The Pennsylvania State University
The Graduate School
Department of Biobehavioral Health

THE EFFECTS OF ACUTE PSYCHOSOCIAL STRESS ON HPA-AXIS AND
SAM-AXIS FUNCTION IN MEN WITH TYPE 2 DIABETES MELLITUS

A Dissertation in
Biobehavioral Health
by
Kimberly N. Walter

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The dissertation of Kimberly N. Walter was reviewed and approved* by the following:

Laura Cousino Klein  
Associate Professor of Biobehavioral Health  
Dissertation Adviser  
Chair of Committee

David M. Almeida  
Professor of Human Development and Family Studies

Elizabeth J. Susman  
Professor of Biobehavioral Health

Sheila G. West  
Associate Professor of Biobehavioral Health

John W. Graham  
Professor of Biobehavioral Health  
Professor-in-Charge of the Biobehavioral Health Graduate Program

*Signatures on file in the Graduate School
Abstract

Type 2 diabetes mellitus is one of the most common and costly chronic diseases in the United States and the world (Whiting et al., 2011). Scientists estimate that by 2050 one in three Americans will have type 2 DM (Boyle et al., 2010). Although type 2 DM related mortality has declined in recent years, it remains a major public health concern (Whiting et al., 2011). The exact etiology of type 2 DM is not known, but scientists have identified multiple genetic and environmental factors (e.g., obesity, diet, socioeconomic status, physical inactivity) that likely contribute to the pathogenesis and pathophysiology of the disease (Codario, 2011). In recent years, scientists have started to investigate the role of psychological factors, such as psychosocial stress, in the pathophysiology of type 2 DM (Nathan et al., 2009). Data from observational studies provide suggestive evidence that psychosocial stress is a factor affecting type 2 DM onset and progression (e.g., Mommersteeg et al., 2012; Pouwer et al., 2010; Toshihiro et al., 2008). However, the stress physiological mechanisms involved in the relationship are unclear, and there is a paucity of research examining stress physiological processes in individuals with type 2 DM.

The primary aim of this dissertation was to determine if men with type 2 DM who do not have major disease-related complications have dysregulated stress systems by examining the effects of acute psychosocial stress exposure on HPA-axis and SAM-axis function. A secondary aim of this dissertation was to investigate if allostatic load, a measure of chronic stress exposure, influences stress physiological processes in men with type 2 DM. This dissertation study used a mixed between- and within subjects-design to examine the effects of type 2 DM and acute psychosocial stress (Trier Social Stress Test)
on stress physiology in a sample of 34 men (20 healthy, 14 type 2 DM) between the ages of 25 and 65. Primary dependent measures included biomarkers of the HPA-axis (salivary cortisol, salivary DHEA-S) and SAM-axis (salivary alpha-amylase), as well as allostatic load parameters (cardiovascular, metabolic, neuroendocrine, anthropometric, immune measures). Results revealed that men with type 2 DM displayed significantly higher total allostatic load levels and significantly lower DHEA-S levels compared to healthy men. Furthermore, men with type 2 DM were able to mount both HPA- and SAM-axis responses to the acute stressor that was comparable to the healthy participants. Overall, the findings suggest that men with type 2 DM without major disease-related complications are able to physiologically respond to acute psychosocial stressors, in spite of having increased total allostatic load and decreased DHEA-S levels.
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<th>Definition</th>
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<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropin hormone</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;g&lt;/sub&gt;</td>
<td>area under the curve with respect to ground</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;i&lt;/sub&gt;</td>
<td>area under the curve with respect to increase</td>
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<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
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<tr>
<td>BIC</td>
<td>Bayesian information criterion</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>CBG</td>
<td>corticosteroid-binding globulin</td>
</tr>
<tr>
<td>CDA</td>
<td>canonical discriminant analysis</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>CES-D</td>
<td>Center for Epidemiological Studies Depression</td>
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<tr>
<td>CHR1R</td>
<td>CRH1 receptor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CO</td>
<td>carbon monoxide</td>
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<tr>
<td>CRC</td>
<td>Clinical Research Center</td>
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<tr>
<td>CRH</td>
<td>corticotropin releasing hormone</td>
</tr>
<tr>
<td>CRP</td>
<td>c-reactive protein</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
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<tr>
<td>DHEA</td>
<td>dehydroepiandosterone</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>dehydroepiandrosterone-sulfate</td>
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<td>DHEA(S)</td>
<td>dehydroepiandrosterone &amp; dehydroepiandrosterone-sulfate</td>
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DM: diabetes mellitus
EASD: European Association for the Study of Diabetes
EDTA: ethylenediaminetetraacetic acid
EPI: epinephrine
FPG: fasting plasma glucose
HbA1c: glycosolated hemoglobin
HDL: high density lipoprotein cholesterol
HPA-axis: hypothalamic-pituitary-adrenal axis
IGT: impaired glucose tolerance
LDL: low density lipoprotein cholesterol
MANOVA: multivariate analysis of variance
MLM: Multi-level modeling
NCEP: National Cholesterol Education Program
NE: norepinephrine
NHBPEP: National High Blood Pressure Education Program
OGTT: oral glucose tolerance test
PNS: parasympathetic nervous system
PNMT: phenylethanolamine N–methyltransferase
POMC: pro-opiomelanocortin
PVN: paraventricular nucleus
sAA: Salivary Alpha-Amylase
SAM-axis: sympathetic-adrenal-medullary axis
SBP: systolic blood pressure
SD: Standard deviation
SEM: standard error of the mean
SNS: sympathetic nervous system
SPSS: Statistical Program for the Social Sciences
SST: serum separator tube
TC: total cholesterol
TIA: transient ischemic attack
TSST: Trier Social Stress Test
WHO: World Health Organization
11β-HSD : 11β-hydroxysteroid dehydrogenase
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CHAPTER 1: INTRODUCTION

Scope of Problem

Type 2 diabetes mellitus (DM) is one of the most common and costly chronic
diseases in the United States and the world (Whiting, Guariguata, Weil, & Shaw, 2011).
In the United States, an estimated 11.5% of adults have DM, and type 2 DM accounts for
over 90% of all DM cases [Centers for Disease Control (CDC) 2011]. Scientists estimate
that by 2050 one in three Americans will have type 2 DM (Boyle, Thompson, Gregg,
Barker, & Williamson, 2010). Although type 2 DM related mortality has declined in
recent years, it remains a major public health concern (Whiting et al., 2011). It
contributes to the death of over 230,000 Americans annually and is a leading cause of
cardiovascular disease, blindness, renal failure, and hospitalizations (CDC, 2011). Type
2 DM also is a huge economic burden, and economists estimated that the total economic
cost of diagnosed DM in 2012 was $245 billion, which was a 41% increase from 2007
[American Diabetes Association (ADA), 2013].

The exact etiology of type 2 DM is not known, but scientists have identified
multiple genetic and environmental factors (e.g., obesity, diet, socioeconomic status,
physical inactivity) that likely contribute to the pathogenesis of the disease (Codario,
2011). The complex nature of type 2 DM has made it difficult for scientists to develop
truly effective prevention and treatment interventions. In recent years, scientists have
started to investigate the role of psychological factors, such as psychosocial stress, in the
pathophysiology of type 2 DM (Nathan et al., 2009).

There is evidence from observational studies that psychosocial stress contributes
to the pathophysiology of type 2 DM (e.g., Mommersteeg, Herr, Zijlstra, Schneider, &
Results from epidemiological cohort studies indicate that psychological distress is predictive of future type 2 DM (Mommersteeg et al., 2012; Pouwer et al., 2010; Toshihiro et al., 2008). Psychosocial stress may also exacerbates the disease by affecting an individual’s glucose control and health behaviors (e.g., diet, exercise, treatment adherence), and as a result can contribute to disease-related complications (e.g., retinopathy, nephropathy, coronary artery disease) (Cox et al., 2013; Gonder-Frederick, Cox, & Clarke, 2009). Although data from observational studies provide suggestive evidence that psychosocial stress is a factor affecting type 2 DM onset and progression, the stress physiological mechanisms involved in the relationship are unclear. A better understanding of the stress physiology in type 2 DM may assist scientists in developing targeted, stress-specific interventions and also may help to identify individuals with type 2 DM who are more physiologically vulnerable to the negative health effects of psychosocial stress.

There is a paucity of research examining stress physiological processes in individuals with type 2 DM. Studies have examined hypothalamic-pituitary-adrenal axis (HPA-axis) function in type 2 DM, but these studies did not examine the effects of acute psychosocial stressors. Rather, they examined HPA-axis function following pharmacological challenges (e.g., dexamethasone suppression test) or in non-stressor settings (e.g., diurnal rhythm, basal circulating cortisol levels) (e.g., Cameron, Kronfol, Greden, & Carroll, 1984; Chiodini et al., 2006; Lederbogen et al., 2011; Roy, Roy, & Brown, 1998). Studies also have examined sympathetic-adrenal-medullary axis (SAM-axis) function in non-stressor settings, but the findings have been inconsistent with some studies finding increased SAM-axis activity and other finding decreased SAM-axis
activity in individuals with type 2 DM (Bottini et al., 1995; Cryer, Silverberg, Santiago, & Shah, 1978; Hilsted, 1995). In general, findings from these studies suggest that individuals with type 2 DM have impaired stress system functioning, but these findings do not provide information about how psychosocial stressors specifically affect stress system functioning (de Kloet, Joels, & Holsboer, 2005).

To date, only one laboratory study specifically has investigated the effects of an acute psychosocial stressor on HPA-axis function in men with type 2 DM (Faulenbach et al., 2012; Sung, Izzo, Dandona, & Wilson, 1999). Although several studies have examined sympathetic nervous system (SNS) responses (e.g., heart rate, blood pressure) to acute stress in individuals with type 2 DM (e.g., Goetsch, VanDorsten, Pbert, Ullrich, & Yeater, 1993; Sung et al., 1999), no studies to date have specifically investigated the effects of an acute psychosocial stressor on SAM-axis function (e.g., salivary alpha-amylase). Given the lack of research on the effects of acute psychosocial stressors on stress physiological processes in type 2 DM, additional research is needed to further elucidate the mechanisms involved in the relationship between acute psychosocial stress and type 2 DM.

This dissertation will address the gap in the literature by examining the effects of acute psychosocial stress exposure on HPA-axis and SAM-axis function in individuals with type 2 DM. The primary aim of this dissertation is to assess the effects of an acute laboratory psychosocial stressor on multiple stress system parameters to determine if men with type 2 DM have alterations in stress system functioning. A secondary aim of this dissertation is to investigate if allostatic load, which is a measure of chronic stress exposure, influences stress physiological processes of men with type 2 DM. The present
chapter will review type 2 DM pathophysiology; provide an overview of stress physiology, chronic stress, and allostatic load; and discuss the literature on stress physiology in type 2 DM.

**Type 2 Diabetes Mellitus**

**Pathophysiology of Type 2 Diabetes Mellitus**

Type 2 DM is a complex metabolic disorder characterized by impaired glucose regulation and hyperglycemia (i.e., impaired glucose homeostasis) (Nolan, Damm, & Prentki, 2009). Prior to discussing glucose regulation in type 2 DM, it is important to review normal glucose regulation. In healthy individuals, glucose homeostasis is controlled by well-coordinated hormonal and neural processes that work together in an intricate manner to maintain glucose levels within a narrow range (70 - 110 mg/dL) (Aronoff, Berkowitz, Shreiner, & Want, 2004). Plasma glucose levels remain relatively constant even following meals, during fasting, and following physiological stressors (e.g., intense exercise) (Grayson, Seeley, & Sandoval, 2013).

Proper glucose regulation ensures that the tissues of body receive adequate fuel (Grayson et al., 2013). The pancreatic hormones, insulin and glucagon, are the main regulators of glucose usage and storage (Grayson et al., 2013). Insulin is an anabolic hormone synthesized and secreted by pancreatic β-cells, and glucagon is a catabolic hormone synthesized and secreted by pancreatic α-cells. Receptors on the α- and β-cells receive input from nutrient, hormonal, and neuronal signals, which enables them to carefully monitor and control glucose levels (Aronoff et al., 2004).

Increases in circulating plasma glucose levels (e.g., following a meal, during stress) stimulate the β-cells to secrete insulin into peripheral circulation. Insulin is
secreted in a biphasic manner, with the first-phase suppressing hepatic glucose production and the second phase involving more sustained insulin release (Codario, 2011). Insulin exerts anabolic effects on many tissues of the body including skeletal muscle, adipose tissue, and the liver (Aronoff et al., 2004). In the skeletal muscle, insulin stimulates the transport of glucose into cells and facilitates the storage of glucose as glycogen, which is the stored form of glucose. In the liver, insulin decreases hepatic glucose production by inhibiting glycogenolysis and gluconeogenesis. Glycogenolysis is the conversion of stored glycogen to glucose, and gluconeogenesis is the production of glucose from non-carbohydrate sources. In adipose tissue, insulin increases glucose uptake, glycerol synthesis, and triglyceride formation (Aronoff et al., 2004).

When blood glucose levels are below normal (e.g., during fasting states, during exercise), the β-cells stop secreting insulin and the α-cells begin secreting glucagon. Glucagon acts in many tissues of the body and has several effects that result in increased blood glucose levels (Aronoff et al., 2004). The catabolic actions of glucagon include accelerating glycogenolysis in the liver and skeletal muscle, increasing the breakdown of fats to fatty acids and glycerol that are used by cells for energy, and stimulating gluconeogenesis in the liver (Aronoff et al., 2004).

Type 2 DM is characterized by hyperglycemia secondary to impaired glucose regulation. Insulin resistance and β-cell dysfunction are the primary pathophysiological mechanisms affecting glucose homeostasis in type 2 DM (Codario, 2011; Ferrannini, 2012). Other processes involved in type 2 DM hyperglycemia include impairments in hepatic glycogenolysis, hepatic gluconeogenesis, and incretin release, but it is beyond the
scope of this review to discuss all processes involved in impaired glucose regulation in type 2 DM. Thus, only insulin resistance and β-cell dysfunction will be discussed here.

Insulin resistance is characterized by reduced sensitivity to insulin’s physiologic effects (Codario, 2011). The reduced sensitivity impairs glucose, lipid, and protein metabolism, as well as vascular endothelial function (Codario, 2011). The tissues demonstrating the highest degree of insulin resistance are skeletal muscle, liver, and adipose tissues. In addition to peripheral tissues, insulin resistance also occurs in the brain and central nervous system (Zhao & Alkon, 2001). Insulin resistance begins several years prior to development of type 2 DM, and scientists estimate that it may start 5 to 10 years prior to impaired glucose tolerance (IGT) (i.e., postprandial hyperglycemia) (Codario, 2011; Narayan, Imperatore, Benjamin, & Engelgau, 2002). Insulin resistance in the context of normal glucose tolerance is classified as pre-diabetes. In pre-diabetes, glucose tolerance remains normal in the face of insulin resistance because the β-cells initially are able to compensate by increasing insulin production (Codario, 2011). Factors such as genetics, diet, obesity, physical inactivity, aging can further worsen insulin resistance and accelerate the transition from pre-diabetes to type 2 DM (Codario, 2011).

Eventually, β-cell insulin secretion is not able to compensate for the worsening insulin resistance and postprandial hyperglycemia begins to develop. The underlying mechanisms leading to β-cell failure are very complex and can vary between individuals (Nolan et al., 2009). The β-cell dysfunction is characterized by delayed first phase insulin release and a blunting of the second phase insulin release (Codario, 2011). Impaired first phase insulin release results in a diminished suppression of hepatic glucose
production, which causes plasma glucose levels continue to rise (Codario, 2011). As type 2 DM progresses, β-cell dysfunction and insulin resistance worsen, blood glucose control becomes severely impaired, and individuals are at greater risk for developing microvascular (e.g., retinopathy, nephropathy, neuropathy) and macrovascular (e.g., coronary artery disease, cerebrovascular disease, peripheral artery disease) complications (Codario, 2011).

**Diagnosis and Treatment**

Type 2 DM can remain undiagnosed for many years because individuals often are asymptomatic or have minimal symptoms early in the disease process (ADA, 2012). An estimated one-fourth of individuals with type 2 DM may be unaware they have it (ADA, 2012). This statistic is alarming because early diagnosis is critical to preventing type 2 DM disease-related complications. To address this issue, the ADA (2012) now recommends that all adults over 45 years of age and obese adults over 18 years of age [body mass index (BMI) $\geq 25$ kg/m$^2$] be screened for type 2 DM and pre-diabetes.

