THE BIOLOGICAL EMBEDDING OF EARLY-LIFE ADVERSITY: USING SALIVARY BIOMARKERS TO EXAMINE THE INFLUENCE OF MATERNAL PSYCHOLOGICAL WELL-BEING ON CHILD NEUROENDOCRINE-IMMUNE FUNCTIONING

by

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ABSTRACT

Background: Neuroendocrine-immune (NEI) regulation is essential for maintaining health. Through repeated activation of the stress response, early-life adversity may lead to dysregulation of the NEI network and increase risk of inflammatory-related disease. Studying NEI functioning during childhood, however, has been limited by the need for biologic data, which has been largely restricted to blood-based measures.

Objectives: Using salivary biomeasures of immune activity (cytokines) this study examined: 1) the nature and correlates of salivary cytokines in children; and 2) the role of early-life adversity in moderating child NEI functioning.

Methods: Data were drawn from the Fetus to Five study, a laboratory-based study of mother-child pairs. Children participated in stress-inducing tasks and provided four saliva samples. Mothers completed a survey about child health, family sociodemographics, and maternal mental health. Saliva was assayed for four inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα) and markers of autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) activity (alpha-amylase (sAA), cortisol). Multilevel mixed models examined relations between cytokines and child health and demographic factors, and associations with sAA and cortisol. Composite scores reflecting socioeconomic status (SES) and maternal distress were created for each child using factor analysis of SES and maternal psychological (depressive symptoms, anxiety, stress) variables. Multilevel mixed models for cortisol examined child cortisol-cytokine relations and interactions between maternal distress and cytokines on cortisol, adjusting for SES.

Results: Cytokines were largely unrelated to health and demographic factors, but were associated with oral health measures. Among boys, cytokines were positively associated with sAA and inversely associated with cortisol. In the full sample, positive maternal distress-cytokine

interactions on cortisol for IL-1β, IL-6 and TNFα indicated that as maternal distress increased, inverse cytokine-cortisol relations became weaker. These interactions were driven by significant interactions among girls.

Conclusions: Salivary cytokines in children reflect oral immune processes. Relations between cytokines and markers of ANS and HPA activity in saliva, however, mirror those in serum, suggesting NEI functioning may be studied using salivary biomeasures. Among girls, regardless of SES, maternal distress was associated with less efficient regulation of child inflammatory activity by cortisol. This desensitization may increase inflammatory-related disease risk and contribute to health disparities.

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CHAPTER ONE

Introduction, Study Aims, and Significance

1.1. Introduction

Eliminating health disparities in the United States has been a national priority for decades. However, efforts toward this goal have made limited progress (1–3). Disadvantaged populations still suffer higher morbidity and mortality rates of diabetes, obesity, depression, stroke, and cardiovascular and respiratory disease (1). In addition to exhibiting similar socioeconomic disparities, these diseases also share a common underlying pathophysiology involving dysfunctional inflammatory processes of the immune system (4–6). Understanding how these inflammatory processes are altered by socioeconomic factors and the health risks associated with these changes is fundamental to understanding and addressing health disparities in the US. Of particular importance is the critical role of the early-life environment in shaping the development and regulation of these inflammatory processes (7).

The goal of this study was to further our understanding of the biologic mechanisms linking low socioeconomic status (SES) and increased disease risk through use of new salivary immune biomeasures. The study focused on the role of early-life adversity in shaping the development of two biologic systems involved in inflammatory processes– the central nervous system (CNS) and the immune system. There were three specific aims, to: 1) explore current salivary analyte data analysis methods and their implications for data quality, and the validity and reliability of study findings; 2) examine the nature and correlates of salivary immune markers (cytokines) in five-year old children, and examine relations between cytokines and demographic and health characteristics, as well as relations with activation of the CNS; 3) examine the role of early-life adversity in moderating the relation between CNS and immune system activity in five-year old children.

1.2. Study Aims and Hypotheses

Aim 1: Explore current salivary analyte data analysis methods, with a focus on pre-analytic data processing techniques, and their implications for data quality, and the validity and reliability of study findings.

Biomeasure data are increasingly important and popular in child health and development research (8). As a cost-effective, socially acceptable, and minimally-invasive method for obtaining biologic data, saliva is well-suited for large-scale public health studies of child health and development. This aim advances the field of salivary bioscience by addressing the challenges inherent in salivary biomeasure data analysis and by proposing standard methods and reporting for salivary biomeasure studies. Despite the wide-spread use of salivary biomeasure data, there is limited information regarding the best practices of salivary data processing and analysis. In particular, differences in pre-analytic data processing methods are important, because these early steps of data processing are largely unreported in published manuscripts. Furthermore, differences in pre-analytic data processing methods between studies limit the validity and reliability of salivary biomeasure results and may underlie inconsistencies in findings throughout the literature.

Aim 2: Examine the nature and correlates of salivary immune markers (cytokines) in five-year old children, and relations between cytokines and demographic and health characteristics, as well as relations with activation of the CNS (including autonomic nervous system (ANS) and hypothalamic-pituitary adrenal (HPA) axis activity).

Hypothesis 2.1. Salivary cytokine activity is positively intercorrelated across a series of cognitive and emotional challenge tasks.

The four salivary cytokines examined in this study are proinflammatory and share regulatory mechanisms (6). Findings from studies examining salivary cytokines in older

participants suggest a coordinated immune response in the oral cavity and strong inter-cytokine correlations; however, to date, studies in young children are lacking (9). It was hypothesized that the inter-cytokine associations in young children are similar to those observed in older participants. Although no studies were identified that examined inter-cytokine correlations after a stressor task, the cytokines examined in this study have common regulatory mechanisms mediated by the activation of the ANS and HPA axis (6). Therefore, the cytokines' response to stress was hypothesized to be related to the stress response of the ANS and HPA axis and to be highly intercorrelated.

Hypothesis 2.2. Salivary cytokine activity is positively associated with oral health issues and unrelated to demographic and systemic health factors.

Immune processes are highly specific to the tissues and immunological threats of the region. Salivary cytokines are produced within the oral cavity to respond to local immunological threats (10). Previous studies show consistent positive associations between oral health problems and baseline salivary cytokine levels in adolescents and adults (11–17). Systemic health factors, however, are largely unrelated to baseline levels of salivary cytokines in healthy adolescents and adults (9,18–20). It was hypothesized that similar relations exist in young children.

Hypothesis 2.3. Salivary cytokines are positively associated with ANS activity and negatively associated with HPA activity in five-year old children.

Systemically, the immune system and the ANS and HPA axis are reciprocally regulated (21). Activation of the ANS by acute stress stimulates the release of proinflammatory cytokines (21). Inflammatory cytokines stimulate the HPA axis to release cortisol, and cortisol inhibits proinflammatory cytokine production (21). The nature of these neuroendocrine-immune interactions in saliva are hypothesized to be similar to those systemically. Although not widely studied, this hypothesis is supported by two investigations that examined associations between one proinflammatory cytokine (interleukin-6) and HPA activity in saliva (22,23). These studies

show that inflammatory activity was inversely related to HPA activity and positively related to ANS activity (22,23). If interactions between salivary ANS, HPA, and inflammatory activity mirror systemic interactions, this finding would support the use of these minimally-invasive biomeasures for the study of systemic neuroendocrine-immune functioning and inflammatory regulation.

Aim 3: Examine the role of early-life adversity in moderating the relation between HPA and immune system activity in five-year old children.

Hypothesis 3.1. Maternal psychological well-being moderates HPA-immune system relations in five-year old children regardless of family socioeconomic status.

Early-life adversity and stress impact the development and calibration of the HPA axis and the immune system (24,25). Caregivers, in particular, play a significant role in both the child's exposure to stress and the impact the exposure has on the developing child's physiology. A supportive caregiver can buffer the child from the negative effects of early-life stressors (26), while children raised by mothers with poor psychosocial health are more likely to experience cognitive, behavioral and physical health problems during childhood and later in life (27). It was hypothesized that coordination between the HPA axis and immune system is altered by maternal psychological well-being and that these changes are measurable using salivary biomeasures of the HPA axis (cortisol) and inflammatory activity (cytokines). Furthermore, it was hypothesized that maternal psychological well-being moderates the relation between salivary cytokines and cortisol regardless of family SES.

1.3. Significance

The financial and societal costs of health disparities in the US are substantial. Over a three year period, the national cost of health disparities climbed to nearly $1.25 trillion, with over $200 billion in direct medical costs (28). Annually, an estimated $1 trillion of forgone economic

value, including wages and years working, is attributed to health disparities in low-SES adult populations (29). Disproportionate disease burden also leads to large disparities in mortality. One study found a 35-year gap between the highest and lowest life expectancies in the US (30), and a high school education alone has been associated with nearly 13 years of additional life expectancy (31).

Advancing efforts to stem health disparities requires an increased understanding of the mechanisms and risk factors of diseases disproportionately affecting low-SES populations (32,33). This dissertation furthers these goals by examining relations between psychosocial and socioeconomic aspects of the early-life environment and neuroendocrine-immune coordination- a critical regulatory mechanism of the inflammatory processes underlying many of the diseases that cluster in low-SES populations. This study recognizes the importance of early-life environments in calibrating these regulatory processes. It focuses on identifying mechanisms and risk factors for health disparities early in life when prevention, intervention, and treatment efforts can have the largest impact.

Findings from this study could inform policies and programs aimed at reducing the burden of inflammation-related diseases. Evidence of biologic changes related to psychosocial stressors early in life would have significant implications for shaping policy and programmatic interventions that focus on early childhood as a critical intervention point for fostering healthy development and future health and well-being. By demonstrating the role of family factors on physiologic functioning, the results from this study can help increase focus on policy and programmatic interventions for parents and families, an area of domestic policy that has traditionally been overlooked (34) but has recently received increasing investments (e.g., home visiting programs). The results can also highlight the integral role of the psychosocial environment in child health, and be used to support community and family interventions to reduce health disparities (35).

Finally, the study's methodology is an important contribution to the field of public health and child development. Biomarkers play an increasingly important role in research and in supporting evidence-based policies and programs (36). However, the collection of blood-based biomeasures is subject to strict regulations and requires considerable financial and personnel resources (36). In addition, obtaining consent for blood collection can be difficult and social acceptability is low, particularly for children (36). Saliva collection is a minimally-invasive, low-cost and socially acceptable method of obtaining biomeasure data (36,37). Little research has focused on immune system salivary analytes, and only two studies were identified that examined these biomeasures in children (38,39). Findings from this study will advance the field of salivary immunologic analyte research and demonstrate the feasibility and value of salivary biomeasure research in public health. Furthermore, study aim 1 will contribute to the field of salivary bioscience by discussing the challenges and best practices of salivary biomeasure data analysis and calling for standardization and disclosure of methodology. This approach will improve the validity, reliability and replicability of study findings in this new and growing area of public health research.

1.4. Organization of the Dissertation

This dissertation includes three independent manuscripts, as well as four chapters that introduce the dissertation, present the overarching theoretical framework and existing knowledge, describe the study methods and design, and discuss the conclusions and implications of the study findings. This chapter presents the study aims, hypotheses and public health significance of the dissertation. The second chapter discusses the theoretical framework underlying the study aims. It also provides basic background information about the biologic and psychosocial concepts examined in the studies, as well as key findings from the literature that informed the study aims and hypotheses. Chapter three presents a detailed description of the study design and methods, including information about the study from which the data used in the dissertation were drawn, as

well as sample selection and statistical analysis details. Chapters four, five and six each contain manuscripts associated with each study aim (aim 1 is addressed in chapter four, aim 2 in chapter five, and aim 3 in chapter six). The final chapter synthesizes the results from the three manuscripts and discusses the conclusions of the studies. This chapter also discusses the implications of the study findings as an integrated work with a focus on public health research, practice and policy.

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CHAPTER TWO

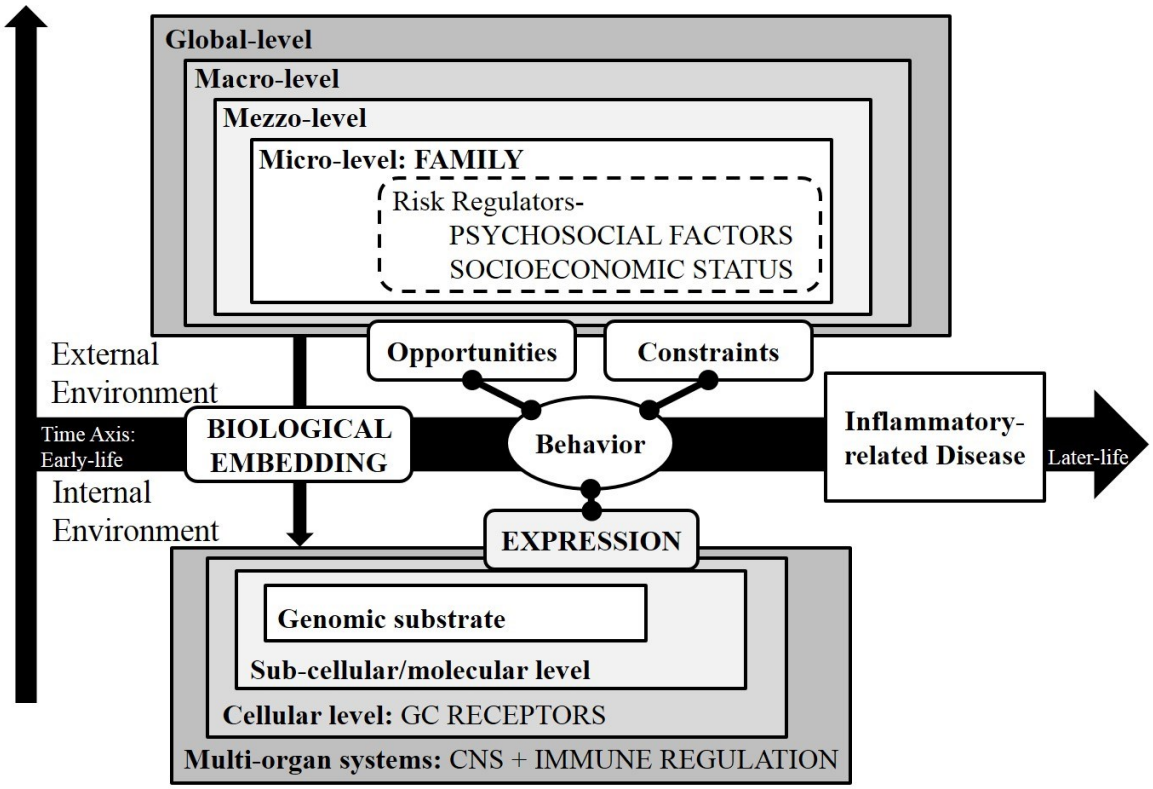
Theoretical Basis and Background

2.1. Theoretical Basis

The conceptual framework for this study (Figure 2.1) is adapted from the multi-level society-behavior-biology framework of Glass and McAtee (2006) and the concept of biological embedding (1,2). Glass and McAtee organize the determinants of health within multiple levels of nested systems (1). There are two sets of systems – one inside the body that ranges from the genetic constitution to multi-organ systems, and one outside the body that ranges from the family to the global context (1). As an individual develops over the life course, the social and built environment influence physiologic systems via embodiment (1). Embodiment is the process by which external forces alter an individual's biology at each level from epigenetic changes to changes in multi-organ interactions (1). Embodiment is also known as biological embedding (2). Like embodiment, biological embedding explains how life experiences and environments "get under the skin" and alter biologic functioning to affect life-long health and vulnerability to disease (2).

Glass and McAtee also discuss the importance of stable characteristics of the social and built environment that influence the likelihood that an individual will be exposed to or engage in risky behaviors (1). These characteristics are called risk regulators (shown in Figure 2.1 as dashed boxes; 1). An individual's social and built environments create opportunities and constraints on behavior (1). Accordingly, an individual's health is influenced by both physiologic bottom-up processes, which are altered through embodiment, and by the opportunities and constraints presented by the individual's external environment, social structures, and institutions (1).

Figure 2.1. Conceptual framework for the study



*Adapted from Glass & McAtee (2006). CNS= central nervous system; GC= glucocorticoid (e.g., cortisol).

Following Glass and McAtee's framework (1), this study examines the embodiment of micro-level (family) adversities, and whether the embodiment of these adversities is expressed as alterations within the neuroendocrine-immune (NEI) network. The study considers socioeconomic and psychosocial family characteristics as risk regulators that affect physiologic regulation of the central nervous system (CNS) and immune system via symbolic inputs (e.g., fear, threat). The multi-level and integrative nature of this study allows for a deeper understanding of the within and across-level relations between psychosocial and biologic factors.

2.2. Background

Early-life Adversity

Early-life adversity is defined as the experience of negative life events in childhood (3). A wide range of events can be categorized as adverse experiences. Events range from physical, sexual, and psychological abuse to a lack of parental responsiveness to the child's needs, dysfunctional family relationships, and the witnessing of violence or death (3). Negative life events are not under the control of the child and are capable of causing harm, suffering, and lasting changes in the development of the child (3). Children in low socioeconomic status (SES) families experience a greater number of negative life events than children in more advantaged families (4–6). Many studies, therefore, use SES as a proxy measure for adversity (e.g., 7,8).

Adversity and Stress

Life events that constitute early-life adversity, such as maltreatment, neglect, harsh or inattentive parenting and chaotic family environments, elicit negative emotional responses such as feelings of fear, threat, danger, and helplessness (3). These emotional responses are coupled with the activation of the physiologic stress response (stimulation of the CNS). The acute activation of the stress response to negative life events can be protective in the short-term and help the child cope with the immediate danger or threat (9). The frequency and severity of negative life events experienced during early-life adversity, however, triggers repeated activation of the stress response, which may result in physiologic damage and dysfunction (9). Childhood adversity is associated with an increased risk of a wide range of childhood and adult diseases including heart disease, chronic respiratory diseases, and depression (10,11). The link between early-life adversity and adult disease is so strong that childhood adversities have been called "the new morbidities of childhood" (12,13). The CNS and immune system's response to stress that accompanies these "new morbidities" (12,13) is an important part of the mechanism underlying the association between early-life adversity and disease.

The Physiologic Response to Stress

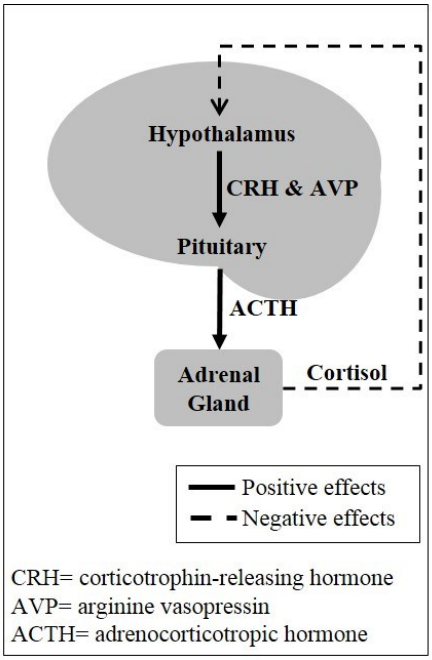
The Central Nervous System

Two interconnected systems within the CNS regulate the body's response to stress – the sympathetic-adrenomedullary (SAM) system (in the sympathetic branch of the autonomic nervous system (ANS)) and the hypothalamic-pituitary-adrenal (HPA) axis. These systems are activated by the brain, specifically the hypothalamus, when physiologic balance within the body is challenged by internal or external stimuli. Once activated, the SAM system and HPA axis respond to the demands of the stimuli and work to restore balance within the body. (14)

The SAM system: When activated by the hypothalamus, the SAM system rapidly responds with an increase in heart rate, respiration, blood pressure and the release of catecholamines (epinephrine (adrenaline) and norepinephrine). This quick response to challenge braces the body for a "fight or flight". The catecholamines mobilize immune cells into the blood stream and trigger the release of immune messengers (cytokines) to protect the body against immediate injury and damage. The SAM stress response is dampened by activation of the parasympathetic nervous system, which decreases the immune system response via the vagus nerve. (15)

The HPA response: Compared to the SAM response, the HPA response is delayed, peaking about 25 minutes after the stressor. The HPA axis involves a cascade of chemical reactions. It begins with the hypothalamus releasing corticotrophin-releasing hormone (CRH) and culminates with the adrenal glands releasing cortisol into the bloodstream. Cortisol affects the function of cells throughout the body and in multiple biologic systems, including the nervous, cardiac, immune, digestive, and reproductive systems by binding to cell receptors. Once bound to cell receptors, cortisol can enter cell nuclei and affect gene transcription (epigenetic effects). Cortisol also crosses the blood-brain barrier. In the

Figure 2.2. HPA negative feedback loop



brain, and in the hippocampus, pituitary, and hypothalamus in particular, cortisol binding inhibits the production of CRH. This response creates a negative feedback loop that is important for regulating the acute response to stress (see Figure 2.2). Keeping the acute HPA response regulated in this manner is critical because it allows the body to establish homeostasis. (14)

The Immune System: A Focus on Inflammation

Like the CNS, the immune system maintains balance within the body and protects it from foreign threats. The immune system is made up of two cooperative systems, the innate and adaptive immune systems. When faced with an immediate threat, both systems react with a coordinated response called inflammation. (16)

Inflammation is a surge in the innate and adaptive immune systems. During inflammation, immune cells rush to the area of infection or threat. Specialized immune cells (B cells) release antibodies that tag invading bodies for destruction, and other immune cells engulf and kill (phagocytosize) foreign bodies and infected cells. Inflammation is facilitated and regulated by chemical messengers called cytokines that are released by immune and somatic cells. Proinflammatory cytokines promote the inflammatory processes by increasing the permeability of vascular and lymphatic endothelial walls and facilitating an influx of blood, immune cells, and cytokines to the site of infection or injury. (16)

Cytokines

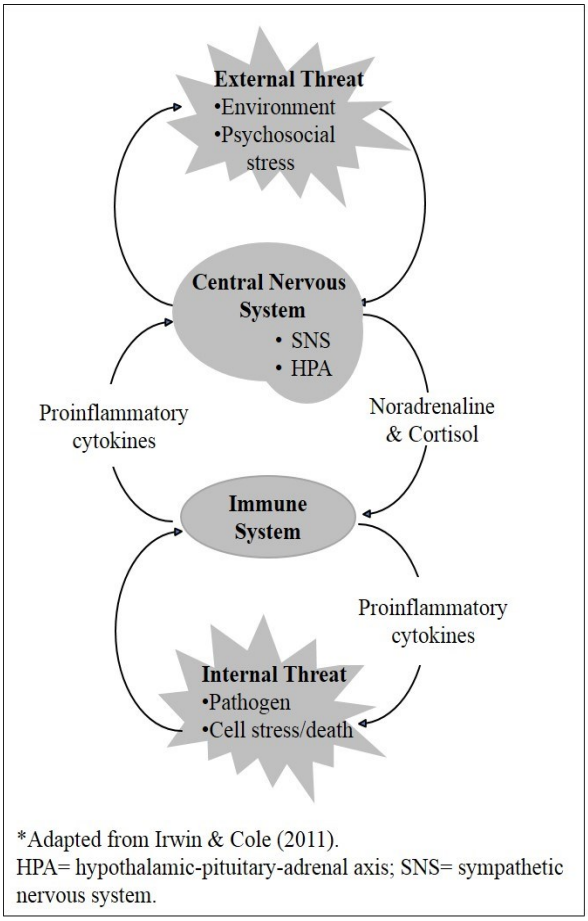
Cytokines are the primary chemical messengers of the immune system (16). In addition to regulating immune processes, cytokines facilitate communication between the immune system and the rest of the body and are critical for development, growth, healing, and tissue maintenance throughout the body (17). Cytokines in the interleukin family, like interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8), act on leukocytes (17,18). Tumor necrosis factors, like tumor necrosis factor alpha (TNFα), are involved in initiating inflammation, maintaining

lymphoid organs and regulating lymphocytes (17,18). IL-1β, IL-6, IL-8 and TNFα have proinflammatory mechanisms; they are released during inflammation and promote inflammatory processes (19).

Interaction between the CNS and Immune System: The Neuroendocrine-Immune (NEI) Network

Both the immune system and the CNS are tasked with adapting the body's internal environment to meet the demands and challenges of the external environment. Communication between these systems is essential for establishing homeostasis, allostasis, and overall health. A set of shared chemical messengers (cytokines) and direct nerve-cell interactions allow for the integration and reciprocal regulation of the CNS and immune system. See Figure 2.3 for an illustration of NEI connections (20,21).

Figure 2.3. The neuroendocrine-immune network



*Adapted from Irwin & Cole (2011).
HPA= hypothalamic-pituitary-adrenal axis; SNS= sympathetic nervous system.

Cytokines and the neuroendocrine-immune network

Cytokines play a critical role in coordinating and regulating NEI activity, including stress-related activity in the ANS and HPA axis (20). ANS activation in response to acute stress stimulates the release of noradrenaline which activates primary and secondary tissues of the immune system (e.g., bone marrow, spleen, lymph nodes; 20). ANS activation also increases the production and circulation of proinflammatory cytokines and mobilizes immune cells (20). The surge in inflammatory cytokines associated with ANS activation stimulates the HPA axis to release cortisol

which then inhibits inflammatory cytokine production (20,22). This creates a negative feedback loop between the immune system and the CNS (20,21).

Damage or dysfunction within this feedback loop can cause imbalance in the immune system and inflammatory processes (19,20). Imbalance may result in chronic inflammation and excessive cytokine secretion which is associated with "sickness behaviors" (e.g., depressed mood and decreased appetite), autoimmune and neurologic disease, and death (19,23,24). On the other hand, imbalance may also result in insufficient immune functioning and potentially increased susceptibility to disease (25).

Cortisol and the neuroendocrine-immune network

The effect of cortisol on the immune system is of particular importance for understanding developmental changes in the NEI network related to early-life adversity. Cortisol binds to glucocorticoid receptors on immune cell membranes. Once bound, cortisol can interfere with the cell's ability to interact with other messengers such as cytokines. Cortisol can also pass through the cell membrane, enter the cell nucleus, and bind to specific gene promoter sites altering the cell's production of cytokines. Inside the cell, cortisol can bind to the cell mitochondria (the powerhouse of the cell) and cause dysregulation in cell energy and cell death. The magnitude of cortisol's effect on immune cells, however, is determined by the cells' sensitivity to circulating cortisol. The number and sensitivity of glucocorticoid receptors on CNS and immune cells, therefore, are critical for regulation of the NEI network. (19,26,27)

Markers of ANS, HPA and Immune System Activity in Saliva

Activity within the ANS and HPA axis can be reliably measured using salivary biomarkers. Salivary cytokines, however, are relatively new salivary biomeasures, and basic questions regarding their levels, correlations with systemic immune activity, and stress sensitivity have yet to be addressed.

Salivary cortisol

Salivary cortisol is an established biomarker of HPA axis activity (28–30), and there are strong serum-saliva correlations for cortisol (31). The HPA response to stress can be measured in salivary cortisol approximately 20 to 40 minutes post-stressor (32–35).

Salivary alpha-amylase

ANS activity can be indexed by salivary alpha-amylase (sAA; 36,37). Specialized cells in the saliva glands produce sAA (37). These cells are innervated by the ANS; norepinephrine released in response to ANS activation stimulates secretion of sAA (37). Stress-related increases of sAA occur approximately five to ten minutes post-stressor and are associated with other indices of ANS arousal, including cardiovascular and skin conductance measures (38). sAA is a byproduct of ANS activation; peaks of sAA reflect activity of the ANS acute stress response and should therefore correlate with a rise in proinflammatory cytokines (20). Although research is limited, one study of adults participating in an acute stress task found salivary IL-6 was positively associated with a marker of ANS activity (heart rate; 39).

Salivary cytokines

Immune processes are highly localized and specific to the tissues and immunological threats of the immediate area (e.g., blood, mucosal tissues, intestines and airways). Given the compartmentalized nature of the immune system and the local production of cytokines within the oral cavity, variation in salivary cytokines may largely represent oral immune processes rather than general immune functioning (40). Therefore, the utility of salivary cytokines for understanding overall health may be limited. Studies of adults and adolescents have found little evidence supporting strong serum-saliva cytokine correlations (41–44). However, serum-saliva correlations for young children are not known.

Also, interactions between salivary cytokines and salivary measures of ANS and HPA activity (sAA and cortisol) have not been examined in children and may provide insight into NEI functioning. Salivary cortisol and alpha-amylase provide important information regarding HPA and ANS activation in response to stress, therefore correlations between these biomarkers and salivary cytokines may provide insight into neuro-immune connections and overall NEI network regulation.

Salivary cytokines and stress and emotions: Few studies have examined associations between salivary cytokines and emotions and their response to stress. While one study was identified that found salivary IL-1β was associated with activation of brain areas involved in emotional processing in adults (45), all other studies identified used salivary IL-6 to examine the relation between salivary immune markers and stress and emotions. In general, higher levels of salivary IL-6 have been associated with negative emotions and mental states, such as fear (46), depression, anxiety, social withdrawal and hopelessness (43,47). In response to social stressors, adults exhibit an increase in salivary IL-6 (48,49).

