effects at assessment

year 3 may be due to smoking in our sample. Older and more pubertally mature girls were more likely to smoke, and therefore, they are more likely to have compromised oral health (Heikkinen, Pajukanta, Pitkaniemi, Broms, Sorsa, Koskenvuo, & Meurman, 2008; Heikkinen, Pitkaniemi, Kari, Pajukanta, Elonheimo, Koskenvuo, & Meurman, 2012; Lee, Taneja, & Vassallo, 2012; Azar & Richard, 2011). Poorer oral health may be especially pronounced at the third assessment year when the sample is the oldest. Although the sample size within each age group was too small to adequately test the effect of smoking on salivary cytokine levels, it is possible that the effects of age and puberty at year 3 were masked by smoking-related changes in salivary cytokine concentrations. Larger studies that follow girls (and boys) through this developmental phase and monitor reproductive hormones and salivary cytokines might prove important for understanding risk for periodontal disease in adolescence.

In conclusion, cytokines in saliva show different levels, stability, inter-relationships, and associations with oral health than serum cytokines. There is little evidence of a serum-saliva association under conditions of general health. Collectively, the pattern of findings suggests that basal levels of salivary cytokines reflect activity of a highly compartmentalized oral mucosal immune system. In these circumstances, the value of salivary cytokines to the study of brain, behavior and immunity may be questionable. Future studies may examine whether this is also true in other physiological states, developmental stages and stressed versus resting conditions. The observed variation in salivary cytokines in these circumstances may be more likely to reflect systemic processes and be reliably associated with child development, behavior and health. In the current study, focusing on a healthy and non-stressed sample of adolescent girls, the findings of our study highlight the importance of measuring salivary cytokines to advance our understanding of risk for oral health and disease during adolescence and raise important

questions about the role of salivary cytokines in intracellular and hormonal signaling within the larger NEI network.

Notes

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Conflict of interest: in the interest of full disclosure, DAG is founder and Chief Strategy and Scientific Advisor at Salimetrics LLC (State College, PA). DAG's relationship with Salimetrics LLC is managed by the policies of the Conflict of Interest Committee at the Johns Hopkins University School of Medicine.

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Table 1. Descriptive statistics of non-transformed salivary cytokines in healthy adolescent girls at each assessment year (pg/mL)

	M	SD	Min	Max	Median	25th percentile	75th percentile
Year 1							
IFNγ	1.14	1.89	0.00	6.96	0.00	0.00	3.14
IL-10	0.35	1.10	0.00	10.09	0.00	0.00	0.00
IL-12p70	0.74	1.03	0.00	7.62	0.61	0.00	0.97
IL-1β	75.84	88.44	1.54	530.61	51.92	23.11	85.59
IL-2	1.76	1.40	0.00	6.92	1.43	0.76	2.21
IL-6	1.40	3.27	0.00	27.12	0.68	0.36	1.18
IL-8	61.26	63.13	0.43	316.86	43.16	17.76	79.29
TNFα	0.90	1.07	0.00	5.77	0.78	0.00	1.35
GM-CSF	3.25	3.45	0.00	17.36	1.96	0.95	4.01
Year 2							
IFNγ	1.94	2.78	0.00	12.20	0.00	0.00	3.69
IL-10	0.18	0.55	0.00	3.45	0.00	0.00	0.00
IL-12p70	0.49	0.74	0.00	5.48	0.51	0.00	0.72
IL-1β	69.88	105.91	1.67	913.53	35.82	19.05	95.56
IL-2	1.57	1.96	0.00	14.41	0.99	0.55	1.95
IL-6	0.89	1.72	0.00	13.03	0.46	0.31	0.66
IL-8	54.95	107.25	1.87	816.83	25.50	10.76	56.92
TNFα	0.65	0.92	0.00	4.59	0.58	0.00	0.93

GM-CSF	2.44	3.36	0.00	26.47	1.34	0.74	3.09
Year 3							
IFNγ	1.71	2.24	0.00	7.44	0.00	0.00	3.56
IL-10	0.44	0.88	0.00	6.49	0.00	0.00	1.03
IL-12p70	0.63	0.76	0.00	5.25	0.59	0.00	0.87
IL-1β	66.18	63.54	4.37	310.25	46.07	21.86	82.23
IL-2	1.70	2.51	0.00	25.60	1.29	0.72	2.03
IL-6	1.04	2.51	0.00	24.29	0.54	0.30	0.98
IL-8	50.16	44.24	0.83	186.77	35.23	16.93	69.49
TNFα	0.87	0.90	0.00	5.99	0.78	0.00	1.28
GM-CSF	2.55	2.37	0.00	11.37	1.79	1.03	2.87

Note. Descriptive statistics were calculated across cohorts. N = 113 for year 1, N = 114 for year 2, N = 107 for year 3.