There are several methods used to diagnosis type 2 DM. Until recently, fasting plasma glucose (FPG) levels and the oral glucose tolerance test (OGTT) were the primary diagnostic methods. Type 2 DM is diagnosed if an individual has FPG levels $\geq 126$ mg/dL or 2-hour post-OGTT plasma blood glucose levels $\geq 200$ mg/dL (ADA, 2012). More recently, glycosolated hemoglobin concentrations (HbA1c) has gained wider acceptance as a type 2 DM diagnostic method. In 2008, a committee comprised of members from the ADA, the European Association for the Study of Diabetes (EASD), and the International Diabetes Federation endorsed the use of HbA1c levels to diagnose type 2 DM (HbA1c $\geq 6.5\%$) (International Expert Committee, 2009).
HbA1c is a more convenient diagnostic measure than FPG and the OGTT because fasting is not required. In addition to being used as a diagnostic tool, HbA1c also is used to assess treatment efficacy, treatment adherence, and disease progression. HbA1c is a measure of the percent of hemoglobin in red blood cells that is glycosylated (i.e., bound by glucose) (Kilpatrick, Maylor, & Keevil, 1998). If an individual experiences frequent hyperglycemia, then more glucose binds to hemoglobin in the red blood cells, and glycosylated hemoglobin concentrations increase. Because red blood cells have a 60 to 120 day lifecycle, HbA1c concentrations at a given time are a reflection of average blood glucose levels over the previous few months. It is important to note that there are individual differences (e.g., gender, race, genetics) in the life cycle of red blood cells, making it difficult to estimate the exact time period that HbA1c reflects (Kilpatrick et al., 1998).

The primary goal of type 2 DM disease management is maintaining glycemic control (HbA1c < 7%). Current guidelines emphasize using a patient-centered approach that includes patient education, lifestyle modifications, and pharmacological therapies (ADA, 2012). Glycemic management can be very complex due to the availability of a wide variety of pharmacological therapies to control glucose regulation. There are treatment algorithms that provide clinicians with guidance on how to use the various pharmacological and lifestyle interventions, but there is limited empirical evidence to support the efficacies of the algorithms, which makes treating type 2 DM very complex for both the clinician and patient (Inzucchi et al., 2012). For a comprehensive outline of current type 2 DM management guidelines see The Standards of Medical Care in Diabetes – 2013 (ADA, 2012).
Acute Stress Physiology

Stress broadly can be defined as experiences (e.g., physical, psychological, social) that result in physiological, psychological, or behavioral changes in the organism (Cannon, 1932; Chrousos & Gold, 1992; McEwen, 1998; Selye, 1936). Stress is caused by stressors, which are internal or external stimuli that affect the stability of the internal physiological environment (i.e., stressors disrupt homeostasis) (Cannon, 1932). A stressor’s effects on physical and psychological well-being are dependent on the degree to which the stressor disrupts homeostasis, the individual’s perception of the stressor, the individual’s perceived ability to cope with the stressor, and the individual’s ability to adapt to the stressor and restore homeostasis (Cannon, 1932; Chrousos & Gold, 1992; McEwen, 2007; Selye, 1936).

When an individual encounters an acute psychosocial or physical stressor, it mounts a complex biobehavioral response that enables the individual to physiologically cope with the stressor (Cannon, 1932; Chrousos & Gold, 1992; McEwen, 2007; Selye, 1936). The acute stress response is necessary for survival because without it the individual would not be able to reestablish a state of physiological homeostasis. An effective physiological stress response involves the rapid activation of the stress response systems followed by quick termination after resolution of the stressful event (Chrousos & Gold, 1992; McEwen, 2007). The term allostasis is used to describe the dynamic processes and adoptions involved in the body’s attempt to reestablish physiological homeostasis following exposure to a stressor (McEwen, 1998).

The acute stress response involves the intricate coordination of numerous brain regions, neural circuits, and peripheral systems that work together to influence the
individual’s perception of the stressor and subsequent biobehavioral stress responses (Chrousos & Gold, 1992; Goldstein & Kopin, 2007; McEwen, 1998). It is beyond the scope of this chapter to discuss every physiological process involved in the stress response. This chapter will focus on the two principal stress systems, the HPA-axis and SNS (Cannon, 1932; McEwen, 1998; Selye, 1936; Tsigos & Chrousos, 2002).

The HPA-axis and SNS receive a variety of neurosensory (e.g., visual, auditory, nociceptive, visceral), blood-borne (e.g., toxins, drugs, bacteria) and limbic (e.g., emotion, memory) signals from the periphery and central nervous system (CNS) (McEwen, 2007; Selye, 1936; Tsigos & Chrousos, 2002). The control centers for these systems are located in the hypothalamus and the brain stem (Kvetnansky, Lu, & Ziegler, 2013; Tsigos & Chrousos, 2002). Additionally, the HPA-axis and SNS interact with higher brain centers. For example, the mesocorticolimbic dopaminergic system influences the anticipatory and affective components of stress perception and response; the amygdala and hippocampus are involved in the initiation, propagation, and termination of the stress response; and the arcuate nucleus is involved with pain perception (de Kloet et al., 2005). Thus, the perception of the stressor and the biobehavioral responses to the stressor are dependent on the complex interaction among many systems.

**HPA-Axis**

Activation of the HPA-axis occurs in response to a stressor, and is controlled by corticotropin releasing hormone (CRH) neurons located in the paraventricular nucleus (PVN) of the hypothalamus (Selye, 1936; Tsigos & Chrousos, 2002). The HPA-axis is activated as a result of CRH1 receptor (CRH1R) stimulation in the PVN (Tsigos &
Chrousos, 2002). Simulation of CRH1Rs leads to the release of CRH and arginine vasopressin (AVP) into hypophyseal portal vessel system circulation. CRH and AVP then travel downstream to the anterior pituitary, where CRH and to a lesser extent AVP stimulate the anterior pituitary gland to synthesize pro-opiomelanocortin (POMC) (Tsigos & Chrousos, 2002). POMC subsequently is converted into adrenocorticotropic hormone (ACTH) (Tsigos & Chrousos, 2002). POMC also is converted into several other peptides including β-lipotropin, β-Melanocyte Stimulating Hormone (β-MSH) and β-endorphin (de Kloet et al., 2005; Tsigos & Chrousos, 2002). Besides ACTH, β-endorphin is the only other anterior pituitary hormone specifically involved in the stress response (de Kloet et al., 2005).

After it is synthesized from POMC, ACTH is released from the anterior pituitary into systemic circulation where it stimulates the release of glucocorticoids (e.g., cortisol) from the adrenal cortex. The glucocorticoid, cortisol, is the end product of HPA-axis activation in humans and plays a critical role in homeostasis and the stress response (McEwen, 2007; Tsigos & Chrousos, 2002). Circulating free cortisol binds to mineralocorticoid receptors and glucocorticoid receptors throughout the periphery and in the CNS, and affects the functioning of nearly every major organ in the body (de Kloet et al., 2005; Sapolsky, Romero, & Munck, 2000).

An important factor affecting circulating free cortisol levels in humans is the plasma protein, corticosteroid-binding globulin (CBG), which is both a transporter and reservoir of cortisol (Moisan, 2013). Under resting, non-stress conditions, the majority of plasma cortisol (80-90%) is bound to CBG, and only 5 to 10% of plasma cortisol is free and bioactive (Gagliardi, Ho, & Torpy, 2010). CBG is a negative acute phase protein and...
levels decrease in response to stress and inflammation (Gagliardi et al., 2010). A decrease in CBG levels results in increased levels of free, bioactive cortisol. Thus, increased cortisol levels following stressor exposure are due to increased cortisol release from the adrenal cortex as well as decreased levels of CBG.

In addition to mediating the stress response, the HPA-axis also has a diurnal rhythm in humans with cortisol levels typically the highest in the morning and the lowest at night (Sapolsky et al., 2000). The physiological effects of low levels of cortisol (i.e., nighttime levels and non-stressed levels) are primarily mediated by mineralocorticoid receptors, whereas the physiological effects of high levels of cortisol (i.e., morning levels and stress levels) are primarily mediated by glucocorticoid receptors (Sapolsky et al., 2000). Cortisol produces different physiological effects depending on the type of receptors it binds to in the periphery or CNS. Typically, cortisol only binds to glucocorticoid receptors when mineralocorticoid receptors are saturated, which only occurs when there are high levels of circulating cortisol (e.g., during times of stress, in the morning) (Sapolsky et al., 2000).

Cortisol has numerous physiological effects that support the stress response including mobilization of energy stores to meet increased metabolic demands, suppression of the local inflammatory response, and suppression of the immune response (de Kloet et al., 2005; Sapolsky et al., 2000). Cortisol also exerts effects in the CNS and influences learning, memory, and emotion (Sapolsky et al., 2000). Furthermore, it is responsible for the termination of the HPA-axis stress response and plays a key role in the HPA-axis negative feedback loop (de Kloet et al., 2005). More specifically, biologically active (i.e, unbound) cortisol in systemic circulation travels upstream to the
hypothalamus, anterior pituitary gland, and hippocampus where it stimulates glucocorticoid receptors to terminate the HPA-axis stress response by inhibiting the release of CRH and ACTH (de Kloet et al., 2005).

In addition to cortisol, other neuroendocrine hormones such as dehydroepiandrosterone (DHEA) and its more stable sulfate ester, DHEA-S, are involved in the HPA-axis stress response. DHEA and DHEA-S are the most abundant steroid hormones in the human body (Maninger, Wolkowitz, Reus, Epel, & Mellon, 2009). Only DHEA is biologically active, and DHEA-S is considered to be the reservoir of DHEA (Nguyen & Conley, 2008). Circulating concentrations of DHEA-S are higher than DHEA because DHEA has a shorter half-life and faster clearance rate than DHEA-S (Nguyen & Conley, 2008).

DHEA(S) primarily are synthesized in the zona reticularis layer of the adrenal cortex, but also are synthesized in the gonads (i.e., ovaries, testes) (Maninger et al., 2009). DHEA is synthesized from pregnenolone after cholesterol is converted into pregnenolone. Pregnenolone is converted into DHEA by the enzyme cytochrome P450c17, and then the enzyme hydroxysteroid sulfotransferase catalyzes the conversion of DHEA to DHEA-S (Maninger et al., 2009; Miller, 2002). Interestingly, rodents are not able to synthesize DHEA(S) in the adrenal cortex because they do not have a zona reticularis layer. Only primates (human and non-human) have a zona reticularis layer in their adrenal glands (Maninger, Capitanio, Mason, Ruys, & Mendoza, 2010). Thus, rodents have very low levels of circulating DHEA(S) compared to humans because they only synthesize and secrete DHEA(S) from their gonads (van Weerden, Bierings, van Steenbrugge, de Jong, & Schröder, 1992).
Like glucocorticoids, ACTH stimulates the release of DHEA(S) from the adrenal cortex. Studies with human and non-human primates have found that DHEA(S) concentrations have a diurnal rhythm similar to cortisol, with higher levels in the morning and lower levels in the evening (e.g., Goncharova, Shmaliy, Bogatyrenko, & Koltover, 2006; Klein, et al., under review; Maninger et al., 2010). In addition to having a diurnal rhythm, DHEA(S) levels also increase following acute stress exposure (e.g., Izawa et al., 2008; Lennartsson, Kushnir, Bergquist, & Jonsdottir, 2012; Maninger et al., 2010; Morgan et al., 2004; Pico-Alfonso et al., 2007)

There is suggestive evidence that DHEA(S) may have positive physiological benefits in humans. DHEA(S) are thought to have cardio-protective, antiobesity, antidiabetic, and immune-enhancing effects (von Mühlen, Laughlin, Kritz-Silverstein, & Barrett-Connor, 2007). Unlike cortisol, which is a catabolic steroid, DHEA(S) are precursors to anabolic steroids, such as testosterone (Labrie, 2004). Studies have shown that DHEA has anti-inflammatory effects and decreases pro-inflammatory cytokine production in rats (tumor necrosis factor-α, interleukin -6) and humans (interleukin -6). DHEA(S) levels also increase following stressor exposure, and this increase is thought to be protective against the negative effects of stress [i.e., DHEA(S) have stress buffering effects]. However, the mechanisms involved in DHEA(S)’ physiological effects are poorly understood. To date, scientists have not been able to identify a CNS steroid receptor with high affinity for DHEA(S) (Maninger et al., 2009).

Scientists also speculate that DHEA(S) have anti-glucocorticoid effects, but the underlying mechanisms involved are not known. There is evidence that DHEA may affect the enzymes 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 and type 2.
11β-HSD type 1 is the enzyme that catalyzes the conversion of cortisone (inactive glucocorticoid) to cortisol (active glucocorticoid), and 11β-HSD type 2 is the enzyme that catalyzes the conversion of cortisol to cortisone. DHEA-treated rats and mice have higher 11β-HSD type 2 enzyme activity (Balazs, Schweizer, Frey, Rohner-Jeanrenaud, & Odermatt, 2008) and lower 11β-HSD type 1 activity (Apostolova, Schweizer, Balazs, Kostadinova, & Odermatt, 2005). These results suggest that DHEA may decrease circulating cortisol levels by affecting 11β-HSD type 1 and type 2 enzyme activities. Additional research is needed to further elucidate the mechanisms involved in stress buffering effects of DHEA(S).

**Sympathetic Nervous System**

The SNS and the parasympathetic nervous system (PNS) are the two branches of the autonomic nervous system, and they play key roles in homestatic regulatory processes (Kvetnansky et al., 2013). The SNS is the dominant branch during stress, whereas the PNS is the dominant branch during resting conditions. Activation of the SNS following stressor exposure commonly is referred to as the “fight or flight” response because activation increases the organism’s ability to physiologically cope with an acute threat (e.g., increased heart rate, increased respiratory rate, dilation of pupils, shunting of blood to muscles, increased cognitive functioning) (Cannon, 1932; McEwen, 2007; Tsigos & Chrousos, 2002).

The SNS innervates tissues in almost every organ system of the body and regulates a diverse set of functions including the stress response. The two primary SNS components involved in the stress response are the SAM-axis and the sympathoneural system (Kvetnansky et al., 2013). The catecholamines, norepinephrine (NE) and
epinephrine (EPI), are the chief neurohormones/neurotransmitters involved in SNS stress responses. NE is the primary catecholamine of the sympathoneural system, and EPI is the primary catecholamine of the SAM-axis. The release of catecholamines from both the SAM-axis and sympathoneural system involve descending pre-ganglionic fibers (McCorry, 2007). In the sympathoneural system, sympathetic preganglionic fibers originating at the T1 to L2 levels of the spinal cord synapse with post-ganglionic fibers that originate in the sympathetic ganglia (McCorry, 2007). Preganglionic neurons release acetylcholine, which then stimulates nicotinic acetylcholine receptors on the postganglionic neurons to release NE. NE then stimulates adrenergic receptors on effector tissues of the SNS (McCorry, 2007)

In the SAM-axis, sympathetic preganglionic fibers originating at the T5 to T9 levels of the spinal cord innervate the chronomaffin cells in the adrenal medulla and stimulate the release of EPI and to a lesser extend NE (Kvetnansky et al., 2013; McCorry, 2007). Catecholamines secreted from the adrenal medulla are released into to systemic circulation, and thus affect a wider array of tissues compared to the NE released following sympathoneural stimulation. In other words, SAM-axis catecholamines affect tissues that are not innervated by sympathetic fibers such as that airway smooth muscle and adipose tissue. Thus, catecholamines released from the adrenal medulla have wider effects than those released by sympathoneural stimulations (McCorry, 2007; Wong et al., 2012).

Circulating levels of catecholamines consist of catecholamines secreted by the adrenal medulla and also catecholamines that “spill over” or diffuse into systemic circulation (i.e., catecholamines that are not locally metabolized or reabsorbed)
(McCorry, 2007). During rest, levels of circulating plasma catecholamines are typically very low because a majority of catecholamines are locally reabsorbed or metabolized and do not spillover into systemic circulation. However, following stress exposure, plasma levels of both NE and EPI significantly increase (Goldstein, 2013).

The SAM-axis and sympathoneural systems do not function as a single unit in response to stressors (Kvetnansky et al., 2013). In other words, stressors can have different effects on SAM-axis and sympathoneural system functioning. The sympathoneural system is more involved in the response to stressors that affect blood pressure homeostasis such as cold exposure, exercise, hypotension, hemorrhage, and hypovolemia (Goldstein, 2013; Kvetnansky et al., 2013; Kvetňanský et al., 1995). Whereas, the SAM-axis plays a larger role in the SNS response to more global stressors including emotional stressors and metabolic stressors (e.g., hypoglycemia) (Goldstein, 2013; Kvetnansky et al., 2013; Kvetňanský et al., 1995). Additionally, the relationship between the SAM-axis and the HPA-axis likely is stronger than the relationship between the sympathoneural system and HPA-axis. For example, ACTH release is highly correlated with EPI release following stress exposure (Goldstein & Kopin, 2008). This close association likely is because the HPA-axis indirectly controls the SAM-axis stress response by stimulating phenylethanolamine N–methyltransferase (PNMT) to synthesize EPI from NE via (Goldstein, 2013).

Activation of the SAM-axis occurs in response to a stressor and is controlled by central catecholaminergic neurons in the locus coeruleus in the pons, which is part of the brain stem (Tsigos & Chrousos, 2002). It is important to note that there are neural connections between the PVN neurons and central catecholaminergic neurons, which
enable the HPA-axis and SAM-axis to interact with each other. Furthermore, the HPA-axis and SAM-axis stress responses interact in the periphery during the stress response. Like the HPA-axis, CRH and ACTH play key roles in the activation of the SAM-axis by stimulating the secretion of EPI from the adrenal medulla (Goldstein, 2013).