The stress-reactivity of IL-6 in children may vary by sex with one study finding a stress-related increase in salivary IL-6 among girls and a decrease in boys (38). This change in salivary IL-6, however, was not associated with age, body mass index (BMI), or ethnicity, and baseline levels of IL-6 did not vary by age, BMI, ethnicity, or sex (38). 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.

Early-life Adversity, NEI Regulation and Biological Embedding

Infancy and early childhood, when the HPA axis and immune system are undergoing developmental change, represents a sensitive period during which stress may have a profound, and possibly lasting, impact on the functioning of the NEI network (50,51). Animal studies have demonstrated the importance of early-life experience on establishing a set-point for the immune

system (50). Chronic or repeated stress early in life floods the developing body with stress hormones. High levels of cortisol during early-life development can alter the CNS, the immune system, and the regulatory mechanisms of the NEI network (2,7,8,52,53).

The SAM response does not habituate to stress (54). Throughout development in infancy and early childhood, repeated psychological stressors elicit steady spikes in SAM hormones, which trigger the HPA axis to release cortisol (54). High levels of circulating cortisol early in life can decrease the number and sensitivity of cortisol receptors on cells throughout the body, including immune and HPA cells involved in the NEI network (55,56). Fewer or less sensitive receptors make the cells less responsive to cortisol, thereby making cortisol a less effective regulator of both inflammatory activity and the HPA response. This response can result in a "defensive phenotype" characterized by an over-active inflammatory and HPA response to challenge (55). In addition, cortisol early in life can shift the polarization of the immune system to favor the inflammatory response (57,58). The combination of a less regulated and inflammation-prone immune system and a less regulated HPA response could put children experiencing chronic early-life stress at greater risk of developing inflammation-based disorders.

Early-life adversity is often experienced within the family, as family is the primary context for early childhood development. Family psychosocial factors, including maternal mental health and family SES are two areas of exposure to early-life adversity that have been linked to biological changes in the NEI network (2,4,8,59–62).

Maternal mental health

A sensitive and attentive adult caregiver is one of the most important protective factors supporting child health (13,28). Maternal mental health is critical for shaping a child's development and laying the foundation for lasting health and well-being (13,28,63,64). Animal studies have shown a potential link between the quality of early-life maternal care and later-life immune functioning (50). In rat studies, dysfunctional maternal behavior (early weaning and care

by humans, rather than mother rats) is associated with an inflammation-prone immune response in offspring (50). Few studies, however, have examined these effects on the immune system at the biologic level in humans.

One such study examined the effect of maternal warmth experienced during childhood among adults who grew up in low-SES families (65). The results showed that adults from low SES families who reported higher levels of maternal warmth had less expression of proinflammatory genes and an attenuated increase in IL-6 to an immune challenge compared to adults from low SES families with less warm mothers during childhood (65). These relations were not mediated by current SES, health behaviors, or BMI (65). These findings support the notion that more supportive and caring mothers during childhood can help buffer changes in NEI network regulation associated with low childhood SES and illustrate the critical importance of maternal factors in child development.

Evidence from a recent study of adults illustrates the protective influence of social support from stress-related physiologic changes within the NEI network. Miller and colleagues found that glucocorticoids (chemicals similar to cortisol) were less effective regulators of IL-6 activity in adults experiencing chronic stress compared to adults not experiencing chronic stress (chronic stress defined as having a child undergoing treatment for cancer compared to parents of medically healthy children) (56). This stress-related desensitization of the IL-6 response to glucocorticoids was mitigated (the IL-6 response was more sensitive to the regulatory effects of glucocorticoids) in adults reporting high levels of support from their friends and family members (56). The protective effect of social support was unique to adults experiencing high levels of stress and was not seen in adults with low levels of stress (56). These findings further demonstrate the importance of social support in NEI network regulation particularly under high stress conditions.

While few studies have examined the influence of maternal factors on NEI network regulation specifically, there is strong evidence that maternal mental health is associated with changes to HPA regulation in children (66–69). The direction of these changes, however, remains unclear. While some studies show a positive relation between maternal depression and children's cortisol levels (69,70), maternal mental health has also been associated with decreased or more variable cortisol (67,70). Furthermore, relations between maternal mental health and child cortisol may vary by sociodemographic factors (68,71). Maternal mental health may also moderate the association between SES and child cortisol levels (66). Observed relations between maternal mental health and child HPA activity may also reflect combined effects of other related adversities, such as low SES, family violence and instability, and poor child care practices (72). Furthermore, the impact of maternal mental health on child HPA regulation depends on many factors, including the nature and severity of mental health problems, the child's developmental stage, the presence of other buffering factors (e.g., fathers and other caregivers), and the timing of the mental health problems (69,72). Given the range of factors that influence the relation between maternal mental health and child HPA regulation, it is not surprising that the effects of maternal mental health on child cortisol remains unclear.

Family socioeconomic status

Socioeconomic disadvantage is associated with an increased number of negative life events, such as increased residential mobility and instability, disruptive family relationships, and witnessing community violence (66-68). Family SES is often used as a proxy for childhood adversity, and studies have found evidence of the biologic embedding of early-life adversities related to low family SES in the regulatory pathways of the HPA axis and immune system (4,6,55,62,73).

Miller and Chen found that low SES during early childhood, particularly at age two, was associated with reduced expression of glucocorticoid receptor genes and increased expression of

genes related to inflammatory processes in adolescent girls (73). The effect of early-life SES was not mediated by current stress, health behaviors, or BMI (73). Also, improvements in SES from early to late childhood did not mitigate the association between early-life disadvantage and the expression of stress and immune-related genes (73). Similar results were found by a study that examined the effect of early-life SES in adults. In this study, compared to adults who grew up in higher-SES families, adults who grew up in low-SES families showed decreased expression of genes coding for cortisol receptors and increased expression of genes coding for proinflammatory cytokines (55). Low early-life SES adults also displayed higher daily salivary cortisol and had a greater increase in IL-6 to immune challenge than adults from higher SES families (55). These early SES-related differences in physiology remained after controlling for current SES and health behaviors (55). Findings from these two studies support the notion that low-SES in early-childhood is associated with lasting changes in elements of NEI network regulation at many levels of physiology (genetic, cell receptors, cortisol production).

Taken together, studies of maternal mental and psychosocial characteristics and family SES support the hypothesis that early-life adversity is associated with a patterned physiologic response by the CNS and immune system that, when repeatedly activated throughout development, may cause biologic changes in the activation and regulation of these systems. Such changes may result in an over-active HPA axis and a poorly-regulated and inflammation-prone immune system. This "defensive phenotype" (55) may put children growing up with family-level adversity at risk of developing inflammation-based disorders (4,60,62,74,75).

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CHAPTER THREE

Study Design and Methods

3.1. Study Design and the Fetus to Five Study

This study used data from the Fetus to Five study. Fetus to Five is a laboratory-based study investigating the influence of maternal psychosocial factors and family socioeconomic status (SES) on the development of cognitive, emotional and regulatory processes in children. The study protocol was approved by the Johns Hopkins Bloomberg School of Public Health Institutional Review Board in 2010 and 2012; the principal investigator was Sara Johnson, PhD MPH.

Participants

Fetus to Five recruited mother/child pairs from March 2011 to July 2013. There were two phases of subject recruitment. Phase 1 data was collected from spring 2011 to summer 2012. Phase 1 participants were recruited from a pool of mothers who participated in the Fetal Development Study of Dr. Janet DiPietro in 2006-2007 (1). For phase 1 participants, the Fetus to Five study is a five-year follow-up to the Fetal Development Study; mother/fetus pairs studied in the 2006/07 Fetal Development Study were recruited to be mother/child pairs in the Fetus to Five study. Fetus to Five collected data from 65% of eligible mother/child pairs from the Fetal Development Study. This sample includes 58 mother/child pairs who came into the laboratory for a study visit, as well as 18 mothers who participated remotely by email or mail surveys (Figure 3.1). The majority of mother participants from phase 1 were middle-class (mean household income: $100,000-$150,000) and non-Hispanic white (82% white, 13% African American, 5% Asian/Pacific Islander, 4% Hispanic). At the time of testing, the mean age of phase 1 mothers was 37.89 (SD= 4.62) years, and the mean age of phase 1 children was 5.35 (SD= 0.28) years. Slightly more than half of phase 1 children were female (59%). Table 3.1 displays demographic

information for the analytic sample (including participants from recruitment phases 1 and 2 for whom complete salivary analyte data were available).

Since phase 1 participants were from mostly stable middle/high-income families, phase 2 of Fetus to Five was designed to replicate the study procedures of phase 1 using a community sample of five-year-old children and their mothers from lower-income families. Phase 2 data collection was conducted from fall 2012 to summer 2013. Mother/child pairs were recruited through fliers posted in Baltimore City (e.g., at churches, community centers, and markets) and at the Johns Hopkins Harriet Lane Pediatric Clinic. Ninety-three mother/child pairs participated in phase 2. Of these, three were unable to complete the study procedures (one due to language barriers, one due to child refusal, and one due to mother refusal; Figure 3.1). The majority of mother participants from phase 2 were low-income (mean household income: $16,000-$24,999) and non-Hispanic African American (90% African American, 9% white, 1% Asian/Pacific Islander, 5% Hispanic). At the time of testing, the mean age of phase 2 mothers was 29.64 (SD=7.04) years, and the mean age of phase 2 children was 5.50 (SD= 0.30) years. Approximately half of phase 2 children were female (48%).

Figure 3.1. Study sample flow chart

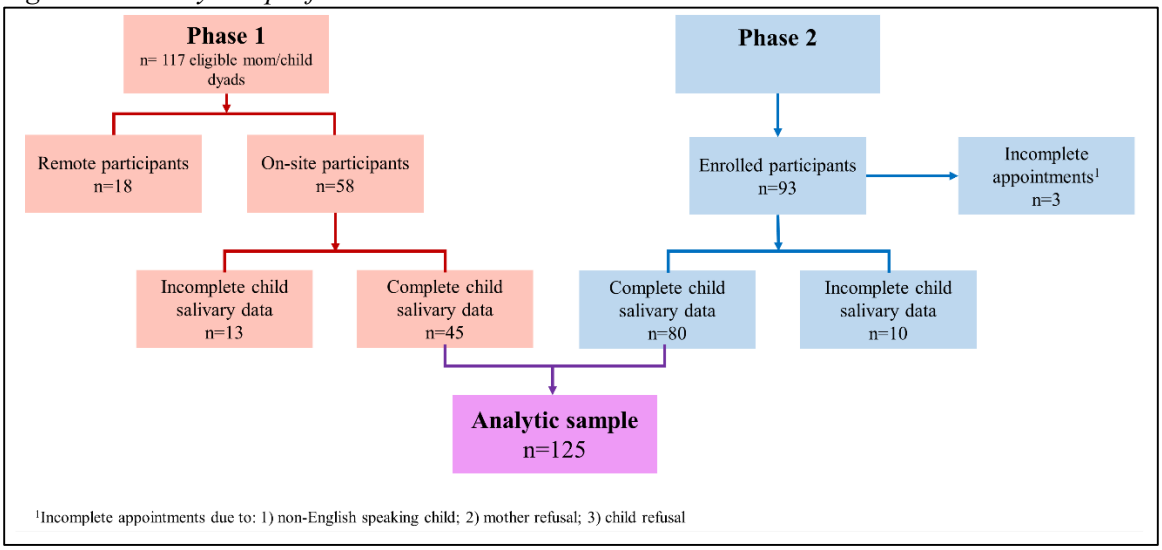


Table 3.1. Demographic information for the study sample (n=125) by phase of recruitment

	Phase 1 (n=45)		**Phase 2** (n=80)	
	Frequency	Percent	Frequency	Percent
Child age, mean years (SD)*	5.35 (0.28)		5.50 (0.28)	
Child race*				
White	35	78%	7	9%
African American	7	16%	73	91%
Asian/Pacific Islander	3	7%	0	0%
Child ethnicity				
Hispanic	1	2%	4	5%
Child sex				
Female	25	56%	39	49%
Maternal age, mean years (SD)*	37.89 (4.62)		29.64 (7.04)	
Family income*				
<$5,000	0	0%	13	16%
$5,000-11,999	0	0%	17	21%
$12,000-15,999	0	0%	8	10%
$16,000-24,999	0	0%	21	26%
$25,000-34,999	0	0%	7	9%
$35,000-49,999	2	4%	2	3%
$50,000-74,999	3	7%	7	9%
$75,000-99,999	3	7%	1	1%
$100,000-149,999	18	40%	3	4%
$150,000+	19	42%	1	1%
Maternal education*				
< High school (HS)	0	0%	8	10%
HS/GED	1	2%	32	40%
Some college	1	2%	30	38%
2-year degree	4	9%	3	4%
4-year degree	16	36%	4	5%
Masters degree	8	18%	3	4%
Doctoral/Professional degree	15	33%	0	0%

*Difference between phases $p < .01$.
SD= Standard deviation; GED= General Equivalency Diploma.

Inclusion and exclusion criteria

To be included in the Fetus to Five study, phase 1 mothers had to have met the inclusion criteria for the Fetal Development Study (being a nonsmoking pregnant mother with a healthy singleton fetus; 1) and expressed interest in follow-up studies in 2006/07. For phase 2, female biological mothers or legal guardians of five-year-old children in the Baltimore City-area were

eligible for enrollment as mother participants. English fluency was required for mother and child participants in both phases of data collection, and all children had to be five-years old. Also, children in both phases were excluded if their mothers reported that they had a significant health condition or developmental disability that impaired cognitive, motor, or regulatory functioning such as cystic fibrosis, autism, or mental retardation.

Procedures

All study visits were conducted on the Johns Hopkins Bloomberg School of Public Health East Baltimore Campus. Figure 3.2 illustrates the Fetus to Five in-lab study procedures for child participants. The 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 (2) and two cognitive challenge tasks that tested inhibitory control (the Silly Sounds Game and Pig Game; 3,4). 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 (5). Study sessions were video and audio recorded.

Four saliva samples were collected from both mother and child participants throughout the study visit (two samples were collected before emotional stressor tasks and two were collected after emotional stressor tasks; see Figure 3.2). Electrophysiologic data (heart rate and skin conductance) were also collected from mothers and children throughout the visit. The procedures for phases 1 and 2 were very similar, with minor changes between phases aimed to increase participation and completion rates in phase 2. Differences in procedures between the phases are noted in the summary below.

Child emotional stressor tasks

Children participated in three emotional stressor tasks: the Disappointing Gift Game, the Not Sharing Game, and Mischel's Delay of Gratification Task (5). The timeline for administration of each task relative to the saliva measures is shown in Figure 3.2. The Disappointing Gift Game assessed a child's response to disappointment (6). Each child ranked six potential gifts (including undesirable items (e.g., a sock)) from favorite to least favorite. Approximately 15 minutes later, the child was given his/her least favorite gift. The child's reaction to the gift was recorded (audio/video and electrophysiologic recording) for one minute. This task has been shown to challenge behavioral control and to elicit negative emotions (e.g., anger) in young children (6–10).

The Not Sharing Game is an emotional stressor and test of emotional regulation (11,12). In this game, an evaluator unevenly distributed candy between herself and the child. The child's thoughts about the unequal sharing were elicited four times during the game via direct questioning (e.g., "Wow! I have a lot more candy than you. How do you feel about that?"). This task has been shown to elicit negative emotions (e.g., frustration) and to increase salivary cortisol in young children (12).

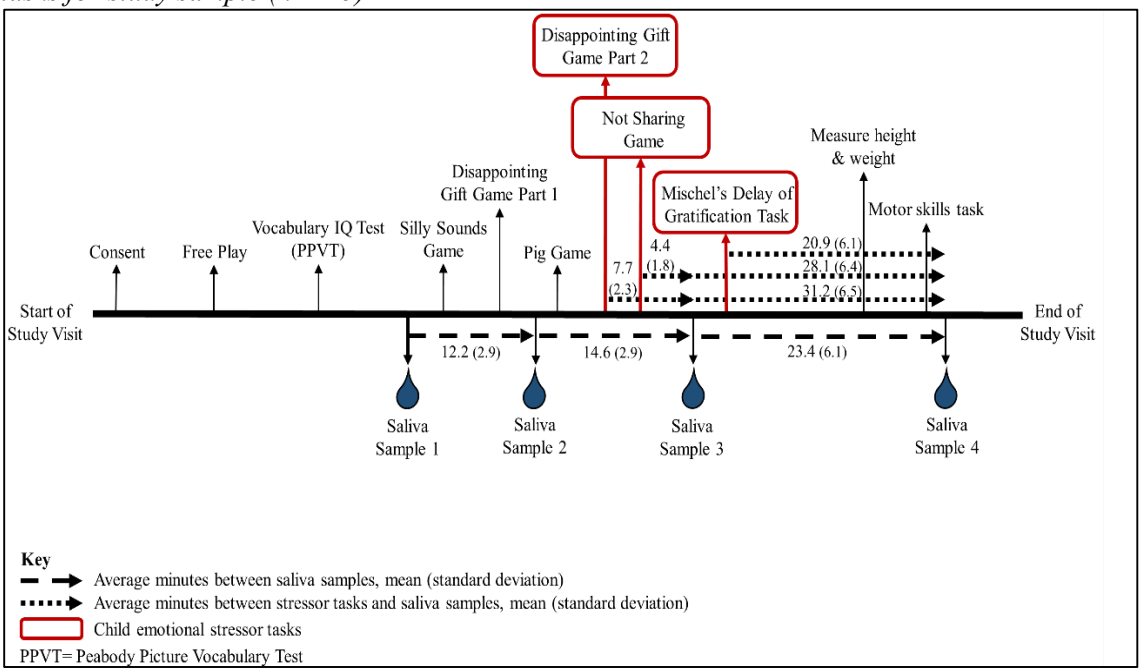
The Delay of Gratification Task is an emotional stressor and test of coping abilities (5,11). The child was left alone in the study room with a treat of the child's choosing (either a marshmallow or a pretzel). The child was told that the evaluator must briefly leave the room, and he/she was asked to not eat the treat while the evaluator was away. A bell was placed in front of the child, and he/she was told that ringing the bell would bring the evaluator back to the room and allow him/her to eat the treat. However, the child was told that if he/she waited for the evaluator to return on her own (without ringing the bell), he/she would be rewarded with two treats. The child was left alone with the treat for eight minutes or until he/she rang the bell. This age-

appropriate stressor task has been shown to elicit negative emotions and correlate with inhibitory control measures in young children (5,10,13).

Child anthropometry

Child height and weight were each measured once by study staff using a wall-mounted yard stick and a scale. Body mass index (BMI) was calculated, and weight status categories (underweight and healthy weight vs. overweight and obese) were assigned to each child using the Centers for Disease Control and Prevention's BMI percentile ranges by sex and age (14,15).

Figure 3.2. Fetus to Five study protocol with elapsed time between saliva samples and stressor tasks for study sample (n=125)



Parent Survey

Mothers completed a survey during the study visit that included questions about sociodemographic and psychosocial factors for the mother, family, and child participants. Psychological instruments were embedded within the survey to assess the mother's level of parenting stress (Parenting Stress Index (PSI; 16)), financial stress, depression (the Center for Epidemiological Study of Depression scale (CES-D-20; 17)), and anxiety (the State-Trait Anxiety

Inventory (STAI; 18)). Questions about family income and maternal education provided objective measures of SES. The mother was also asked to report her marital status, smoking status (yes/no), and the number of times the family moved in the past five years. Appendix A includes portions of the Parent Survey used in the dissertation.

Saliva Collection and Storage

Four in-laboratory whole saliva samples were collected using passive drool. The timing of saliva samples was linked to the study tasks completed by the child and therefore determined by the length of time the child took to complete the tasks. Each mother synchronized her samples with her child's samples (see Figure 3.2 for average timing of saliva samples and tasks). During each saliva collection, participants drooled into a vial for three minutes or until 0.75 milliliters of fluid was collected. Saliva samples were stored at -20 degrees Celsius until assayed.

Determination of Salivary Analytes

Saliva assays were performed at the Center for Interdisciplinary Salivary Bioscience Research at the Johns Hopkins School of Nursing and the Institute for Interdisciplinary Salivary Bioscience Research at Arizona State University. Saliva samples were assayed following Granger and colleagues (19) for salivary alpha-amylase (sAA) by kinetic reaction assay. Salivary alpha-amylase results were computed in U/mL. For sAA, intra-assay variation (CV) 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, and intra- and inter-assay coefficients of variation less than 5 and 10% respectively. Salivary cortisol data were expressed in micrograms per deciliter (μg/dL).

Salivary cytokines were measured following Riis and colleagues (20) using 96-well format multiplex electrochemiluminescence immunoassays manufactured by Meso Scale Discovery (MSD, Gaithersburg, MD). Each well was coated with capture-antibodies to IL-1β, IL-6, IL-8 and TNFα. Detection antibodies were coupled to SULFOTAG^{TM} labels that emit light when electrochemically stimulated via carbon-coated electrodes in the bottom each microwell. The MSD 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 (CV) were: 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%). For all analytes, concentrations below the assay's LLD were estimated by the saliva processing laboratory using the curve model for the plate.

Associations between sAA and cytokine concentrations and salivary flow rate were tested using Spearman's rank correlations and regression analyses with robust variance. No consistent associations between flow rate and raw analyte concentrations were found, so data were not adjusted for salivary flow rate (21). Due to the timing of subject recruitment and saliva laboratory processing, the effect of batch (i.e., assay plate assignment) was confounded by phase effects in these data and could not be fully examined (although laboratory estimates of inter-assay CV were within the acceptable range).

3.2. Study Sample

This study uses data from the 125 mother/child pairs that had complete salivary analyte data for child participants in the Fetus to Five study (Figure 3.1). This number includes 45 mother/child pairs from phase 1 and 80 pairs from phase 2.

Phase 1: Remote vs. On-site Participants

Fetal Development Study participants from 2006/07 who were interested in participating in Fetus to Five in 2011/12, but were unable to participate on-site were offered remote participation via mail and email. No saliva data were collected for remote participants. Eighteen phase 1 subjects participated remotely. On average, on-site and remote participants for phase 1 were similar in terms of child sex, race, ethnicity, current sickness and periodontal problems, and maternal age, smoking, education and marital status and family income. Remote child participants were older than on-site participants ($t(74)=-3.43$, $p< .01$), because the age enrollment criterion for children (i.e., 5 years of age) was relaxed for remote participants since no biologic data were collected remotely.

Sample With and Without Full Salivary Analyte Data

All children who participated in complete study appointments on-site (n=148) provided saliva samples. However, 23 children had incomplete salivary analyte data due to insufficient saliva volume for processing (n= 9), contamination during the saliva processing (n=2), or use of an alternate saliva collection method (salivette) that did not allow for cytokine analysis (n=12). There were no differences between children with (n=125) and without full salivary analyte data (n=23) in terms of age, sex, race, ethnicity, current sickness or periodontal problems, recent health, body mass index category, maternal age, smoking, education or marital status, family income, or the timing of study tasks relative to saliva samples. Overall, children with full analyte data were awake for longer when the study appointment began compared to those without full analyte data ($t(146)=2.00$, $p< .05$). Differences in time since waking are important given the circadian pattern of salivary cortisol. Within study phase, there were no differences between children with and without full analyte data for any sociodemographic or health factor, and there were no within-phase differences in time since waking.

Phase 1 vs. Phase 2: Demographic and Health Characteristics

Phase 2 of subject recruitment was designed to diversify the phase 1 sample of stable middle/high socioeconomic status (SES) families. As such, there were significant differences between the phases for sociodemographic variables (see Table 3.1.). Compared to phase 1 mothers, phase 2 mothers were younger ($t(123)=7.05$, $p< .001$), and they were less likely to be married ($\chi^2(1)=73.25$, $p< .001$) and more likely to smoke ($\chi^2(1) =16.47$, $p< .001$). Phase 2 was majority African American, while phase 1 was majority white ($\chi^2(1)=61.51$, $p< .001$), however, participants in both phases were majority non-Hispanic. Phase 2 mothers had less education ($\chi^2(7)=85.79$, $p< .001$) and lower family incomes ($\chi^2(9)= 74.50$, $p< .001$).

Overall, there were no differences between children in phase 1 and 2 in terms of sex, current sickness and periodontal problems, and measures of recent health. The timing of study tasks relative to saliva samples was similar for phases 1 and 2. Although all children were five years old, phase 2 children were older than phase 1 children ($t(123)= -2.95$, $p< .01$; Table 3.1), and phase 2 children were also more likely to be overweight/obese than phase 1 children ($\chi^2(1)=25.10$, $p< .001$). Also, children from phase 2 were, on average, awake for longer when the study appointment began compared to phase 1 children ($t(123)= -4.23$, $p< .001$).

3.3. Salivary Analyte Non-detects and Duplicate Data

Salivary biomeasure data from children were examined in this study. Cytokine testing was performed with 15% duplicates, and the average of the duplicate measures was used as the raw concentration. Eight microplates were used to test child saliva samples for cytokines, and the LLD varied by microplate and cytokine. Of the 500 samples (4 samples each for 125 children), there were three non-detected samples of IL-6 and 25 of TNFα. Non-detects were estimated by the laboratory using the fit curve. Two of the non-detects were estimated as zero by the laboratory (one for IL-6 and one for TNFα). All samples of IL-1β and IL-8 were measureable above the LLD.

Alpha-amylase was tested in singulate with 30 duplicate tests for intra-assay validity calculations (duplicate assays run on the same plate). When assayed in duplicate, the mean of the two values was used as the raw concentration. The LLD for alpha-amylase was 0.4 U/mL for all microplates. One sAA concentration was below the LLD and was estimated as zero by the laboratory. Cortisol was tested in duplicate (i.e., all samples measured twice). The average of the duplicate measures was used as the raw concentration for each sample. The LLD for cortisol was 0.007 μg/dL for all microplates. Thirty-eight non-detects (7.6%) were estimated by the laboratory for cortisol, and nineteen of these values were estimated as zero.

3.4. Study Aim 1 Variables and Analytic Methods

Variables

Study aim 1 explored current salivary analyte pre-analytic data processing techniques and their implications for data quality, and the validity and reliability of study findings. TNFα and cortisol data from the study sample of 125 five-year old children were used to demonstrate the impact of different pre-analytic data processing methods on analyte mean levels by sex. Sex differences in mean TNFα and cortisol were compared when data were subjected to different, commonly used pre-analytic processing techniques. Child sex was based on maternal report.

Exploratory Data Analysis and Missing Data

Salivary analyte data displayed a strong positive skew. Multiple non-linear transformations (inverse, cubic, inverse cubic, square, inverse square, squared-root, inverse of the squared-root, and natural log) were tested for each analyte. The natural log transformation was applied to both analytes, because it provided the least skewed distributions (zero values were handled in various ways to allow for the data transformation, discussed below). To allow for the comparison of results using data subjected to different pre-analytic data processing decisions, no

data used in study aim 1 were Winsorized, meaning outlying data points were included as measured. There were no missing child sex data.

Pre-analytic Processing of Analyte Data and Statistical Analyses

The purpose of study aim 1 analyses was to demonstrate the impact of different pre-analytic data processing methods on salivary analyte data and analyte mean levels by child sex. Therefore, the main statistical analyses for study aim 1 were pre-analytic data processing methods. Analyses included comparing five methods for handling censored data (non-detects) and four methods for handling non-real zero values. Sex differences in raw mean cortisol concentrations were compared when non-detects were: 1) dropped from the data; 2) replaced with half the LLD; 3) replaced with the LLD; 4) included as censored points in Tobit regression models with robust variance; and 5) included as censored points in quantile regression. These comparisons illustrate the impact of different methods for handing censored data on group means, including commonly employed and simple substitution methods, as well as less commonly used regression models designed for censored data (Tobit models) and non-parametric modeling approaches (quantile regression). There are currently no universally-accepted best practices for handling zero values in salivary analyte data, accordingly, sex differences in mean log-transformed TNFα and cortisol were compared when: 1) two was added as a constant to all raw data points to eliminate zero values; 2) 0.001 was added as constant to eliminate zero values; 3) zero values were replaced with 0.0001; and 4) zero values were dropped from the data. These comparisons illustrate how typically-employed techniques for handling zero values in salivary analyte data impact analyte group means. For these analyses, laboratory-estimates of all censored data were used.

3.5. Study Aim 2 Variables and Analytic Methods

Dependent Variables

Study aim 2 tested three hypotheses regarding salivary cytokines (IL-1β, IL-6, IL-8 and TNFα) in five-year old children: 1) salivary cytokine activity is positively intercorrelated across a series of cognitive and emotional challenge tasks; 2) salivary cytokine activity is positively associated with oral health issues and unrelated to demographic and systemic health factors; and 3) salivary cytokines are positively associated with ANS activity and negatively associated with HPA activity. The dependent variables were the four salivary cytokines across the four saliva samples.

Early data processing of salivary cytokines

Laboratory-estimated concentrations of non-detects for IL-6 and TNFα were included in the data. All cytokine data distributions were very positively 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). Similar to other studies using salivary analyte data, cytokine data were log-transformed and Winsorized to adjust for a small number of outliers (22). To allow for log-transformation, zero values were replaced with 0.0001. Winsorization was performed by cytokine and saliva sample separately for 0.6% of ILβ-1, 0.6% of IL-6, 0.8% of IL-8, and 1.0% of TNFα concentrations. Individual trajectories across saliva samples were maintained during the Winsorization process. All values were Winsorized within three standard deviations of the mean. 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).