Table 2. Descriptive statistics of non-transformed serum cytokines in healthy adolescent girls at each assessment year (pg/mL)

	M	SD	Min	Max	Median	25th percentile	75th percentile
Year 1							
IFNγ	9.60	46.34	0.00	411.78	0.00	0.00	2.90
IL-10	32.93	120.17	0.00	1127.08	7.17	3.91	11.97
IL-12p70	30.04	115.13	0.00	1001.60	1.35	0.00	6.83
IL-1β	12.28	43.61	0.00	417.37	1.88	0.00	6.05
IL-2	19.34	62.37	0.00	613.79	5.44	1.14	11.17
IL-6	10.35	27.12	0.00	173.81	1.41	0.71	4.35
IL-8	8.09	7.49	0.00	62.42	6.92	4.94	9.02
TNFα	9.75	7.56	0.70	51.70	8.60	6.06	10.98
GM-CSF	98.91	285.31	0.00	2353.39	16.40	0.00	65.35
Year 2							
IFNγ	3.37	17.36	0.00	172.78	0.00	0.00	0.67
IL-10	17.72	53.17	0.37	506.25	6.28	4.26	11.42
IL-12p70	12.70	49.77	0.00	467.98	0.59	0.00	5.42
IL-1β	9.83	53.07	0.00	558.43	1.17	0.00	4.20
IL-2	13.99	67.94	0.00	720.55	3.60	0.63	9.00
IL-6	7.31	26.19	0.00	244.17	1.76	0.96	4.32
IL-8	7.36	6.34	1.49	64.69	6.36	4.99	7.79
TNFα	8.32	4.27	2.02	27.73	7.52	5.29	10.26

GM-CSF	79.03	336.62	0.00	3493.67	0.00	0.00	54.45
Year 3							
IFNγ	187.04	349.14	0.00	1688.08	53.02	5.44	197.28
IL-10	52.94	307.86	0.00	2872.73	2.99	1.45	8.20
IL-12p70	266.86	887.06	0.00	5276.33	7.37	1.06	44.73
IL-1β	10.96	60.92	0.00	584.28	0.33	0.00	2.62
IL-2	30.33	111.41	0.00	694.30	2.16	0.32	9.23
IL-6	38.62	96.83	0.00	560.63	7.48	1.54	20.57
IL-8	54.39	74.84	3.75	319.26	24.07	9.21	57.02
TNFα	17.29	42.28	2.24	362.34	8.05	5.69	12.79
GM-CSF	55.82	264.95	0.00	2187.26	0.00	0.00	11.14

Note. Descriptive statistics were calculated across cohorts. $N = 113$ for year 1, $N = 114$ for year 2, $N = 107$ for year 3.

Table 3. Intercorrelations within salivary cytokines and within serum cytokines at the first assessment year, controlling for age.

	IL-12p70	IL-1β	IL-2	IL-6	IL-8	TNFα	GM-CSF
IL-12p70	-------	.58**	.61**	.89**	.33**	.22*	.50**
IL-1β	.54**	-------	.82**	.48**	.37**	.35**	.76**
IL-2	.77**	.75**	-------	.49**	.36**	.31**	.75**
IL-6	.57**	.51**	.63**	-------	.31**	.20*	.39**
IL-8	.71**	.72**	.93**	.61**	-------	.53**	.39**
TNFα	.66**	.57**	.65**	.54**	.66**	-------	.24*
GM-CSF	.75**	.71**	.91**	.68**	.89**	.65**	-------

Note. Partial correlations for the salivary cytokines can be found below the diagonal; the serum correlations are presented above the diagonal. *p<.05, **p < .01. N=113

Table 4. Correlations of cytokine concentrations over time in serum and saliva, controlling for age.