**Immune System**

The immune system interacts with both the SAM-axis and HPA-axis during the stress response. During the early phase of the stress response, the SAM-axis stimulates immune processes in the periphery and CNS (Padgett & Glaser, 2003). In the periphery, immune system activation results in immune cell proliferation, pro-inflammatory cytokine production (e.g., tumor necrosis factor-α, interleukin -6), antibody production, cytolytic activity, and cell trafficking (Padgett & Glaser, 2003). In the CNS, the SAM-axis stimulates microglia to produce pro-inflammatory cytokines (O’Connor et al., 2003).

The HPA-axis also interacts with the immune system during the stress response. Glucocorticoids released by the HPA-axis in response to stress exert immunosuppressive and anti-inflammatory effects (Sorrells & Sapolsky, 2007). The immunosuppressive effects of glucocorticoids are counter intuitive because it does not seem adaptive for the immune system to be suppressed during times of stress when proper immune functioning is essential to survival (Frank, Watkins, & Maier, 2011). Scientists have hypothesized that the immunosuppressive effects of glucocorticoids assist the organism in recovering from the stressor (Munck, Guyre, & Holbrook, 1984; Sapolsky et al., 2000). In other words, glucocorticoids dampen the immune stimulating effects of the acute stress response, which enables the organism to recover from the stressor.
Chronic Stress and Allostatic Load

Although the catabolic and immunosuppressieve effects of the stress response are protective and adaptive in the short-term, they have pathological consequences in the long-term (McEwen, 1998; Wong et al., 2012). Chronic stress occurs when an individual is exposed to physical or psychosocial stressors (present or remembered) for prolonged periods of time (continuous exposure or repeated exposure) (Baum, Cohen, & Hall, 1993). Chronic stress is associated stress system dysregulations and is linked to the pathogenesis of many disease states including cardiovascular disease, depression, hypertension, and diabetes (McEwen, 2007; Wong et al., 2012).

Chronic stress exposure leads to chronic activation of the stress systems, which ultimately lead to long-term impairments in stress system functioning (e.g., not activated when needed, not turned off following termination of stressor) (Baum et al., 1993; McEwen, 1998; Miller, Chen, & Zhou, 2007). The HPA-axis and cortisol are thought to play a greater role in the long-term consequences of chronic stress, and as a result a majority of research studies examining the effects of chronic stress on stress physiology have focused on the effect of chronic stress on the HPA-axis (Wong et al., 2012). However, the SNS also is affected by chronic stress exposure, and chronic stress exposure leads to a state of sympathoneural and SAM-axis hyperactivity (Baum et al., 1993; Wong et al., 2012).

Most research on chronic stress and stress system functioning has focused on the effect of chronic stress on the HPA-axis (Strahler, Berndt, Kirschbaum, & Rohleder, 2010). Studies examining chronic stress commonly assessed HPA-axis functioning by measuring daily cortisol output or by assessing the functioning of HPA-axis negative
feedback loop. Fewer studies have examined HPA-axis response to acute stressors in chronically stressed individuals. A recent meta-analysis examining HPA-axis activity and chronic stress reported mixed results with some studies suggesting chronic stress leads to HPA-axis hypoactivity (e.g., low daily cortisol output) and some studies suggesting HPA-axis hyperactivity (e.g., high daily cortisol output) (Miller et al., 2007). Miller and colleagues (2007) determined that the variability in findings between studies could be explained by the variability in type of chronic stressor in the studies. They conclude that chronic stressors that still were present, perceived as uncontrollable, and physical in nature were associated with increased daily cortisol secretion and diminished diurnal cortisol pattern, which are indicative of HPA-axis dysregulation (Miller et al., 2007).

Fewer studies have specifically examined the effects of chronic stress exposure on SNS reactivity in humans. Most of the work examining the effect of chronic stress on SNS functioning has focused on sympathoneural effects (e.g., blood pressure, heart rate). For example, data from a study examining the relationship between social class and cardiovascular outcomes found decreased heart rate variability in individuals of lower social class, which is indicative of autonomic nervous system dysregulation (Lampert, Ickovics, Horwitz, & Lee, 2005). Similar results were reported from a study examining the effect of chronic psychosocial stress (i.e., daily hassles in the past month) on heart rate variability (Schubert et al., 2009). This study found that higher levels of chronic stress were associated with decreased heart rate variability (Schubert et al., 2009). Findings from these studies provide suggestive evidence that chronic psychosocial stress is associated with decreased heart rate variability. Studies also have examined the
relationship between chronic psychosocial stress and other measures of SAM-axis functioning. A longitudinal study found that caregivers of dementia patients had age-independent decreases in β2-adrenergic receptor sensitivity and that increases in caregiver stress from baseline were associated with decreased receptor sensitivity (Mausbach et al., 2008). These findings provide suggestive evidence that chronic stress is associated with sympathetic nervous system hyperactivity.

There also is suggestive evidence that chronic stress affects SAM-axis function. Data from studies using rodent models of chronic stress indicate that chronic stress exposure increases the synthesis and secretion of epinephrine and decreases the synthesis of NE from the adrenal medulla as a result of increased PNMT activity (Wurtman, 2002). NE is converted into EPI by PNMT, and PNMT activity in the adrenal medulla is controlled by ACTH and glucocorticoids. In chronic stress states characterized by increased levels of ACTH and glucocorticoids, the adrenal medulla increases the synthesis and secretion of EPI, which decreases adrenal medulla NE stores (Wurtman, 2002). Furthermore, studies examining chronic stress in humans have shown that exposure to chronic environmental stressors (e.g., nuclear disasters) is associated with increased urinary EPI and NE levels (Baum, Gatchel, & Schaeffer, 1983). Similarly, a study examining chronic stress in caregivers of individuals with Alzheimer’s Disease found that caregivers had significantly higher plasma catecholamine levels compared to healthy controls (Mills et al., 1997).

The pathophysiological effects of chronic stress vary considerably between types of chronic stressors and between individuals. Multiple factors such as the intensity of the stressor, duration of the stressor, and the individual’s ability to cope with the stressor
influence how chronic stress affects health and well-being (Baum et al., 1993; McEwen & Seeman, 1999). The allostatic load model is a conceptual framework used to assess the cumulative effects of chronic stress exposure (McEwen, 1998). The model proposes that repeated and prolonged exposure to stressors and stress system activations lead to dysregulations of multiple physiological systems (e.g., neurological, cardiovascular, metabolic, immune, neuroendocrine, autonomic) (McEwen & Seeman, 1999; McEwen, 1998).

As previously discussed, an effective biobehavioral stress response involves the rapid activation of the stress response systems followed by quick termination after resolution of the stressful event (Chrousos & Gold, 1992; Goldstein & Kopin, 2007; McEwen, 2007). Allostatic load occurs when the processes involved in the stress response are impaired. McEwen (1998) proposed four stress response profiles associated with chronic stress and allostatic load: 1) repeated exposure to multiple stressors; 2) lack of adaptation to stressors (i.e., failure to habituate to stressors; 3) prolonged response following stressor termination (i.e., impaired recovery); 4) dampened stress system response (i.e., impaired reactivity) and hyperactivity of other physiologic systems (e.g., heightened pro-inflammatory cytokine response).

The pathophysiological consequences of chronic stress exposure are due to increases in multiple biophysiological stress mediators (McEwen & Seeman, 1999; McEwen, 1998; Wong et al., 2012). The primary mediators of allostatic load include stress neurohormones (e.g., cortisol, epinephrine), pro-inflammatory cytokines (e.g., interleukin-6, tumor necrosis factor-α), and anti-inflammatory cytokines (e.g, interleukin-4, interleukin-10) (Juster, McEwen, & Lupien, 2010; McEwen, 2007). Primary mediators
have protective effects in the short-term because they help the individual physiologically cope with the stressor. However, they can have damaging effects when chronically elevated (McEwen, 2007; Wong et al., 2012).

There is an adaptive purpose for the stress system dysregulations seen in chronically stressed populations (Juster et al., 2010; McEwen, 2007; Wong et al., 2012). For example, in order to enhance survival during periods of chronic stress, it is necessary to maintain high cortisol levels, yet high cortisol levels result in HPA-axis adaptations that disrupt the cortisol negative feedback loop (McEwen, 2007). In the short term, high levels of circulating cortisol enable the individual to physiologically respond to the chronic stressor, but, over time, elevated cortisol levels can have deleterious effects on health (e.g., depression, cardiovascular disease) (McEwen, 2007). Prolonged cortisol elevations can alter physiological responses to acute stressors due to chronic stress-related dysregulations in the stress systems. More specifically, chronic stress exposure leads to diminished acute stress reactivity and also negatively influences the emotional and cognitive appraisal of stressors due to glucocorticoid receptors dysregulations in the hippocampus and amygdala (McEwen, 2007).

Over time, chronic elevations in primary stress mediators and dysregulations in stress system function lead to elevations in secondary mediators of the metabolic (e.g., elevated insulin, glucose, total cholesterol, triglycerides, low density lipoprotein), cardiovascular (e.g., elevated systolic blood pressure, elevated diastolic blood pressure), and immune (e.g., elevated C-reactive protein, elevated interleukin-6, elevated TNF-α) systems (Juster et al., 2010). Ultimately, the physiological dysregulations caused by primary and secondary mediators can lead to chronic disease states (e.g., type 2 DM,
cardiovascular disease, metabolic syndrome) (McEwen & Seeman, 1999). For example, chronic elevations in cortisol can lead to insulin resistance, visceral adiposity, dyslipidemia, impaired glucose tolerance, and hypertension (Chrousos & Gold, 1992; Tsigos & Chrousos, 2002). Chronic elevations in catecholamines result in elevated blood pressure, heart rate, endothelial dysfunction, and increased coagulation factors (Hauss, Bauch, & Schulte, 1990; Mavrogiannis, Trambakoulos, Boomsma, & Osmond, 2002; Plante, 2002). Furthermore, chronic stress system activation leads to chronic elevation elevations in pro-inflammatory cytokines, which further damage vascular walls and accelerate the progression of atherosclerosis, coronary artery disease, and diabetes. Pro-inflammatory cytokines also further activate the HPA-axis and SAM-axis, which perpetuates the cycle of chronic stress (Frostegård, 2013).

**Type 2 Diabetes Mellitus and Stress**

There is suggestive evidence that psychosocial stress contributes to the development and progression of type 2 DM (e.g., Gonder-Frederick et al., 2009; Mommersteeg et al., 2012; Pouwer et al., 2010; Toshihiro et al., 2008). Type 2 DM also is considered to be a chronic metabolic stressor that impairs stress system functioning and is associated with abnormal levels of primary and secondary allostatic load mediators (Reagan, 2012). Thus, there is a bi-directional relationship between type 2 DM and stress (i.e., stress affects type 2 DM pathophysiology and type 2 DM affects stress system functioning). The subsequent sections will further discuss the bi-directional relationship between stress and type 2 DM.
Psychosocial Stress and Type 2 DM

Psychosocial stress has been implicated in the development and progression of type 2 DM (Mommersteeg et al., 2012; Pouwer et al., 2010; Toshihiro et al., 2008). Results from several longitudinal studies demonstrate that psychological stress is associated with future onset of type 2 DM. For example, a study using data from the British Household Panel Survey, which is a large prospective cohort study, examined the relationship between psychological distress at baseline and future development of type 2 DM during the 18-year follow up period. Findings from this study revealed that higher baseline levels of psychological distress were associated with a 33% increased risk of developing diabetes in both men and women (Mommersteeg et al., 2012). Similarly, a study using data from the Copenhagen City Heart Study found that men in the high perceived stress group were twice as likely to develop diabetes at follow-up compared to men in the low perceived stress group (Rod, Grønbaek, Schnohr, Prescott, & Kristensen, 2009). Interestingly, high perceived stress at baseline was not associated with future type 2 DM in women suggesting that there are possible sex differences in the effects of psychosocial stress on type 2 DM pathogenesis (Rod et al., 2009). In contrast, a population-based prospective cohort study of women found that stressful life events at baseline predicted the development of metabolic syndrome, which is a major risk factor for diabetes, during the 15 year follow up period (Räikkönen, Matthews, & Kuller, 2007). These studies demonstrate that there is a link between psychosocial stress and type 2 DM onset, but additional research is needed to further explore the differential effects between men and women.
Stress also can affect glucose homeostasis and glucose control, and thus can contribute to progression of type 2 DM. HPA-axis and SNS stress mediators (e.g., cortisol, catecholamines) affect glucose control by mobilizing energy stores in the body. Cortisol increases glucose levels by enhancing gluconeogenesis in the liver and antagonizing the anabolic effects of numerous hormones (e.g., growth hormone, thyroid hormone, insulin, sex steroids). Furthermore, chronic stress exposure can result in hypercortisolemia, which affects glucose control by increasing visceral obesity, increasing insulin resistance, and increasing pro-inflammatory cytokine levels (e.g., interleukin-6, tumor necrosis factor-α) (Kyrou & Tsigos, 2009). The catecholamines also affect glucose homeostasis by affecting glucose metabolism. They directly affect glucose metabolism by increasing gluconeogenesis and glycogenolysis. They indirectly affect glucose homeostasis by inhibiting insulin secretion, decreasing insulin action, and stimulating cortisol and glucagon release. (Barth et al., 2007). Although there is evidence that stress contributes to the pathophysiology of type 2 DM, there has been little research on the underlying stress physiological processes involved in the relationship between psychosocial stress and type 2 DM.

**Type 2 DM and Stress Physiology**

There is evidence that type 2 DM is associated with stress system dysregulations. There likely is a bi-directional relationship between stress system dysregulations and type 2 DM. More specifically, stress system dysregulations may contribute to the pathogenesis of type 2 DM, and type 2 DM disease processes may further impair stress system functioning (Alrefai, Allababidi, Levy, & Levy, 2002; Surwit, Schneider, & Feinglos, 1992). Due to the complex nature of type 2 DM pathophysiology, it has been difficult for
scientists to identify the exact patterns of stress system impairments and to disentangle the mechanism involved in these impairments (Alrefai et al., 2002). However, researchers speculate that chronic autonomic nervous system activation may lead to HPA-axis and SAM-axis hyperactivity. Stress system hyperactivity results in reduced negative feedback sensitivity to glucocorticoids at different levels of the HPA-axis (e.g., pituitary, hypothalamus, hippocampus), increased secretion of and sensitivity to CRH, and changes in 11β-HSD enzyme activity (Alrefai et al., 2002; Surwit et al., 1992).

Data from studies using rodent models of diabetes indicate that type 2 DM is characterized by elevated basal glucocorticoid levels, elevated ACTH responses to stressors, blunted glucocorticoid responses to stressors, and delayed recovery from stressors in male rodents (i.e., HPA-axis does not turn off following termination of stressor) (e.g., Chan, Inouye, Vranic, & Matthews, 2002; Magariños & McEwen, 2000; Plotsky, Thrivikraman, Watts, & Hauger, 1992; Winocur et al., 2005). Scientists also have found that male rats with experimentally-induced type 2 DM have reduced adrenal cortex mass and impaired glucocorticoid synthesis, which may be a pathway involved in the diminished glucocorticoid response and increased ACTH response to stressors (Noguchi, Ohno, & Aoki, 2007; Takao, Tojo, Nishioka, & Hashimoto, 2000).

Human studies examining HPA-axis functioning in type 2 DM patients report that type 2 DM is associated with dysregulated HPA-axis functioning. Overall, data from human studies indicate that type 2 DM is characterized by elevated basal cortisol levels, loss of negative feedback control, and a flattened diurnal cortisol rhythm (Cameron et al., 1984; Chiodini et al., 2006; Roy et al., 1998). More specifically, studies have shown that individuals type 2 DM have elevated cortisol levels following a dexamethasone
suppression test, which is indicative of loss of HPA-axis negative feedback control (Chiodini et al., 2006; Cameron et al., 1984; Hudson et al., 1984). Studies also have found that type 2 DM patients have elevated plasma ACTH levels, which may further demonstrate a loss of negative feedback control or impaired adrenal glucocorticoid synthesis (Cameron et al., 1984; Vermes, Steinmetz, Schoorl, van der Veen, & Tilders, 1985). Furthermore, when compared to healthy individuals, type 2 DM patients have a flattened diurnal cortisol rhythm characterized by elevated afternoon and evening cortisol concentrations (Lederbogen et al., 2011). Findings from human studies on HPA-axis function in individuals with type 2 DM are consistent with the findings from rodent studies and provide strong evidence that individuals with type 2 DM have dysregulated HPA-axis function.