Independent Variables

Study aim 2 examined the relations between cytokine concentrations and activation of the ANS and HPA axis (indexed by sAA and cortisol, respectively), as well as demographic and health characteristics. The impact of circadian rhythm and elapsed time between samples was also examined.

Markers of ANS and HPA activation

Early data processing: Laboratory-estimates of non-detected values of sAA and cortisol were used. Alpha-amylase and cortisol displayed strong positive skews (range across the four samples (skew, kurtosis) for sAA: 1.96-4.13, 7.95-29.52; cortisol: 2.26-12.00, 4.37-10.97), so data were log-transformed. To allow for transformations, zero values of sAA and cortisol were replaced with 0.0001. After transformation, sAA and cortisol data were Winsorized by analyte and sample. Twenty-two cortisol values (4.4%) were Winsorized to be within 3.7 standard deviations of the mean. Ten sAA values (2.0%) were Winsorized to be within three standard deviations from the mean. Individual trajectories were maintained during the Winsorization process. Winsorization and transformation improved the distribution of the analytes (range across the four samples (skew, kurtosis) for sAA: -0.61- -0.33, 3.28-4.56; cortisol: -1.81- -1.03, 4.72-6.55).

Health and demographic data

Information about health and demographic variables included in study aim 2 is shown in Table 3.2. Child height (inches) and weight (pounds) were used to calculate body mass index (weight (lb.) / [height (in)] 2 x 703), and weight status (underweight or healthy weight vs. overweight or obese) was assigned to each child using the Centers for Disease Control and Prevention's age- and sex-specific criteria (14,23).

Maternal smoking, child race, child medication use, child oral health problems, and child health were based on maternal report on the Parent Survey. Mothers listed all prescription and over-the-counter medications taken by her child in the two days prior to the study visit. Two dummy variables coding for use of any medication, and use of allergy and asthma medication were created. Current child periodontal/dental issues were assessed with the question "Does your child currently have any dental problems such as cuts or sores in his or her mouth, very loose teeth, a tooth lost in the last 48 hours, bleeding gums while brushing, or untreated cavities?" (yes/no). Child current illness was assessed with the question "Is your child currently feeling sick

Table 3.2. Study aim 2 demographic, health, and timing variables

Variable	Variable Type	Data source	Question
Child age	Continuous: in months	Direct questioning of mother	"What is your child's birthday"
Child sex	Dichotomous: male, female	Parent Survey	"What is your child's gender?" (male/female)
Child race	Dichotomous: white, non-white	Parent Survey	"How would you describe your child's race? (Check all that apply)?" (White/Black or African American/Asian or Pacific Islander/ Native American or Alaskan Native/Other, please specify)
Child body mass index status	Dichotomous: overweight/ obese, healthy weight/ underweight	Direct measurement of child height & weight	
Child currently sick	Dichotomous: yes, no	Parent Survey	"Is your child currently feeling sick or ill (i.e., runny nose, fever, cough aching, etc.)?" (yes/no)
Child recent health	Dichotomous: excellent-good, fair/poor	Parent Survey	"Compared to others his or her age, would you say your child's health in the last 2 days (48 hours) has been:" (excellent/very good/good/fair/poor)
Child taken any medicine in last two days	Dichotomous: yes, no	Parent Survey	"Has your child taken any prescription or over-the-counter medications in the last 2 days (48 hours)?" (yes/no); If yes, please list the names of these medications:"
Child taken allergy/ asthma medicine in last two days	Dichotomous: yes, no	Parent Survey	
Child current oral health issues	Dichotomous: yes, no	Parent Survey	"Does your child currently have any dental problems such as cuts or sores in his or her mouth, very loose teeth, a tooth lost in the last 48 hours, bleeding gums while brushing, or untreated cavities?" (yes/no)
Maternal smoking status	Dichotomous: yes, no	Parent Survey	"Do you smoke cigarettes?" (yes/no)
Time since waking	Continuous: in hours	Parent Survey & study staff notes	"What time did your child wake up this morning? (Please write the hour, minute, and whether this was AM/PM)" & time of study appointment
Task timing	Continuous: in minutes	Study staff notes & video recording of study session	

or ill? (e.g., runny nose, fever, cough, aching, etc.)" (yes/no), and recent health was assessed with the question "Compared to others his or her age, would you say your child's health in the last two days (48 hours) has been: (responses: excellent; very good; good; fair; poor)". Recent health categories were dichotomized to compare children with excellent to good health to those whose mothers reported their health to be fair or poor. Child race was reported by mothers and data were dichotomized (white vs. non-white), because of a low percentage of Asian/Pacific Islanders in the sample (see Table 3.1.).

Timing data

The impact of potential circadian patterns in cytokine levels was examined using the elapsed time from waking to the study appointment. Elapsed time from waking was calculated using maternal report of child's wake time and the study visit start time for each child. A task timing variable, defined as the elapsed time from the Disappointing Gift Part 2 task and each saliva sample, was used to account for the differential timing of emotional stressor tasks across child participants.

Analytic Methods

Exploratory data analysis

Exploratory data analysis examined the distributions and means of all continuous variables and frequency distributions of all categorical variables. These analyses tested the normality of continuous variables and informed the dichotomization of categorical variables. Multiple non-linear transformations (inverse, cubic, inverse cubic, square, inverse square, squared-root, inverse of the squared-root, and natural log) were tested for each analyte. The natural log transformation was selected for all analytes, because it provided the least skewed distributions for the majority of the analytes.

Missing data

Missingness was examined for each variable. There were low rates of missing data for most variables due to incomplete survey responses or staff omissions (missingness ranged from 0-2.4% for all variables except child age). Parent-reported child age in months was missing for 8.8% of the sample. Missing data for age, BMI, sample and stressor times, time since waking, and survey data were obtained from evaluator's notes and video recordings of study appointments (16% of the missing data) or imputed using mean values (84% of missing data).

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 the first hypothesis (i.e., salivary cytokines are positively intercorrelated), 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.

The second hypothesis was that salivary cytokine activity is positively associated with oral health and unrelated to demographic and systemic health. To test this 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 children 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. Sample size restrictions required

model parsimony to avoid overfitting, therefore, backwards stepwise regression with a retention threshold of $p < .05$ was used to create parsimonious adjusted models for each cytokine.

The nonlinearity of the cytokine concentrations across the study visit was examined using a spline knot to explore the extent and nature of task-related change in cytokine levels. With no a priori hypotheses about the timing of task-related change in cytokine levels, the appropriateness of a knot placed at sample 2 or 3 in both null and parsimonious adjusted models was tested. Spline models used a parameterization describing the intercept, 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 vs. last samples. Differences between slope terms were examined using post-estimation tests, and significant differences suggested a task-related change in cytokine slope beginning at the spline knot. For each cytokine, log likelihood estimates 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).

The third hypothesis was that salivary cytokines are positively associated with ANS activity and negatively associated with HPA activity. To test this hypothesis, sAA and cortisol were added separately as independent variables to the final model for each cytokine. Based on literature that suggests that the NEI relations may vary by sex, the interaction between sex and sAA or cortisol on each cytokine was examined (24,25).

Checks of model fit and sensitivity analyses

Model fit was assessed for each final model using residual plots and added variable plots (added variables plots were perfomed using simple linear regression that ignored the nested nature of the data). Influential points were identified and models were performed without influential cases to test the robustness of the findings. Additional sensitivity analyses were performed on complete case data to test the impact of data imputation methods. Sensitivity analyses also examined the interaction between each covariate and each cytokine reactivity slope

or overall trajectory (slope from sample 2-4 or 3-4, or overall trajectory from sample 1-4). The only significant effects were seen for TNFα which had a non-significant positive slope from sample 2-4 that was steeper for older children and less steep slope for children of smoking mothers. The findings for TNFα from these models were similar to those without the covariate by slope interaction terms. The robustness of sAA and cortisol findings was tested by including, along with sAA or cortisol, all relevant and statistically significant health and demographic covariates for sAA and cortisol in final models. Furthermore, to examine the nature of sAA and cortisol effects on cytokine levels, the individual mean and trajectory effects of sAA and cortisol were examined simultaneously as separate terms in each final model. These models revealed no significant difference between individual mean and trajectory effects of either sAA or cortisol. Therefore, final models tested cytokine relations with sAA and cortisol as single combined effects.

To assess the impact of censoring in the cortisol data and the laboratory-estimated values used to replace the non-detects, final models were performed using a substitution method for cortisol non-detects (i.e., replace non-detects with half the LLD (0.0035 μg/dL)). Also, random effects Tobit models with bootstrapped variance were performed for each final model with the cytokine and cortisol variables swapped, so that cortisol was the dependent variable and cytokines were the independent variables (in separate models). This approach allowed for the specification of a single lower limit of censoring (i.e., the natural log of the LLD of the cortisol assay (-4.961845)) in Tobit models.

3.6. Study Aim 3 Variables and Analytic Methods

Dependent Variables

Study aim 3 examined the role of maternal psychological well-being in modifying the relation between HPA and immune system activity in the oral cavity. The dependent variable was cortisol concentrations at the four saliva samples.

Early data processing of salivary cortisol

Substitution methods were used to handle censored cortisol values. The 38 non-detected cortisol values (7.6%) were replaced with half the LLD of the cortisol assay (0.0035 µg/dL). Cortisol data were very positively skewed (range across the four samples (skew, kurtosis): 1.13-2.27, 4.37-11.02). To improve the normality of the distribution, data were log-transformed. Three data points from one outlier were Winsorized so that all values were within three standard deviations of the sample mean. The individual's cortisol trajectory across the study visit was maintained during the Winsorization process. Winsorization and transformation improved the distribution of the data for the full sample (range across the four samples (skew, kurtosis): -1.20- -0.84, 3.04- 4.64).

Independent Variables

Study aim 3 examined the modifying role of maternal psychological well-being in the relation between cortisol and cytokine activity. The main independent variables were the cytokine concentrations at the four saliva samples, a factor score indexing maternal distress, and the interaction between each cytokine and the maternal distress factor score. A factor score of family financial hardship was included as a covariate, so that the effect of maternal distress could be examined after adjusting for financial factors. In addition, fully-adjusted models tested the influence of health and demographic covariates, as well as a measures of circadian rhythm and elapsed time between samples. Statistically significant covariates were retained in the final models.

Salivary cytokines

The same salivary cytokine data used in study aim 2 analyses were used in study aim 3 analyses.

Maternal distress and family financial hardship

Maternal psychological well-being and family financial hardship were indexed by composite scores generated from a factor analysis of eight variables related to maternal mental and psychosocial health and family SES (detail follows). Table 3.3 shows the variables included in the factor analysis. Family income in the last 12 months, maternal education and marital status, and the number of moves in the child's life were assessed with questions on the parent survey. Instruments embedded within the parent survey were used to create the other four variables used in the factor analysis, including: 1) maternal financial stress; 2) maternal depressive symptoms; 3) maternal anxiety; and 4) maternal parenting stress.

Maternal financial stress was measured using a six item instrument adapted from Essex and colleagues (26) assessing the frequency of financial stressors in mothers' lives in the last 3 months including difficulty paying bills, thoughts about money problems, fears of losing her home and job, and feelings of limited opportunities due to finances. Each item was scored on a five point Likert scale from "never" to "always" with higher scores indicating more financial stress. The sum score of the six items was used as a measure of maternal financial stress.

Maternal depressive symptoms in the last week were measured using the Center for Epidemiologic Studies Depression Scale (CES-D; 17). The CES-D is a 20 item scale. Each item is scored on a Likert scale from "rarely or none of the time" (score of 0) to "most or all of the time" (score of 3) with higher scores indicating more depressive symptoms. The sum CES-D score was used to index maternal depressive symptoms.

Maternal trait-anxiety was measured using the Speilberger State-Trait Anxiety Inventory-Form Y2 (STAI; 18). The STAI has 20 items. Responses range from "almost never" (score of 1) to "almost always" (score of 5) with higher scores associated with more anxiety. The sum STAI score was used to index maternal anxiety.

Table 3.3. Family SES and maternal mental and psychosocial health variables used in factor analysis for aim 3

Variable	Variable Type	Data source	Question or Instrument
Family income	Categorical: <$5,000- 11,999, $12,000- 24,999, $25,000- 49,999, $50,000- 99,999, $100,000+	Parent Survey	"For the past 12 months, which category best describes your total combined family income? Include income before taxes and from all sources. (Ex. wages, rent from properties, social security, disability, veteran's benefits, unemployment benefits, worker's compensation, help from relatives, child support payments, alimony, etc.)" (12 response options including "Don't Know")
Maternal education	Categorical: <HS, HS /GED, some college, 2-year college, 4-year college, masters, doctoral or professional	Parent Survey	"What is the highest level of education you have completed?" (<HS/HS or GED/Some College/2-year College Degree/4-year College Degree/Master's Degree/ Doctoral Degree/Professional Degree (JD, MD)
Maternal marital status	Dichotomous: married, not married	Parent Survey	"What is your current marital status?" (Single, never married/Married/Divorced/ Separated/Widowed/Other)
Number of moves during child's life	Right-censored continuous	Parent Survey	"Since this child was born, how many times have you moved to a different house or apartment?" (0/1/2/3/4/5/6 or more)
Maternal financial stress	Continuous	Parent Survey	Financial Stress Index
Maternal depressive symptoms	Continuous	Parent Survey	Center for Epidemiologic Studies Depression Scale
Maternal anxiety	Continuous	Parent Survey	State-Trait Anxiety Inventory
Maternal parenting stress	Continuous	Parent Survey	Parenting Stress Index (Short Form)

HS=high school; GED=general educational development degree; JD= juris doctor; MD=medical doctor.

Maternal parenting and life stress was measured using the Parenting Stress Index (PSI; 16). Phase 1 mothers completed the PSI Long-form and phase 2 mothers completed the PSI Short-form. Both forms include Likert scale response-type and multiple choice questions, and higher scores indicate higher levels of parenting and life stress. The correlation between total scores on the Long and Short-form PSI is 0.94 (16), and the PSI Short-form is a subset of Long-

form items. Both forms of the PSI have subscales that measure different domains of parent and life stress; domain scores can be summed to create a total stress score for each form. PSI Short-form total scores were calculated for each participant to index parenting stress.

Interaction between cytokine concentrations and early-life adversity factor score

The main independent variable in study aim 3 analyses was the interaction term for salivary cytokine concentrations by maternal distress factor score. This term represents the moderating effect of maternal psychological well-being on the relation between cytokine and cortisol activity.

Key covariates: health, demographic and timing data

The health, demographic, and timing variables examined in study aim 2, except for child race, were used in study aim 3 as potential covariates (see Table 3.2). Child race was not assessed as a potential covariate because race and SES/adversity are highly correlated in this sample. In addition to these 11 covariates, interaction terms for time since waking by saliva sample and child sex by cytokine concentrations were included in fully-adjusted models based on findings from the literature suggesting a blunted cortisol response to stress in the morning vs. afternoon (27) and sex-related differences in salivary cytokine activity (24,25,28).

Analytic Methods

Exploratory data analysis

In addition to the exploratory data analysis for salivary cytokine, demographic, health and timing data performed for study aim 2, the distributions and means of family SES and maternal mental and psychosocial health variables (Table 3.3) were examined. These analyses examined the distribution of all variables and informed the recategorization of ordinal variables.

Missing data

The missingness methods employed for study aim 2 were used in study aim 3 for all demographic, health, and timing variables. Before using family SES and maternal mental and psychosocial health variables, missingness was also examined for these variables. There were no missing data for maternal education, marital status, or the number of moves during child's life. Family income data were incomplete for 10.4% of the study sample. All incomplete data for family income were from phase 2 participants with seven missing responses and seven participants selecting the "Don't know" response option. "Don't know" responses and missing data were substituted with the mean family income for phase 2 ($16,000-$24,999/year).

Item-level missingness on instruments: Item-level missingness for each of the instruments (financial stress index, CES-D, STAI, PSI) was examined and imputed before instrument sum scores were used in factor analyses. Financial stress data were missing for 6.4% of the study sample. While all missing data for this measure were from phase 2 participants, no participant was missing more than one item. CES-D data were incomplete for 9.6% of the study sample (two phase 1 participants, and 10 phase 2 participants). Only one participant was missing more than one item on the CES-D. STAI data were missing for 16% of the study sample (one phase 1 participant, and 19 phase 2 participants), but only two participants were missing more than one item. Missing items on the financial stress index, CES-D and STAI were imputed with the mean score of the completed items.

The PSI Short-form items were incomplete for 17.6% of the study sample. A survey formatting error resulted in 14 phase 1 participants missing one item from one of the PSI subscales, and two of these participants were also missing one item from another PSI subscale. Eight phase 2 participants were missing items on the PSI, however only one participant was missing more than one item per subscale. Missing items on the PSI were imputed with the mean of the subscale scores, and subscale scores were summed to create the total stress index score.

Statistical analyses

Factor analyses: Polychoric exploratory factor analysis (EFA) was conducted with the eight family SES and maternal mental and psychosocial health variables (Table 3.3) using maximum likelihood estimation with robust standard errors, oblique (geomin) rotation, and one to four factors. Continuous variables were standardized before use in the EFA and the number of times the child moved was specified as a continuous right-censored variable. A two factor solution was selected based on information criteria, factor loadings, and residual variances (Akaike and Bayesian Information Criteria (AIC, BIC) and sample-size adjusted Bayesian Information Criteria: 1 factor= 2450.34, 2532.36, 2440.65; 2 factor= 2306.05, 2407.87, 2294.03; 3 factors= 2303.65, 2422.44, 2289.63; 4 factors= 2311.55, 2444.49, 2295.86). The three-factor pattern had low residual variances and the same loading pattern as the two-factor solution except with an additional third factor comprised of only one variable (moves during child's life). The two-factor pattern had low residual variances for each input variable (residual variance ranged from 0.10-0.62), and oblique rotation resulted in two factors with correlation=0.57 ($p< .001$). Factor loadings indicated one factor represented financial hardship and the other factor represented maternal distress (see Table 3.4). Factor scores, derived using the posterior mode method, provided a score for financial hardship and maternal distress for each child. Higher financial hardship scores indicated lower family SES and higher maternal distress scores indicated poorer maternal psychological well- being. Financial hardship and maternal distress factor scores were positively correlated ($r(123)=.63$, $p< .0001$).

Table 3.4. Factor loadings and parameter estimates from factor analyses with a two-factor solution for family SES and maternal mental and psychosocial health variables

Variable	Financial Hardship Factor		Maternal Distress Factor	
	Factor Loading	Factor Parameter Estimate	Factor Loading	Factor Parameter Estimate
Family income in last 12 months	**.95***	.93**	.00	
Maternal education	**.75***	.82**	.13	
Maternal marital status	**1.04***	.89**	-.23	
Number of moves in child's life	**.59***	.61**	.04	
Financial stress	**.51***	.68**	.30*	
Maternal CES-D score	.08		**.86***	.90**
Maternal STAI score	.00		**.89***	.89**
Maternal PSI score	-.20*		**.74***	.62**

EFA= exploratory factor analysis; CES-D= Center for Epidemiologic Studies Depression Scale; STAI= Speilberger State-Trait Anxiety Inventory-Form Y2; PSI= Parenting Stress Index- Short Form. Bolded factor loadings indicate factor structure used in to generate factor scores. Factor loadings are rotated and parameter estimates are standardized.
*p< .05, **p< .001.

Multilevel mixed models for cortisol: Cortisol was modeled as an outcome using multilevel mixed models with robust variance, a random intercept, and a random slope to account for repeated sampling within individuals and varying cortisol trajectories across the study visit between individuals. An independent covariance and residual errors structure were specified, and the residual variance was modeled separately for each saliva sample to allow for unequal variance across samples. Model specification was informed by examination of the model AIC/BIC estimates as well as theoretical considerations given the study's reactivity paradigm and expected variation in cortisol stress responses across children.

Separate multilevel mixed models assessed the relation between cortisol and each of the 11 health, demographic, and timing variables. The relation between cortisol and each potential covariate was also assessed in separate multilevel mixed models adjusted for each cytokine. Statistically significant and conceptually important variables were retained for use in fully-adjusted models. Four separate fully-adjusted models for cortisol were performed, each with only one cytokine as a predictor. Table 3.5 displays the variables included in the fully-adjusted and

final models for each cytokine model. The main independent variables in fully-adjusted models were: the cytokine of interest, the maternal distress factor score, and the interaction between cytokine concentrations and the maternal distress factor score (Table 3.5). Health and demographic covariates in fully-adjusted models included: child age, sex, BMI category, current sickness and oral health issues, maternal smoking status, family financial hardship factor score, the time from child waking to appointment start time, the elapsed time from stressor task to saliva sample, and the interactions between time from waking and sample, and between cytokine and child sex[1] (Table 3.5). Four parsimonious final models were created by subjecting fully-adjusted models to backwards stepwise selection of covariates using a $p < .05$ retention criterion. The main independent variables were retained for use in the final models and not subjected to the selection procedures.

In addition to controlling for the family financial hardship factor score, post-hoc models examined the specificity of the modifying effect of maternal distress on the cytokine-cortisol relation by testing the significance of a cytokine by family financial hardship interaction term in final models with maternal distress included as a covariate.

Checks of model fit and sensitivity analyses

Model fit was assessed for each final model using residual plots and added variable plots (added variables plots were perfomed using simple linear regression that ignored the nested nature of the data). Influential points were identified, and models were fit without influential cases to test the robustness of the findings. The robustness of the interaction between cytokine concentrations and maternal distress was scrutinized by using a three-level categorical maternal

[1] Maternal smoking status, time from waking to appointment, child current sickness and BMI category were statistically significant in preliminary tests of significance. Other covariates included as conceptually important variables.

distress variable instead of the continuous factor score. The distributions of model residuals by interaction terms were also examined.

Table 3.5. Variables included in fully-adjusted and final models for aim 3

Fully-adjusted model for each cytokine	Final Model with IL-1β	Final Model with IL-6	Final Model with IL-8	Final Model with TNFα
Dependent Variable:				
Cortisol	Cortisol	Cortisol	Cortisol	Cortisol
Main Independent Variables:				
Cytokine concentration	IL-1β	IL-6	IL-8	TNFα
Maternal distress factor score	✓	✓	✓	✓
Cytokine by maternal distress	✓	✓	✓	✓
Covariates:				
Sample	✓	✓	✓	✓
Child age				
Child sex	✓	✓	✓	✓
Child sex by cytokine	✓	✓	✓	✓
Child body mass index category				✓
Child currently sick	✓	✓	✓	✓
Child current oral health issues				
Maternal smoking status				
Family financial hardship	✓	✓	✓	✓
Time since waking	✓	✓	✓	✓
Time since waking by sample	✓	✓	✓	✓
Task timing				

✓= Included in final model

To assess the impact of substitution methods for censored cortisol data, final models were performed using an alternate substitution (i.e., the LLD (0.007 μg/dL)), as well as using laboratory estimates of cortisol values below the LLD (with zero values replaced with 0.0001). Also, random effects Tobit models with a lower limit of censoring set at the natural log of the LLD of the cortisol assay (-4.961845) and bootstrapped variance were performed.

Sensitivity analyses also tested the use of individual maternal distress and family financial variables in final models instead of factor scores. The impact of imputation methods for missing data was also assessed in sensitivity analyses performed on complete case data.

Furthermore, to examine the nature of cytokine effects on cortisol levels, the individual mean and trajectory effects of each cytokine were examined simultaneously as separate terms in each final model. These models revealed no significant difference in the individual mean and trajectory effects of any cytokine, therefore final models tested cortisol relations with cytokines as single combined effects.

3.7. Statistical Software

All analyses, except factor analyses performed for aim 3, were conducted in Stata/SE 12.1 (StataCorp LP, College Station, TX). Factor analyses and factor score computations for aim 3 were conducted in MPlus Version 7.11 (Muthen and Muthen, Los Angeles, CA).

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CHAPTER FOUR

Pre-Analytic Considerations and Best Practices for Studies Involving Salivary Analyte Data

4.1. Abstract

Objective: This review discusses the methodological challenges and pitfalls in salivary analyte data analysis. The paper focuses on pre-analytic data processing procedures that are essential for data validity and reliability, but are rarely described in the literature, including: flow-rate adjustments, censored data points, and data transformations.

Methods: A review of in-laboratory and pre-analytic data processing steps is presented. Techniques for addressing data processing and analytic challenges drawn from current salivary analysis studies and literature from other fields encountering similar issues are then discussed. Finally, the impact of different pre-analytic methods choices on data parameters and study results is illustrated using sample salivary analyte data.

Results: A set of best practice recommendations is proposed to improve validity, reliability, precision, and across-study consistency in the pre-analytic phase of salivary analyte data analysis.

Conclusions: Salivary biomeasures present exciting opportunities for advancing health research and interdisciplinary studies examining mechanisms linking brain, behavior, and health. To maximize this potential, this paper highlights the need for researchers to discuss pre-analytic data processing choices and methods, and for collaboration across multiple fields to establish a set of comprehensive best practices and accepted methodology.

4.2. Introduction

Multidisciplinary biobehavioral research is critical to enhancing the understanding of health and development of children. In their strategic plans, the National Institutes of Health tasked investigators to advance the use and development of cross-systems biologic data for disease screening, diagnosis, and treatment, as well as monitoring normative development and aging (e.g., 1). With a national focus on transdisciplinary biobehavioral research intensifying, a growing number of investigators are incorporating biologic data collection and analysis into their studies.

Saliva offers a minimally-invasive, safe, accurate, time- and cost-efficient, and ecologically valid method for obtaining biologic data (e.g., 2). Analytes and biomarkers in oral fluids are advancing research on the mechanisms underlying cognitive function, behavior, emotional and mental disorders (e.g., 3,4), as well as the biomonitoring of environmental exposures and stress (5,6). Within the past 20 years, collection devices and assay reagents specifically designed for saliva have become commercially available and widely accessible. With minimal training, research participants and patients can self-collect, handle, transport and store saliva specimens. Using saliva, researchers are able to collect repeated samples and assay multiple analytes per sample without high participant burden.

There are methodological challenges associated with the collection, handling, transport, and assay of saliva as a research specimen. These challenges, and strategies to address them, are well-documented and extensively discussed in the literature (2). This paper draws attention to more subtle challenges beginning in the early steps of sample handling and assay, as well issues related to pre-analytic data processing. Pre-analytic data processing encompasses handling duplicate, extreme, censored and zero values; addressing kurtotic and skewed distributions; evaluating data transformations; and computing indicators of individual differences. Choices investigators make in the pre-analytic data processing phase can impact the reliability and validity

of the data and study findings. However, guidance about pre-analytic decision-points is sparse in the existing literature.

The purpose of this paper is to review pre-analytic data processing steps, highlight specific challenges, and present best practices for salivary analyte data processing. This paper uses sample data to illustrate how variations of these practices impact the nature, interpretation and implications of study results. Decisions made by technicians in saliva processing laboratories that have the potential to impact data validity are briefly discussed. The focus, however, is on the choices an investigator must make after receiving analyte data from the laboratory. Basic analytic methods that are particularly useful for salivary analyte data analysis, such as transforming data and modeling group parameters, are also discussed.

4.3. In-laboratory Saliva Processing Steps

A basic understanding of in-laboratory sample processing helps to inform pre-analytic data processing decisions, as well as determinations of data quality, validity, and reliability. Saliva samples are typically donated and prepared in the field, transported to the laboratory under controlled temperatures, and then processed for the determination of analytes using high complexity assays (e.g., immunoassay or kinetic reaction) designed for use with oral fluid. These assay protocols often employ microtiter plate technology. Each microtiter plate has a finite number of microwells, and a portion of each saliva sample is placed in the microwells. On each plate, some of the microwells are reserved for a series of specimens known as calibrators or standards. These samples have known concentrations of the analyte of interest and are used to construct the *standard curve*.

Controls are also tested on each assay plate. Controls are created by an external source and their levels reflect the lower and upper expected range of participant specimens. During laboratory processing, color change in each well is determined in measurement units of optical density. Through comparisons with the standard curve, optical density units are converted into

standard units. Assay protocols include strict criteria to ensure the consistency of liquid handling, the standard curve, and the reliability and precision of each assay (for more detail the reader is referred to 7,8). Raw data generated by this process typically include: determinations for the analyte of interest (in singlet, duplicate or triplicate), a metric to indicate salivary flow rate (i.e., volume or volume estimated by weight, and time), and the indices of inter- and intra-assay precision (i.e., coefficients of variation).