	Serum			Saliva		
	Years 1 and 2	Years 2 and 3	Years 1 and 3	Years 1 and 2	Years 2 and 3	Years 1 and 3
IL-12p70	.72**	.49**	.41**	.24**	.09	.22*
IL-1β	.75**	.29**	.45**	.37**	.36**	.46**
IL-2	.73**	.54**	.57**	.31**	.26**	.31**
IL-6	.70**	.40**	.50**	.21*	.30**	.19
IL-8	.53**	.17	.16	.38**	.28**	.45**
TNFα	.39**	.10	.29**	.33**	.18	.35**
GM-CSF	.53**	.29**	.39**	.25**	.28**	.43**

Note. Years 1 and 2, N=113; years 2 and 3, N=107; years 1 and 3, N=106. *$p < .05$, **$p < .01$.

Table 5. Correlations of serum and salivary cytokines with adiponectin for the first assessment year, controlling for age.

	Serum	Saliva
IL-12p70	-.08	.51**
IL-1β	-.01	.77**
IL-2	.00	.69**
IL-6	-.01	.62**
IL-8	-.07	.71**
TNFα	.00	.46**
GM-CSF	-.05	.68**

Note. N= 113. ** $p < .01$

Table 6. Correlations between salivary and serum cytokines at the first assessment year, controlling for age.

	Correlation with serum levels
IL-12p70	-.11
IL-1β	.11
IL-2	-.01
IL-6	.07
IL-8	-.05
TNFα	-.08
GM-CSF	-.07

Note: $N = 113$

Table 7. Regression analyses for each cytokine predicting salivary levels from serum levels, controlling for age, adiponectin and smoking (first assessment year)

		B	SE	β	*t*	*p*	R^2	*F*
IL-12p70	Step 1						0.31	19.30**
	Age	-0.01	0.01	-.12	-1.27	.21		
	Adiponectin	0.13	0.02	.49	7.03	<.01		
	Smoking	-0.07	0.05	-.12	-1.33	.19		
	Step 2						0.32	14.59**
	Serum levels	-0.01	0.01	-.06	-0.87	.39		
IL-1β	Step 1						0.60	61.12**
	Age	-0.03	0.03	-.07	-1.01	.32		
	Adiponectin	0.92	0.07	.76	12.80	<.01		
	Smoking	-0.05	0.16	-.02	-0.28	.78		
	Step 2						0.61	48.48**
	Serum levels	0.11	0.07	.12	1.61	.11		
IL-2	Step 1						0.52	51.78**
	Age	-0.03	0.01	-.16	-1.96	.05		
	Adiponectin	0.27	0.02	.66	11.58	<.01		
	Smoking	-0.06	0.06	-.07	-0.89	.38		
	Step 2						0.52	39.58**
	Serum levels	0.00	0.02	-.01	-0.09	.93		
IL-6	Step 1						0.44	19.56**
	Age	-0.02	0.01	-.12	-1.04	.17		
	Adiponectin	0.23	0.03	.60	7.08	<.01		
	Smoking	-0.09	0.05	-.12	-1.87	.06		
	Step 2						0.45	14.42**
	Serum levels	0.02	0.02	.08	0.96	.34		
IL-8	Step 1						0.55	74.03**
	Age	-0.09	0.04	-.19	-2.35	.02		

	Adiponectin	0.79	0.05	.68	14.73	<.01		
	Smoking	-0.15	0.19	-.07	-0.79	.43		
	Step 2						0.55	60.38**
	Serum levels	-0.01	0.12	-.01	-0.12	.91		
TNFα	Step 1						0.29	25.27**
	Age	-0.04	0.01	-.26	-2.63	.01		
	Adiponectin	0.16	0.03	.44	5.80	<.01		
	Smoking	-0.01	0.08	-.02	-0.15	.88		
	Step 2						0.29	18.88**
	Serum levels	-0.06	0.08	-.08	-0.73	.47		
GM-CSF	Step 1						0.56	50.83**
	Age	-0.06	0.02	-.24	-3.43	<.01		
	Adiponectin	0.40	0.04	.61	11.03	<.01		
	Smoking	-0.19	0.08	-.15	-2.46	.02		
	Step 2						0.56	40.07**
	Serum levels	-0.01	0.02	-.04	-0.66	.51		

Note. The statistics are derived from the second step of each regression model. $N = 113$, $^{**}p < .01$