Human studies also have found that circulating levels of DHEA(S) are decreased in type 2 DM. For example, data from observational studies have found that men with type 2 DM have significantly lower plasma DHEA levels compared to healthy men (e.g., Barrett-Connor, Khaw, & Yen, 1986; Yamauchi et al., 1996). Another study found that plasma DHEA levels were inversely related to insulin resistance, such that lower levels of DHEA were associated with higher insulin resistance (Kawano et al., 2003). Furthermore, decline in DHEA-S levels are associated with development of type 2 DM. For example, data from a population cohort study demonstrated that serum DHEA-S levels predicted the development of type 2 DM 5 years later, such that men with a greater decrease in DHEA-S levels form baseline to follow-up were more likely to develop type 2 DM (Kameda et al., 2005).
Only one study to date has investigated the effects of an acute psychosocial stressor on HPA-axis in individuals with type 2 DM (Faulenbach et al., 2012). The purpose of this study was to examine the effects of an acute psychosocial stressor on glucose levels of men and women with type 2 DM. The researchers also assessed salivary cortisol levels, blood pressure, and heart rate before and after the psychosocial stressor, which consisted of a speech and math task (Trier Social Stress Test). They found that salivary cortisol levels, heart rate, and blood pressure increased significantly from baseline to post-stressor. However, it is difficult to draw conclusions about the stress system response patterns from these findings because this study did not include a group of healthy, non-type 2 DM participants for comparison. Although, the type 2 DM participants had significant increases in cortisol, heart rate, and blood pressure, it is possible that the type 2 DM participants would have had a dampened stress system responses (i.e, dampened reactivity and recovery) compared to healthy individuals. Thus, additional research is needed to further explore the effect of acute psychosocial stress on stress system response patterns in individuals with type 2 DM.

The findings on SAM-axis and sympathoneural functioning in type 2 DM are less consistent than the findings on the HPA-axis. Although it is accepted that type 2 DM is associated with increased SNS activity as demonstrated by elevated blood pressure and heart rate, findings on SAM-axis and adrenal medulla secretion of EPI and NE are inconsistent. Some studies have found increased plasma EPI and NE in individuals with type 2 DM (Bottini et al., 1995; Cryer et al., 1978), while other studies have found no difference in circulating catecholamine levels between individuals with type 2 DM and healthy controls (e.g., Beretta-Piccoli, Weidmann, Ziegler, Glück, & Keusch, 1979;
There is evidence that decreased epinephrine secretion from the adrenal medulla may contribute to the development of insulin resistance because lower levels of circulating epinephrine are associated with higher insulin resistance (Landsberg, 1999; Ward et al., 1994). There also is suggestive evidence that type 2 DM is associated with decreased SAM-axis response to hypoglycemia, which is a metabolic stressor (Ramanathan & Cryer, 2011).

Laboratory based studies have also found that acute stress exposure is associated increased sympathoneural function in individuals with type 2 DM (e.g., blood pressure, heart rate). Studies have revealed that systolic blood pressure and heart increased significantly following acute psychosocial stress exposure (Goetsch et al., 1993; Goetsch, Wiebe, Veltum, & van Dorsten, 1990; Sung et al., 1999). However, no studies to date have examined the effects of acute psychosocial stress on SAM-axis function (e.g., EPI, salivary alpha-amylase). Additional research is needed that further investigates SAM-axis alternations in individuals with type 2 DM. To date, no studies have assessed both SAM-axis and HPA-axis responses to an acute psychosocial stressor in individuals with type 2 DM.

**Type 2 DM and Allostatic Load**

Individuals with type 2 DM are chronically stressed due to the physical and psychological burdens of the disease. As discussed earlier in this chapter, type 2 DM affects multiple physiological systems, and clearly is a chronic metabolic stressor. Type 2 DM also is a chronic psychological stressor and is associated with increased levels of psychological distress (Anderson, Freedland, Clouse, & Lustman, 2001; Grigsby, Anderson, Freedland, Clouse, & Lustman, 2002; Li et al., 2009). Given that individuals
with type 2 DM are chronically stressed, they likely have elevated levels of primary and secondary mediators of allostatic load, which put them at increased risk for additional chronic diseases.

There is evidence that type 2 DM is associated with abnormal levels of many primary and secondary mediators of allostatic load. As discussed in the previous section, individuals with type 2 DM have increased basal levels of cortisol and decreased basal levels of DHEAS. Individuals with type 2 DM also have elevated levels of pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-6, and interleukin-1β (Alexandraki et al., 2006; Guest, Park, Johnson, & Freund, 2008). Increased levels of interleukin-6 and CRP also have been linked to the development of diabetes (Barzilay et al., 2001; Doi et al., 2005; Pradhan, Manson, Rifai, Buring, & Ridker, 2001). Furthermore, there is suggestive evidence that increased inflammation begins years prior to type 2 DM diagnosis (Alexandraki et al., 2006). Individuals with type 2 DM also have abnormal levels of secondary mediators of allostatic load including increased plasma insulin levels, increased plasma glucose levels, increased triglycerides levels, low HDL cholesterol levels, and increased LDL cholesterol levels compared to individuals without type 2 DM (Codario, 2011; Kannel, 2011).

Type 2 Diabetes Mellitus, Allostatic Load, and Stress Physiology

Overall, the information presented in this chapter provides suggestive evidence that individuals with type 2 DM have dysregulated stress systems. Stress system dysregulations may make them more vulnerable to acute psychosocial stressors because they are not able to physiologically respond to and recover from acute psychosocial stressors. Additionally, allostatic load, which is a cumulative index of chronic stress, may
The relationship between type 2 DM and stress system dysregulations. More specifically, individuals with higher allostatic load levels likely have greater impairments in stress system functioning, which may make them more vulnerable to acute psychosocial stressors and puts them at greater risk for disease-related complications. To date, there have been few studies specifically investigating the effects of acute psychosocial stressors on HPA-axis and SAM-axis function in individuals with type 2 DM. This dissertation addressed the gap in the literature by examining the effects of an acute psychosocial stressor (i.e., Trier Social Stressor Task) on both HPA-axis and SAM-axis function in men with type 2 DM. The primary aim of this dissertation is to investigate the effects of an acute laboratory psychosocial stressor on multiple stress physiological parameters, and the secondary aim of this dissertation is to explore the relationship between allostatic load and stress physiology.
CHAPTER 2: METHODS

Overview

This study used a mixed between- and within subjects-design to examine the effects of type 2 DM and acute psychosocial stress on stress physiology in men (N = 34). The between-subjects factor was type 2 DM status (healthy vs. type 2 DM diagnosis) and the within-subjects factor was time (baseline, stress, 15-minutes post-stressor, 45-minutes post-stressor). Primary dependent measures included biomarkers of the HPA-axis and SAM-axis (cortisol, DHEA-S, salivary alpha-amylase) and allostatic load parameters (cardiovascular, metabolic, neuroendocrine, anthropometric, immune measures). Eligible participants attended 2 laboratory sessions, an initial 1-hour health screening session to confirm eligibility and a 3-hour test session to assess stress reactivity and stress recovery in response to an acute psychosocial stressor (Trier Social Stress Test). HPA- and SAM-axis functioning were assessed to determine if type 2 DM participants had alterations in their stress physiologies compared to healthy control participants. Additionally, allostatic load parameters were assessed to determine if type 2 DM participants had higher levels of allostatic load compared to healthy participants and if allostatic load was associated with alterations in stress physiology. All of the following procedures were reviewed and approved by the Pennsylvania State University Institutional Review Board (IRB #39698) (see Appendix A for IRB approval letter).

Hypotheses

Hypothesis 1: Salivary Free Cortisol

Overview. I hypothesized that the salivary free cortisol profiles would differ significantly between the type 2 DM group and healthy control group. Specifically, the
type 2 DM group would have higher baseline cortisol levels, dampened cortisol reactivity, and dampened cortisol recovery in response to the Trier Social Stress Test (TSST) task compared to the healthy control group.

**Rationale.** The TSST reliably produces significant increases in cortisol levels in healthy individuals (Kirschbaum, Pirke, & Hellhammer, 1993). Whereas in many clinical populations (e.g., depression, chronic fatigue syndrome, fibromyalgia, asthma), the cortisol response to the TSST is significantly smaller than healthy participants (e.g., Buske-Kirschbaum, von Auer, Krieger, Weis, Rauh, Hellhammer, 2003; Burke, Davis, Otte, Mohr, 2005; Kudielka & Wuest, 2010; Wingenfeld, Heim, Schmidt, Wagner, Meinschmidt, Hellhammer, 2008). Several studies also have found that type 2 DM patients have HPA-axis dysregulations characterized by elevated basal cortisol levels, loss of cortisol negative feedback control, and flattened diurnal cortisol rhythm, which may contribute to a dampened cortisol response to the TSST (Cameron, Kronfol, Greden, & Carroll, 1984; Chiodini et al., 2006; Lederbogen et al., 201; Roy, Roy, & Brown, 1998).

**Hypothesis 2: Salivary DHEA-S**

**Overview.** I hypothesized that salivary DHEA-S profiles would differ significantly between the type 2 DM group and the healthy control group. Specifically, the type 2 DM group would have lower baseline DHEAS levels, dampened DHEAS reactivity, and dampened DHEAS recovery in response to the TSST compared to the healthy control group.

**Rationale.** The TSST produces significant increases in DHEA-S levels in healthy individuals (Izawa et al., 2008; Lennartsson et al., 2012). The DHEA-S stress response
has not been examined in clinical populations such as diabetes. However, there is suggestive evidence that prolonged stress exposure inhibits the production of DHEA-S (Hornsby, 1997; Mason, 1968; Theorell, 2009). Thus, because type 2 DM is a chronic physiological stressor, it is possible that the type 2 DM group will have lower baseline levels of DHEA-S and a dampened DHEA-S release following the TSST compared to the healthy control group.

**Hypothesis 3: Salivary Alpha-Amylase (sAA)**

**Overview.** I hypothesized that salivary alpha-amylase (sAA) profiles would differ significantly between the type 2 DM group and the healthy control group such that the type 2 DM group would have higher baseline sAA levels, dampened sAA reactivity, and dampened sAA recovery in response to the TSST compared to the healthy control group.

**Rationale.** There is suggestive evidence that salivary alpha-amylase (sAA) is a valid and reliable indirect biomarker of sympathetic nervous system (SNS) activity (Nater & Rohleder, 2009). Data from several studies with healthy subjects consistently demonstrate that sAA levels increase in response to acute laboratory stressors (Nater & Rohleder, 2009). Although far fewer studies have examined sAA stress reactivity in clinical populations, existing data indicate that sAA reactivity in response to acute laboratory stressors is attenuated in clinical populations and chronically stressed populations (Nater et al., 2010).

**Hypothesis 4: Allostatic Load**

**Overview.** I hypothesized that the type 2 DM group and the healthy control group would differ significantly on indicators of allostatic load in the following ways:
1. Cardiovascular indicators: Higher baseline systolic blood pressure and diastolic blood pressure

2. Metabolic indicators: Higher triglycerides, total cholesterol, low density lipoprotein cholesterol (LDL), HbA1c, FPG, fasting plasma insulin and lower high density lipoprotein cholesterol (HDL) levels.

3. Neuroendocrine indicators: Higher baseline salivary cortisol and sAA levels and lower baseline salivary DHEA-S levels

4. Anthropometric indicators: Higher BMI and waist-to-hip ratio

5. Immune indicators: Higher c-reactive protein levels

**Hypothesis 5: Effect of Allostatic Load on Stress Reactivity and Stress Recovery**

**Overview**

5a. **Main effects of allostatic load.** I hypothesized that higher total allostatic load scores would be associated with stress system response patterns in the following ways:

1. Dampered cortisol reactivity (baseline to stress) and dampened cortisol recovery (stress to recovery)

2. Dampered DHEA-S reactivity (baseline to stress) and dampened DHEA-S recovery (stress to recovery)

3. Dampered sAA reactivity (baseline to stress) and dampened sAA recovery (stress to recovery)

5b. **Allostatic load by type 2 diabetes status interaction.** I hypothesized that the magnitude of the effect of allostatic load on stress reactivity and stress recovery would depend on type 2 DM status (i.e., there would be a significant group X allostatic load interaction).
interaction). I hypothesized that allostatic load would be a stronger predictor of stress system response patterns in the type 2 DM group compared to the healthy control group.

**Rationale.** The allostatic load model proposes that chronic stress exposure and activation of the stress systems can cause physiological impairments and neurobiological adaptations that contribute to negative health outcomes (Juster et al., 2010; McEwen, 2007). A comprehensive assessment of allostatic load indicators can be used to predict disease susceptibility (McEwen & Seeman, 1999). There are multiple biophysiological mediators involved in allostatic load (Juster et al., 2010). Primary mediators include stress hormones (e.g., cortisol, DHEA-S, salivary alpha-amylase), pro-inflammatory cytokines (e.g., interleukin-6, tumor necrosis factor-alpha), and anti-inflammatory cytokines (McEwen, 2007). Prolonged secretion of primary mediators result in subclinical impairments in metabolic (e.g., elevated insulin, glucose, total cholesterol, triglycerides, low density lipoprotein), cardiovascular (e.g., elevated systolic blood pressure, elevated diastolic blood pressure), and immune (e.g., elevated C-reactive protein) system functioning.

**Participants**

**Participant Characteristics**

Participants in the final study sample were 34 males between the ages of 25 and 65 who were recruited into one of two groups: 1) type 2 DM group (n=14); 2) healthy control group (n=20). We only included men in this study because menstrual cycle status and sex can alter stress physiology (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). A total of 39 participants participated in the health screening session, but one participant did not complete the health screening session due to blood pressure
values. Four (type 2 DM = 2, healthy = 2) of the 38 participants who completed the health screening session did not complete the test session.

Table 1 provides details on demographic variables for the two groups [mean ± standard error of the mean (SEM)]. On average, participants in the type 2 DM group (55.71 ± 2.75 years) were significantly older than participants in the healthy group (37.25 ± 2.30 years) [F(1,32) = 26.50, p < 0.05]. Additionally, participants in the type 2 DM group had significantly greater BMIs (34.60 ± 1.25) compared to participants in the healthy group (24.71 ± 1.05) [F(1,32) =36.67, p < 0.05]. Racial composition of the type 2 DM group included: White/Caucasian, 14 (100%); Native American/Alaska native, 2 (7.1%). Racial composition of the healthy group included: White/Caucasian, 19 (90%); “other” 1 (10%). Of the type 2 DM participants, 71% were employed full-time, and 71.4% had an average family income between $50,000 and $100,000. Of the healthy participants, 50% were employed full-time, and 60% had an average family income between $15,000 and $50,000. Of type 2 DM participants, 42.9% percent were college graduates, with their education levels ranging from completion of high school to completion of a graduate degree. One-hundred percent of the healthy participants completed college, with their education levels ranging from completion of college to completion of a graduate degree.

Table 2 provides details on clinical characteristics for the two groups [mean ± SEM]. The two groups did not significantly differ on self-reported symptoms of depression [F(1,32)=0.10,  p =.76]. There were a significantly greater number of participants with diagnosed hypertension in the type 2 DM group than in the healthy group [χ²(1)=8.76,  p <.05]. Similarly, there were a significantly greater number of
participants with diagnosed high cholesterol in the type 2 DM than in the healthy group 
$[\chi^2(1) = 16.61, p < .05]$. On average, the type 2 DM participants were diagnosed with type 
2 DM 6 years ago. Over 70% of type 2 DM participants reported taking Metformin, 
making it the most common type 2 DM medication.

**Recruitment**

**Advertisements.** Participants were recruited through the use of flyers posted 
around the local Sate College, PA community (e.g., Weis, Starbucks, Panera, local 
restaurants, churches) and on the Pennsylvania State University campus (see Appendix 
B). Participants also were recruited from advertisements placed on craigslist.com, on the 
Pennsylvania State University research website ([http://www.research.psu.edu/volunteer](http://www.research.psu.edu/volunteer)), 
and in central Pennsylvania newspapers (e.g., Centre Daily Times) (see Appendix B). 
Radio advertisements also were aired on central Pennsylvania radio stations (e.g., B94.5, 
Eagle 93.7) (see Appendix B). All advertisements instructed interested individuals to call 
or email the Biobehavioral Health Studies Laboratory ([diasmoke@psu.edu](mailto:diasmoke@psu.edu)) in the 
Department of Biobehavioral Health at the Pennsylvania State University and provide 
their name, telephone number, and a convenient time for a research team member to call 
them.

**Medical offices.** Informational letters and study flyers were mailed to 30 central 
Pennsylvania medical offices asking them to post the study flyers in the offices (see 
Appendix B). Physicians and clinicians (e.g., doctors, physician assistants, nurse 
practitioners, diabetes nurse educators) from Mount Nittany Medical Center and Penn 
State Hershey Medical Group assisted with recruitment by distributing study flyers to 
potential participants. Additionally, Dr. Franklin Berkey from Penn State Hershey
Medical Group Windmere Centre recruited potential participants using an approved script and provided their contact information to KNW (see Appendix B).