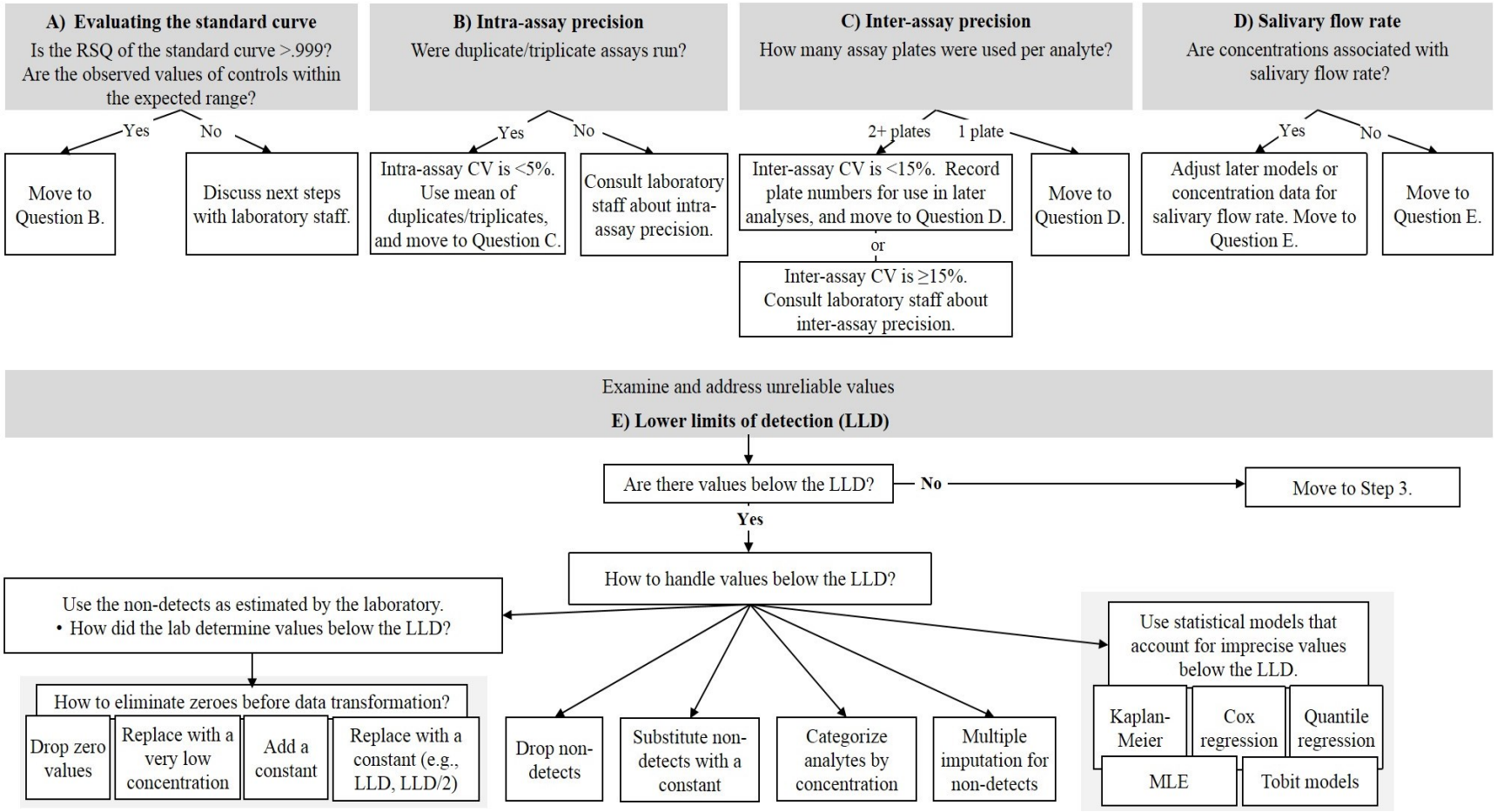
4.4. Pre-analytic Data Processing

Once received by the investigator, the raw data are reviewed, evaluated, and prepared for use in statistical models. These pre-analytic steps include: 1) examining data parameters related to in-laboratory saliva processing procedures; 2) examining and addressing unreliable values; and 3) examining the distribution of the data. Each step is discussed in detail below, and references are provided for further reading. Figures 4.1 and 4.4 illustrate these steps and important questions and decision points that arise during pre-analytic data processing.

Step 1: Examine data parameters related to in-laboratory saliva processing procedures

A) Evaluating the standard curve. The shape and slope of the standard curve is critical to the precision, reliability and validity of salivary analyte determinations. The slope of the standard curve must be nearly perfectly linear to enable the precise computation of standard units from the microplate reader output. The assay's standard curve RSQ (r-squared) should be greater than 0.999 to ensure high quality data (Figure 4.1). Values from the standard curve for the high and low controls must be used to verify that the curve is valid in the range expected of the study samples. Laboratory technicians should provide investigators with the expected and observed values of control samples and ensure that the observed values of control samples are within the expected range (Figure 4.1). For detailed information about standard curves, the reader is referred to The Handbook of Immunoassay (8).

Figure 4.1. Pre-analytic data processing steps 1 and 2: 1) Examine data parameters related to in-laboratory saliva processing procedures; and 2) Examine and address unreliable values



B) Intra-assay precision. Intra-assay precision represents how well the assay was performed on the samples in the hands of the assay's operator. The intra-assay coefficient of variation (CV) indexes the reliability of the assay for individual samples as well as the study as a whole. To calculate the intra-assay CV, all, or a subset of, samples are run in duplicate (or triplicate). On an individual sample basis, the CV is used to determine whether the individual sample's assay results are reliable or whether the test should be repeated. The criterion by which investigators judge duplicate values as reliable is rarely reported. Adequate reliability across an entire study is indexed by an intra-assay CV that is less than 5%, on average across all samples (7). When samples are assayed in duplicate/triplicate, the average of the concentrations should be used in the analysis.

C) Inter-assay precision. The number of unique samples that can be run on an assay plate varies according to the number of wells on the microplate, the percent of duplicates/triplicates run, and the number of wells dedicated to controls (including non-specific binding wells) and standards. Typically each microplate will assay 36 unknown samples in duplicate, and studies with more than 36 samples need multiple microplates. Inter-assay precision is calculated across microplates, typically using the high and low controls tested on each microplate, as a measure of reliability. Immunodiagnostic industry standards expect the inter-assay CV to be less than 15% (7). With up to 15% variation in assay results accepted as normative between plates, investigators should ensure that all samples collected from the same participant are placed on the same microplate to minimize within-subject error variance. However, the impact of plate (or batch) on between-subject variance remains an issue. Investigators should request plate numbers, and values for high and low controls in order to examine the impact of microplate assignment (Figure 4.1).

D) Salivary flow rate. Levels of analytes that move from circulation into the oral fluid or are secreted by the salivary glands may be influenced by salivary flow rate (e.g., alpha-amylase; 9 secretory Immunoglobulin A; 10, and dehydroepiandrosterone-sulfate; 11). Salivary flow rate is

calculated using laboratory measurements of sample volume (flow rate= sample volume/sample collection duration) and is expressed in units of mL/minute. It is important to determine whether flow rate is associated with analyte levels. If there is an association, or if prior research illustrates the importance of flow rate on the analyte's concentration, the effect of flow rate should be examined in the main analyses (Figure 4.1). As an alternative to statistical covariation, a correction can be computed by multiplying concentration/volume by volume/minute. This corrected index is expressed in units of concentration/minute or units of activity/minute and is referred to as "output". Failing to account for salivary flow rate can significantly restrict the utility of some salivary analyte data.

E) Lower limits of detection (LLD). The LLD is the lowest concentration of a salivary analyte that the assay can reliably discriminate from zero. The reliability of determinations below an assay's LLD depends on the laboratory's procedures for setting the assay LLD and the microplate and reader technology. Investigators should ask the laboratory about their procedures for setting assay LLDs and estimating concentrations below the LLD for each analyte. Depending on the reliability of laboratory estimates of values below the LLD (i.e., *non-detects*), the investigator can chose from several methods for handling these low concentrations. These methods are detailed below and shown in Figure 4.1.

Step 2. Examine and address unreliable values

A. Methods for handling values below the LLD. Investigators can chose from several methods for handling values below the LLD, including: 1) dropping all non-detected values; 2) substituting non-detects with a constant (e.g., the LLD concentration, a percentage of the LLD concentration, or zero); 3) using non-detects in the data as they were estimated by the laboratory; 4) including non-detects in the data and categorizing all analyte data; 5) using multiple imputation to fill in non-detects; and 6) using statistical methods that account for imprecise values below the LLD. When choosing a method for handling censored data, the sample size, the percent of

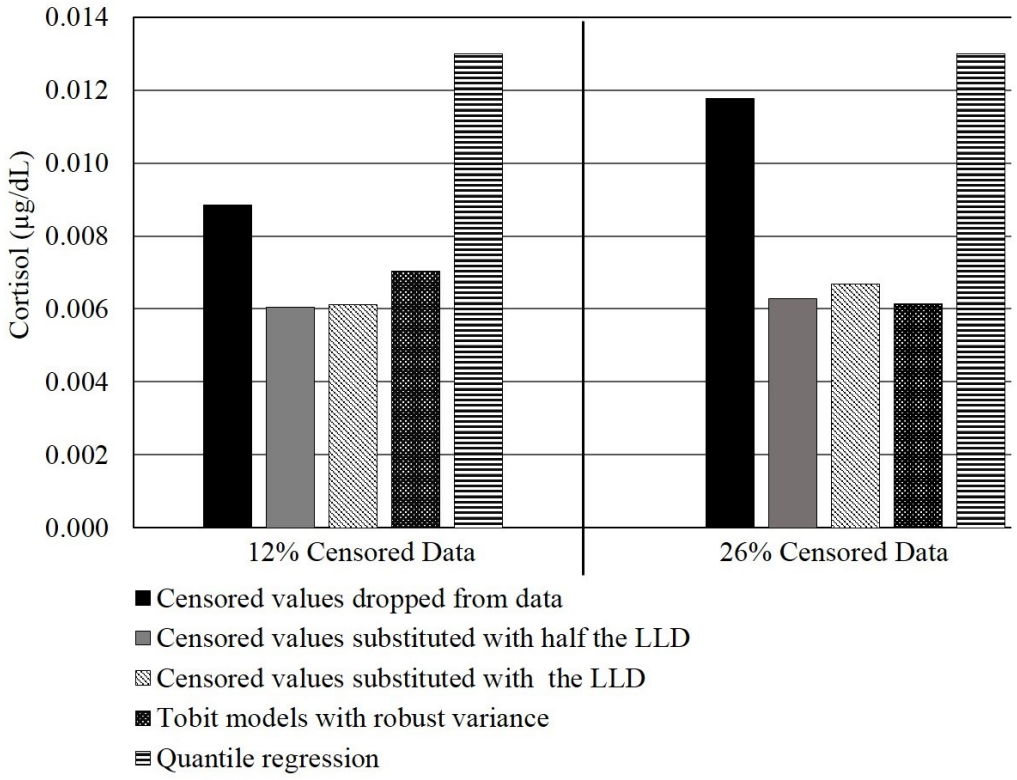
censoring, the LLD concentration relative to the observed data, and laboratory procedures for estimating values below the LLD should all be considered. Detailed discussions and comparisons of these six methods (and others) are available in the literature (for reviews see 12–14). This report will only briefly discuss key advantages/disadvantages of methods typically employed by salivary analyte researchers. Interested readers are referred to the National Institute of Child Health and Human Development (NICHD) Epidemiology Branch (15), for more detailed descriptions of emerging methods for analyzing censored data.

The impact of different censored data methods on group parameters and how this impact changes by level of censoring is illustrated in Figure 4.2 using sample data. Sex differences in raw salivary cortisol for 125 children (61 male) are shown under the true percent of censoring (12%) and with censoring artificially increased to 26% using five different censored data methods discussed below and presented in Figure 4.1. Figure 4.2 illustrates the impact dropping non-detects has on sex differences in mean cortisol compared to when substitution or Tobit models are used. Furthermore, the impact of these methods varies by level of censoring. Quantile regression uses median values, however, and group parameters are therefore insensitive to censoring levels when the percent of non-detects is less than 50%.

In the literature, the most common approaches to handling non-detects involve dropping or substituting censored data points, and categorizing analyte data (Figure 4.1). Dropping censored data points is not generally recommended, because it introduces bias, sacrifices data, and decreases power (13,16,17). Substituting non-detects with a constant (e.g., the LLD or a percent of the LLD) also introduces bias and inappropriately reduces variance (13,17,18). The amount of bias associated with substitution methods depends on the distribution of the data, the level of measurement error, the percent of censored data, and the LLD relative to the observed concentrations (14,19–21). Substitution may be an acceptable method of handling censored data

when the percent of censoring is low and between-subject variance is the focus of the study (18,22).

Figure 4.2. Difference between male and female mean raw cortisol (µg/dL) using five different methods for handling censored values in salivary analyte data with 12 and 26% censoring[a]



[a]12% censoring represents the percent of non-detects under the true LLD (0.007 µg/dL). 26% censoring assumes a hypothetical LLD of 0.028 µg/dL. Quantile regression results are sex differences in median cortisol which are insensitive to the percent of censoring if there are less than 50% non-detects .
LLD= lower limit of detection

Another common option is to use non-detects as estimated by the laboratory (Figure 4.1). Modern microplate reader software extrapolates values for samples below the LLD. Laboratories often estimate values below the LLD and include them as flagged values in the data report. It is important to recognize, however, that the reliability of these values depends on the assay technology and the quality of the laboratory procedures, and estimates may not be reliable upon

re-assay. Thus, laboratory estimates of these values may be no more valid than other substitutions (12).

Finally, categorizing analyte data by concentration (e.g., into quartiles or quintiles) is an attractive method for handling non-detects, because it allows the investigator to avoid the problem of censored data since all non-detects are grouped into the same category. However, categorization sacrifices information and may limit further statistical modeling strategies and complicate interpretation of model parameters (19).

The last two approaches for handling non-detects, multiple imputation and statistical modeling, account for the imprecision of censored data. Multiple imputation techniques estimate censored data points through regression models and substitute each non-detect with a set of imputed values (18,19). Statistical models can then be performed on the imputed data, and results are pooled across imputations (19). Modeling of the non-detects with multiple imputation assumes a normal distribution of the underlying data (16). If this assumption is violated, the validity of the imputed data is compromised (16).

Finally, pre-analytic data processing steps for filling in censored data can be bypassed if statistical analysis methods that account for imprecision below the LLD are employed. Unlike the methods discussed above, these models do not replace non-detects, rather they recognize the censored data and incorporate the imprecision introduced by censoring into the model parameters. There are non-parametric and parametric modeling approaches (Figure 4.1).

Non-parametric modeling options for non-correlated data include Kaplan-Meier methods and Cox and quantile regression. Kaplan-Meier methods are particularly useful for estimating means in the presence of multiple LLDs (23,24). Cox and quantile regression can compute trends in the data and examine the influence of covariates (25,26). The reader is referred to Gillespie and colleagues (23) for a review of the Kaplan-Meier method, Eilers and colleagues (25) for more information about quantile regression, and Dinse and colleagues (26) for a review of Cox regression for censored biomarker data.

Parametric modeling approaches for continuous censored data include maximum likelihood estimation (MLE) and Tobit models (13,14,18). Robust variations of MLE may be more appropriate for use with salivary analyte data that tend to violate these model assumptions of data normality and homoscedasticity (14). Extended versions of MLE are also available for use with correlated data (e.g., repeated measures data; 22).

Tobit models are regression models designed for modeling censored dependent variables by fixing a limit of censoring (i.e., the LLD) for the outcome (18). Tobit models assume a normal distribution, homoscedasticity, and independence of observations (27). However, Tobit model extensions designed to handle heteroscedasticity (28), and longitudinal and correlated data (29,30) are available. Tobit models only account for censoring in the dependent variable and are most appropriate when there is a single, constant limit of censoring. If there are multiple LLDs (e.g., if LLD varies by microplate) or if analyte data have been combined into composite measures (e.g., proportion change, slope, area under the curve) Tobit models may not be appropriate because there is no constant limit of censoring.

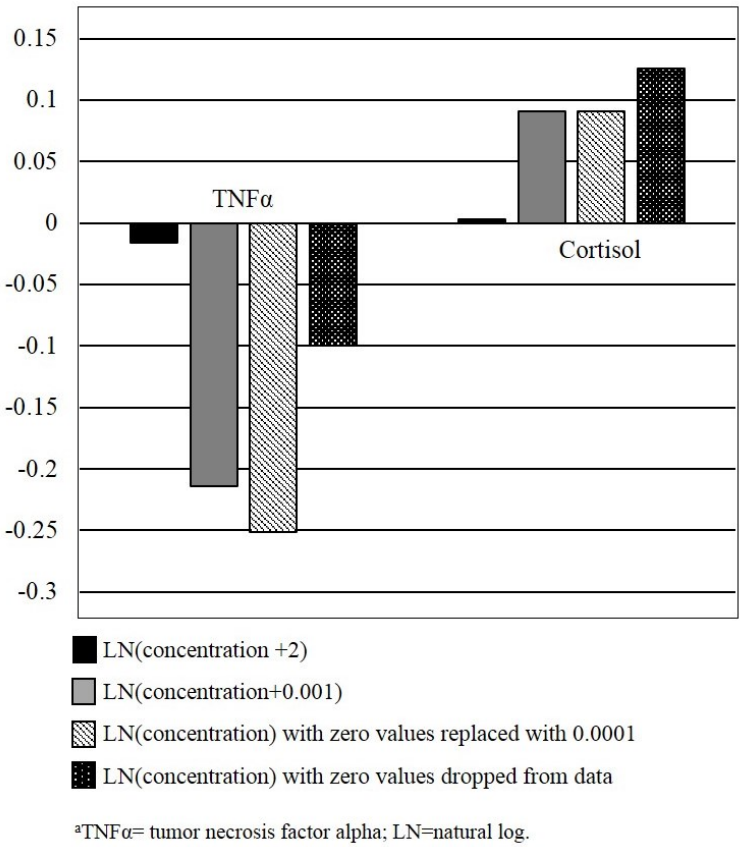
B) Techniques to handle zeroes. Another important step in addressing values below the LLD is the handling of zero values (Figure 4.1). Zero values are below the LLD; therefore they are neither reliable concentrations nor true zeroes. Zeros also complicate data transformation techniques that are typically necessary before performing parametric statistical tests with salivary analyte data. For example, to perform a log-transformation data cannot include zero values. Zeroes can be handled by: 1) dropping zero values from data; 2) replacing zero values with a very low concentration; 3) adding a constant to all data; or 4) replacing zero values with a constant (e.g., the LLD or half the LLD). These options should be considered alongside the question of how to handle non-detects, as replacement methods should be consistent and logical across all values below the LLD. There is no accepted standard method for handling zero values;

investigators should consider the percent of zero values and evaluate the impact of zero values and the methods for handling them on data distributions and parameters.

Important details must also be considered for each method for handling zero values. For example, if replacing zeroes with a constant, should the constant be the same across analytes or should it vary by analyte or plate? If adding a constant to the data, what is an appropriate constant? Also, if examining multiple analytes, what constant is appropriate to add for all the data (as the constant must be the same for each analyte if comparisons are planned)? These questions are important to keep in mind prior to data transformation, because non-linear data transformations (e.g., log transformation) will result in differential spacing between transformed values depending on their magnitude (low values are stretched out and higher values are compressed together).

Figure 4.3 uses sample data to illustrate the impact of different methods for handling zeroes on group parameters. Sex differences in mean salivary tumor necrosis factor-alpha (TNFα) and cortisol are shown using the four different methods for handling zeroes discussed above. For this figure, saliva for 125 children (61 males) was assayed for TNFα and cortisol; non-detects were included as estimated by the laboratory and data were log-transformed. Only one sample of TNFα was estimated as zero by the laboratory, and ten were estimated as zero for cortisol. Figure 4.3 illustrates the importance of choosing an appropriate constant; the difference between male and female TNFα and cortisol means varies by the choice of constant (two or 0.001). This figure also shows the impact of dropping zeroes from the data even when there are few zero values. For example, TNFα only has one zero value, however, dropping this value markedly changes the sex difference.

Figure 4.3. Difference between male and female mean log-transformed TNFα (pg/mL) and cortisol (µg/mL) using four different methods for handling zeroes in salivary analyte data[a]



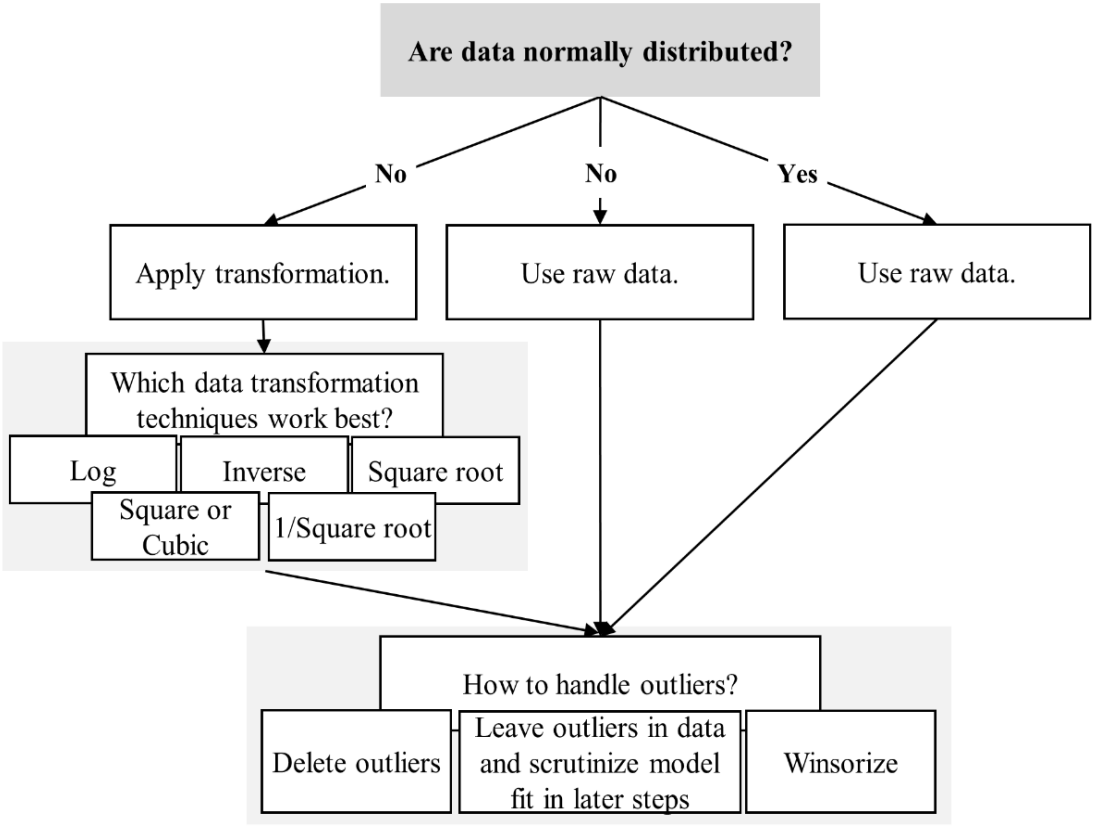
[a]TNFα= tumor necrosis factor alpha; LN=natural log.

Step 3: Examine the distribution of the data (Figure 4.4)

Salivary analyte data typically display a strong positive skew with a disproportionate amount of low concentrations and a long tail of high concentrations. Many data transformation techniques can be employed to correct the skew of the distribution (e.g., log-transformation, inverse-square root, square root), so that data can be analyzed using parametric statistical models (Figure 4.4). The impact of each of these approaches should be tested for each analyte. In choosing data transformation techniques, it is important to consider future analyses that may include more than one analyte or comparisons across analytes. Making small compromises in normality and choosing a single transformation technique for all analytes will keep analytes on the same scale and improve interpretability.

Even after transforming the data, there may still be outlying data points. Outlying data points may be plausible data or may be due to sample contamination or laboratory processing errors. To the extent possible, implausible or contaminated values should be scrutinized and flagged or dropped from the data. There are many techniques available for dealing with plausible outliers including: 1) deleting all outliers from the data; 2) Winsorizing the outliers (31); and 3) keeping plausible outliers in the data and examining model fit in future analyses (Figure 4.4). Dropping plausible outliers reduces information and introduces bias in the data. Winsorizing the data, or replacing outlying data points with values equal to the most extreme plausible data point, will improve the data distribution and reduce variance (31). If Winsorization techniques are applied, a series of questions should be addressed. For example, it is important to consider which groups of data (e.g., participant groups, time points) should be Winsorized together and to set an acceptable range of variation from the mean. When Winsorizing longitudinal data by time point, individual trajectories should be taken into account; for example, if an individual's raw value at time one is greater than the raw value at time two, the Winsorized value at time one should also be greater than the Winsorized value at time two. Dropping and Winsorizing outliers both introduce biases. However, including unadjusted outliers in the data can impact model fit and may not provide additional information about the overall nature of the data. See Wilcox and Keselman (32) for a review of identifying and handling outliers.

Figure 4.4. Pre-analytic data processing step 3: Examine the distribution of the data



4.5. Beyond Pre-analytic Data Processing

Every decision made in the pre-analytic phase of data processing impacts the data that will be used in more complex statistical models to answer the specified research question. The goal of pre-analytic data processing is to generate a dataset that best represents the nature of the data and can be used in statistical models to produce valid and reliable study findings. Common statistical modeling approaches for salivary analytes include modeling: within-individual change over time, coordination among multiple analytes, and concordance within and between subject groups (e.g., families, mother-child dyads). The quality of the results of these more complex statistical analyses is contingent upon the validity and reliability of the raw analyte data, as well as pre-analytic decisions which determine the data distribution, variance, and bias, and define the

range of statistical approaches available to the investigator (e.g., non-parametric vs. parametric modeling, linear vs. ordinal regression). A detailed discussion of complex statistical methods in these areas is beyond the scope of this paper. However, key methods are presented below to demonstrate the link between pre-analytic processing decisions and statistical model building.

A) Within-individual analyte change

There are many ways to examine change over time in salivary analyte data. Discussion of complex methods, such as latent curve modeling (33), multilevel mixed models (34), generalized estimating equations for repeated measures (35) and structural equation modeling (36) is outside the scope of this paper. These statistical approaches, however, are powerful methods that should be considered when modeling change in analytes over time.

Comparing "responders" to "non-responders". Often studies aim to examine the psychological or health correlates of a specific analyte's response to a stimulus (e.g., the response of salivary alpha-amylase to a laboratory stressor). One common and straightforward approach is to divide the sample into "responders" and "non-responders" and compare these groups on a range of selected factors. However, there are no established standards for distinguishing groups, and differences in the number of groups and cut-offs for group membership makes it difficult to compare results across studies. Granger and colleagues (2) suggest that a threshold for meaningful change in salivary analytes should be based on twice the average inter-assay coefficient of variation and an absolute difference twice the LLD. Other studies have used difference scores, proportion change percentiles, cluster analysis, and mean and standard deviation parameters to determine group cut-offs (37–39).

When deciding how to distinguish groups of responders, it is important to consider thresholds used by previous researchers, as well as the distribution of the data. If the data are censored and data parameters are to be used as group membership thresholds, statistical modeling methods for parameter estimation (e.g., Kaplan-Meier, Tobit models) rather than simple

substitution methods may be appropriate. Statistical modeling approaches for parameter estimation are generally more appropriate for highly censored data, and errors in parameter estimation will be perpetuated if parameters (e.g., mean, standard deviation) are used as group cut-offs. Although methodologically simple, comparing responders and non-responders sacrifices information as continuous data is categorized and participants may be dropped from analyses if their responses do not meet group thresholds.

Examining composite measures of change. Another commonly used and simple approach to examining change in analytes over time is to use composite measures of change, such as proportion change, difference scores, slope, and area under the curve with respect to ground (AUCg) and with respect to increase (AUCi; 40). Composite measures express concentration changes with a single value, thereby avoiding the complexities of modeling repeated measures and correlated data points. Proportion change and difference scores summarize analyte change across two samples. Slope measures further incorporate the elapsed time between samples.

AUCg and AUCi are composite measures that express change in concentration over many samples and account for the time between samples (40). AUCg is a measure of total analyte output, while AUCi measures output across time with respect to the initial concentration (40). Pruessner and colleagues (40) standardized the formula for AUCg and AUCi to improve across-study comparisons. However, pre-analytic data processing steps, such as handling of non-detects and zeroes, and data transformations, impact the data that are used in the computation of composite measures and can cause variation in these measures across studies. For example, it is important to keep the scale of the data in mind when calculating composite measures. If log transformations were used, the data will likely contain negative values, so composite measures' formulae must be modified to account for transformed data and negative values or raw data should be used to compute the composite measures.

B) Coordination among multiple analytes

Modern microplate technology allows investigators to assay multiple analytes per sample. While this can provide a wealth of information about individual and cross-system functioning, simultaneous analysis of multiple analytes presents challenges in both the pre-analytic (e.g., decisions regarding non-detects, choice of a constant, and data transformations) and analytic phases of data analysis. In the analytic phase, common techniques for examining analyte coordination include: conducting simple correlations, principal component analysis (41), cross-correlations (42), ratios (e.g., alpha-amylase to cortisol ratios; 43), and hierarchical linear modeling (44).