**Diabetes Registry.** The Diabetes Institute at Penn State Hershey performed a Diabetes Registry search and sent recruitment letters to type 2 DM males who were between ages 25 to 65 years. The search was restricted to State College and surrounding communities (e.g., Tyrone, Altoona, Bellefonte, Lewistown). The Diabetes Registry is a custom built application that assists in tracking clinical outcomes for diabetes patient and has over 17,000 patient registrants (see Appendix B).

**Listservs.** Emails were sent to staff assistants in departments and colleges at the University Park and Altoona campuses of the Pennsylvania State University. The email requested that the staff assistant send an email with study information to faculty, staff, and graduate student listservs (see Appendix B).

**Research labs.** Male type 2 DM participants from studies conducted by Dr. Sheila West in the Vascular Health Interventions Lab in the Department of Biobehavioral Health at the Pennsylvania State University were contacted to inquire if they were interested in participating in this study. Participants from past studies only were contacted if they had indicated that they would like to be informed about future research studies for which they may be eligible to participate. In these cases, members of the study team called these individuals to inform them about this study.

**Penn State Extension.** KNW attended three days of the 2013 Pennsylvania State University Ag Progress Days and distributed flyers at the Dining with Diabetes informational exhibit.
Community leaders. A community leader in Mifflinburg, PA distributed flyers to members of his community and also posted flyers at various community locations (e.g., churches, restaurants, recreation centers).

Telephone screening

A trained interviewer contacted the interested individuals and conducted the telephone screening to determine initial eligibility (see Appendix C). The telephone screening interview included questions about the participant’s recent history of medical or psychiatric disorders, prescription and over-the-counter medication use, type 2 DM history, cigarette smoking history, and chronic health condition history. At the end of the telephone screening interview, the interviewer informed the participant that he would be contacted about study eligibility. Study investigators reviewed the responses to the telephone screening interview to determine eligibility. Participants met the type 2 DM criteria if they reported that they were diagnosed with type 2 DM by a qualified medical professional (e.g., medical doctor, nurse practitioner, physician assistant) and did not take insulin.

Participants who did not meet the initial telephone screening criteria were sent an ineligibility letter via email or mail (see Appendix D). Those who met the initial telephone screening criteria were invited to a health screening session at the Clinical Research Center (CRC) at the Pennsylvania State University (see “Health Screening Session” section on page 43 for details about the screening session). Eligible individuals were sent an email confirmation message or were mailed a letter with information about the time and location of the health screening session (see Appendix E). The purpose of the health screening session was to confirm eligibility and ensure that the participant was...
capable of safely completing the test session. Individuals whose eligibility was confirmed during the health screening session were contacted to schedule the 3-hour test session at the CRC.

Exclusionary Criteria

Potential participants were excluded from the study if they had insulin-dependent diabetes, stroke, or other neurological disorders, or a recent history of depression as screened by self-reported answers on the Center for Epidemiological Studies Depression inventory (Radloff, 1977) (i.e., CES-D > 16). Potential participants also were excluded if they had significant health conditions or took medications that prevented them from safely completing the laboratory test session. These conditions include neurological disorders such as stroke, transient ischemic attack (TIA); history of functional heart murmur, enlarged heart, heart attack, blood clotting disorder, pulmonary emphysema, cancer or malignant tumor, ulcer; history of major mental illness (e.g., bipolar disorder, panic disorder, PTSD or flashbacks); cognitive or attentional disorders such as attention deficit/hyperactivity disorder; current use of medications that would interfere with normal hormonal and cognitive functioning such as oral and injected corticosteroids use in the previous 3 months, psychotropic medication use in the previous 8 weeks, and current inhaled beta-agonist use. Additionally, people were excluded if they donated blood or plasma during the 4 weeks prior to the study or plan to donate within 4 weeks after their participation has ended.

Participants with hypertension, high cholesterol, and coronary heart disease were allowed to participate in the study due to the comorbidity of these conditions with type 2 DM. However, participants with unstable coronary heart disease (e.g., history of
myocardial infarction, angina) or uncontrolled hypertension (blood pressure > 160/100 mmHg during health screening session; \( n = 1 \) type 2 DM) were excluded from the study. Participants also were excluded if they quit smoking within the past 5 years, currently used or within the past 5 years have used cigarettes or other tobacco products (e.g., chewing tobacco, snuff, pipes, cigars), or currently used or within the past 5 years had used nicotine replacement products (e.g., patch, gum).

**Type 2 Diabetes Mellitus Status**

Type 2 DM status was determined during the initial telephone screening interview (see Appendix C). Potential participants who did not meet the exclusion criteria met the type 2 DM criteria if they reported that a qualified medical professional (e.g., medical doctor, nurse practitioner, physician’s assistant) diagnosed them with type 2 DM. Type 2 DM participants also must have reported that they did not take insulin (Faulenbach et al., 2012).

**Protocol**

**Health Screening Session**

Following the telephone screening interview, eligible participants (\( n = 38; 16 \) type 2 DM, 22 healthy) were scheduled to come to the Clinical Research Center (CRC) on the University Park Campus of PSU for a 1-hour health screening session to further confirm eligibility and health status. Participants were instructed not eat anything after midnight the night before the visit, to abstain from exercising for 12 hours prior to the visit, and to abstain from drinking alcohol for 48 hours prior to the visit because these factors can affect blood lipid results and blood pressure. Figure 1 presents a timeline of the health screening session.
Participants provided written consent at the beginning of the health screening session prior to completing any additional study procedures (see Appendix F for the informed consent form and Appendix G for the health screening session procedures). Following consent, participant’s systolic blood pressure, diastolic blood pressure, heart rate, height, weight, and carbon monoxide via expired air (CO; Micro+™ Smokerlyzer®; coVita, Haddonfield, NJ) were measured. Exhaled CO commonly is used to assess smoking status in clinical and research settings. Participants were excluded \( (n = 0) \) from participating in the laboratory session if their exhaled CO levels were greater than 6.5 ppm (mean of 2 readings) because a CO level greater than 6.5 ppm suggests recent cigarette smoke exposure (Deveci, Deveci, Açik, & Ozan, 2004). Additionally, participants \( (n = 1 \text{ type 2 DM}) \) whose resting blood pressure (mean of 2 readings) was greater than 160/100 mmHg were excluded from participating in the laboratory test session because they likely had uncontrolled hypertension (Barengo, Kastarin, Antikainen, Nissinen, & Tuomilehto, 2009).

A CRC clinician then conducted a clinical interview to gain further information about participant’s health history and current health status. Following the interview, a clinician collected blood via venipuncture in three vacutainer blood collection tubes [7.7 mL serum separator tube (SST), 4 mL ethylenediaminetetraacetic acid (EDTA) tube, and 2 mL heparin tube] using standard antiseptic techniques. The SST and the EDTA tubes were sent to Quest Diagnostics (Pittsburgh, Pennsylvania) for assessment of HbA1c (EDTA tube), insulin (SST), and lipids (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides; SST). Additionally, whole blood collected in the heparin tube was used to assess fasting plasma glucose levels. Plasma glucose levels were measured
immediately following the health screening session using a TRUEresult blood glucose monitor (Nipro Diagnostics, Fort Lauderdale, FL, USA) (see “Biological sample collection and storage” section on page 62 for details about sample processing).

A 12-lead EKG was ordered at the CRC physician or nurse’s discretion based on the clinical interview and screening values of blood pressure. If a potential participant had an abnormal 12-Lead EKG, he was excluded from participating in the laboratory test sessions. Five participants had a 12-lead EKG and zero participants were excluded based on the results of the EKG. Following the blood draw and 12-Lead EKG, participants were provided a snack and then completed a set of demographic and psychosocial questionnaires using an online survey management website (www.surveymonkey.com) (e.g., demographic form; medication use form; tobacco use history) (see Appendix H for all health screening session measures). At the end of the health screening session, participants were compensated $10 for their time and informed that a research team member would contact them via telephone about the test session

**Test Session**

Following the health screening session, participants (n = 34; 14 type 2 DM, 20 healthy) who remained eligible and interested were scheduled to attend the 3-hour test session at the CRC. The test session was scheduled at 2 pm to control for the diurnal fluctuations in neuroendocrine hormones (Van Cauter, 1990). Figure 2 presents a timeline of the test session and Appendix I contains the test session procedures.

Upon arrival at the CRC, all participants provided two breath samples to assess expired CO (Micro+™ Smokerlyzer®; coVita, Haddonfield, NJ). Similar to the screening assessment, participants (n = 0) were excluded from participating in the test session if
their exhaled CO levels were greater than 6.5 ppm (mean of 2 readings) (Deveci et al., 2004). Following CO assessment, participants provided a saliva sample using a cotton Salivette® (Sarstedt, Newton, NC). Participants were instructed to roll the cotton Salivette in their mouths, without chewing on it, for 2 minutes to ensure that it was saturated with saliva before putting it back in the tube.

Participants then were taken to the study room and a Polar® RS800CX™ heart rate monitor chest strap transmitter and wrist receiver (Polar®, Kempele, Finland) were placed on the participant following the instructions in the user manual. The chest strap was fixed tightly just below the pectoral muscles. The participant was informed that his heart rate would be measured continuously with the heart rate monitor during the entire session. The participant then was instructed to sit in a chair, and a Dinamap (Model 1847-SX; Critikon, Tampa, FL) blood pressure cuff was placed on the participant’s non-dominant arm.

**Acclimation.** The participant sat quietly in the chair for 5 minutes and had two acclimation blood pressure readings taken. The cuff was placed on the non-dominant arm because the participant used his dominant hand to move the mouse when completing questionnaires on the computer. If the mean of the two blood pressure readings was below 160/100 mmHg, the participant was allowed to continue with the session. If the mean was greater than 160/100, the participants \( n = 0 \) were excluded from completing the remainder of the study session because these values are indicative of uncontrolled hypertension and it may not have been safe for the participant to continue the session (Barengo et al., 2009). These participants were provided with their blood pressure levels and instructed by a CRC nurse to follow up with their physician.
**Questionnaire Set 1.** During the 10-minute questionnaire set 1 period, the participant continued to have his heart rate assessed continuously with the heart rate monitor and his blood pressure and heart rate were collected at 2-minute intervals with the automated blood pressure monitor. While sitting quietly, participants completed a set of psychosocial measures using an online survey management website (www.surveymonkey.com). These measures included the Mood Assessment Scale and Positive and Negative Affect Scale (see Appendix J for all Questionnaire Set 1 measures). The participant sat quietly in the chair after completing the questionnaires for the remainder of the 10-minute period.

**Baseline.** Following the questionnaire set 1 period, the participant continued to sit quietly in the chair for a 10-minute baseline period. During the baseline period, the participant continued to have his heart rate assessed continuously with the heart rate monitor and his blood pressure and assessed at 2-minute intervals with the automated blood pressure monitor. At the end of the 10-minute baseline period, the participant provided a saliva sample for later assessment of salivary free cortisol, salivary alpha-amylase, and salivary DHEA-S. A trained CRC nurse then collected blood via venipuncture. Blood was collected into two, 10 mL anticoagulant free red top tubes and one, 2 mL heparin tube. The blood samples in the red top tubes were processed for later assaying of c-reactive protein. The blood sample in the heparin tube was used to measure blood glucose levels. After the blood draw, a trained research assistant measured blood glucose levels using the TRUEresult blood glucose monitor (Nipro Diagnostics, Fort Lauderdale, FL, USA).
**Stressor task.** During the test session, participants completed the TSST task (Kirschbaum et al., 1993). The TSST is a laboratory based stress protocol involving a public speaking task and serial subtraction arithmetic tasks (see Appendix K for TSST procedures). Researchers have found that the TSST reliably stimulates biomarkers of psychosocial stress (e.g., cortisol) (Dickerson & Kemeny, 2004) and can be used to detect dysregulations of the neuroendocrine stress response (e.g., HPA-axis, SAM-axis) (Hellhammer, Wüst, & Kudielka, 2009). This study used a similar protocol to the protocol described by Kirschbaum, Pirke, et al. (1993).

In this study, the TSST was administered by a panel consisting of two female study team members, who prior to the test session had never interacted with the participant. Prior to being introduced to the panel, the primary experimenter had the participant watch a TSST instructional DVD. The DVD provided basic instructions about the speech task and informed the participant he would be giving a speech explaining why he was a good candidate for a job as a customer service representative at an airline company. The DVD informed the participant that a panel of psychologists would be evaluating his performance and that they were trained at analyzing verbal and nonverbal behavior. The DVD also informed participants that the speech task would be video recorded, and the psychologists would be analyzing the video tape later. In actuality, the speech was not videotaped and was not analyzed by psychologists. Following the DVD, the primary experimenter left the room and allowed the participant to mentally prepare for 10 minutes. After 10 minutes, the panel members who were dressed professionally and wore white laboratory coats, entered the room.
The speech task consisted of a 10-minute preparation period and 5-minute speech delivery period in front of a female panel comprised of a lead evaluator (doctoral student) and a secondary evaluator (undergraduate research assistant). Upon entering the study room, the lead evaluator set up the video camera to make it appeared to the participant that he would be videotaped. After setting up the video camera, the lead evaluator instructed the participant to begin, and she put the camera on “standby mode” rather than pressing the “record” button. Thus, the participant was not actually videotaped.

If the participant stopped talking during the 5-minute speech period, the lead evaluator used a standardized set of responses (e.g., “Your time is not up, please continue speaking.”, “I will tell you when to stop speaking.”, Please continue with your speech.”). Immediately following the speech task, the participant watched the second part of the instructional DVD that provided information about the mental arithmetic task. The mental arithmetic task consisted of two serial subtraction task, which involved first counting backwards from 9081 by 7’s and then counting backwards 6027 by 13’s. Participants had 4 minutes for each serial subtraction task, and if the participant had an incorrect response, the lead evaluator stopped the participant and instructed him to start from the last correct response.

During both the speech task and the mental arithmetic task, the panel treated the participant in a cold and reserved manner. They were trained to be serious and stoic, but not hostile, towards the participant. They were instructed to maintain eye contact, and not to smile, nod their heads, or provide affirmative verbal responses, such as “mm-hmm.” During the speech, the evaluators also were instructed to act as if they were evaluating the participant and they looked down approximately every 20 seconds to mark
checkboxes on a mock evaluation form. They also were instructed to write 3 comments throughout the 5 minutes on the form. Following the TSST, the panelists completed a feedback form where they answered questions about the participant’s behavior during the speech and math tasks.

**Task period measures.** During the TSST, heart rate was assessed continuously with the heart rate monitor and blood pressure and heart rate were collected at 2-minute intervals using the automated blood pressure monitor.

**Rest.** After the TSST, participants sat quietly for 15 minutes to allow the stress hormones (e.g., cortisol) to rise. Heart rate was assessed continuously with the heart rate monitor and blood pressure and heart rate were collected at 2-minute intervals with the automated blood pressure monitor. While sitting quietly, participants completed a second set of psychosocial measures on the computer. These measures included the, Post-task appraisal questionnaire, Mood Assessment Scale, and Positive and Negative Affect Scale (see Appendix L for all Questionnaire Set #2 measures). After 15 minutes, participants provided another saliva sample for later assessment of salivary free cortisol, salivary DHEA-S, and sAA. A trained CRC nurse then collected blood via venipuncture. Blood again was collected into two, 10 mL anticoagulant free red top tubes and one, 2 mL heparin tube. The blood samples in the red top tubes were processed for later assaying of biomarkers. The blood sample in the heparin tube was used to measure blood glucose levels. After the blood draw, a trained research assistant measured blood glucose levels using the TRUEresult blood glucose monitor (Nipro Diagnostics, Fort Lauderdale, FL, USA).
Recovery. Following the blood draw, participants continued to sit quietly for 30 minutes. Heart rate was assessed continuously with the heart rate monitor and blood pressure and heart rate were collected at 2-minute intervals with the automated blood pressure monitor. Participants also completed a set of psychosocial measures. These measures included the Mood Assessment Scale and Positive and Negative Affect Scale (see Appendix M for all Questionnaire Set #3 measures). After 30 minutes, participants provided a final saliva sample for later assessment of salivary free cortisol, salivary DHEA-S, and sAA. After confirming that vital signs had returned to baseline, the blood pressure cuff and heart rate monitor were removed from the participant and then the participant’s waist circumference and hip circumference were measured. The experimenter then debriefed the participant about the study (see Appendix N), and the participant was given $55 in cash for completing the test session.

Biological Sample Collection and Storage

Blood collection and storage. Approximately 60 mL of blood was collected during the study. Blood was collected one time during the health screening session and two times during the test session (immediately before the stressor, 15 minutes post-stressor). During the health screening session, blood was collected into three vacutainer blood collection tubes [7.7 mL SST tube, 4 mL EDTA tube, and 2 mL heparin tube). The SST and the EDTA tubes were sent to Quest Diagnostics (Pittsburgh, PA) for assessment of HbA1c (EDTA tube), insulin (SST), and lipids (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides; SST). Additionally, the experimenter measured fasting blood glucose levels in the whole blood collected in the heparin tube. The experimenter
used a TRUEresult blood glucose monitor (Nipro Diagnostics, Fort Lauderdale, FL, USA) to measure blood glucose levels.

During the test session, the first blood draw occurred at the end of the baseline period (immediately before the stressor), and the second blood draw occurred at the end of the rest period (15 minutes post-stressor). At each blood draw, blood was collected into two, 10 mL anticoagulant free red top tubes and one, 2 mL heparin tube. The blood samples in the anticoagulant free red top tubes sat at room temperature for 30 minutes before centrifugation (1500 X g at 4°C for 15 minutes). Following centrifugation, trained research assistants aliquoted serum into separate 2000 µL microtubes; cryotubes then were placed in a low-temperature freezer at -80°C for later assay of biomarkers. Blood samples in the heparin tubes were used to measure baseline and post-stressor glucose levels. After the blood draws, a trained research assistant measured blood glucose levels using the TRUEresult blood glucose monitor (Nipro Diagnostics, Fort Lauderdale, FL, USA) (see Appendix O for blood processing protocol).

Saliva collection and storage. Participants provided a total of 4 saliva samples using a cotton Salivette® during the study. After the test session, saliva samples were frozen at -80 °C for later assay of salivary free cortisol, salivary DHEA-S, and sAA by the Biomarker Core Laboratory at Penn State University Park (see Appendix P for assay protocol information).

Biomarker Assay Procedures

Assays were conducted at Quest Diagnostics (Pittsburgh, Pennsylvania; HDL, LDL, total cholesterol, insulin, triglycerides, HbA1c) and at the Biomarker Core
Laboratory at Penn State University Park (serum CRP, salivary free cortisol, salivary DHEA-S, and sAA assays) (see Appendix P for assay protocol information).

**Salivary free cortisol assessment.** Baseline, stress (15 minutes post-stressor), and recovery (45 minutes post-stressor) saliva samples were assayed for salivary free cortisol in duplicate in a single assay batch at the Biomarker Core Laboratory via a commercially available enzyme immunoassay (EIA; Salimetrics LLC, State College, PA). The sample test volume was 25 μl of saliva (for singlet determinations). The assay had a range of sensitivity from 0.007 to 1.8 μg/dl, with average inter- and intra-assay covariances of less than 10% and 5%, respectively (see Appendix P for salivary free cortisol assay protocol information). Cortisol values were converted from μg/dl to nmol/L by multiplying by 27.59 (see Appendix P for salivary free cortisol assay protocol).

**Salivary DHEA-S assessment.** Baseline, stress (15 minutes post-stressor), and recovery (45 minutes post-stressor) saliva samples were assayed for salivary DHEA-S at the Biomarker Core Laboratory using a commercially available enzyme immunoassay kits (EIA; DRG International, Springfield, NJ). The sample test volume was 50 μL. The assay had a lower limit of sensitivity of 0.05 ng/ml, with an average inter- and intra-assay covariance of less than 15% and 8%, respectively (see Appendix P for salivary DHEA-S assay protocol).

**sAA assessment.** Baseline, stress (15 minutes post-stressor), and recovery (45 minutes post-stressor) saliva samples were assayed for salivary alpha amylase (sAA) at the Biomarker Core Laboratory using a commercially available kinetic reaction assay kit (Salimetrics LLC, State College, PA). The Salimetrics sAA kit has been validated for the measurement of sAA in saliva (Granger et al., 2006). The assay uses a chromogenic
substrate, 2-chloro-p-nitrophenol, linked to maltotriose (Granger et al., 2006). The enzymatic action of sAA on the substrate yields 2-chloro-p-nitrophenol, which can be spectrophotometrically measured at 405 nm using a standard laboratory plate reader. The amount of sAA activity present in the sample is directly proportional to the increase (over a 2-min period) in absorbance at 405 nm. Results are computed in units per milliliter of sAA using the formula: \[ \text{absorbance difference per minute} \times \text{total assay volume (328 ml)} \times \text{dilution factor (200)} \div [\text{millimolar absorptivity of 2-chloro-p-nitrophenol (12.9)} \times \text{sample volume (0.008 ml)} \times \text{light path (0.97)}]. \] Intraassay variation (CV) computed for the mean of 30 replicate tests was less than 7.5%. Interassay variation computed for the mean of average duplicates for 16 separate runs was less than 6% (Granger et al., 2006). (see Appendix P for sAA assay protocol).

**Serum C-reactive protein assessment.** Baseline blood samples were assayed for serum CRP in duplicate in a single assay batch at the Biomarker Core Laboratory via a commercially available enzyme immunoassay (EIA; DRG International Inc., Mountainside, NJ). The sample test volume was 10 μL of serum (for singlet determinations). (see Appendix P for CRP assay protocol).

**Lipid panel, HbA1c, & serum insulin.** The fasting blood sample from the health screening was sent to a clinical laboratory (Quest Diagnostics, Pittsburgh, PA) for assessment of HbA1c, fasting serum insulin, and lipids (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides) (see Appendix P for Quest Diagnostics assay information).
**Fasting plasma glucose.** Health screening session fasting plasma glucose levels were assessed using the TRUEresult blood glucose monitor (Nipro Diagnostics, Fort Lauderdale, FL, USA) (see Appendix O for blood processing protocol).

**Physiological procedures**

**Body mass index (BMI).** Height and weight were assessed during the health screening session. A digital weight scale and stadiometer were used to measure the participant’s weight and height (Deteco 6855; Webb City, MO). Weight was recorded in pounds and converted to kilograms in SPSS during data cleaning. Height was recorded in inches and converted to meters in SPSS during data cleaning. BMI was calculated using the following formula: kg/m²

**Waist-to-hip ratio.** Waist circumference and hip circumference were measured at the end of the test session. Waist circumference (inches) was measured two times by placing the tape in a horizontal plane around the abdomen at the level of the iliac crest. The hip circumference (inches) was measured two times by placing the tape around the widest portion of the buttocks. Before reading the tape measurements, the experimenter ensured that the tape was snug, did not compress the skin, and was parallel to the floor. The waist to hip ratio was calculated by dividing the mean waist circumference by the mean hip circumference (WHO, 2008).

**Self-Report Measures**

Participants completed several psychosocial and demographic self-report measures during the health screening session and laboratory test session. Only measures used in the primary analyses for this dissertation study will be discussed here.
Appendices H, J, L, & M contain all measures administered during the various study periods (i.e., health screening session and test session baseline, rest, and recovery).

**Demographic form.** The demographic form has been used by Dr. Klein in her previous research studies (e.g., Bennett, Rodrigues, & Klein, 2013; Klein, Bennett, Whetzel, Granger, & Ritter, 2010). The purpose of this form is to collect basic demographic information such as age, marital status, occupation, education, race, ethnicity, and income. Participants completed the demographic form during the health screening session (see Appendix H for health screening session measures).

**Medication use form.** Because some medications and over-the-counter supplements can affect neuroendocrine hormones (e.g., cortisol) (Granger, Hibel, Fortunato, & Kapelewski, 2009), medication information was collected on the medication use form. The medication use form asked participants to write all medication and supplements (prescription and over the counter) that they take on a regular basis. For each medication or supplement, the participant was instructed to write the frequency and the dose of the medication or supplement. Participants completed the medication use form during the health screening session (see Appendix H for health screening session measures).

**Stress levels.** Participants reported their current stress levels at the beginning of the baseline period, immediately following the stressor period, and at the beginning of the recovery period. Self-reported stress level was an item on the Mood Assessment Scale and was assessed using a 7-point Likert Scale (1 = not at all, 7 = very much) (see Appendices J, L, & M).
Data Analytic Strategy

Data Preparation

**Data entry and cleaning.** Physiological data (e.g., blood pressure, height, weight, carbon monoxide) from the health screening and laboratory test sessions were entered into the Statistical Program for the Social Sciences [version 21; SPSS (SPSS; Chicago, IL)] by KNW and trained undergraduate research assistants. Data were entered into two datasets and then the datasets were compared using SAS® software’s (version 9.3; SAS Institute Inc.; Cary, NC) PROC COMPARE procedure to find any discrepancies between the two datasets due to data entry errors. All discrepancies were compared against the raw data by KNW to determine the correct value. Self-report data collected using surveymonkey.com were downloaded as a SPSS file and cleaned in SPSS. The final SPSS dataset was saved as a SAS dataset for statistical analyses.

**Calculated variables.** Mean SBP, DBP, and heart rate values were calculated for each test session period by averaging the readings taken every 2 minutes during each of the test session periods as described by Llabre and colleagues (1988). Mean baseline SBP, DBP, and heart rate were calculated by averaging the 5 measurements that were taken during the 10-minute baseline period. Mean stressor period SBP, DBP, and heart rate were calculated by averaging the 15 readings collected during the TSST task (i.e., speech preparation, speech task, and 2 math tasks). Mean rest period SBP, DBP, and heart rate were calculated by averaging the 7 readings taken during the 15-minute rest period. Mean recovery period SBP, DBP, and heart rate were calculated by averaging the 15 readings taken every during the 30-minute recovery period.
Stress reactivity and stress recovery variables were calculated for cortisol, DHEA-S, and sAA data. Stress reactivity variables were calculated by subtracting baseline levels from stress levels. Stress recovery variables were calculated by subtracting recovery levels from stress levels. Positive stress reactivity change scores indicate that biomarker levels increased from baseline to stress, and positive recovery change scores indicate that biomarker levels decreased from stress to recovery.

Area under the curve with respect to increase (AUC$_i$) and area under the curve with respect to ground (AUC$_g$) variables were calculated for each biomarker. AUC$_i$ and AUC$_g$ are commonly used in stress physiology research to estimate the overall secretion of a stress biomarker over specific time periods (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). The AUC variables were calculated using the trapezoid formulas presented in Pruessner and colleagues (2003)’s paper. AUC$_g$ is believed to be reflective of total hormonal output, and zero is used as the line of reference in the formula. AUC$_i$ is believed to be reflective of stress system sensitivity, and the participants baseline stress biomarker level is used as the line of reference in this formula (Pruessner et al., 2003).

**Allostatic load calculations.** Methods similar to those described by Corwin and colleagues (2006) were used to calculate allostatic load scores for each allostatic load parameter (Corwin, McCoy, Whetzel, Ceballos, & Klein, 2006). Participants were assigned a value of 0 (low), 1 (moderate), or 2 (high) for each allostatic load parameter. When available, values were based on either established guidelines or clinical recommendations (SBP, DBP, HDL, LDL, triglycerides, total cholesterol, fasting glucose, BMI, CRP, HbA1c). Explanations for calculating the allostatic load parameters scores based on existing clinical guidelines can be found below.
When guidelines or clinical recommendations were not available, values were assigned based on the how far the participants deviated from the sample mean for that parameter. For fasting insulin, baseline salivary cortisol, baseline sAA, and waist-to-hip ratio, a value of 0 (low risk) was assigned to participants with scores from zero to 0.5 SD above the mean; a value of 1 (moderate risk) was assigned to participants with scores from 0.5 to 1.0 SD above the mean; and a value of 2 (high risk) was assigned to participants with scores greater than or equal to 1 SD above the mean. For DHEA-S, a value of 0 (low risk) was assigned to participants with scores from zero to 0.5 SD below the mean; a value of 1 (moderate risk) was assigned to participants with scores from 0.5 to 1.0 SD below the mean; and a value of 2 (high risk) was assigned to participants with scores greater than or equal to 1 SD below the mean. The total allostatic load score was calculated by summing the scores of the 15 individual allostatic load parameters. Three additional total allostatic load variables were created: 1) a total allostatic load score variable without cortisol; 2) a total allostatic load score without DHEA-S; 3) and a total allostatic load score variable without sAA. These allostatic load score variables were used in the analyses that had the associated biomarker as the outcome variable.

**Systolic blood pressure.** SBP allostatic load values were determined using recommendations outlined in the National High Blood Pressure Education Program (NHBPEP) (2004) report. If the mean baseline SBP was less than 120 mmHg, then a value of 0 (low risk) was assigned. If the mean baseline SBP was between 120 and 139, a value of 1 (moderate risk) was assigned. If mean baseline SBP was greater than or equal to 140 than a value of 2 (high risk) was assigned.
**Diastolic blood pressure.** Diastolic blood pressure (DBP) allostatic load values were determined using recommendations outlined in the NHBPEP (2004) report. If the mean baseline DBP was less than 80 mmHg, a value of 0 (low risk) was assigned. If the mean baseline DBP was between 80 to 89 mmHg, a value of 1 (moderate risk) was assigned. If mean baseline DBP was greater than or equal to 90 mmHg than, a value of 2 (high risk) was assigned.

**Low density lipoprotein.** Low density lipoprotein (LDL) allostatic load values were determined using recommendations outlined in the National Cholesterol Education Program (NCEP) (2002) report. If the LDL was less than 130mg/dL, a value of 0 (low risk) was assigned. If LDL was greater than or equal 130 or less than 160 mg/dL, a value of 1 (moderate risk) was assigned. If LDL was greater than or equal to 160 mg/dL, a value of 2 (high risk) was assigned.

**High density lipoprotein.** High density lipoprotein (HDL) allostatic load values were determined using recommendations outlined in the NCEP (2002) report. If HDL was greater than or equal to 60 mg/dL, a value of 0 (low risk) was assigned. If HDL was greater or to 40 and less than 60 mg/dL, a value of 1 (moderate risk) was assigned. If the HDL was less than or 40 mg/dL, a value of 2 (high risk) was assigned.

**Total cholesterol.** Total cholesterol (TC) allostatic load values were determined using recommendations outlined in the NCEP (2002) report. If the TC was less than 200 mg/dL, a value of 0 (low risk) was assigned. If TC was greater than or equal to 200 or less than 240 mg/dL, a value of 1 (moderate risk) was assigned. If TC was greater than or equal to 240 mg/dL, a value of 2 (high risk) was assigned.
Triglycerides. Triglyceride allostatic load values were determined using recommendations outlined in the NCEP (2002) report. If the triglycerides were less than 150 mg/dL, a value of 0 (low risk) was assigned. If triglycerides were greater than or equal to 150 or less than 200 mg/dL, a value of 1 (moderate risk) was assigned. If triglycerides were greater than or equal to 200 mg/dL, a value of 2 (high risk) was assigned.

C-reactive protein. C-reactive protein allostatic load values were determined using recommendations of the American Heart Association and CDC (Pearson et al., 2003). If the triglycerides were less than 1 mg/L, a value of 0 (low risk) was assigned. If triglycerides were between 1 and 3 mg/L, a value of 1 (moderate risk) was assigned. If triglycerides were greater than 3 mg/L, a value of 2 (high risk) was assigned.

Body mass index. BMI allostatic load values were determined using recommendations of the Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults (National Institutes of Health, 1998). If the BMI was less than 25 kg/m², a value 0 (low risk) was assigned. If BMI was between 25 and 29.9 kg/m², a value of 1 was assigned (moderate risk). If BMI was greater than or equal to 30 kg/m², a value of 2 was assigned (high risk).

Glycosylated hemoglobin. HbA1c allostatic load values were determined using recommendations of the ADA. If the HbA1c level was less than 5.7%, a value of 0 (low risk) was assigned. If HbA1c level was greater than or equal to 5.7% and less than 6.5%, a value of 1 (moderate risk) was assigned. If the HbA1c level was greater than or equal to 6.5%, a value of 2 (high risk) (ADA, 2009).
**Fasting plasma glucose.** FPG allostatic load values were determined using recommendations of the American Diabetes Association. If the FBG level was less than 100 mg/dl, a value of 0 (low risk) was assigned. If the FBG level was between 100 mg/dl and 125 mg/dl, a value of 1 (moderate risk) was assigned. If the FBG level was greater than or equal to 126 mg/dl, a value of 2 (high risk) (ADA, 2009)

**Data transformations.** Data was assessed to ensure that the dependent variables met the assumption of normality (e.g., skewness, stem-and-leaf plot, frequency distribution, outliers). One participant in the healthy group was excluded from sAA analyses because his sAA values were extreme outliers at all 3 test session time points (i.e., greater than 4 SD from the mean). Removing him from the analyses of other outcome variables did not change the results so he was kept in the other analyses. Several variables were not normally distributed, and separate transformations were performed on these data to normalize the distribution so parametric tests could be conducted

A natural logarithmic transformation was applied to the cortisol and DHEA-S data from the 3 test session time points, as well as the sAA reactivity score, FPG, CRP, triglyceride, and insulin data. A square root transformation was applied to the sAA data from the 3 test session time point, the sAA AUC_g data, and sAA AUC_i data. It was necessary to add a constant to the sAA reactivity and sAA AUCi data prior to applying the square root transformations because there were negative values, and you cannot take the square root of a negative number. Adding a constant of 1 plus the absolute value of the minimum sAA reactivity value to all sAA reactivity data resulted in all values being greater than or equal to 1. The same procedure was applied to the sAA AUCi data. All
data analyses were conducted on transformed data. However, mean values ± SEM are reported in the text for clarity.