C) Concordance within and between subject groups

Common statistical strategies to model concordance between individuals fall in two major categories – multilevel modeling (45) and latent growth curve modeling (46). Both modeling techniques operationalize the degrees of concordance by estimating the strength of the association between two participants' analyte concentrations at baseline and in the rate change over time. Often, researchers are interested in examining factors that influence the level of concordance between subjects (e.g., how does stress impact cortisol concordance between mother/child pairs?). These questions can be addressed by including moderators in the models. In most cases, multilevel modeling and latent growth curve modeling tend to yield comparable parameter estimates (47). However, the multilevel framework has been more commonly employed due to its flexibility in incorporating both categorical and continuous moderators (48,49).

Furthermore, researchers interested in establishing temporal precedence and directionality of influence between participants can use a time-lagged approach (50). A recent development in statistical analysis, the actor-partner interdependent model (APIM), allows researchers to examine the bidirectional effect of analytes between the members in a dyad (51).

Finally, these modeling techniques can be applied to study the concordance among multiple participants to paint a more colorful picture of the role of salivary analytes in family or group dynamics (50). Novel applications of social network analysis to salivary analyte data (e.g., 52) also provide new opportunities for examining associations between social relationships and biologic functioning within complex social groups.

4.6. Best Practices in Pre-analytic Data Processing

Salivary analyte data hold great promise for advancing health and development research. Salivary data can help elucidate mechanisms underlying mental, emotional, and behavioral disorders, track toxic exposures, and monitor health and growth. However, in order to fully realize the role of salivary analyte data in furthering our understanding of psychology, health, and the interplay of experience, environments and multi-system functioning, better dissemination of information regarding pre-analytic data processing is needed. Pre-analytic data processing is a neglected phase of data analysis for which no standard protocols are followed and limited information is provided in published manuscripts. Variations in pre-analytic data processing methods complicate across-study result comparisons and undermine the reliability and replicability of salivary analyte study results.

The best practices of pre-analytic data processing likely vary by analyte, data distribution, censoring level, and research question. However, establishing reporting requirements for pre-analytic salivary analyte data processing methods, similar to the Consolidated Standards of Reporting Trials, would facilitate across-study comparisons and improve interpretation of the internal and external validity of biomeasure findings. Explicit detailing of pre-analytic data processing methods in manuscripts would also provide researchers with a toolkit of pre-analytic data processing methods and promote consistency as well as innovation in these methods.

Collaborations among psychology, health, biostatistics, epidemiology, and environmental health researchers to establish standards for pre-analytic salivary analyte data processing methods

would greatly advance biomarker research. The Epidemiology Branch of the NICHD is currently collaborating with the Biostatistics and Bioinformatics Branch to develop methods for analyzing biomarker data. However, there is no standard set of practices available for health research. While some fields have proposed recommendations for some steps of pre-analytic data processing (e.g., the Environmental Protection Agency provides recommendations for censored data), these recommendations are not typically followed by health researchers. Consistent data analysis methods across multiple areas of health research would maximize the potential of salivary analyte research in promoting fruitful research contributions and collaborations.

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CHAPTER FIVE

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

5.1. Abstract

Background and Objective: 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.

Methods: 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)).

Results: Cytokines were positively intercorrelated and task-sensitivity varied. Except IL-8, cytokines were elevated in children with oral health issues and exposure to tobacco smoke. Among boys, cytokines were positively related to sAA and negatively related to cortisol.

Conclusions: 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.

5.2. 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 (1–3). 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 (1). Understanding the influence of early-life adversity and stress on NEI functioning may be foundational to understanding and addressing the development and progression of inflammatory-related diseases in later life (1,4,5).

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 (6). 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 7).

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. Only two studies have examined salivary cytokines in pre-pubertal youth, and these studies only examined interleukin-6 (8,9). 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. The present study begins 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 (10,11). Communication between the CNS and immune system is essential for establishing homeostasis and allostasis (1,12). 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 the 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 (13,14).

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 (15). 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 (15,16). Rising levels of

inflammatory cytokines stimulate the HPA axis to release cortisol which then inhibits inflammatory cytokine production (15,17). This negative feedback loop between the immune system and the CNS is critical to regulating inflammation and maintaining health (15,18). 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 (19,20). On the other hand, insufficient immune functioning increases susceptibility to disease (21).

The Present Study

This paper represents the first systematic exploration of the nature and correlates of salivary cytokines in early childhood. This study examined cytokine intercorrelations 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, the associations between cytokines and salivary alpha-amylase (sAA) and cortisol were also examined. Three hypotheses were tested. In addition to these hypotheses, changes in cytokine levels across a series of cognitive and emotional challenge tasks were explored 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 (22). Findings from studies examining salivary cytokines in older youth suggest a coordinated immune response in the oral cavity and strong inter-cytokine correlations (23). It was 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 (22). 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 (24). Not surprisingly, previous studies show consistent positive associations between oral health problems and salivary cytokine levels in older youth and adults (25–31). Systemic health and demographic factors, however, are largely unrelated to levels of salivary cytokines (23,32–34). It was hypothesized that cytokine levels in the saliva of healthy young children 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 (15). Although not widely studied, two investigations reported negative associations between IL-6 and HPA activity in the oral cavity in adults (35,36). Izawa and colleagues (35) 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 (37–40). 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 (35,41,42). Higher levels of salivary IL-6 are also associated with negative emotions and mental states (9,33,36). In addition, salivary IL-1β has been associated with the activation of brain areas involved in emotional processing in adults (43). 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; 8). 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.

5.3. 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 44). Mothers from the fetal development study were self-referred and mostly well-educated, married and white

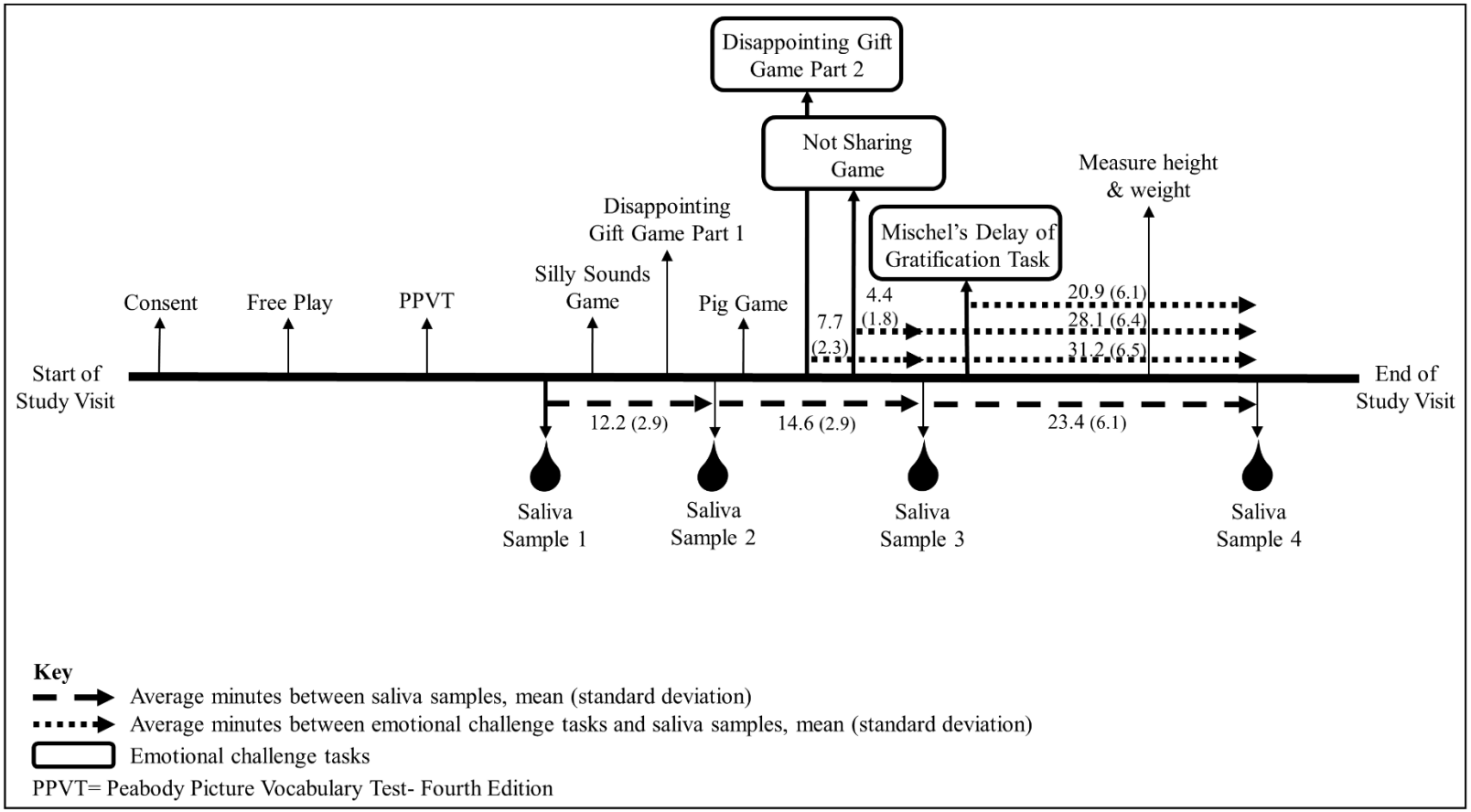
(44). To increase the diversity of the sample, mother-child pairs were also recruited from Baltimore City through community postings and fliers. To be enrolled in the current study, children had to be five years old, and mothers and children had to be fluent in English. Children with significant health 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, and body mass index (BMI). Approximately half the sample was male, about a third was white, and the majority was in good health (Table 5.1). 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 5.1). 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 5.1).

Procedures

The Johns Hopkins Bloomberg School of Public Health Institutional Review Board approved the study protocol. Mothers provided written informed consent. Figure 5.1 shows the study protocol for children. The 90 minute 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 (45) and two cognitive challenge tasks that tested inhibitory control (the Silly Sounds Game and Pig Game; 46,47). Children also participated in three age-appropriate emotional challenge tasks: the Disappointing Gift Game, the Not Sharing Game, and Mischel's

Figure 5.1. Study protocol for children with elapsed time between saliva samples and stressor task (n=125)



Delay of Gratification Task (48) (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 (except overweight and obese children were awake for longer when the appointment began compared to underweight and children of a healthy weight ($t(123)= -3.95$, $p< .01$)).

Table 5.1. Demographic and health characteristics of the sample of 125 five-year old children

Demographic Characteristics		
Age, mean years (SD)	5.45 (0.29)	
	Frequency	**Percent**
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%

[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.)".
SD= standard deviation.

Emotional challenge tasks: The Disappointing Gift Game assessed the child's response to disappointment (49). 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 minutes 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 (49–53).

The Not Sharing Game is an emotional stressor and test of emotional regulation (54,55). 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 (54).

The Delay of Gratification Task is an emotional stressor and test of coping abilities (48,55). 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 eight minutes 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 (48,53,56).

Health and demographic data: An evaluator measured children's 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 standards (57,58). Maternal smoking, children's medication use in the last 48 hours, and children's 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 5.1). 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 hours) 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 5.1 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 and colleagues (59), whole unstimulated saliva was collected by passive drool into a 2 mL cyrogenic vial for three minutes or until 0.75 milliliters of fluid was collected. Saliva samples were stored at -20 degrees Celsius until assayed. Saliva samples were assayed following Granger and colleagues (39) 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 (23) using a 4-plex 96-well format electrochemiluminescence 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 95-100% 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 (60). 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 (7), data were not adjusted for salivary flow rate. First, the raw data were explored, 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 the 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 the 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, to explore the extent and nature of task-related change in cytokine levels, the nonlinearity of cytokine trajectories across the study visit was examined using a spline knot. With no a priori hypotheses about the timing of task-related change in cytokine levels, the appropriateness of a knot placed at sample 2 or 3 was tested 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 vs. 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 estimates 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 the 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, the interaction between sex and sAA or cortisol on each cytokine was also examined (61,62).

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 obtain 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).

5.4. Results

Salivary Cytokine Concentrations

The descriptive statistics for raw concentrations of each cytokine by saliva sample are shown in Table 5.2. 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 ($t(123)= 47.42\text{-}74.45$, $p< .008$ for all). Analyte levels were highly variable across participants but 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).

Table 5.2. 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

[a] IL-1β= interleukin-1 beta; IL-6= interleukin-6; IL-8= interleukin-8; TNFα= tumor necrosis factor alpha; SD= standard deviation.

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 Bonferroni-corrected significance level (corrected $\alpha= .017$). Using a 10% threshold for meaningful change (7), 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: Cytokine Intercorrelations

Table 5.3 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 5.3). 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 $\alpha =.008$).

Table 5.3. Correlations between salivary cytokines at each saliva sample (n=125)[a]

Sample 1			
	IL-1β	**IL-6**	**IL-8**
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*

[a] IL-1β= interleukin-1 beta; IL-6= interleukin-6; IL-8= interleukin-8; TNFα= tumor necrosis factor alpha. *p<.008 (Bonferroni-corrected significance level)

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 5.4-5.7. 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 5.4 and 5.6). 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 5.7). 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 5.5).

Hypothesis 2: Associations between Salivary Cytokines and Demographic and Health Factors

The results from final models for each cytokine are shown in Tables 5.4-5.7. IL-1β and IL-6 were higher in children with current oral health issues compared to those without oral health issues (Table 5.4 and 5.5, respectively). TNFα was higher in children with smoking mothers than those with non-smoking mothers (Table 5.7). IL-6 was also positively associated with time since waking and age (Table 5.5). IL-8 was the only cytokine not related to any demographic or health variable.

Table 5.4. Results from final mixed models 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 5.5. Results from final mixed models 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 5.6. Results from final mixed models 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 5.7. Results from final mixed models 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.

Hypothesis 3: Associations 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 Appendix B Supplemental Tables S.5.1- S.5.4). 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 relation between ANS or HPA activity and cytokine levels, the significance of sex by sAA or sex by cortisol interactions was tested in each final cytokine model. The interaction between sAA and sex was significant for IL-1β, IL-8 and TNFα (IL-1β: β= -0.40, *z*= -2.82, *p*< .01; IL-8: β= -0.30, *z*= -2.51, *p*<.05; TNFα: β= -0.49, *z*= -2.62, *p*<.01), and it was marginally significant for IL-6 (β= -0.44, *z*= -1.96, *p*= .05). These interactions indicated significant positive relations between cytokines and sAA among boys (effect of sAA on IL-1β: β= 0.43, *z*= 4.39, *p* <.001; IL-6: β=0.46, *z*= 2.68, *p* <.01; IL-8: β=0.30, *z*= 3.53, *p* <.001; TNFα: β=0.49, *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= 0.25, *z*= 2.69, *p*< .01; IL-6 β = 0.33, *z*= 2.46, *p*< .05; IL-8: β = 0.25, *z*= 3.50, *p*< .001; TNFα: β = 0.30, *z*= 2.69, *p*< .01). These interactions indicated significant inverse relations between cytokines and cortisol among boys (effect of cortisol on IL-1β: β = -0.17, *z*= -3.24, *p*< .01; IL-6: β = -0.25, *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, the effects of sAA and cortisol were examined separately in sex-stratified final models for each cytokine. The results from stratified models are shown in Appendix B Supplemental Tables S.5.5- S.5.8. Among boys, sAA was positively associated with each cytokine (IL-1β: β= 0.43, *z*= 4.62, *p*< .001; IL-6: β= 0.53, *z*= 3.87, *p*< .001; IL-8: β= 0.32, *z*= 3.66, *p*< .001; TNFα: β= 0.46, *z*= 3.33, *p*< .01) and cortisol was negatively associated with each cytokine (IL-1β: β= -0.17, *z*= -4.28, *p*< .001; IL-6: β= -0.27, *z*= -4.74, *p*< .001; IL-8: β= -0.21, *z*= -5.16, *p*< .001; TNFα: β= -0.26, *z*= -4.34, *p*< .001). Among girls, there were no significant associations between cytokines and sAA or cortisol (Appendix B Supplemental Tables S.5.5- S.5.8).

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= -0.54, z= -3.68, p<.001, with cortisol: β= -0.53, z= -3.63, p<.001; for IL-8 with sAA: β= -0.36, z= -2.11, p<.05, with cortisol: β= -0.35, z= -2.18, p<.05; Appendix B Supplemental Tables S.5.5 and S.5.7). 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= -0.16, z= -2.65, p<.01, with cortisol: β= -0.15, z= -2.56, p<.05; Appendix B Supplemental Table S.5.6). 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= 0.42, z= 2.19, p<.05, with cortisol: β= 0.43, z= 2.22, p<.05; Appendix B Supplemental Table S.5.8).

5.5. 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 (23,26,28,63), levels of IL-1β 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 (64–66). Despite varying concentrations, the cytokines were significantly positively intercorrelated at each saliva sampling time point. These findings support the first hypothesis; they add to findings from studies in older participants and suggest strongly coordinated immune processes within the oral cavity (23,67).

Associations between Salivary Cytokines and Demographic and Health Factors

It was 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 (25–28), and in adolescents with dental caries (29). The current 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 (30,68) and adolescents (31), and second-hand smoke exposure has been linked to elevated levels of salivary IL-1β in adults (30). 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 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 (69). 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 (8,9). In a study of adolescent girls, age and puberty were related to salivary cytokine levels, however there were inverse associations in this older sample (23). Early childhood is a period of complex neurologic and immunologic development (1), therefore the positive associations between age and IL-6 found in the current study may represent developmental differences in neuro-immune 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 the study were visually contaminated with blood, future investigations should examine whether blood contamination may confound the relation between salivary cytokines and oral health among children (7). 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. 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.

Associations between Salivary Cytokines and ANS and HPA Activity

The hypothesis that cytokine activity is positively associated with ANS and negatively associated with HPA activity was partially supported. Surprisingly, 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 (61,62,70,71). 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 (61,62,71). 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 and increase (61,62). 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.

This is the first study to demonstrate these sex differences in NEI relations in children and using salivary markers of HPA and immune activity. The 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 (72), and sex differences in maternal-fetal relations, placental function, and neuroendocrine-neuroimmune interactions may have lasting effects on NEI relations (73). The findings may help explain those of El-Sheikh and colleagues (8) who found stress-related increases in salivary IL-6 in girls and decreases in boys. Although El-Sheikh and colleagues (8) did not measure sAA or cortisol, the current findings suggest that sex-related changes in IL-6 may have be influenced by relations with ANS or HPA activity. The 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

Changes in cytokine levels across a series of cognitive and emotional challenge tasks were examined 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 (69,74). 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 the study 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 (39,40,75), tasks involving emotional and social evaluative threat are typically needed to initiate an HPA stress response (76). This study used emotional challenge tasks shown to be effective stressors for young children (49,54,56). 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 (76), and the observed increases in sAA indicate ANS activation in response to the emotional tasks. This increases 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.

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 and colleagues (77) 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.

5.6. 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 the findings is bolstered by the tight controls afforded by laboratory-based sampling and repeated measures within individuals. The 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 neuroendocrine-immune 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.

5.7. References

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CHAPTER SIX

Maternal Distress and Child Neuroendocrine-Immune Regulation

6.1. Abstract

Background and Objectives: Early-life adversity may cause dysregulation in the neuroendocrine-immune network through repeated activation of the stress response, thereby increasing disease risk. It was hypothesized that, regardless of family socioeconomic status, maternal psychological well-being moderates neuroendocrine-immune relations in children.

Methods: Five-year old children (n=125) and their mothers participated in a laboratory study. Children engaged in emotional stressor tasks and provided four saliva samples. Mothers completed a survey and psychological assessments. Saliva was assayed for markers of immune function (cytokines: IL-1β, IL-6, IL-8, TNFα) and hypothalamic-pituitary-adrenal activity (cortisol). A composite score for depressive symptoms, anxiety, and parenting stress characterized maternal distress. Multilevel mixed models for cortisol examined cortisol's relations with cytokine levels, maternal distress, and the interaction between cytokines and maternal distress. Models were adjusted for family socioeconomic status.

Results: Significant cytokine by maternal distress interactions indicated that as maternal distress increased, expected inverse cytokine-cortisol relations within children became weaker for IL-1β, IL-6, and TNFα (β's=0.13-0.21, SE's=0.05-0.06, $p < .05$ for all). Sex-stratified models revealed that these interactions were only significant among girls (β's=0.18-0.27, SE's=0.07, $p < .01$ for all). Among boys, there were inverse cytokine-cortisol relations for all cytokines (β's=-0.18--0.34, SE's=0.05-0.08, $p < .01$ for all), and, while in the same direction as observed among girls, the cytokine by maternal distress interactions were non-significant.

Conclusions: Maternal distress influences child neuroendocrine-immune functioning by altering the sensitivity of inflammatory immune processes to cortisol's inhibitory effects. This desensitization may place the child at risk for inflammatory diseases.

6.2. Introduction

Early-life adversity contributes to poorer mental and physical health throughout life (1–3). Increasingly, pediatricians are expected to play a role in identifying and preventing early-life adversity (4–6). Developing effective interventions requires advanced understanding of the mechanisms that link adversity to disease. Repeated activation of the stress response, as a consequence of childhood adversity, may disrupt the critical balance required for healthy neuroendocrine-immune (NEI) network function. Such disruptions may render the body vulnerable to imbalanced NEI activity, and increase disease risk later in life. For example, adversity may make inflammatory immune processes less sensitive to inhibitory signals from the central nervous system (i.e., cortisol), thereby increasing risk for chronic inflammation (7). Previous research suggests, however, that the negative effects of adversity on health may be buffered by a supportive caregiver (8–10). Better understanding of the role caregivers play in moderating stress-related damage within the NEI network may illuminate a link between early-life adversity and health, which, in turn, may contribute to more effective screening tools and interventions.

This study uses salivary biomeasures to examine whether the coordination between hypothalamic-pituitary-adrenal (HPA) axis and immune system functioning varies by level of maternal distress in five-year old children from diverse socioeconomic backgrounds. This paper addresses two gaps in current knowledge. First, unlike prior studies that do not adequately parse financial and psychosocial aspects of adversity (11), this paper used several markers of maternal psychological well-being and socioeconomic status (SES) to separate the effects of caregivers from SES. Second, rather than examining a single biologic system, this paper assessed relations between the HPA and immune systems to gain a nuanced understanding of the biologic consequences of early-life adversity. It was hypothesized that maternal psychological well-being moderates HPA-immune system relations regardless of SES.

6.3. Methods

This study used data from the Fetus to Five study (12,13).

Participants

Mother-child pairs were recruited from 2011 to 2013 as reported previously (12,13). Approximately a third of the sample (58 mother-child pairs) was recruited from mothers who participated in a fetal development study in 2006-2007. Enrollment in the fetal development study was limited to low-risk, healthy women (13). To increase the diversity of the sample, an additional 93 participant pairs were enrolled from Baltimore, Maryland using flyers in libraries, schools, and grocery stores. English fluency was required, and child participants had to be five-years old. Mother-child pairs were excluded if mothers reported that the child had a health or developmental condition impairing cognitive, motor, or regulatory functioning.

Procedures

The Johns Hopkins Bloomberg School of Public Health Institutional Review Board approved the study protocol. Mothers provided written consent. During a 90-minute study visit, five-year old children (mean=5.45 years, SD=0.29) completed neuropsychological and behavioral assessments and participated in emotional stressor tasks. Four saliva samples were collected from children; two before and two after the stressor tasks. Mothers provided sociodemographic and child health information, and completed a battery of psychological assessments.

Emotional stressor tasks: Children participated in three age-appropriate emotional stressor tasks including: the Disappointing Gift Game, the Not Sharing Game, and Mischel's Delay of Gratification Task (14). These tasks elicit negative emotions (e.g., anger and frustration) and challenge behavioral and emotional regulation (14–21). The Not Sharing Game also elicits an increase in salivary cortisol in young children (20). Detailed descriptions of the tasks were previously published (12).

Family sociodemographic data: Sociodemographic data included: family income, maternal education, marital status, and the number of moves during the child's life. Financial stress was measured using a six-item instrument adapted from Essex and colleagues (22) assessing the frequency of financial stressors in the last three months (e.g., difficulty paying bills, fears of losing home/job). (Table 6.1)

Maternal psychological measures: Maternal psychological well-being was measured using three commonly-used, validated instruments. Maternal depressive symptoms in the last week were measured using the Center for Epidemiologic Studies Depression Scale (CESD-20; 23). Trait-anxiety was assessed using the Speilberger State-Trait Anxiety Inventory-Form Y2 (STAI; 24). Maternal parenting and life stress were measured using the Parenting Stress Index-Short Form (PSI; 25). (Table 6.1)

Key covariates: Salivary biomarkers can be affected by illness and contamination with blood. Thus, mothers provided information on whether children were experiencing symptoms of illness as well as whether they had loose or recently shed teeth or had other dental issues. Information about other potential confounders of cortisol and cytokine concentrations, including child age, sex, and maternal smoking status (smoking/non-smoking), was based on maternal report.

Body mass index (BMI) was determined using evaluators' measurements of children's height and weight (26). Weight status (underweight or healthy weight vs. overweight or obese) was assigned using the Centers for Disease Control and Prevention's BMI percentile ranks by age and sex (27).

The impact of diurnal patterns in cortisol was examined using elapsed time from mother reports of child waking to study appointment. To account for the differential timing of stressor tasks relative to saliva samples across children, a task timing variable, defined as the time between the beginning of the stressor tasks and each sample, was calculated.

Biomeasure data: Whole saliva was collected using passive drool. Children pooled saliva in their mouths and drooled it into a collection vial. Samples were stored at -20 degrees Celsius until assayed. Saliva was assayed for cortisol, a marker of HPA activity, and for four proinflammatory cytokines: interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor alpha (TNFα).

Table 6.1. Family sociodemographic and maternal psychological characteristics (n=125)

Sociodemographic Characteristics[a]		
	Frequency	**Percent**
Family income in last 12 months		
<$5,000-$11,999	30	24%
$12,000-$24,999	29	23%
$25,000-$49,999	11	9%
$50,000-$99,999	14	11%
≥$100,000	41	33%
Maternal education		
<High school (HS)	8	6%
HS degree/General Equivalency Diploma	33	26%
Some college	31	25%
2-year degree	7	6%
4-year degree	20	16%
Masters degree	11	9%
Doctoral/professional degree	15	12%
Maternal marital status		
Married	56	45%
Moves during child's life		
0	35	28%
1	38	30%
2	17	14%
3	21	17%
4	7	6%
5	2	2%
6+	5	4%
Maternal financial stress (mean (SD), range)[b]	14.62 (6.87)	6-30
Maternal Psychological Characteristics[c]		
	Mean (SD)	**Range**
Maternal depressive symptoms[d]	13.62 (10.91)	0-47
Maternal anxiety[e]	37.39 (9.62)	20-62
Maternal parenting and life stress[f]	67.67 (17.26)	36-113

SD= Standard deviation. [a]Variables included in financial hardship factor. [b]Cronbach's α=.91 for this sample of 125 mothers. [c]Variables included in maternal distress factor. Correlation between measures: depressive symptoms with anxiety: Spearman's rho=.84; depressive symptoms with stress: Spearman's rho=.53; anxiety with stress: Spearman's rho=.60 (p<.001 for all). [d]Cronbach's α=.90 for this sample of 125 mothers. [e]Cronbach's α=.85 for this sample of 125 mothers. [f]Cronbach's α=.90 for this sample of 125 mothers.

Determination of Salivary Analytes

Saliva assays were performed at the Center for Interdisciplinary Salivary Bioscience Research at the Johns Hopkins School of Nursing. Cortisol was analyzed in duplicate using a commercially available enzyme immunoassay (Salimetrics, Carlsbad, CA). Cortisol assay sensitivity ranged from 0.007 to 3.0 μg/dL. The intra-assay coefficient of variation (CV) was less than 5%, and the inter-assay CV was less than 10%. Eight percent of cortisol concentrations were below the lower limit of detection (LLD); these values were substituted with half the LLD (0.0035 μg/dL).

Salivary cytokines were measured following Riis and colleagues (28) using multiplex electrochemiluminescence immunoassays by Meso Scale Discovery (Gaithersburg, MD). MSD 4-plex Multi-Spot Array assays were run following the manufacturer's protocol. 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 and intra-assay CVs were: 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%. All IL-1β and IL-8 concentrations were measureable above the LLD. A small number of non-detects for IL-6 (<1%) and TNFα (5%) were replaced with laboratory-estimated concentrations. Associations between cytokine concentrations and flow rate were tested using Spearman's rank correlations and regression analyses with robust variance. No consistent relations were found, so data were not adjusted for salivary flow rate.

All analyte data were positively skewed. Data were log-transformed and Winsorized (29) (by analyte and sample) to improve the normality of the distributions. For each analyte, ≤1% of concentrations were Winsorized, and all values were brought within three standard deviations of the sample mean. Transformed and Winsorized data were used in all statistical analyses.

Statistical Analyses

Factor analyses: The first step in data analysis was to determine whether the eight SES indicators and maternal psychological scale scores (Table 6.1) could be combined into distinct measures. This was achieved by polychoric exploratory factor analysis using maximum likelihood estimation with robust standard errors, oblique rotation, and one to four factors. A two factor solution was selected based on information criteria, factor loadings, and residual variances; one factor represented financial hardship and one represented maternal distress (Tables 6.1, 6.2). Oblique rotation resulted in two factors with correlation=0.57 ($p < .001$). Greater financial hardship was associated with greater maternal distress. Factor scores, derived using the posterior mode method, provided a score for financial hardship and maternal distress for each child.