**Statistical Analysis Plan**

Statistical analyses were performed using SAS version 9.3. All statistical tests were two-tailed and significance was determined at the alpha= 0.05 level. Means are reported as least squares mean ± SEM. Primary dependent variables were: 1) salivary free cortisol, salivary DHEA-S, and sAA at each test session time point (i.e., baseline, stress, recovery); 2) constructed stress biomarker variables including area AUC, stress reactivity change scores, and stress recovery change scores; 3) measures of allostatic load.

**One-way analysis of variance (ANOVA).** Separate one-way ANOVAs (continuous variables) and chi-square tests (categorical variables) were conducted to determine baseline differences between the two groups (type 2 DM, healthy control) on psychosocial and demographic variables. Results of these analyses were reported in the Participant Characteristics section on page 43 of this chapter. Separate one-way ANOVAs also were performed to test group differences in baseline stress biomarker levels, AUC$_g$, AUC$_i$, stress reactivity change scores, and stress recovery change scores, and total allostatic load. The SAS PROC MIXED procedure was used to conduct the one-ways ANOVAs, and the SAS PROC FREQ procedure with the CHISQ option was used to conduct chi-square tests.

**Linear mixed models.** Multi-level modeling (MLM) methods were employed to examine stress system response patterns during the test session, as well as to confirm that the stressor successfully elicited a stress response by testing the SBP, DBP, HR, and self-reported stress response patterns. MLM methods allowed me to test the effects of type 2
DM status on stress system response patterns, while taking into account the within-subject correlations (Singer & Willett, 2003). Level 1 of the models represented the individual’s change in a given measure across the session time points, and level 2 of the models represented the between-subject differences in the stress biomarker response patterns (e.g., group differences, total allostatic load score). Below, I provide equations for each level of the multi-level model, and a brief explanation of the variables included at each level.

At Level 1, \( Y_{ti} \) is the stress biomarker level at time \( t \) for participant \( i \), \( \beta_{oi} \) is the average biomarker level for participant \( i \), and \( e_{ti} \) is the time level error term.

\[
\text{Level 1: } Y_{ti} = \beta_{oi} + e_{ti}
\]

At Level 2, \( \beta_{0i} \) represents the participant’s average stress biomarker level, \( Y_{00} \) represents the grand mean of the stress biomarker across all time points and participants. \( \text{Group}_i \) is a between-subject predictor, and represents type 2 DM status. \( Y_{01} \) is the regression coefficient associated with \( \text{Group}_i \), and \( \mu_{0i} \) represents the individual level error term.

\[
\text{Level 2: } \beta_{0i} = Y_{00} + Y_{01}\text{Group}_i + \mu_{0i}At
\]

All linear mixed models were tested using the SAS PROC MIXED procedure with a compound symmetry (CS) covariance structure. The outcome variables in the models were stress biomarker levels. Time (baseline, stress, and recovery), group (type 2 DM, healthy), and the time–by-group interaction terms were treated as fixed effects, and participants were treated as a random effect in the models. Each set of biomarker analyses also tested models that included person-level covariates such as age (grand mean centered) and BMI (grand mean centered). Additionally, group-by-total allostatic load
and time-by-total allostatic load interaction terms were tested in the allostatic load analyses. The fit of models tested in each set of analyses were compared to each other, and model selection was based on optimizing fit statistics using the Bayesian information criterion (BIC). Post hoc analyses were adjusted for multiple comparisons using the Tukey-Kramer adjustment.

**Linear regression modeling.** Separate linear regression models were tested to investigate if total allostatic load predicted the AUCi, AUCg, stress reactivity score, and stress recovery score associated with each stress biomarker. The SAS PROC REG procedure was used to conduct the linear regression analyses.

**Multivariate analysis of variance.** A one-way multivariate analysis of variance (MANOVA) was performed to analyze group differences in the individual allostatic load parameters. All 15 allostatic load parameters could not be included in the MANOVA model because the number of dependents variables in a model cannot exceed the number of participants in smallest cell (Stevens, 2009). To address this issue, canonical discriminant analysis (CDA) was employed to determine which allostatic load parameters should be included in the MANOVA model.

CDA is a dimension reduction technique that is similar to principal component analysis and canonical correlation, and can be used in conjunction with a one-way MANOVA (Stevens, 2009). For this dissertation study, the goal was to use CDA to assist in reducing the number of allostatic load parameters to eight. The pooled within canonical structure coefficients used to determine which allostatic load parameters should be retained or removed from the model. Several models were tested separately, and allostatic load parameters were added to and removed from model in a stepwise manner.
based on their canonical coefficients. The greater the size of the absolute value of the coefficient, the greater the two groups likely differed on that variable compared with variables with lower coefficient absolute values. The SAS PROC DISRIM procedure with the CANONICAL option was used to conduct the CDA.

The first model included the seven allostatic load parameters that were thought to be the most strongly associated with type 2 DM status (HbA1c, FPG, insulin, BMI, waist-to-hip ratio, SBP, and DBP), and the one variable thought to be most weakly associated to type 2 DM status (sAA). After reviewing the canonical coefficients associated with each variable, the variable with the smallest coefficient was removed from the model and another variable was added. Subsequent models were tested and additional variables were added to the model one at a time based on how strongly they were believed to be associated with type 2 DM status, with the weaker variables added first.

Following the CDA, a one-way MANOVA was conducted to test group differences in the eight allostatic load parameters. The SAS PROC GLM procedure was used to conduct the one-way MANOVA. Prior to conducting the MANOVA, the multicollinearity of the allostatic load parameters was tested by examining the correlations between the 8 allostatic load parameters. If two variables were highly correlated (i.e., greater than 0.8), one of the variables was removed from the model (Stevens, 2009). Additionally, Pillai’s Trace multivariate test was used because it is believed to be the most reliable and protective against Type I errors when the sample size is small. If the multivariate test was significant, the results of the univariate $F$-tests for each variable were examined. Furthermore, the coefficients produced by the SAS PROC
DISCRIM procedure were used to rank the importance of each allostatic load parameter in predicting type 2 DM status.
CHAPTER 3: RESULTS

Stressor Confirmation

Self-Reported Stress

Figure 3 presents mean self-reported stress levels ± SEM during the baseline, stress, and recovery periods. A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, revealed a significant main effect of time \( F(2, 62.7) = 31.41, \ p < 0.001 \). Post-hoc analysis with Tukey-Kramer adjustments for multiple comparisons revealed that self-reported stress levels significantly increased from baseline to stress \([1.58, \ p < 0.001]\) and significantly decreased from stress to recovery \([-1.62, \ p < 0.001]\). There was not a significant group-by-time interaction \( F(2, 62.7) = 0.30, \ n.s. \) or main effect of group \( F(1, 32.8) = 0.60, \ n.s. \). Additional models were run with age and BMI included as covariates. Neither age nor BMI were significantly related to self-reported stress levels, nor did the overall fit of the model change after adding them to the model.

Systolic Blood Pressure

Figure 4 presents mean SBP (mmHg) ± SEM during the baseline, stress, rest, and recovery periods. A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, revealed a significant main effect of time \( F(3, 96) = 104.86, \ p < 0.001 \). Post-hoc analysis with Tukey-Kramer adjustments for multiple comparisons revealed that SBP significantly increased from the baseline to stress \( (28.23 \pm 1.69 \text{ mmHg}, \ p < 0.001) \), significantly decreased from stress to rest \( [-15.15 \pm 1.69 \text{ mmHg}, \ p < 0.001] \), and significantly decreased from rest to recovery \( [-7.56 \pm 1.69 \text{ mmHg}, \ p < 0.001] \). There also was a
significant main effect of group \[ F(1,32) = 7.21, p < 0.05 \], such that the type 2 DM group had significantly higher SBP levels during every time period. There was not a significant group-by-time interaction \[ F(3,96) = 0.35, \text{n.s.} \]. Additional models were run with age and BMI included as covariates. The main effect of group was no longer significant after including age and BMI as covariates in the same model \[ F(1,30) = 0.11, \text{n.s.} \], or when including age \[ F(1,31) = 1.03, \text{n.s.} \] and BMI \[ F(1,31) = 0.80, \text{n.s.} \] as covariates in separate models. Neither age nor BMI were significantly related to SBP in any of the models.

**Diastolic Blood Pressure**

Figure 5 presents mean DBP (mmHg) ± SEM during the baseline, stress, rest, and recovery periods. A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, revealed a significant main effect of time \[ F(3,96) = 66.93, p<0.001 \]. Post-hoc analysis with Tukey-Kramer adjustments for multiple comparisons revealed that DBP significantly increased from the baseline to stress (10.02 ± 0.76 mmHg, \( p < 0.001 \)), significantly decreased from the stress to rest (-7.11 ± 0.76 mmHg, \( p < 0.001 \)), and significantly decreased from rest to recovery (-1.26 ± 0.76 mmHg, \( p < 0.001 \)). There also was a significant main effect of group \[ F(1,32) = 6.16, p < 0.05 \], such that the type 2 DM group had significantly higher DBP levels during every time period. There was not a significant group-by-time interaction \[ F(3,96) = 1.30, \text{n.s.} \]. Additional models were run with age and BMI included as covariates. The main effect for group was no longer significant after including age and BMI as covariates in the same model \[ F(1,30) =0.47, \text{n.s} \] or when including age \[ F(1,31) = 1.02, \text{n.s.} \] and BMI \[ F(1,31) =0.14, \text{n.s.} \] as
covariates in separate models. Age was not a significant covariate in any of the models, and BMI was not a significant covariate when it was included as the only covariate in the model. However, BMI was significantly related to DBP when it was included in the model with age \[F(1,30) = 4.78, \ p < 0.05\].

**Heart Rate**

Figure 6 presents the mean HR (bpm) ± SEM during the baseline, stress, rest, and recovery periods. A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, revealed a significant main effect of time \[F(3,96) = 65.40, \ p < 0.001\]. Post-hoc analysis with Tukey-Kramer adjustments for multiple comparisons revealed that HR significantly increased from the baseline to stress period (12.20 ± 1.07 bpm, \(p < 0.001\)), significantly decreased from the stress to rest (-9.62 ± 1.07 bpm, \(p < .001\)), and significantly decreased from the rest to recovery (-3.84 ± 1.07 bpm, \(p < 0.001\)). There was not a significant main effect of group \[F(1,32) = 1.27, \text{n.s.}\] nor a significant group-by-time interaction \[F(3,96) = 0.61, \text{n.s.}\]. Additional models were run with age and BMI included as covariates. Age was not a significant covariate in the models. BMI was significantly related to HR \[F(1,31) = 9.64, \ p < 0.05\], but it did not improve the fit of the model.

**HPA-axis stress physiology**

**Salivary Cortisol**

**Baseline.** Table 2 contains the mean raw baseline cortisol (nmol/L) levels ± SEM for the two groups. Results of a one-way ANOVA did not show that the type 2 DM group had significantly higher baseline cortisol levels compared to the healthy group. Although the type 2 DM group had higher baseline cortisol levels (6.06 ± 0.88 nmol/L) compared
to the healthy group (5.28 ± 0.75 nmol/L), the difference was not statistically significant \[ F(1,32) = 0.70, \text{n.s.} \]. Additional models were run with age and BMI included as covariates. Neither age nor BMI were significantly associated with cortisol levels, nor did the fit of the model change after adding them to the model.

**Stress response.** Figure 7 presents the mean raw cortisol (nmol/L) levels ± SEM at baseline, stress, and recovery for the two groups. A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, revealed a significant main effect of time \[ F(2,64) = 37.17, \ p < 0.001 \]. Post-hoc analysis with Tukey-Kramer adjustments for multiple comparisons revealed that cortisol levels significantly increased from baseline to stress (8.99 nmol/L, \( p < 0.001 \)) and significantly decreased from stress to recovery (-8.29 nmol/L, \( p < 0.001 \)). There was not a significant main effect of group \[ F(1,32) = 0.76, \text{n.s.} \] nor a significant group-by-time interaction \[ F(2,64) = 0.27, \text{n.s.} \]. Additional models were run with age and BMI included as covariates. Neither age nor BMI were significantly related to cortisol levels, nor did the overall fit of the model improve after adding them to the model.

**Stress reactivity and recovery.** Table 3 contains the mean cortisol stress reactivity and stress recovery change scores ± SEM for the two groups. Positive stress reactivity change scores indicate that cortisol levels increased from baseline to stress, and positive recovery change scores indicate that cortisol levels decreased from stress to recovery. Five participants had negative cortisol reactivity scores (2 type 2 DM, 3 healthy), but no participants had negative cortisol recovery scores.

Results from the separate one-ANOVARs did not show that men in the type 2 DM group displayed dampened cortisol reactivity \[ F(1,32) = 2.29, \ p = 0.14 \]. The type 2 DM
displayed steeper cortisol reactivity (10.40 ± 2.38 nmol/L) compared to the healthy group (7.58 ± 2.58 nmol/L), but the difference was not statistically significant \[ F(1,32) = 0.83, p = 0.37 \]. Similarly, the type 2 DM had a steeper cortisol recovery (9.97 ± 1.71 nmol/L) compared the healthy group (6.60 ± 2.43 nmol/L), but the difference was not statistically significant \[ F(1,32) = 2.29, p = 0.14 \]. Additional models were run with age and BMI included as covariates. Neither age nor BMI were significant predictors of cortisol reactivity or recovery, nor did the fit of the model improve after adding them to the model.

**Area under the curve.** Table 3 contains the mean cortisol AUC\(_g\) and AUC\(_i\) concentrations ± SEM for the two groups. Results of the separate one-way ANOVAs did not show that the type 2 DM group had significantly higher AUC\(_g\) or significantly lower AUC\(_i\) compared to the healthy group. Although the type 2 DM group had higher AUC\(_g\) (18.30 ± 2.58 nmol/L) than the healthy group (14.80 ± 2.16 nmol/L), the difference was not statistically significant \[ F(1,32) = 1.08, \text{n.s.} \]. Similarly, although the type 2 DM group had higher AUC\(_i\) (10.72 ± 2.29 nmol/L) than the healthy group (8.20 ± 1.91 nmol/L), the difference was not statistically significant \[ F(1,32) = 0.71, \text{n.s.} \]. Additional models were run with age and BMI included as covariates. Neither age nor BMI were significantly associated with cortisol AUC\(_g\) or AUC\(_i\), nor did the fit of the model improve after adding them to the model.

**Salivary DHEA-S**

**Baseline.** Table 3 contains the mean raw baseline DHEA-S (ng/ml) levels ± SEM for the two groups. Results from a one-way ANOVA did not show that the type 2 DM group had significantly lower baseline DHEA-s levels compared to the healthy group.
Although the type 2 DM group had lower baseline DHEA-S levels (3.78 ± 0.45μg/mL) compared to the healthy group (4.85 ± 0.38 ng/ml), the difference was not statistically significant \[F(1,32) = 2.99, p = 0.09\]. Additional models were run with age and BMI included as covariates. Neither age nor BMI were significantly associated with baseline DHEA-S levels, nor did fit of the model improve after adding them to the model.

**Stress response.** Figure 8 presents the mean raw DHEA-S (ng/ml) levels ± SEM at baseline, stress, and recovery for the two groups. A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, did not show a significant main effect of time \[F(2,64) = 2.48, p = 0.09\] or group \[F(1,32) = 2.62, p = 0.12\], or a significant group-by-time interaction effect \[F(2,64) = 1.47, n.s.\]. Additional models were run with age and BMI included as covariates. BMI was not significantly associated with DHEA-S levels when it was the only covariate in the model. When age was included as the only covariate in the model, group became significant, such that the type 2 DM group had significantly lower DHEA-S levels \[F(1,31) = 4.44, p < 0.05\]. Age was not significantly associated with DHEA-S in this model. The significant main effect of group disappeared when both age and BMI were included as covariates in the same model \[F(1,30) = 0.54, n.s.\].

**Stress reactivity and recovery.** Table 2 contains the mean DHEA-S (ng/ml) stress reactivity and stress recovery change scores ± SEM for the two groups adjusted. Positive stress reactivity change scores indicate that DHEA-S levels increased from baseline to stress, and positive recovery change scores indicate that DHEA-S levels decreased from stress to recovery. Eighteen participants (12 healthy, 6 type 2 DM) had
negative DHEA-S reactivity scores, and 13 participants (11 healthy, 2 type 2 DM) had negative DHEA-S recovery scores.

Results from the one-ANOVA did not show that the type 2 DM group had dampened DHEA-S reactivity (i.e., smaller reactivity score) compared to the healthy group [$F(1,31) = 1.24, n.s.$]. This model included age as a covariate because it was a significant predictor of DHEA-reactivity, such that increased age was associated with increased reactivity [$F(1,31) = 4.81, p < 0.05$]. The group-by-age interaction was also tested, but it was not significantly associated with DHEA-S reactivity. When age was added as a covariate in the model, the direction of the mean DHEA-S reactivity changed for both groups. In the model without age as a covariate, the type 2 DM group had a positive mean DHEA-S reactivity score (0.13 ± 0.46 ng/ml), and the healthy group had a negative mean DHEA-S reactivity score (-0.14 ± 0.38 ng/ml). Whereas when age was included as a covariate, the type 2 DM group displayed a negative mean DHEA-S reactivity score (-0.53 ± 0.53 ng/ml) and the healthy group had a positive mean DHEA-S reactivity score (0.32 ± 0.42 ng/ml).