Table 6.2. Factor loadings and parameter estimates from factor analyses with a two-factor solution for family sociodemographic and maternal psychological characteristics[a]

Variable	Financial Hardship Factor		Maternal Distress Factor	
	Factor Loading	Factor Parameter Estimate	Factor Loading	Factor Parameter Estimate
Family income in last 12 months	**.95***	.93**	.00	
Maternal education	**.75***	.82**	.13	
Maternal marital status	**1.04***	.89**	-.23	
Moves during child's life	**.59***	.61**	.04	
Maternal financial stress[b]	**.51***	.68**	.30*	
Maternal depressive symptoms[c]	.08		**.86***	.90**
Maternal anxiety[d]	.00		**.89***	.89**
Maternal parenting and life stress[e]	-.20*		**.74***	.62**

[a]Two-factor solution selected using information criteria, factor loadings and residual variances of solutions with one to four factors. Akaike and Bayesian Information Criteria and sample-size adjusted Bayesian Information Criteria: 1 factor= 2450.34, 2532.36, 2440.65; 2 factors= 2306.05, 2407.87, 2294.03; 3 factors= 2303.65, 2422.44, 2289.63; 4 factors= 2311.55, 2444.49, 2295.86. The two-factor solution had low residual variances (residual variance ranged from 0.10- 0.62). The three-factor solution had low residual variances with the same loading pattern as the two-factor solution except with an additional third factor comprised of only one variable (moves during child's life).
[b]Measured with the Financial Stress Index. [c]Measured with Center for Epidemiologic Studies Depression Scale. [d]Measured with Speilberger State-Trait Anxiety Inventory-Form Y2. [e]Measured with Parenting Stress Index-Short Form.
Bolded factor loadings indicate factor structure used in to generate factor scores. Factor loadings are rotated and parameter estimates are standardized. *$p < .05$, **$p < .001$.

Multilevel Mixed Models: The next step was to examine the effect of cytokine concentrations, maternal distress, and cytokine by maternal distress interactions on cortisol values. Cortisol was

modeled as an outcome using multilevel mixed models with a random intercept and a random slope to account for repeated sampling within individuals and varying cortisol trajectories across the visit between individuals. Robust variance and an independent covariance and residual errors structure were specified. Residual variance was modeled separately for each saliva sample to allow for unequal variance across samples.

First, bivariate analyses explored relations between cortisol and each independent variable. Next, four separate fully-adjusted models for cortisol were performed, each with only one cytokine predictor. The independent variables in fully-adjusted models included: the cytokine of interest, maternal distress score, cytokine by maternal distress interaction, all key covariates, and the financial hardship score. Fully-adjusted models also included a time since waking by saliva sample interaction term based on literature showing blunted cortisol responses to acute stress in the morning vs. afternoon (30). Sex differences in cytokine-cortisol relations in these data were reported previously (12), so a sex by cytokine interaction term was also included in fully-adjusted models. The main independent variables of interest in fully-adjusted models were the cytokine of interest, maternal distress score, and cytokine by maternal distress interaction. Four parsimonious final models were created by subjecting fully-adjusted models to backwards stepwise selection of covariates using a $p < .05$ retention criterion. The main independent variables were retained for final models and not subjected to the backwards stepwise selection. Model fit was assessed for each final model using residual and added variable plots.

In addition to including financial hardship as a covariate, post-hoc models examined the specificity of the moderating effect of maternal distress on cytokine-cortisol relations by testing the significance of cytokine by financial hardship interaction terms in final models with maternal distress included as a covariate.

All analyses, except factor analyses, were conducted in Stata/SE 12.1 (StataCorp LP, College Station, TX). Factor analyses were conducted in MPlus Version 7.11 (Muthen and Muthen, Los Angeles, CA).

Missingness: There were no missing data for maternal education, marital status, or the number of moves. Family income data were missing or classified as "don't know" for 10% of the sample. Incomplete responses were imputed with the mean response for the sample group (e.g., fetal development follow-up or community participants). Item-level missingness for the financial stress index, CES-D, STAI and PSI ranged from 10-18%, but only six participants were missing more than one item per instrument. Thus, item-level missingness was imputed with the mean of the completed items in the scale (or subscale for PSI).

There were low rates of missingness for most demographic, health and timing data (0-<3% for all variables except child age). Parent-reported child age in months was missing for 9% of the sample (although all children were five-years old). Missing demographic, health, and timing data were obtained using evaluator's notes (14%) or imputed with mean values (86%).

Sensitivity Analyses: Sensitivity analyses performed on complete case data assessed the impact of missingness imputations. Also, the impact of substitution methods for cortisol non-detects was examined by testing an alternate substitution and using random effects Tobit models. Results from these sensitivity analyses (estimates, *p*-values) did not significantly differ from the reported results.

6.4. Results

Data were collected from 151 mother-child pairs. Three children were unable to complete the appointment, and 23 children had insufficient salivary data. Analyses were performed on the remaining 125 pairs. Sociodemographic and health measures did not differ between children with and without full salivary data. Approximately half the children in the

sample were female, and slightly more than a third were overweight (Table 6.3). On average, children were awake for seven hours when the study appointment began (Table 6.3). A wide range of SES families were represented in the sample; annual incomes ranged from less than $5000 to greater than $100,000 (Table 6.1). There was also a wide range of maternal psychological distress in the sample (assessed using the CES-D, STAI and PSI; Table 6.1).

Table 6.3. Demographic and health characteristics (n=125)[a]

	Frequency	Percent
Child age, mean years (SD)	5.45 (0.29)	
Female	64	51%
Current illness	24	19%
Current periodontal/dental issues	14	11%
Mother smokes	28	22%
Child overweight or obese	44	35%
Time from waking to study appointment, mean hours (SD)	7.05 (2.41)	

SD=Standard deviation. [a]Missing data were <3% for all variables except child age. Although all children were five years old, age in months was missing for 9% of the sample. Missing data were imputed with mean values.

Multilevel Mixed Models for Cortisol

Backward stepwise selection of covariates: Seven covariates were retained in each of the four final models (sample, child sex, sex by cytokine, current illness, financial hardship, time since waking, time since waking by sample). In addition to these covariates, child BMI category was significantly associated with cortisol in models with TNFα as the main cytokine predictor. Thus, BMI was included in this final model.

Final model results for covariates: Results from the final models are displayed in Table 6.4. Cortisol decreased across the study visit in all models, and as time since waking increased, the decline in cortisol across the visit became steeper. Cortisol was higher in sick children compared to healthy children. Also, there were significant inverse relations between family financial hardship and cortisol indicating that as financial hardship increased, cortisol levels decreased. In the model with TNFα as a main predictor, overweight and obese children had lower cortisol levels than underweight children and children of a healthy weight. There were no sex differences in cortisol at mean cytokine levels in any model, however, there was a significant interaction

between sex and cytokine in every model indicating stronger inverse relations between cytokine and cortisol concentrations in boys compared to girls.

Neuroendocrine-immune relations and moderation by maternal distress: IL-6 and IL-8 were inversely associated with cortisol (Table 6.4). While there was no main effect of maternal distress on cortisol in any model, there were significant interactions between cytokines and maternal distress for IL-1β, IL-6 and TNFα (Table 6.4). Inverse relations between cortisol and IL-1β, IL-6 and TNFα became less strong with increasing maternal distress (Figure 6.1).

Post-hoc analyses tested the specificity of cytokine by maternal distress interaction effects on cortisol by examining the significance of cytokine by financial hardship interaction terms in final models with maternal distress included as a covariate. The cytokine by financial hardship interaction was not significantly associated with cortisol for IL-6, IL-8 and TNFα. The interaction between IL-1β and financial hardship was significantly positively associated with cortisol (β=0.03, z=2.42, p< .01) indicating that the inverse relation between IL-1β and cortisol became less strong as financial hardship increased, similar to the relation for maternal distress.

The role of child sex: There were significant sex by cytokine interactions in every model indicating stronger inverse cytokine-cortisol relations in boys than girls (Table 6.4). To explore sex-specific relations, stratified analyses for each final model were performed using a homogeneous independent residual structure and regular (not robust) variance given the smaller sample sizes.

Cytokine-cortisol relations varied by sex with significant inverse relations between cortisol and all cytokines among boys, but not girls (Table 6.5). Among girls, IL-1β, IL-6 and IL-8 were positively related to cortisol, although these relations were only marginally significant (Table 6.6). Similarly, cytokine by maternal distress interactions varied by sex for IL-1β, IL-6 and TNFα with significant positive interactions among girls, but not boys (although t-tests of

Table 6.4. Adjusted relations between cortisol, cytokines, and maternal distress among five-year old children using multilevel mixed models (n=125)

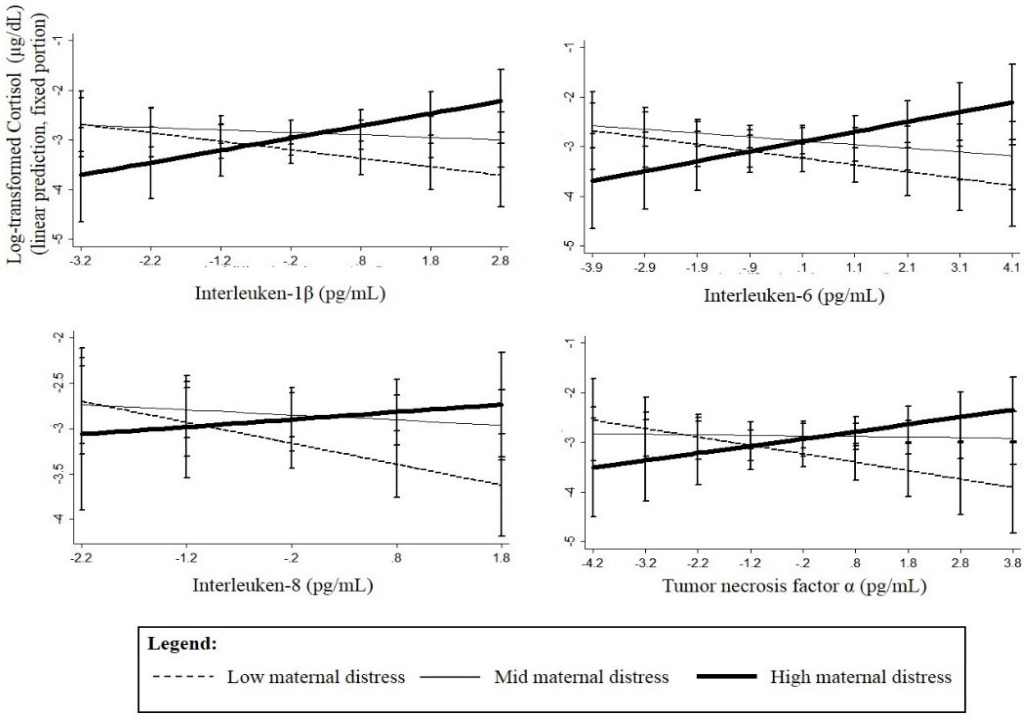
	Cytokine Main Independent Variable: IL-1β			**Cytokine Main Independent Variable: IL-6**			**Cytokine Main Independent Variable: IL-8**			**Cytokine Main Independent Variable: TNFα**		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	-3.00	0.13	<.001	-3.00	0.13	<.001	-2.97	0.13	<.001	-2.88	0.13	<.001
Main Independent Variables:												
Cytokine concentration	-0.13	0.10	.19	-0.15	0.07	<.05	-0.27	0.12	<.05	-0.11	0.09	.22
Maternal distress	0.04	0.13	.76	0.04	0.13	.75	0.07	0.13	.57	0.07	0.13	.59
Cytokine*maternal distress	0.21	0.05	<.001	0.14	0.06	<.05	0.13	0.08	.09	0.13	0.05	<.05
Covariates:												
Sample	-0.10	0.03	<.01	-0.12	0.02	<.001	-0.13	0.03	<.001	-0.11	0.03	<.001
Child sex (female)	-0.09	0.15	.56	-0.06	0.15	.69	-0.08	0.15	.58	-0.07	0.14	.64
Child sex*cytokine	0.27	0.12	<.05	0.28	0.11	<.05	0.40	0.15	<.01	0.20	0.10	<.05
Child overweight or obese[a]	-	-	-	-	-	-	-	-	-	-0.38	0.18	<.05
Child currently sick	0.46	0.16	<.01	0.44	0.15	<.01	0.47	0.16	<.01	0.51	0.16	<.01
Family financial hardship	-0.08	0.02	<.01	-0.08	0.02	<.01	-0.08	0.02	<.001	-0.07	0.02	<.01
Time since waking	-0.05	0.03	.12	-0.03	0.03	.30	-0.05	0.03	.11	-0.03	0.03	.29
Time since waking*sample	-0.02	0.01	<.05	-0.02	0.01	<.01	-0.02	0.01	<.01	-0.02	0.01	<.05
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Variance(intercept)	0.60	0.10	0.44-0.82	0.60	0.10	0.43-0.84	0.59	0.10	0.43-0.81	0.58	0.09	0.42-0.78
Variance(sample)	0.02	0.02	0.01-0.09	0.02	0.02	0.00-0.10	0.02	0.02	0.01-0.09	0.02	0.02	0.01-0.09
Residual variance												
Sample 1	0.16	0.07	0.07-0.36	0.17	0.07	0.08-0.36	0.17	0.07	0.07-0.38	0.16	0.07	0.07-0.36
Sample 2	0.13	0.05	0.06-0.28	0.13	0.05	0.06-0.29	0.14	0.06	0.06-0.31	0.14	0.05	0.06-0.30
Sample 3	0.28	0.10	0.13-0.58	0.26	0.10	0.13-0.54	0.27	0.09	0.14-0.54	0.28	0.10	0.14-0.55
Sample 4	0.43	0.12	0.25-0.75	0.42	0.13	0.23-0.76	0.41	0.12	0.24-0.72	0.42	0.12	0.23-0.75

SE= robust standard error; CI= confidence interval.

[a]Child body mass index category was only significantly associated with cortisol in models with TNFα, so body mass index category was only retained in the final model with TNFα as the cytokine main independent variable.

model coefficients revealed no significant differences between the interaction effects for boys and girls; Table 6.6). Among girls, as maternal distress increased, the positive cytokine-cortisol relations became stronger, mirroring the relations in the full sample. Again, the specificity of cytokine by maternal distress interactions were tested in boys and girls separately in post-hoc analyses. No significant cytokine by financial hardship interactions were found among boys. Among girls, the only significant cytokine by financial hardship interaction was seen for IL-1β (β=0.03, z=2.43, p< .05) and the direction of this effect was the same as that observed in the full sample.

Figure 6.1. Interaction between salivary cytokines and maternal distress on estimated mean log-transformed salivary cortisol in five-year old children (n=125)[a]



[a]Cortisol and cytokines are Winsorized and log-transformed. Cytokines are centered. Models were adjusted for significant child health and demographic factors and family socioeconomic status. Maternal distress categorized using tertiles.

Sensitivity analyses: Two sets of sensitivity analyses assessed the robustness of the cytokine by maternal distress interactions on cortisol. First, the maternal distress variable was categorized, and the significance of interactions between cytokines and a three-level maternal distress variable was assessed. Findings from these models were similar to those reported above (p-values and

estimates). Second, influential data points were excluded from the final models. In these models, the cytokine by maternal distress interaction findings were similar to those reported above for IL-1β, IL-6 and IL-8. However, the TNFα by maternal distress interaction was only marginally significant when influential data points were excluded from analyses of the full sample and of girls (in full sample: β=0.09, z=1.74, p< .08; in girls only: β=0.15, z=1.76, p< .08).

6.5. Discussion

Moderating Role of Maternal Distress on Neuroendocrine-immune Relations

Findings from this study provide insight into the sensitivity of child NEI relations to adversity. In the sample of young children from varied SES families, relations between inflammatory immune and HPA activity (measured by cortisol) in saliva varied by maternal distress. Regardless of SES, as maternal distress increased, inverse relations between cytokine and cortisol activity in children became weaker. Systemically, cortisol regulates inflammation by inhibiting the activity of inflammatory cytokines (31). This regulatory mechanism is important for preventing excessive and potentially dangerous inflammation (31,32). The findings suggest that maternal distress is associated with less efficient regulation of inflammatory mechanisms in children. These findings are consistent with the notion that adversity can result in a "defensive phenotype" characterized by an over-active inflammatory response (7,33). While potentially adaptive in the short-term, prolonged up-regulation of inflammatory mechanisms damages cells and increases disease risk (32).

Interestingly, the significant interactions between cytokines and maternal distress were driven by significant interactions among girls. While cytokine by maternal distress interactions observed among boys were in the same direction as those in girls and there were no significant differences in interaction effects by sex, all interaction terms were non-significant among boys. These sex differences may be related to sex differences in cytokine-cortisol relations. As previously reported (12), there were inverse relations between cortisol and every cytokine for

Table 6.5. Adjusted relations between cortisol, cytokines, and maternal distress among five-year old boys using multilevel mixed models (n=61)

	Cytokine Main Independent Variable: IL-1β			**Cytokine Main Independent Variable: IL-6**			**Cytokine Main Independent Variable: IL-8**			**Cytokine Main Independent Variable: TNFα**		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	-2.97	0.13	<.001	-2.99	0.13	<.001	-2.96	0.13	<.001	-2.93	0.16	<.001
Main Independent Variables:												
Cytokine concentration	-0.23	0.08	<.01	-0.21	0.05	<.001	-0.34	0.08	<.001	-0.18	0.06	<.01
Maternal distress	0.28	0.18	0.11	0.27	0.18	.13	0.29	0.17	.09	0.32	0.17	.07
Cytokine*maternal distress	0.11	0.09	0.22	0.11	0.07	.11	0.11	0.10	.25	0.08	0.07	.29
Covariates:												
Sample	-0.15	0.04	<.001	-0.12	0.04	<.01	-0.17	0.04	<.001	-0.13	0.04	<.001
Child overweight or obese[a]	-	-	-	-	-	-	-	-	-	-0.22	0.26	.41
Child currently sick	0.39	0.28	0.18	0.43	0.28	.13	0.46	0.28	.10	0.53	0.28	.06
Family financial hardship	-0.08	0.04	<.05	-0.07	0.04	.06	-0.09	0.04	<.05	-0.08	0.04	<.05
Time since waking	-0.07	0.05	0.16	-0.04	0.05	.44	-0.07	0.05	.14	-0.06	0.05	.25
Time since waking *sample	-0.03	0.01	<.05	-0.03	0.01	<.05	-0.03	0.01	<.05	-0.03	0.01	<.05
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Variance(intercept)	0.76	0.15	0.52-1.11	0.77	0.15	0.52-1.13	0.73	0.14	0.50-1.07	0.74	0.15	0.50-1.09
Variance(sample)	0.03	0.01	0.01-0.08	0.04	0.01	0.02-0.08	0.03	0.01	0.01-0.07	0.04	0.01	0.02-0.08
Residual variance	0.22	0.03	0.17-0.28	0.20	0.03	0.16-0.26	0.21	0.03	0.16-0.27	0.21	0.03	0.16-0.27

SE= standard error; CI= confidence interval.

[a]Child body mass index category only retained in the final model with TNFα as the cytokine main independent variable.

Table 6.6. Adjusted relations between cortisol, cytokines, and maternal distress among five-year old girls using multilevel mixed models (n=64)

	Cytokine Main Independent Variable: IL-1β			**Cytokine Main Independent Variable: IL-6**			**Cytokine Main Independent Variable: IL-8**			**Cytokine Main Independent Variable: TNFα**		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	-3.09	0.09	<.001	-3.06	0.09	<.001	-3.04	0.10	<.001	-2.90	0.11	<.001
Main Independent Variables:												
Cytokine concentration	0.11	0.06	.07	0.09	0.05	.06	0.12	0.07	.07	0.06	0.05	.25
Maternal distress	-0.21	0.12	.08	-0.21	0.13	.10	-0.15	0.13	.26	-0.19	0.13	.13
Cytokine*maternal distress	0.27	0.07	<.001	0.20	0.07	<.01	0.12	0.09	.20	0.18	0.07	<.01
Covariates:												
Sample	-0.07	0.04	.06	-0.11	0.04	<.01	-0.10	0.04	<.01	-0.10	0.04	<.01
Child overweight or obese[a]	-	-	-	-	-	-	-	-	-	-0.49	0.19	<.05
Child currently sick	0.45	0.21	<.05	0.41	0.22	.07	0.42	0.23	.06	0.41	0.22	.06
Family financial hardship	-0.07	0.02	<.01	-0.07	0.02	<.01	-0.07	0.03	<.05	-0.05	0.02	<.05
Time since waking	-0.02	0.04	.58	-0.01	0.04	.86	-0.02	0.04	.59	0.00	0.04	.90
Time since waking*sample	-0.02	0.01	.14	-0.03	0.01	.08	-0.02	0.02	.10	-0.02	0.02	.16
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Variance (intercept)	0.35	0.07	0.23-0.52	0.37	0.08	0.24-0.56	0.39	0.08	0.26-0.59	0.35	0.07	0.23-0.53
Variance (sample)	0.03	0.10	0.10-0.08	0.03	0.01	0.01-0.08	0.03	0.02	0.01-0.08	0.03	0.02	0.01-0.08
Residual variance	0.24	0.03	0.19-0.31	0.25	0.03	0.20-0.32	0.25	0.03	0.20-0.32	0.25	0.03	0.20-0.32

SE= standard error; CI= confidence interval.

[a]Child body mass index category only retained in the final model with TNFα as the cytokine main independent variable.

boys but not girls. Among girls, cytokines were positively associated with cortisol (although relations were non-significant). Similar sex differences in serum cytokine-cortisol relations have been reported in adults (34–37). These studies suggest that inflammatory cytokines in males show increased sensitivity to the inhibitory effects of cortisol after acute stress, while the same cytokines in females show no change or decreased sensitivity (35–37). These differences may make females more vulnerable to the moderating effect of maternal distress on NEI regulation. If cytokines are predisposed to exhibit decreased sensitivity to cortisol, the desensitization of inflammatory mechanisms associated with adversity may be magnified or observed at lower levels of adversity or earlier in the lifecourse among females.

The cytokine by maternal distress interaction was significant for every cytokine except IL-8. Previous investigations of these same data found that, of all four cytokines, IL-8 was present at the highest concentrations and was the only cytokine not associated with any demographic or health factor (12). Further investigation is needed to fully understand salivary IL-8 and its sensitivity to demographic, health and psychosocial health factors. Future studies should also examine the robustness of the TNFα-maternal distress interaction as this relation was only marginally significant when influential cases were excluded.

Cortisol Relations with Covariates

Results from this study are also consistent with previous findings regarding salivary cortisol activity in young children. The observed decline in cortisol across the study visit and the impact of time since waking on this decline are consistent with the known diurnal pattern of salivary cortisol (38). Also, the increased cortisol levels found among children with a cold/fever on the day of the visit reflect the body's response to infection or injury which is typically characterized by elevations in cortisol (39). Finally, overweight and obese children had lower cortisol than underweight children and those of a healthy weight. The relation between cortisol and BMI in children is unclear with studies showing both increases and decreases in cortisol after

a weight gain (40,41). In the current study, overweight and obese children were more likely to be from lower SES families with more maternal distress. The observed relation between cortisol and BMI may have been confounded by family SES and maternal distress. Furthermore, cortisol and BMI were only significantly related in models that included TNFα, and stratified analyses revealed significant relations only among girls. Further research is needed to fully understand the relation between BMI and NEI network activity, as well as the influence of SES, early-life adversity and sex on these relations.

Family financial hardship was inversely associated with cortisol, indicating that as financial hardship increased cortisol levels decreased. These results are consistent with studies showing blunted physiologic stress responses among low-SES children (42,43). SES-related differences in cortisol likely reflect a combination of psychosocial and environmental effects. While the models accounted for the distinct effects of maternal distress, other unmeasured SES-related factors likely contributed to the financial hardship relations observed in this study.

Importantly, the moderating effect of maternal distress on cytokine-cortisol relations was significant after adjusting for financial hardship. Also, financial hardship did not moderate NEI relations for IL-6, IL-8 or TNFα. The only cytokine-cortisol relation moderated by financial hardship was the IL-1β-cortisol relation, and this interaction was in the same direction as cytokine-maternal distress interactions. Additional research is needed to fully understand the role of financial, psychosocial, and environmental factors in modifying NEI relations in children. However, these results suggest that maternal psychological well-being plays a unique role in moderating child NEI relations and these effects cannot be explained by SES.

There are several strengths to the study. First, the sample size is large and includes children with diverse SES backgrounds. Also, several aspects of SES were measured and included as covariates. The discrete effect of maternal psychological well-being was examined using validated instruments. The findings are further strengthened by repeated within-subject

sampling and tightly controlled laboratory procedures. Finally, the use of salivary biomeasures represents a novel approach to studying the effects of adversity which allowed for the examination of several measures of NEI activity. By examining immune system and HPA activity simultaneously, the findings provide a new, more complex understanding of the physiologic effects of adversity. This study demonstrates the potential of using minimally-invasive methods to study, detect, and treat the physiologic effects of early-life adversity.

Despite the strengths, limitations regarding the study design and sample warrant discussion. While the acute stress response was not the focus of this investigation, the findings likely reflect acute stress-related activity within the NEI network. Stress-related change in cortisol concentrations across the study visit were examined in a previous study (12). The acute stress response of cortisol varied across children with about 44% of children showing an increase and 40% showing a decrease in cortisol after the stressor tasks (12). These changes in post-stressor cortisol, however, did not significantly impact the linearity of cortisol across the visit, and preliminary analyses assessing the need for non-linear modeling of cortisol were not significant. The linear trajectory of cortisol observed across the study visit may be partially due to the differential timing of stressor tasks and saliva samples in the study protocol. While the order of study tasks was the same for each participant, the timing of saliva samples relative to the stressor tasks depended on the speed at which the child completed the tasks. Therefore, task-related changes in analytes are considered cumulative responses to all tasks. Variation in cortisol trajectories after an acute stressor is typical for young children (44), and, in the current study, a random slope was included in statistical models to account for varying cortisol trajectories across the study visit. The effect of task timing on cortisol was also examined and there were non-significant relations in every model.

The study sample included a wide range of SES families. However, income and maternal education were not evenly distributed. There were large percentages of low- and high-income

families and few middle-income families. Studies with equal representation across socioeconomic strata are needed to fully understand the effects of SES on NEI relations among children. Also, a maternal distress factor score was used to examine the combined effect of various dimensions of functioning without overloading models with covariates. This approach limits the clinical significance of the findings as accepted cut-offs for individual instruments are not distinguished. Finally, longitudinal studies are needed to clarify the direction of the observed relations and the meaning of altered NEI relations in saliva for health and disease risk. Additional research of salivary cytokines, including serum-saliva studies, is also needed to increase our understanding of the meaning and interpretation of salivary cytokines in healthy children.

6.6. Conclusion

This study advances understanding of potential mechanisms linking early-life adversity and later life disease, as well as aids in the identification of adversity risk factors. While prior studies reported significant relations between maternal mental health and altered HPA activity in children (22,45,46), this is the first study examining the moderating effect of maternal distress on cross-system NEI relations. The findings suggest that maternal distress can impact child NEI functioning by altering the sensitivity of inflammatory immune processes to the inhibitory effects of cortisol. This desensitization may increase risk for chronic inflammation and disease (1).

The findings support the use of maternal mental health screenings for identifying children at risk of toxic stress, regardless of socioeconomic status. Pediatricians play an increasing role in identifying, preventing, and treating toxic stress in children (5,47), and maternal depression screening programs have been successfully integrated into some pediatric care sites (48). The findings support these and other efforts that facilitate the early detection and treatment of at-risk mothers to protect maternal as well as child health and well-being.

6.7. References

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CHAPTER SEVEN

Conclusions and Implications of the Study

7.1. Conclusions

The key findings from this dissertation suggest that salivary biomeasures of the immune system and CNS can be used to study neuroendocrine-immune (NEI) activity and sensitivity to environmental factors in children. Furthering this line of research, the findings demonstrate the role of mothers in child NEI functioning. Changes in NEI relations associated with maternal distress were suggestive of a poorly regulated inflammatory response. These disruptions in NEI regulation may increase the child's risk of developing inflammatory diseases. The studies included in this dissertation are the first to use salivary data to examine cross-system NEI functioning in children. The findings offer new opportunities for advancing the study of stress, adversity, health, and development in public health research.

The dissertation's three study aims laid the foundation for these main findings. This chapter reviews the findings associated with each study aim. The results are discussed in the context of the existing literature, and important strengths and limitations of each study are presented. The implications of the study findings for public health research, programs, and policies are presented in the final section of the chapter.

Study Aim 1 Conclusions and Discussion

Study aim 1 explored current salivary analyte data analysis methods and the implications of these methods on data quality and the validity and reliability of study findings. This aim focused on pre-analytic data processing as a neglected phase of data analysis that has significant consequences for data quality. The review revealed that many practices commonly employed by salivary bioscience investigators are not endorsed by experts who encounter similar issues in

other fields (e.g., with serum data analysis in environmental and occupational health). The review also identified areas of data processing and analysis for which clear standard protocols are needed. Advantages and disadvantages of a variety of pre-analytic data processing techniques were discussed, and the impact of these techniques on parameter estimates was illustrated using sample data. The review called for standardization and disclosure of pre-analytic data processing procedures in all published manuscripts. By identifying and addressing the limitations of current salivary analyte analysis methods, this review helps advance the field of salivary bioscience and maximize the potential of salivary analyte data in public health research.

Study Aim 2 Conclusions and Discussion

Study aim 2 examined the nature and correlates of salivary cytokines in five-year old children. It examined cytokine intercorrelations, and relations with demographic and health characteristics, as well as relations with autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) axis activity. The findings revealed new information about the measurement and characteristics of salivary cytokines. Cytokines were reliably measured in the saliva of young children. High inter-cytokine correlations were found at each saliva sampling point. These findings are consistent with studies of salivary cytokines in older participants and suggest highly coordinated inflammatory immune processes within saliva (1,2).

Analyses assessing associations between salivary cytokines and health and demographic factors revealed positive associations between cytokines and poor oral health factors (periodontal issues, maternal smoking) and no overall association between cytokines and systemic health and demographic factors. While the sample size is large for a biomeasure study of children, there were few children with fair/poor health. The heterogeneity and size of the sample may not have been adequate to find differences in cytokines levels by recent health. The results, however, are consistent with studies of adults and adolescents that link elevated levels of salivary markers of inflammation with gingivitis, periodontal disease, dental carries and second-hand smoke (3–10).

While additional research is needed (e.g., comparing cytokine levels in serum and saliva), these results suggest that immune system activity is compartmentalized and salivary cytokines may reflect local oral, rather than systemic health. The results also confirmed previous findings of a diurnal pattern in interleukin-6 (IL-6; 11) and found evidence of age-related changes in IL-6.

Changes in salivary cytokine concentrations associated with acute stress were examined in this study without a priori hypotheses regarding the nature and magnitude of stress-related change. Interleukin-1 beta (IL-1β) and interleukin-8 (IL-8) showed stress-related declines in concentrations with a time lag similar to cortisol. Tumor necrosis factor alpha (TNFα) showed no overall change in slope after the emotional stressor tasks and IL-6 showed a steady declining (but non-significant) trajectory across the entire study visit.

Interpreting the meaning of changes in analytes across the study visit is complicated by the order and timing of the stressor tasks used in the study and the limited information available about salivary cytokine task-sensitivity and associations with other unmeasured salivary analytes released in response to the study tasks. Additional research on salivary cytokine sensitivity to tasks, stress, and other salivary proteins (12), and the time course of these changes is needed to fully understand the nature of salivary cytokine environmental sensitivity. Furthermore, cytokine trajectories varied by sex; compared to boys, girls exhibited flatter cytokine trajectories across the visit. These differences may be due to sex differences in immune responses or in cytokine-cortisol and cytokine-alpha-amylase relations.

Among boys, cytokines were significantly positively related to alpha-amylase (sAA) and inversely related to cortisol. These relations were all not significant among girls. These findings are consistent with adult studies using serum biomeasures of immune and HPA activity (13–16) and they extend these findings to include immune-ANS relations. The sex differences observed in this study suggest that sexual differentiation of NEI relations begins before puberty (e.g., through intrauterine sex hormones and genetic factors) and that these differences are measurable in saliva.

The results from this study provide unique information regarding the concentrations and correlates of salivary cytokines in young children, and the interplay of these immunologic analytes with markers of ANS and HPA activity across a series of stressor tasks. The findings demonstrate the feasibility and reliability of collecting and measuring salivary cytokine data in a young population. Furthermore, they demonstrate the potential of using salivary biomeasures to study NEI functioning and the mechanisms linking brain, immunity and behavior during development.

Study Aim 3 Conclusions and Discussion

Building on the results of study aim 2, study aim 3 examined the role of early-life adversity in moderating the relation between HPA and immune system activity in the oral cavity in five-year old children. Results from this study revealed significant interactions between maternal distress and salivary cytokines on cortisol indicating that, regardless of family socioeconomic status (SES), as maternal distress increased, the expected inverse relation between cytokines and cortisol became weaker. Systemically, cortisol plays an essential role in reducing inflammation and preventing excessive and dangerously prolonged inflammatory processes (17). The observed effects suggest that poor maternal mental and psychosocial health may be associated with less efficient regulation of child inflammatory processes by the inhibitory effects of cortisol, which may place the child at risk of developing inflammatory diseases. These findings are consistent with the notion that early-life adversity can result in a "defensive phonotype" (18,19) characterized by a hyperactive inflammatory response.

Sex-stratified analyses revealed significant maternal distress-cytokine interactions within the sample were driven by relations among girls. While no significant maternal distress-cytokine interactions were observed among boys, the interactions were in the same direction as those observed among girls and there were no significant sex differences in the interaction effects. These results may be related to sex-specific differences in cytokine-cortisol relations; as

demonstrated in study aim 2, boys exhibited inverse cytokine-cortisol relations for all cytokines, while these relations among girls were generally positive. These differences may make females more vulnerable to the moderating effect of maternal distress on NEI relations. If cytokines are predisposed to exhibit decreased sensitivity to regulatory signals from cortisol, the desensitization of inflammatory mechanisms associated with early-life adversity may be magnified and observed at lower levels of adversity or earlier in the life course among females.

The cytokine by maternal distress interaction was significant for every cytokine except IL-8. Of all four cytokines, IL-8 was present at the highest concentrations and was the only cytokine not associated with any demographic or health factor. Further investigation is needed to fully understand salivary IL-8 and its sensitivity to demographic, health and psychosocial health factors. Future studies should also examine the robustness of the TNFα by maternal distress interaction as this relation was only marginally significant when influential cases were excluded.

Importantly, maternal distress by cytokine interactions were significant after adjusting for family SES. Consistent with prior studies examining the effect of SES on the acute stress response in children, family SES was inversely associated cortisol in this study (20,21). However, unlike maternal distress, family SES did not interact with IL-6 or TNFα to affect cortisol. The only exception was the significant interaction observed between IL-1β and family SES. This interaction was in the same direction as the maternal distress-cytokine interactions. While, additional research on the effects environmental, financial, and psychosocial factors on NEI functioning in young children is needed, these results suggest maternal mental and psychosocial health may play a unique and important role in moderating child NEI relations and that these effects cannot be explained by SES.

Many strengths of the study sample and design make this investigation a valuable contribution to the literature. The study used a large sample of children from a wide range of SES backgrounds. Unlike earlier studies (22), this study used several markers of family SES and

maternal mental and psychosocial health. Maternal distress was indexed using three validated psychological instruments. This multidimensional measurement of SES and adversity allowed for the examination of the distinct effects of SES and maternal distress on child physiologic functioning, within the constraints imposed by the measures. The use of salivary biomeasures is also an important strength of the study; it allowed for the simultaneous examination of activity within the immune system and HPA axis and provided a new and more complex understanding of the physiologic effects of early-life adversity.

Despite these strengths, limitations regarding the study protocol and sample warrant discussion. While the acute stress response was not the focus of this investigation, the NEI activity examined likely reflects stress-related changes in cytokine and cortisol concentrations. Preliminary analyses assessing the need for non-linear modeling of cortisol were all not significant, perhaps due to the variable time from stressor tasks to saliva samples. The task timing variable was included in saturated models, but no significant relations were found between task timing and cortisol. Therefore, task timing was not included in final models and cortisol was modeled linearly with a random slope to account for varying cortisol trajectories across the study visit. Future studies with more precise timing between stressor tasks and saliva samples should examine the nature of NEI relations at baseline and post-stressor in children. These studies will help clarify the sensitivity of NEI relations in saliva to environmental factors.

There were also limitations regarding the study sample. While a wide range of SES families were represented, family income and maternal education were not evenly distributed throughout the sample. There was a large percentage of low- and high-income families and few middle-income families. Future studies with a more equal representation across socioeconomic strata are needed to understand the effects of SES on NEI relations among children. Similarly, scores on the financial stress index and Center for Epidemiologic Studies Depression Scale (CES-D) were positively skewed, and robust estimation procedures were employed to handle the non-

normal distributions. Maternal distress was indexed by a factor score representing depressive symptoms, anxiety, and parenting stress, allowing for the examination of multidimensional maternal functioning without overloading models with covariates. This approach, however, limits the clinical significance of the findings as accepted cut-offs for individual instruments are not distinguished. Practically, maternal distress factors are typically correlated and the current findings demonstrate the importance of maternal mental and psychosocial functioning at all levels. Finally, longitudinal studies are needed to clarify the direction of the observed relations and the meaning of altered NEI relations in saliva for health and disease risk, and additional research of salivary cytokines is needed to increase our understanding of the meaning and interpretation of salivary cytokines in healthy children.

The study findings address two important gaps in the early-life adversity literature. First, they illuminate a potential link between early-life adversity and disease. Second, by demonstrating the effect caregivers may have on child inflammatory regulation, this study aids in the identification of early-life adversity risk factors and supports efforts to increase early detection and treatment of at-risk mothers to protect maternal as well as child health and well-being.

7.2. Implications for Public Health Research

The findings from this dissertation have clear implications for public health research. In particular, they present opportunities for advancing salivary analyte research and the study of SES and health.

Salivary Analyte Research

This study advances salivary bioscience for public health researchers in two ways. First, the methodological review in study aim 1 provides a set of best practices for future salivary analyte researchers. The review also calls for the standardization and disclosure of analysis

methods to facilitate across-study comparisons and meta-analyses. While current best practices are presented, further research is needed to establish comprehensive data analysis protocols, accepted methodology, and reporting requirements. Public health researchers are well-suited to lead the way in advancing salivary biomeasure analysis methods; collaborations across a range of health sciences researchers (e.g., epidemiology, occupational and environmental health sciences, and biostatistics) are needed to develop universal analytic standards. Study aim 1 begins this effort by calling attention to inconsistencies in analytic strategies and the impact these differences may have on study findings.

In addition to advancing salivary bioscience methods, this dissertation includes the first systematic examination of new salivary immune markers (cytokines) in young children and demonstrates the utility of these markers in studying environmental impacts on health. The results lay the foundation for future studies investigating the utility of salivary cytokines as measures of oral disease, and indicators of exposure to toxins and acute stress. Future research should continue to examine relations between salivary cytokines and biomarkers from other physiologic systems (e.g., HPA, ANS, metabolic system) and the meaning of these relations for overall health. Furthermore, research comparing serum and salivary cytokines, and studies conducted with unhealthy participants will help clarify the meaning of immune activity in the oral cavity. While additional research is needed, the finding that interactions between immune, ANS, and HPA activity in saliva mirrors those found in serum, presents exciting opportunities for studying NEI functioning and the biologic impact of adversity in children and on a large scale. Future studies investigating these biomarkers, and others, will advance the use of biologic data for identifying and treating early-life adversity in children.

Population-level biomarker data play an increasingly important role in policy-making; biologic data can help garner public and political support and can be used to objectively reframe social problems and identify effective policy and program solutions. Just as brain science was

used to reframe mental health and substance abuse issues to allow for passage of the Mental Health Parity Act, findings from biomarker studies could help reframe issues regarding the effect of early-life adversity on child health and development. By expanding knowledge of the physiologic effects of early-life adversity and helping identify specific mechanisms by which adversity affects health, biomarker research can be used to increase support for and innovation in early childhood programs. Salivary biomarkers provide a minimally-invasive, low cost, and socially-acceptable method for obtaining biologic data from populations of all ages on a large-scale. By helping advance salivary biomarker research toward the population-level study of environmental impacts on health, the dissertation helps further the use of biologic data in public health policy and practice.

Socioeconomic Status and Health Research

The findings from study aim 3 also have implications for the study of SES and health. The relation between low-SES and poor health is well established. However, many studies of this relation are limited by the confounding of psychosocial and financial aspects of SES, and by the examination of biomeasures from one physiologic system in isolation (22). Study aim 3 addressed these limitations. First, the findings showed the distinct contributions of financial and psychosocial factors on child physiologic functioning. While correlated, maternal distress and SES influenced HPA and immune system functioning in distinct ways. Future studies should continue to examine the separate effects of financial and psychosocial factors on health in order to gain a better understanding of the mechanisms underlying SES and health relations.

Second, findings from study aim 3 also demonstrate the potential of salivary biomarkers for expanding the study of SES and health to include biologic data from multiple physiologic systems. This shift towards a multisystem, integrative approach to studying health, development, and environmental impacts on physiology has been highlighted by clinical and public health professionals as an important step in advancing knowledge and promoting well-being (23,24).

The findings from study aim 3 illustrate the importance of examining cross-system functioning; while there were no main effects of maternal distress on cortisol, maternal distress played an important role in NEI network relations. Future research should examine biomarkers from multiple systems and the interactions between them to gain a more detailed understanding of physiologic functioning and the impact of environmental factors on health. Furthermore, longitudinal studies are needed to examine the directionality of the observed effects and the meaning and consequences of changes in NEI relations in saliva during childhood. Questions regarding associations between NEI regulation measured in saliva and current health and disease, as well as later-life disease risk are important for advancing the study of early-life adversity using salivary biomarkers. Finally, results from study aim 3, and others (25–30), suggest sex differences in biologic sensitivity to early-life adversity. This presents exciting opportunities for future studies examining these differences and the mechanisms underlying them.

7.3. Implications for Programs and Policies

The finding that maternal mental and psychosocial health moderates child NEI relations adds to a large body of research indicating the critical role of caregivers on child health (e.g., 30–35). The findings endorse the use of family and community-based programs and policies that support caregivers and promote child health and well-being. These approaches include: maternal-child interventions, maternal depression screening programs, integrated care, and financial and policy reforms.

Maternal-Child Interventions

Poor maternal mental health is associated with impaired parenting behaviors and disruptions in maternal-child relations (36). Rather than treating maternal health problems in isolation, comprehensive interventions for mother-child dyads that focus on improving maternal-child relations and increasing maternal capacity and social support have sustained impacts on maternal, as well child and family outcomes (32,37). Two popular early intervention programs

providing integrated services to mothers and their children include home visiting programs and other Early Head Start programs.

Home visiting: Home visiting programs serve pregnant and parenting mothers with young children (38). They are a growing component of early intervention plans for high-risk families with recent support stemming from dedicated funds provided by the Patient Protection and Affordable Care Act (PPACA) of 2010 (38). While program effects vary by approach and population, home visiting programs have been shown to increase family stability, positive parenting behaviors, and utilization of health and education services, as well as improve child behavior and cognitive outcomes, and reduce maternal stress, psychological problems, and depression (39–43). Programs successful in improving maternal mental health have adopted several different approaches, including mother-child relationship-based therapy (39), connecting families to community-based and medical services (39,40,42), cognitive behavioral therapy (36,44) and parent training (43).

In addition to connecting clients with mental health resources in the community, home visiting programs can embed mental health workers within the program to better identify and treat maternal mental health issues and improve the link between home visiting and clinical care (36). Specific mental health curricula and training for home visiting staff, as well as program monitoring and evaluation are essential to ensuring home visiting programs provide effective support for maternal mental health (45). Continued support of evidence-based programs for improving maternal mental health and reducing parenting stress is needed.

Early Head Start: Early Head Start (EHS) is a community-based program serving low-SES families with pregnant women, infants or toddlers (46). EHS provides home visiting, center-based, and mixed model (both home and center-based) services (46). EHS has been shown to decrease maternal depressive symptoms by promoting child cognitive and behavior development, improving parenting behaviors and reducing family conflict and parenting stress (47,48). Like

other home visiting programs, EHS programs can further target maternal mental health by embedding maternal depression strategies within the EHS framework. For example, Boston's EHS program implemented the Family Connections project to strengthen staff understanding and treatment capacity for maternal depression and anxiety (36). Also, EHS programs can include peer support groups, such as Sister Circles (36) and group-based parent training programs shown to be effective in reducing maternal depression, anxiety and stress (49).

Maternal Depression Screening Programs and Integrated Care

The programs discussed thus far serve mostly low-income, high-risk women and their families. While maternal depressive symptoms are higher in disadvantaged populations, rates among all pregnant and parenting women may reach up to 25% (36). Given the elevated risk of depression among mothers from all social strata, and the current finding that maternal distress impacts child NEI relations regardless of family SES, programs to identify and treat maternal mental health problems must extend beyond those implemented with high-risk families. Maternal depression screening programs embedded within pediatrician offices are one approach for monitoring and treating women across all socioeconomic strata. While these programs have been successful in identifying and referring mothers with depressive symptoms to specialized care (50,51), their ability to improve outcomes for mothers is unclear (52,53).

The American Academy of Pediatrics recommends screening for post-partum depression as part of standard well child care (54). However, recently, pediatricians have been called upon to increase their role in identifying and treating maternal mental health issues (55,56). Challenges to expanding pediatrician-based maternal mental health programs include, a lack of training among pediatricians in maternal mental health issues, limited knowledge of community resources, and reimbursement for parent services. To address pediatrician training, education programs can improve pediatricians' self-efficacy and knowledge about identifying and treating maternal depression (57).

Beyond building pediatrician capacity and increasing pediatrician screenings and referrals for maternal mental health issues, integrated models of physical and mental health care are an increasingly important and effective approach to treating mental health conditions (36,52,58). Funds allocated to the creation of patient-centered medical homes through the PPACA could be used to support the creation of these integrated healthcare sites for families throughout the US (59). Family medicine and patient-centered medical homes are two examples of integrated care, however the level of care and care coordination varies by site; one study found only 40% of patient-centered medical homes had behavioral health professionals onsite and only half used evidenced-based approaches for mental/behavioral health problems (60).

Better integration of physical and mental health care could be achieved by the adoption of the Collaborative Care (CC) model. This model is a cost-saving, evidenced-based approach to care integration that has demonstrated effectiveness in treating depression, improving physical and social functioning, and increasing quality of life (52,58). This approach uses primary care physicians, care management staff, and psychiatric consultants to provide mental/behavioral health screenings, brief interventions, mental health treatment and referrals, and to proactively track and monitor patients (52).

A key component of the CC model is the use of care management staff to facilitate communication among physicians, psychiatric consultants, and patients (52). The use of a care management team addresses several of the barriers typically faced when integrating physical and mental health care including, communication problems between physical and mental health providers, a shortage of mental health providers, and problems delegating new patient navigation and tracking responsibilities (61,62).

The PPACA expanded coverage of mental health treatment and mandates no-cost depression screening, however reimbursement for services from integrated care models and for multidimensional treatments (e.g., mother-child interventions) remains a significant barrier to

providing these services (52,59,60). Replacing fee-for-service with capitated or case-rate reimbursement approaches, as well as adding incentive-based payments, could aid in the adoption of integrated care models (52) and increase the provision of comprehensive mother-child services to families from all socioeconomic strata.

Financial and Policy Reforms

While the findings from this study suggest maternal mental and psychosocial health may have a unique influence on child health, there was also evidence of family financial stain influencing child HPA activity and, to a lesser extent, NEI relations. Given these findings and the interrelated nature of financial hardship and maternal distress, interventions that aim to increase family financial security warrant discussion. These programs and policies can alleviate caregiver stress and provide flexibly with time and energy which can improve maternal mental health and maternal-child relations.

Programs that provide direct financial support can promote maternal mental health. The Earned Income Tax Credit (EITC) provides payments to low-income working Americans. A recent study found that, in addition to increasing employment and social mobility, increases in EITC payments were associated with improved self-reported health, fewer poor mental health days, and decreases in concentrations of biomarkers of cardiovascular disease, obesity and inflammation among mothers with two or more children (63). Community outreach to increase EITC enrollment among low-income families could help maximize these benefits.

Temporary Assistance for Needy Families (TANF) is another source of financial support for low-income families. TANF provides block grants to states for the provision of cash assistance, child care support, and welfare programs (64). TANF cash assistance levels, however, are very low; benefit levels are not enough to bring families above half the Federal Poverty Line in any state (65). Increasing cash assistance levels and implementing TANF reforms, such as easing work requirements for parents of young children and increasing the time allowed in mental

health and substance abuse programs, could support maternal-child relations and maternal health in this vulnerable population (66). The impact of these reforms, however, may be hindered by the low benefit levels currently provided by TANF.

Beyond providing direct financial support, several programs and policies can be promoted to support family stability and maternal mental health. For example, food insecurity and child care are significant stressors that place mothers at risk of poor mental health (67,68). Continued support of the Women, Infants and Children and Supplemental Nutrition Assistance Programs, as well as child care subsidies could help alleviate these stressors and their toll on maternal mental health. Furthermore, efforts to increase the minimum wage and provide paid parental leave are important options for helping support family financial stability and ease parental stress.

Community-focused Approaches

While the focus of this dissertation was the role of mothers and families in child well-being, it is important to remember that families are embedded within communities. Building community capacity supports caregiver, child, and family health and could help increase the effectiveness of the individual and family-based intervention's discussed above (53,69). For example, safe, clean and accessible community spaces (e.g., parks, recreation/child care centers) can provide time for mother-child interactions, as well as increase opportunities for social interactions among mothers (69). Mothers with greater social support and strong social networks have lower rates of mental health problems, including postpartum depression (70,71).

Supportive community networks can also increase the impact of health messaging campaigns and increase maternal knowledge of community resources and health behaviors (53). In addition, safe and well-cared for neighborhoods are associated with better mental health and these community-level factors can support the effectiveness of maternal mental/behavioral health treatments and increase the odds of recovery (71–73). While the home visiting and clinical

approaches discussed above can provide social support and help link mothers with community resources, efforts to promote communitywide social cohesion and support systems for mothers can augment these individual/family-based approaches and help lay the foundation for maternal and child well-being (53,69).

7.4. Concluding Remarks

Nearly half of all American children live in low-income families, and one of every five children live in families with incomes below the Federal Poverty Line (74). While children growing up in low-SES families face several challenges, a supportive and attentive caregiver can buffer them from the negative impacts of early-life adversity that may otherwise increase their risk of poor mental and physical health later in life. Unfortunately, maternal mental health problems are common in low-income populations with estimates of maternal depressive symptoms reaching nearly 60% (36). Targeted improvements in maternal mental health, social support, and capacity can promote healthy child development and may protect the child's life-long health. The work included in this dissertation aims to promote a new line of research into the effects of early-life adversity using multisystem salivary biomeasures to examine physiologic functioning in children. Advancing this research may help identify, prevent and treat the physiologic effects of early-life adversity during childhood, and promote life-long health and well-being.

7.5. References

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APPENDIX A

Fetus to Five Study Parent Survey Items and Instruments

A.1. Parent Survey Items

Demographic and Health Characteristics

Child sex (Phase 2 only)

Your child's gender:

- ☐ Male
- ☐ Female

Child race

How would you describe your child's race? (Check all that apply)

- ☐ White
- ☐ Black or African American
- ☐ Asian or Pacific Islander
- ☐ Native American or Alaskan Native
- ☐ Other, please specify: _____

Child ethnicity

How would you describe your child's ethnicity?

- ☐ Hispanic/Latino
- ☐ Not Hispanic/Latino

Child current sickness

Is your child currently feeling sick or ill (ex. runny nose, fever, cough, aching, etc.)?

- ☐ Yes
- ☐ No

Child recent health

Compared to others his or her age, would you say your child's health in the last 2 days has been:

- ☐ Excellent
- ☐ Very good
- ☐ Good
- ☐ Fair
- ☐ Poor

Child taken any medication in the last two days

Has your child taken any prescription or over-the-counter medications in the last 2 days?

- ☐ Yes
- ☐ No
  - ➔**If yes**, please list the names of these medications:
    - Medication 1: _____
    - Medication 2: _____
    - Medication 3: _____
    - Medication 4: _____

Child current periodontal or dental issues

Does your child currently have any dental problems such as cuts or sores in his or her mouth, very loose teeth, a tooth lost in the last 48 hours, bleeding gums while brushing, or untreated cavities?

- ☐ Yes
- ☐ No

Maternal smoking status

Do you smoke cigarettes?

- ☐ Yes ➔How many cigarettes did you smoke yesterday & today? (All together)_____
- ☐ No

Maternal age

Your age: _____years

Question for Timing Data

Wake time

What time did your child wake up this morning? (Write the hour : minute, and circle whether this was AM or PM)

_____:_____AM/PM

Socioeconomic Status and Adversity Items

Family income

For the past 12 months, which category best describes your total combined family income? Include income before taxes and from all sources. (Ex. wages, rent from properties, social security, disability, veteran's benefits, unemployment benefits, worker's compensation, help from relatives, child support payments, alimony, etc.)

- ☐ Less than $5,000
- ☐ $5,000 to $11,999
- ☐ $12,000 through $15,999
- ☐ $16,000 through $24,999
- ☐ $25,000 through $34,999
- ☐ $35,000 through $49,999
- ☐ $50,000 through $74,999
- ☐ $75,000 through $99,999
- ☐ $100,000 through $150,000
- ☐ 150,000 and greater
- ☐ Don't know

  ➔How much, all together, do you and the others in your family earn in one month before taxes are taken out?_____ (Phase 2 only)

Maternal education

What is the highest level of education you have completed? (Check one)

- ☐ Less than High School
- ☐ High School / GED
- ☐ Some College
- ☐ 2-year College Degree
- ☐ 4-year College Degree
- ☐ Master's Degree
- ☐ Doctoral Degree
- ☐ Professional Degree (JD, MD)

Maternal marital status

What is your current marital status?

- ☐ Single, never married
- ☐ Married
- ☐ Divorced
- ☐ Separated
- ☐ Widowed
- ☐ Other, please specify: _____

Number of moves during child's life

Since this child was born, how many times have you moved to a different house or apartment?

- ☐ 0
- ☐ 1
- ☐ 2
- ☐ 3
- ☐ 4
- ☐ 5
- ☐ 6 or more

A.2. Parent Survey Instruments

Financial Stress Index

In the last 3 months, how often have you:

	Never	Rarely	Sometimes	Often	Always
Had difficulty making your monthly payments?	□	□	□	□	□
Thought about your money problems?	□	□	□	□	□
Had difficulty paying your bills?	□	□	□	□	□
Feared you might lose your home due to lack of money?	□	□	□	□	□
Feared you might be laid off?	□	□	□	□	□
Felt your opportunities were limited due to lack of finances?	□	□	□	□	□

Center for Epidemiologic Studies Depression Scale

Below is a list of feelings and behaviors. Please tell us how often you have felt or behaved this way during the past week.

**During the past week:*	Rarely or none of the time (less than 1 day)	Some or a little of the time (1-2 days)	Occasionally or a moderate amount of time (3-4 days)	Most or all of the time (5-7 days)
1. I was bothered by things that usually don't bother me.	□	□	□	□
2. I did not feel like eating; my appetite was poor.	□	□	□	□

3. I felt that I could not shake off the blues even with help from my family or friends.	□	□	□	□
4. I felt I was just as good as other people.	□	□	□	□
5. I had trouble keeping my mind on what I was doing.	□	□	□	□
6. I felt depressed.	□	□	□	□
7. I felt that everything I did was an effort.	□	□	□	□
8. I felt hopeful about the future.	□	□	□	□
9. I thought my life had been a failure.	□	□	□	□
10. I felt fearful.	□	□	□	□
11. My sleep was restless.	□	□	□	□
12. I was happy.	□	□	□	□
13. I talked less than usual.	□	□	□	□
14. I felt lonely.	□	□	□	□
15. People were unfriendly.	□	□	□	□
16. I enjoyed life.	□	□	□	□
17. I had crying spells.	□	□	□	□
18. I felt sad.	□	□	□	□
19. I felt that people dislike me.	□	□	□	□
20. I could not get "going."	□	□	□	□

State-Trait Anxiety Inventory

A number of statements which people have used to describe themselves are given below. Read each statement and then check the appropriate box to the right of the statement to indicate how you generally feel. There are no right or wrong answers. Do not spend too much time on any one statement, but give the answer which seems to describe how you generally feel.

	Almost Never	**Sometimes**	**Often**	**Almost Always**
1. I feel pleasant.	□	□	□	□
2. I feel nervous and restless.	□	□	□	□
3. I feel satisfied with myself.	□	□	□	□
4. I wish I could be as happy as others seem to be.	□	□	□	□

5.	I feel like a failure.	□	□	□	□
6.	I feel rested.	□	□	□	□
7.	I am "calm, cool, and collected."	□	□	□	□
8.	I feel that difficulties are piling up so that I cannot overcome them.	□	□	□	□
9.	I worry too much over something that really doesn't matter.	□	□	□	□
10.	I am happy.	□	□	□	□
11.	I have disturbing thoughts.	□	□	□	□
12.	I lack self-confidence.	□	□	□	□
13.	I feel secure.	□	□	□	□
14.	I make decisions easily.	□	□	□	□
15.	I feel inadequate.	□	□	□	□
16.	I am content.	□	□	□	□
17.	Some unimportant thought runs through my mind and bothers me.	□	□	□	□
18.	I take disappointments so keenly that I can't put them out of my mind.	□	□	□	□
19.	I am a steady person.	□	□	□	□
20.	I get in a state of tension or turmoil as I think over my recent concerns and interests.	□	□	□	□

Parenting Stress Index (Short-Form)

The next group of questions focuses on your experience as a parent. Since every child is different, answer based on your experience as a parent of this child.

Read each statement carefully. For each statement, focus on the child you brought here today. Circle the response that best represents your opinion. While you may not find a response that exactly states your feelings, please circle the response that comes closest to describing how you feel. Your first reaction to each statement should be the answer you circle.

		Strongly Agree	**Agree**	**Not Sure**	**Disagree**	**Strongly Disagree**
1.	I often have the feeling that I cannot handle things very well.	SA	A	NS	D	SD
2.	I find myself giving up more of my life to meet my children's needs than I ever expected.	SA	A	NS	D	SD
3.	I feel trapped by my responsibilities as a parent.	SA	A	NS	D	SD
4.	Since having this child, I have been unable to do new and different things.	SA	A	NS	D	SD
5.	Since having a child, I feel that I am almost never able to do things that I like to do.	SA	A	NS	D	SD

6.	I am unhappy with the last purchase of clothing I made for myself.	SA	A	NS	D	SD
7.	There are quite a few things that bother me about my life.	SA	A	NS	D	SD
8.	Having a child has caused more problems than I expected in my relationship with my spouse (or male/female friend).	SA	A	NS	D	SD
9.	I feel alone and without friends.	SA	A	NS	D	SD
10.	When I go to a party, I usually expect not to enjoy myself.	SA	A	NS	D	SD
11.	I am not as interested in people as I used to be.	SA	A	NS	D	SD
12.	I don't enjoy things as I used to.	SA	A	NS	D	SD
13.	My child rarely does things for me that make me feel good.	SA	A	NS	D	SD
14.	Sometimes I feel my child doesn't like me and doesn't want to be close to me.	SA	A	NS	D	SD
15.	My child smiles at me much less than I expected.	SA	A	NS	D	SD
16.	When I do things for my child, I get the feeling that my efforts are not appreciated very much.	SA	A	NS	D	SD
17.	When playing, my child doesn't often giggle or laugh.	SA	A	NS	D	SD
18.	My child doesn't seem to learn as quickly as most children.	SA	A	NS	D	SD
19.	My child doesn't seem to smile as much as most children.	SA	A	NS	D	SD
20.	My child is not able to do as much as I expected.	SA	A	NS	D	SD
21.	It takes a long time and it is very hard for my child to get used to new things.	SA	A	NS	D	SD

For number 22, check one statement from the 5 choices.

22. I feel that I am:
- ☐ not very good at being a parent.
- ☐ a person who has some trouble being a parent.
- ☐ an average parent.
- ☐ a better than average parent.
- ☐ a very good parent.

Answer based on your experience as a parent of this child. Circle the response that best represents your opinion. Your first reaction to each statement should be the answer you circle.

		Strongly Agree	**Agree**	**Not Sure**	**Disagree**	**Strongly Disagree**
23.	I expected to have closer and warmer feelings for my child than I do and this bothers me.	SA	A	NS	D	SD
24.	Sometimes my child does things that bother me just to be mean.	SA	A	NS	D	SD
25.	My child seems to cry or fuss more often than most children.	SA	A	NS	D	SD
26.	My child generally wakes up in a bad mood.	SA	A	NS	D	SD
27.	I feel that my child is very moody and easily upset.	SA	A	NS	D	SD
28.	My child does a few things which bother me a great deal.	SA	A	NS	D	SD

29. My child reacts very strongly when something happens that my child doesn't like.	SA	A	NS	D	SD
30. My child gets upset easily over the smallest thing.	SA	A	NS	D	SD
31. My child's sleeping or eating schedule was much harder to establish than I expected.	SA	A	NS	D	SD

For number 32, check one statement from the 5 choices.

32. I have found that getting my child to do something or stop doing something is:
- ☐ much harder than I expected.
- ☐ somewhat harder than I expected.
- ☐ about as hard as I expected.
- ☐ somewhat easier than I expected.
- ☐ much easier than I expected.

For number 33, choose your response from the choices.

33. Think carefully and count the number of things which your child does that bother you. For example: dawdles, refuses to listen, overactive, cries, interrupts, fights, whines, etc.
- ☐ 10+
- ☐ 8-9
- ☐ 6-7
- ☐ 4-5
- ☐ 1-3

Answer based on your experience as a parent of this child. Circle the response that best represents your opinion. Your first reaction to each statement should be the answer you circle.

	Strongly Agree	**Agree**	**Not Sure**	**Disagree**	**Strongly Disagree**
34. There are some things my child does that really bother me a lot.	SA	A	NS	D	SD
35. My child turned out to be more of a problem than I had expected.	SA	A	NS	D	SD
36. My child makes more demands on me than most children.	SA	A	NS	D	SD

APPENDIX B

Chapter 5 Supplemental Tables

Table S.5.1. Adjusted relations between IL-1β and alpha-amylase (model 1) and cortisol (model 2) in the full sample[a]

	IL-1β Model 1 (n=125)			IL-1β Model 2 (n=125)		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	4.78	0.13	<.001	4.78	0.15	<.001
Task Timing	0.00	0.01	.58	0.00	0.01	.71
Sample 1-3	-0.13	0.03	<.001	-0.13	0.04	<.01
Sample 3-4	-0.58[b]	0.14	<.001	-0.58[c]	0.15	<.001
Current Oral Health Issues	0.58	0.18	<.01	0.58	0.19	<.01
Alpha-amylase	0.16	0.07	<.05			
Cortisol				-0.03	0.06	.66
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Intercept:						
Variance(Intercept)	0.70	0.12	0.51-0.97	0.68	0.12	0.48-0.95
Residual: AR(1)						
Rho	0.48	0.08	0.30-0.62	0.51	0.08	0.33-0.65
Within-subject Variance	0.28	0.06	0.18-0.42	0.30	0.06	0.20-0.45

SE= robust standard error; CI= confidence interval.

[a]IL-1β, alpha-amylase, and cortisol were log-transformed and Winsorized.

[b]Difference between slopes from sample 1-3 and 3-4: β= -0.45, z= -3.45, p< .01.

[c]Difference between slopes from sample 1-3 and 3-4: β= -0.45, z= -3.37, p< .01.

Table S.5.2. Adjusted relations between IL-6 and alpha-amylase (model 1) and cortisol (model 2) in the full sample[a]

	IL-6 Model 1 (n=125)			IL-6 Model 2 (n=125)		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	1.41	0.13	<.001	1.42	0.13	<.001
Task Timing	0.00	0.00	.40	0.00	0.00	.28
Sample	-0.07	0.04	.08	-0.08	0.05	.09
Current Oral Health Issues	1.02	0.38	<.01	1.02	0.39	<.01
Age	0.10	0.04	<.01	0.09	0.04	<.01
Time Since Waking	0.10	0.04	<.05	0.10	0.04	<.05
Alpha-amylase	0.16	0.10	.11			
Cortisol				-0.06	0.09	.48
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Intercept:						
Variance(Intercept)	1.50	0.22	1.13-1.99	1.48	0.21	1.11-1.95
Residual: AR(1)						
Rho	0.33	0.09	0.14-0.50	0.36	0.09	0.17-0.52
Within-subject Variance	0.38	0.08	0.24-0.58	0.39	0.09	0.26-0.60

SE= robust standard error; CI= confidence interval.

[a]IL-6, alpha-amylase, and cortisol were log-transformed and Winsorized.

Table S.5.3. Adjusted relations between IL-8 and alpha-amylase (model 1) and cortisol (model 2) in the full sample[a]

	IL-8 Model 1 (n=125)			**IL-8 Model 2 (n=125)**		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	6.32	0.13	<.001	6.35	0.14	<.001
Task Timing	0.10	1.51	.13	0.00	0.01	.79
Sample 1-3	-0.06	0.03	.07	-0.07	0.04	.06
Sample 3-4	-0.34[b]	0.16	<.05	-0.35[c]	0.16	<.05
Alpha-amylase	0.10	0.07	.13			
Cortisol				-0.05	0.05	.29
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Intercept:						
Variance(Intercept)	0.41	0.07	0.30-0.57	0.41	0.07	0.29-0.58
Residual: AR(1)						
Rho	0.25	0.11	0.04-0.45	0.27	0.10	0.06-0.46
Within-subject Variance	0.23	0.04	0.16-0.32	0.23	0.04	0.16-0.33

SE= robust standard error; CI= confidence interval.

[a]IL-8, alpha-amylase, and cortisol were log-transformed and Winsorized.

[b]Difference between slopes from sample 1-3 and 3-4: β= -0.28, z= -1.91, p= .06.

[c]Difference between slopes from sample 1-3 and 3-4: β= -0.28, z= -1.98, p< .05.

Table S.5.4. Adjusted relations between TNFα and alpha-amylase (model 1) and cortisol (model 2) in the full sample[a]

	TNFα Model 1 (n=125)			**TNFα Model 2 (n=125)**		
Fixed Effects	**Parameter**	**SE**	***P***	**Parameter**	**SE**	***p***
Intercept	0.55	0.18	<.01	0.59	0.20	<.01
Task Timing	-0.02	0.01	<.05	-0.02	0.01	<.05
Sample 1-2	-0.24	0.06	<.001	-0.24	0.06	<.001
Sample 2-4	0.10[b]	0.11	.34	0.08[c]	0.11	.50
Maternal Smoking	0.49	0.22	<.05	0.48	0.22	<.05
Alpha-amylase	0.16	0.09	.07			
Cortisol				-0.09	0.07	.19
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Intercept:						
Variance(Intercept)	0.85	0.13	0.62-1.15	0.83	0.14	0.60-1.15
Residual: AR(1)						
Rho	0.34	0.12	0.10-0.55	0.38	0.12	0.13-0.58
Within-subject Variance	0.47	0.10	0.31-0.71	0.49	0.11	0.32-0.76

SE= robust standard error; CI= confidence interval.

[a]TNFα, alpha-amylase, and cortisol were log-transformed and Winsorized.

[b]Difference between slopes from sample 1-2 and 2-4: β= 0.34, z= 2.69, p< .01.

[c]Difference between slopes from sample 1-2 and 2-4: β= 0.32, z= 2.52, p< .05.

Table S.5.5. Adjusted relations between IL-1β and alpha-amylase and cortisol in boys (models 1 and 2) and girls (models 3 and 4) separately[a]

	IL-1β Model 1 Boys (n=61)			**IL-1β Model 2 Boys (n=61)**			**IL-1β Model 3 Girls (n=64)**			**IL-1β Model 4 Girls (n=64)**		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	4.72	0.17	<.001	4.70	0.17	<.001	4.87	0.20	<.001	4.84	0.02	<.001
Task Timing	0.01	0.01	.11	0.01	0.01	.16	0.00	0.01	.83	0.00	0.01	.86
Sample 1-3	-0.16	0.04	<.001	-0.15	0.04	<.001	-0.12	0.06	<.05	-0.11	0.06	.07
Sample 3-4	-0.70[b]	0.16	<.001	-0.68[c]	0.16	<.001	-0.47[d]	0.25	.06	-0.47[e]	0.24	.06
Current Oral Health Issues	0.52	0.39	.19	0.63	0.40	.12	0.55	0.32	.08	0.59	0.32	.06
Alpha-amylase	0.43	0.09	<.001				0.01	0.08	.85			
Cortisol				-0.17	0.04	<.001				0.08	0.04	.07
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Intercept:												
Variance(Intercept)	0.76	0.15	0.51-1.12	0.79	0.16	0.53-1.17	0.50	0.16	0.27-0.92	0.49	0.15	0.27-0.90
Residual: AR(1)												
Rho	0.36	0.14	0.07-0.59	0.40	0.14	0.09-0.64	0.54	0.13	0.24-0.74	0.55	0.13	0.25-0.75
Within-subject Variance	0.16	0.03	0.11-0.24	0.17	0.04	0.11-0.26	0.39	0.11	0.23-0.67	0.39	0.11	0.23-0.68

SE= standard error; CI= confidence interval.

[a]IL-1β, alpha-amylase, and cortisol were log-transformed and Winsorized.

[b]Difference between slopes from sample 1-3 and 3-4: β= -0.54, z= -3.68, p<.001.

[c]Difference between slopes from sample 1-3 and 3-4: β= -0.53, z= -3.63, p<.001.

[d]Difference between slopes from sample 1-3 and 3-4: β= -0.35, z= -1.57, p= .12.

[e]Difference between slopes from sample 1-3 and 3-4: β= -0.36, z= -1.64, p= .10.

Table S.5.6. Adjusted relations between IL-6 and alpha-amylase and cortisol in boys (models 1 and 2) and girls (models 3 and 4) separately[a]

	IL-6 Model 1 Boys (n=61)			**IL-6 Model 2 Boys (n=61)**			**IL-6 Model 3 Girls (n=64)**			**IL-6 Model 4 Girls (n=64)**		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	1.38	0.18	<.001	1.41	0.18	<.001	1.41	0.16	<.001	1.40	0.16	<.001
Task Timing	0.01	0.00	.19	0.01	0.00	.26	0.00	0.01	.58	0.00	0.01	.61
Sample	-0.16	0.06	<.01	-0.15	0.06	<.05	-0.03	0.07	.62	-0.02	0.07	.81
Current Oral Health Issues	0.71	0.58	.22	0.79	0.59	.18	1.40	0.43	<.01	1.44	0.43	<.01
Age	0.05	0.05	.32	0.06	0.05	.20	0.11	0.04	<.05	0.11	0.04	<.05
Time Since Waking	0.20	0.07	<.01	0.17	0.07	<.05	-0.01	0.06	.92	0.00	0.06	.96
Alpha-amylase	0.53	0.14	<.001				0.00	0.10	.98			
Cortisol				-0.27	0.06	<.001				0.09	0.05	.09
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Intercept:												
Variance(Intercept)	1.60	0.31	1.09-2.34	1.65	0.32	1.13-2.42	1.11	0.25	0.72-1.72	1.12	0.24	0.73-1.72
Residual: AR(1)												
Rho	0.25	0.12	0.01-0.46	0.29	0.12	0.04-0.51	0.42	0.14	0.12-0.65	0.41	0.14	0.11-0.64
Within-subject Variance	0.31	0.05	0.23-0.42	0.31	0.05	0.22-0.43	0.44	0.10	0.28-0.69	0.43	0.09	0.28-0.66

SE= standard error; CI= confidence interval.

[a]IL-6, alpha-amylase, and cortisol were log-transformed and Winsorized.

Table S.5.7. Adjusted relations between IL-8 and alpha-amylase and cortisol in boys (models 1 and 2) and girls (models 3 and 4) separately[a]

	IL-8 Model 1 Boys (n=61)			**IL-8 Model 2 Boys (n=61)**			**IL-8 Model 3 Girls (n=64)**			**IL-8 Model 4 Girls (n=64)**		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	6.37	0.15	<.001	6.40	0.15	<.001	6.31	0.19	<.001	6.28	0.19	<.001
Task Timing	0.00	0.01	.65	0.00	0.01	.72	-0.01	0.01	.50	-0.01	0.01	.48
Sample 1-3	-0.12	0.05	<.05	-0.12	0.05	<.05	-0.03	0.06	.59	-0.02	0.06	.77
Sample 3-4	-0.47[b]	0.18	<.05	-0.47[c]	0.18	<.01	-0.23[d]	0.25	.37	-0.21[e]	0.25	.40
Alpha-amylase	0.32	0.09	<.001				0.00	0.07	.95			
Cortisol				-0.21	0.04	<.001				0.07	0.04	.10
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Intercept:												
Variance(Intercept)	0.37	0.08	0.24-0.56	0.38	0.08	0.25-0.58	0.40	0.10	0.25-0.64	0.38	0.09	0.24-0.62
Residual: AR(1)												
Rho	0.19	0.13	-0.06-0.42	0.23	0.13	-0.03-0.47	0.31	0.14	0.02-0.55	0.30	0.14	0.14-0.54
Within-subject Variance	0.18	0.03	0.13-0.24	0.17	0.03	0.13-0.24	0.27	0.51	0.19-0.39	0.27	0.05	0.19-0.39

SE= standard error; CI= confidence interval.

[a]IL-8, alpha-amylase, and cortisol were log-transformed and Winsorized.

[b]Difference between slopes from sample 1-3 and 3-4: β= -0.36, z= -2.11, p<.05.

[c]Difference between slopes from sample 1-3 and 3-4: β= -0.35, z= -2.18, p<.05.

[d]Difference between slopes from sample 1-3 and 3-4: β= -0.19, z= -0.87, p>.20.

[e]Difference between slopes from sample 1-3 and 3-4: β= -0.20, z= -0.87, p>.20.

Table S.5.8. Adjusted relations between TNFα and alpha-amylase and cortisol in boys (models 1 and 2) and girls (models 3 and 4) separately[a]

	TNFα Model 1 Boys (n=61)			**TNFα Model 2 Boys (n=61)**			**TNFα Model 3 Girls (n=64)**			**TNFα Model 4 Girls (n=64)**		
Fixed Effects	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***	**Parameter**	**SE**	***p***
Intercept	0.48	0.23	<.05	0.47	0.23	<.05	0.70	0.21	<.01	0.69	0.22	<.01
Task Timing	-0.01	0.01	.25	-0.01	0.01	.18	-0.02	0.01	<.05	-0.02	0.01	<.01
Sample 1-2	-0.24	0.10	<.05	-0.25	0.10	<.05	-0.25	0.09	<.01	-0.24	0.10	<.05
Sample 2-4	-0.01[b]	0.14	.93	0.00[c]	0.13	.98	0.17[d]	0.14	.23	0.18[e]	0.14	.21
Maternal Smoking	0.67	0.33	<.05	0.92	0.33	<.01	0.13	0.28	.63	0.15	0.28	.59
Alpha-amylase	0.46	0.14	<.01				0.01	0.09	.90			
Cortisol				-0.26	0.06	<.001				0.03	0.05	.58
Random Effects	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**	**Estimate**	**SE**	**95% CI**
Intercept:												
Variance(Intercept)	0.84	0.19	0.54-1.30	0.88	0.21	0.55-1.41	0.71	0.16	0.45-1.12	0.72	0.16	0.46-1.13
Residual: AR(1)												
Rho	0.32	0.12	0.08-0.53	0.43	0.12	0.17-0.64	0.33	0.13	0.06-0.55	0.33	0.13	0.06-0.55
Within-subject Variance	0.47	0.08	0.33-0.65	0.50	0.10	0.34-0.75	0.45	0.08	0.32-0.63	0.44	0.08	0.31-0.63

SE= standard error; CI= confidence interval.

[a]TNFα, alpha-amylase, and cortisol were log-transformed and Winsorized.

[b]Difference between slopes from sample 1-2 and 2-4: β= 0.23, z= 1.20, p> .20.

[c]Difference between slopes from sample 1-2 and 2-4: β= 0.24, z= 1.37, p= .17.

[d]Difference between slopes from sample 1-2 and 2-4: β= 0.42, z= 2.19, p< .05.

[e]Difference between slopes from sample 1-2 and 2-4: β= 0.43, z= 2.22, p< .05.

CURRICULUM VITAE

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EDUCATION

2010-2015 **PhD**, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Department: Population, Family, and Reproductive Health
Track: Child and Adolescent Health, and Development
Concentration: Neurodevelopment, Behavior, and Health
Advisors: Cynthia S. Minkovitz, MD, MPP; Sara B. Johnson, PhD, MPH
Dissertation: The biological embedding of early-life adversity: Using salivary biomarkers to examine the influence of maternal psychological well-being on child neuroendocrine-immune functioning

2008-2010 **MHS**, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Department: Population, Family, and Reproductive Health
Track: Child and Adolescent Health, and Development
Concentration: Neurodevelopment, Behavior, and Health
Advisor: Sara B. Johnson, PhD, MPH

2001-2005 **BA**, Boston College, Boston, MA
Major: Psychology
Concentration: Biology

HONORS & AWARDS

2011-2015 *Hopkins Sommer Scholar* awarded by Johns Hopkins Bloomberg School of Public Health

2013 *Bernard and Jane Guyer Scholarship* awarded by Johns Hopkins Bloomberg School of Public Health

2012 *Donald Cornely Scholarship* awarded by Johns Hopkins Bloomberg School of Public Health

2011 *Willian Endowment for Excellence in Science Award* awarded by Johns Hopkins Bloomberg School of Public Health

2005 *Phi Beta Kappa Society* elected by Boston College

RESEARCH & PROFESSIONAL EXPERIENCE

2011-Present **Study Director**, Fetus to Five Study, Johns Hopkins Bloomberg School of Public Health
Research on the physiologic aspects of maternal-child bonding and child development

- Collected biologic and behavioral data on mother-child dyads using a lab-based protocol
- Assist with study development, instrument assessment, data analysis, and manuscript development

2010-2012 **Research Assistant**, The Women's and Children's Health Policy Center, Johns Hopkins Bloomberg School of Public Health
Research examining the relations between state health policies and childhood obesity and second-hand smoke exposure

- Composed an original manuscript, serving as first author
- Analyzed and interpreted large-scale survey and policy data

2009 **Intern,** Majority Health Office of the Health, Education, Labor and Pensions Committee, United States Senate
Legislative work on US health insurance reform, preventive health, food safety and H1N1 flu policy

- Attended Congressional briefings and hearing and composed memorandums for health policy staff
- Performed research on the US health insurance system, coverage and cost

2009 **Research Assistant,** Dyson Initiative National Evaluation Project, The Women's and Children's Health Policy Center, Johns Hopkins Bloomberg School of Public Health
Evaluation of a national training initiative promoting pediatrician participation in community health programs

- Analyzed and interpreted evaluation survey data
- Consulted with colleagues in the preparation of briefing materials and study reports

2009 **Research Assistant/Intern**, Center for Adolescent Substance Abuse Research, Children's Hospital Boston
Clinically- and policy-oriented research on the treatment of opioid- and alcohol-addicted adolescents

- Helped incorporate neuroimaging techniques in the evaluation of addiction treatment for adolescents
- Assisted in the preparation of manuscripts, abstract presentations and federal grant proposals

2005-2008 **Research Assistant**, Division of Behavioral and Cognitive Neurology, Brigham and Women's Hospital
Event-related potential (ERP) research on neurophysiologic and cognitive correlates of healthy and diseased aging

- Collaborated with colleagues on manuscripts, serving as first author on two
- Presented research projects at the Annual Meetings of The Cognitive Neuroscience Society
- Consulted with colleagues to develop neuropsychological test batteries and ERP testing paradigms
- Administered neuropsychological tests, depression scale surveys, and ERP tests

TEACHING & ACADEMIC EXPERIENCE

2012, 2013 & 2014 **Teaching Assistant**, Translating Research into Public Health Programs, Johns Hopkins Bloomberg School of Public Health

2011, 2012, 2013, & 2014 **Teaching Assistant**, The Social Context of Adolescent Health and Development, Johns Hopkins Bloomberg School of Public Health

2012- Present **Founder/Leader**, Biological Embedding Journal Club, Johns Hopkins Bloomberg School of Public Health
Student-run journal club focused on the influence of experience on the development of disease, and the integration of biologic research and methods with public health

2012-Present **Member**, Neurodevelopment and Public Health Collaborative Curriculum Working Group, Johns Hopkins Bloomberg School of Public Health
Interdisciplinary and inter-institutional group of professors working to develop an integrative curriculum for neurodevelopment, policy and public health

2011 **Organizer**, Reproductive Health in Crisis Forum, Johns Hopkins Bloomberg School of Public Health
Expert panel discussion of family planning policy in the US featuring speakers from the Guttmacher Institute, Planned Parenthood and the Maryland Department of Health and Mental Hygiene

2010-2011 **President**, Departmental Student Alliance, Johns Hopkins Bloomberg School of Public Health
Student-run group serving as a liaison between faculty and students, promoting student cohesion, and coordinating department philanthropic and social events

2010 **Student Member**, Masters of Health Science Admissions Committee, Johns Hopkins Bloomberg School of Public Health

RESEARCH PUBLICATIONS

1. Michelson N, **Riis JL**, Newton EK, Johnson SB. Maternal subjective social status, psychological distress and parent-child relationship: The moderating role of race. (In preparation).
2. Li M, **Riis JL**, Johnson SB. Laboratory measured cognitive and emotional self-regulation and its correlates with family and child characteristics among high and low income five-year-olds. (In preparation).
3. **Riis JL**, Granger DA, Minkovitz CS, Bandeen-Roche K, DiPietro JA, Johnson SB. Maternal distress and child neuroendocrine and immune regulation. (In preparation).
4. **Riis JL**, Granger DA, Minkovitz CS, Yeung EW, Johnson SB. Pre-analytic considerations and best practices for studies involving salivary analyte data. (In preparation).
5. Johnson SB, **Riis JL**. State of the art review: Poverty and the developing brain. *Pediatrics* (submitted Jan. 2015).
6. **Riis JL**, Granger DA, DiPietro JA, Bandeen-Roche K, Johnson SB. Salivary cytokines as a minimally-invasive measure of immune functioning in young children: Correlates of individual differences and sensitivity to laboratory stress. *Developmental Psychobiology,* 2015.
7. **Riis JL**, Out D, Dorn LD, Beal SJ, Denson LA, Pabst S, Jaedicke K, Granger DA. Salivary cytokines in healthy adolescent girls: Intercorrelations, stability, and associations with serum cytokines, age, and pubertal stage. *Developmental Psychobiology*, 2013.
8. Johnson SB, Riley AW**,** Granger DA, **Riis JL**. The science of early life toxic stress for pediatric practice and advocacy. *Pediatrics*, 2013; 131(2): 319-327.
9. **Riis JL**, Grason H, Strobino D, Minkovitz C. State school policies and youth obesity. *Maternal and Child Health Journal*, 2012: 1-8.
10. Daffner KR, Chong H, Sun X, Tarbi EC, **Riis JL**, McGinnis SM, Holcomb PJ. Mechanisms underlying age-and performance-related differences in working memory. *Journal of Cognitive Neuroscience*, 2011; 23(6): 1298-1314.
11. Daffner KR, Sun X, Tarbi EC, Rentz DM, Holcomb PJ, **Riis JL**. Does compensatory neural activity survive old-old age? *Neuroimage*, 2011; 54(1): 427-438.
12. **Riis JL**, Chong H, McGinnis SM, Tarbi, EC, Sun X, Holcomb PJ, Rentz DM Daffner KR. Age-related changes in early novelty processing as measured by ERPs. *Biological Psychology*, 2009; 82(1): 33-44.
13. Wolk DA, Sen NM, Chong H, **Riis JL**, McGinnis SM, Holcomb PJ Daffner KR. ERP Correlates of item recognition memory: Effects of age and performance. *Brain Research*, 2009; 23(1250): 218-231.

14. Chong H, **Riis JL**, McGinnis SM, Williams DM, Holcomb PJ, Daffner KR. To ignore or explore: Top-down modulation of novelty processing. *Journal of Cognitive Neuroscience*, 2008; 20(1): 120-134.
15. **Riis JL**, Chong H, Wolk DA, Rentz DM, Holcomb PJ, and Daffner KR. Compensatory neural activity distinguishes different patterns of normal cognitive aging. *NeuroImage*, 2008; 39(1): 441-454.
16. Daffner KR, Chong H, **Riis JL**, Rentz DM, Wolk DA, Budson AE, Holcomb PJ. Cognitive status impacts age-related changes in attention to novel and target events in normal adults. *Neuropsychology*, 2007; 21: 291-300.

RESEARCH PRESENTATIONS

1. **Riis, JL**, Granger, DA, Minkovitz, CS, Bandeen-Roche, K, DiPietro, JA, Johnson, SB. Maternal distress and child neuroendocrine and immune regulation. *Shaping the Developing Brain: Prenatal through Early Childhood Fifth Annual Aspen Brain Forum* 2014.
2. Johnson SB, **Riis JL**, Around Him D, Bui V, DiPietro JA. Biological embedding of early experience in self-regulation: The Fetus to Five study. *Robert Wood Johnson meeting* 2012.
3. Ma S, Knauer H, **Riis J**, Strobino D, Minkovitz C. Changes in children's exposure to secondhand smoking across states, 2003 to 2007. *Pediatric Academic Societies Annual Meeting* 2012.
4. **Riis JL**, Grason H, Strobino D, Minkovitz C. State school policies and youth obesity. *Association of Maternal and Child Health Programs, Annual Conference* 2012.
5. **Riis JL**, Grason H, Strobino D, Minkovitz C. State school policies and youth obesity. *Johns Hopkins School of Public Health, Department of Population, Family and Reproductive Health Research Day* 2011.
6. **Riis JL**, McGinnis SM, Chong H, Wolk DA, Rentz DM, Holcomb PJ, and Daffner KR. Does compensatory neural activity survive old-old age? *Cognitive Neuroscience Society, Annual Meeting* 2008.
7. **Riis JL**, Chong H, Wolk DA, McGinnis SM, and Daffner KR. Age-related changes in early processing of visual novelty oddball stimuli as indexed by the P2 component. *Cognitive Neuroscience Society, Annual Meeting* 2007.