Although the two groups did not significantly differ in DHEA-S recovery, the difference approached significance [$F(1,32) = 3.44, p=0.07$]. Similar to DHEA-S reactivity, the direction of the mean DHEA-S recovery score differed between the two groups, such that the type 2 DM group had a positive mean DHEA-S recovery score (0.68 ± 0.34 ng/ml) and the healthy group had a negative DHEA-S recovery score (-0.01 ± 0.27 ng/ml). Furthermore, when additional models were run with age and BMI included as covariates, neither age nor BMI were significant predictors of DHEA-S recovery, nor did the overall fit of the model change after adding them to the model.
**Area under the curve.** Table 3 contains the mean DHEA-S (ng/ml) AUC$_g$ and AUC$_i$ concentrations $\pm$ SEM for the two groups. Results of the separate one-way ANOVAs did not show that the type 2 DM group had significantly lower AUC$_g$ or AUC$_i$ compared to the healthy group. Although the type 2 DM group had smaller mean AUC$_g$ (4.68 $\pm$ 0.46 ng/ml) than the healthy group (5.96 $\pm$ 0.55 ng/ml), the difference was not significant [$F(1,32) = 3.13, p = .09$]. Additionally, although the type 2 DM a greater mean AUC$_i$ (-0.05 $\pm$ 0.36 ng/ml) compared to the healthy group (-0.13 $\pm$ 0.30 ng/ml), the difference was not significant, [$F(1,32) = .71, n.s.$]. When additional models were run with age and BMI included as covariates, neither age nor BMI were significantly related to AUC$_g$. Age was significantly related to AUC$_i$ [$F(1,30) = 5.24, p < .05$], such that increased age was associated with increased AUC$_i$. BMI was not significantly related to AUC$_i$.

**SAM-Axis Stress Physiology**

**Salivary $\alpha$-amylase**

**Baseline.** Table 3 contains the mean raw baseline sAA(U/ml) levels $\pm$ SEM for the two groups. Results from the one-way ANOVA did not show that the type 2 DM group had significantly lower baseline sAA compared to the healthy group. Although the type 2 DM group had lower baseline sAA levels (115.50 $\pm$ 22.17 U/ml) compared to the healthy group (128.89 $\pm$ 19.03 U/ml), the difference was not statistically significant [$F(1,32)=0.07, n.s.$]. Additional models were run with age and BMI included as covariates. Neither age nor BMI were significantly associated with sAA levels, nor did the fit of the model improve after adding them to the model.
**Stress response.** Figure 9 presents the mean raw sAA(U/ml) levels ± SEM at baseline, stress, and recovery. A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, revealed a significant main effect of time [$F(2,62) = 3.57, \ p < 0.05$]. *Post-hoc* analysis with Tukey-Kramer adjustments for multiple comparisons revealed that sAA significantly increased from baseline to stress (29.69 U/ml, $p < 0.05$), but did not significantly decrease from stress to recovery (-9.96 U/ml, n.s.). There was not a significant main effect of group [$F(1,31) = 0.02, \ n.s.$] nor a significant group-by-time interaction [$F(2,62) = 0.08, \ n.s.$]. When additional models were run with age and BMI included as covariates, neither age nor BMI were significant predictors of sAA, nor did the model improve after adding them to the model.

**Stress reactivity and recovery.** Table 3 contains the mean raw sAA(U/ml) stress reactivity and stress recovery change scores ± SEM for the two groups. Positive stress reactivity change scores indicate that sAA levels increased from baseline to stress, and positive recovery change scores indicate that sAA levels decreased from stress to recovery. Ten participants (6 healthy, 4 type 2 DM) had negative sAA reactivity scores, and 12 participants (6 healthy, 6 type 2 DM) had negative sAA recovery scores.

Results from the one-ANOVA did not show that the type 2 DM group had dampened sAA reactivity (i.e., smaller reactivity score) compared to the healthy group [$F(1,31) = 0.02, \ n.s.$]. The sAA recovery scores also did not significantly differ between the two groups [$F(1,31) = 0.07, \ n.s.$]. When additional models were run with age and BMI included as covariates, neither age nor BMI were significant predictors of sAA reactivity or recovery, nor did the fit of the model improve after adding them.
Area under the curve. Table 3 contains the mean sAA (U/ml) AUC\textsubscript{g} and AUC\textsubscript{i} concentrations ± SEM for the two groups. Results of the one-way ANOVA did not show that the type 2 DM group had significantly higher sAA AUC\textsubscript{g} compared to the healthy group. The type 2 DM group had a lower AUC\textsubscript{g} (174.18 ± 30.13 U/ml) than the healthy group (178.27 ± 25.87 U/ml), but the difference was not statistically significant [F(1,31) = 0.01, n.s.]. When additional models were run with age and BMI included as covariates, neither age nor BMI were significant predictors of sAA, nor did the overall fit of the model change after adding them to the model.

Results of the one-way ANOVA also did not show that the two groups had statistically significant differences in AUC\textsubscript{i} concentrations. Although the difference was not statistically significant, it approached significance [F(1,30) = 3.64, \( p = .06 \)]. The type 2 DM group had a greater AUC\textsubscript{i} (29.80 ± 14.56 U/ml) compared to the healthy group (17.17 ± 12.49 U/ml). Additionally, the this AUC\textsubscript{i} model included BMI as a covariate because it was a significant predictor of AUC\textsubscript{i}, such that higher BMI was associated with higher AUC\textsubscript{i} [F(1,30) = 4.49, \( p < 0.05 \)]. The group-by-BMI interaction was also tested, but it was not significantly associated with AUC\textsubscript{i}. Furthermore, age was not significantly related to sAA AUC\textsubscript{i} in any of the models, nor did it change the fit of the model.

### Allostatic Load

#### Group Differences in Allostatic Load

Table 4 contains the means ± SEM for the 15 allostatic load parameters, the percentage of participants in each allostatic load parameter risk category (low risk, moderate risk, high), and the total allostatic load scores for each group. Table 5 contains
the contains for Pearson product moment correlation coefficients for the correlations between the 15 allostatic load parameters. Results from the one-way ANOVA revealed that the type 2 DM group had significantly higher total allostatic load (12.64 ± 0.90) compared to the healthy group (4.75 ± 0.75) \[F(1,32) = 45.48, p<0.001\]. Additional models were run with age included as a covariate. Age was not a significant predictor of total allostatic load, nor did it improve the overall fit of the model so it was not included in the final model.

Table 6 contains the pooled within canonical structure coefficients for the 7 models tested in the CDA. Prior to conducting the one-way MANOVA, CDA was used to determine which parameters should be included in the final MANOVA model. The variables were added to the model based on how strongly they were conceptually believed to be associated with type 2 DM status. The first CDA model included the 7 allostatic load parameters that were believed to be the most strongly related to type 2 DM status (hemoglobin A1c, fasting plasma glucose, insulin, BMI, waist-to-hip ratio, SBP, and DBP) and the one parameter least likely to be associated with type 2 DM status (sAA). Then the canonical coefficients of these parameters were examined to determine which one should be removed from the model. In the first CDA analysis, sAA had the smallest canonical structure coefficient, and thus was removed from the model. Subsequent models were run by adding the remaining variables in a one at a time in the following order: cortisol, DHEA-S, CRP, LDL, total cholesterol, triglycerides, and HDL. The final model included HbA1c, FPG, insulin, BMI, waist-to-hip ratio, diastolic blood pressure, triglycerides and HDL cholesterol. A one-way MANOVA then was conducted to test group differences in these 8 allostatic load parameters.
Table 7 contains results for the multivariate and univariate tests from the allostatic load parameter one-way MANOVA. Results of the one-way MANOVA revealed that the multivariate test was significant [Pillai’s Trace =0.79, F(8,25) = 12.3, p < 0.001], indicating that there were significant differences between the type 2 DM group and healthy group. Results of the follow-up univariate F-tests showed significant group differences on 8 allostatic load parameters.

The results of the CDA also provided descriptive information about the allostatic load parameters included in the one-way MANOVA. The canonical structure coefficients were used to rank the importance of each allostatic load parameter in predicting type 2 DM status. A high coefficient suggested that the two groups differed a lot on that variable. HbA1c was most strongly associated with type 2 DM status followed by FPG, insulin, BMI and waist-hip ratio. DBP, HDL cholesterol, and triglycerides were the weakest predictors of type to DM status. Additionally, the PROC DISCRIM procedure provided information about group membership and misclassified participants. The results revealed that four participants should be reclassified based on the discriminant function created from the allostatic load parameters. More specifically, the results reclassified three type 2 DM participants as healthy and one healthy participant as type 2 DM.

Allostatic Load and Stress System Physiology

Salivary cortisol. A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, revealed a significant main effects of time [$F(2,66) = 0.27, p <0.001$], which was discussed in the previous section on the cortisol stress response. There was not a
significant main effect for total allostatic load \([F(1,32) = 0.55, n.s.]\). Two additional models also were tested that included a total allostatic load-by-time term or a allostatic load-by-group term, but they did not reveal a significant interaction for total allostatic load-by-time \([F(2,64) = 1.71, n.s.]\) or total allostatic load-by-group \([F(1,31) = 1.14, n.s.]\). Furthermore, when models were run with age and BMI included as covariates, neither age nor BMI were significant predictors of cortisol, nor did the overall fit of the model change after adding them to the model.

Results from the separate linear regression analyses did not reveal that total allostatic load predicted cortisol reactivity, AUC\(_i\), or AUC\(_g\). Although total allostatic load did not significantly predict cortisol reactivity, the relationship approached statistical significance \([R^2=10.36, \beta = 0.55, t(32)=1.92, p=0.06].\) Similarly, total allostatic load was not a significant predictor of AUC\(_i\) \([\beta = 0.50, t(32) =1.81, p =0.08]\) or AUC\(_g\) \([\beta = 0.46, t(32) =1.45, p =0.16]\), but the relationships approached statistical significance. Total allostatic load did significantly predict cortisol recovery, such that higher allostatic load was associated greater cortisol recovery (i.e., larger decrease in cortisol from stress to recovery) \([R^2=12.29, \beta =0.44, t(32)=2.12, \ p<0.05].\)

**Salivary DHEA-S.** A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, revealed a significant main effects of time, did not reveal significant main effects of time \([F(2,66) = 2.08, n.s.]\) or total allostatic load \([F(1,32) = 1.70, n.s.]\). Two additional models also were tested that included a total allostatic load-by-time term or a total allostatic load-by-group term, but they did not reveal a significant total allostatic load-by-time interaction \([F(2, 64) =0.90, n.s.]\) or allostatic load-by-group \([F(1, 31) = 0.45, n.s.]\).
Furthermore, when models were run with age and BMI included as covariates, neither age nor BMI were significant predictors of DHEA-S, nor did the overall fit of the model change after adding them to the model.

The results of the separate linear regression analyses did not show that total allostatic load significantly predicted DHEA-S reactivity \( R^2=0.02, \beta=0.02, t(32)=0.29, \text{n.s.} \) or recovery \( R^2=0.03, \beta=0.04, t(32)=1.06, \text{n.s.} \). Similarly, the results of the separate linear regression analyses did not show that total allostatic significantly predicted AUC \( R^2=0.00, \beta=-0.006, t(32)=0.11, \text{n.s.} \) or AUC \( R^2=0.06, \beta=-0.10, t(32)=-1.40, \text{n.s.} \).

**Salivary \( \alpha \)-amylase.** A linear mixed model with group, time, and group-by-time interaction terms treated as fixed effects, and participants treated as a random effect, revealed a significant main effects of time, revealed a significant main effect of time \( F(2,64) = 3.61, p < 0.05 \), which was described in the previous section on sAA stress physiology. There was not a significant main effect of total allostatic load \( F(1, 31) = 0.22, \text{n.s.} \). Two additional models also were tested that included either a allostatic load-by-time term or an allostatic load-by-group term, but they did not demonstrate a significant total allostatic load-by-time interaction \( F(2, 62) = 0.97, \text{n.s.} \) or total allostatic load-by-group interaction \( F(1,29) = 0.8, \text{n.s.} \). Furthermore, when models were run with age and BMI included as covariates, neither age nor BMI were significant predictors of sAA, nor did the overall fit of the model change after adding them to the model.

The results of the separate linear regression analyses did not show that total allostatic load significantly predicted sAA reactivity \( R^2=0.05, \beta=-0.04, t(32)=-1.27, \text{n.s.} \) or recovery \( R^2=-5.31, \beta=0.04, t(32)=-1.75, \text{n.s.} \). Similarly, the results of the separate linear regression analyses did not show that total allostatic load significantly
predicted $AUC_i [R^2=0.00, \beta=0, t(32)=-0.02, \text{n.s.}]$ or $AUC_g [R^2=0.02, \beta=-0.01, t(32)=-0.60, \text{n.s.}]$.

**Summary of Hypotheses**

**Hypothesis 1: Salivary free cortisol**

I hypothesized that the salivary free cortisol profiles would differ significantly between the type 2 DM group and healthy control group such that the type 2 DM group would have higher baseline cortisol levels, dampened cortisol reactivity, and dampened cortisol recovery in response to the TSST task compared to the healthy control group. This hypothesis was not supported. The type 2 DM had higher baseline cortisol levels, but the difference was not statistically significant. In regards to cortisol response patterns, however, there was a significant main effect of time, with cortisol significantly increasing from baseline to stress and significantly decreasing from stress to recovery. However, there was neither a significant main effect of group nor a significant time-by-group interaction, which indicates that the cortisol response patterns of the groups were not different. Furthermore, the analyses examining cortisol reactivity and recovery change scores showed that the type 2 DM had steeper cortisol reactivity and recovery compared to the healthy group, but the differences was not statistically significant. Results also did not show that the type 2 DM group had significantly higher $AUC_g$ or significantly lower $AUC_i$ compared to the healthy group. Although the type 2 DM group had higher $AUC_g$ and $AUC_i$ than the healthy group, the differences were not statistically significant.

**Hypothesis 2: Salivary DHEA-S**

I hypothesized that salivary DHEA-S profiles would differ significantly between the type 2 DM group and the healthy control group such that the type 2 DM group would
have lower baseline DHEAS levels, dampened DHEAS reactivity, and dampened DHEAS recovery in response to the TSST compared to the healthy control group. This hypothesis was partially supported. In regards to baseline DHEA-S, there was a marginally significant difference ($p = 0.09$) between the two groups such that the type 2 DM group had lower levels compared to the healthy group. The DHEA-S response pattern analyses revealed a statistically significant main effect of group such that the type 2 DM group had lower DHEA-S levels across all time points. However, there was not a significant group-by-time interaction, indicating that the response pattern did not significantly differ between the two groups.

There also was a marginally significant group difference in DHEA-S recovery changes scores ($p = 0.07$). The type 2 DM group had a larger recovery score (i.e., greater decrease in DHEA-S levels from stress to recovery) compared to the healthy group. Results did not show group differences in DHEA-S reactivity change scores, but age was significantly related the DHEA-S reactivity change score such that increased age was associated with increased reactivity change scores. Additionally, when age was added as a covariate in the model, the direction of the mean DHEA-S reactivity change score changed for both groups. In the model without age as a covariate, the type 2 DM group had a positive mean DHEA-S reactivity score, and the healthy group had a negative mean DHEA-S reactivity score. Whereas when age was included as a covariate, the type 2 DM group displayed a negative mean DHEA-S reactivity score, and the healthy group displayed a positive mean DHEA-S reactivity score.

The results also did not reveal that the type 2 DM group had significantly lower AUC$_g$ or AUC$_i$ compared to the healthy group. Although the type 2 DM group had
smaller mean $\text{AUC}_g$ and greater mean $\text{AUC}_i$ than the healthy group, the differences were not significant. Age was significantly related to $\text{AUC}_i$ such that increased age was associated with increased $\text{AUC}_i$.

**Hypothesis 3: Salivary alpha-amylase (sAA)**

I hypothesized that sAA profiles would differ significantly between the type 2 DM group and the healthy control group such that the type 2 DM group would have higher baseline sAA levels, dampened sAA reactivity, and dampened sAA recovery in response to the TSST compared to the healthy control group. The hypothesis was not supported. Results did not show that the type 2 DM group had significantly lower baseline sAA compared to the healthy group. In regards to the sAA response pattern, there was neither a significant group-by-time interaction nor main effect of group. However, there was a significant main effect of time, such that sAA levels significantly increased from baseline to stress. Additionally, there were not significant group differences in sAA reactivity change scores or recovery changes scores. Results also did not show that the type 2 DM group had significantly higher $\text{AUC}_g$ compared to the healthy group. However, there was a marginally significant group difference ($p = 0.06$) in $\text{AUC}_i$ such that the type 2 DM group had greater $\text{AUC}_i$ compared to the healthy group.

**Hypothesis 4: Allostatic load**

I hypothesized that the type 2 DM group and the healthy control group would differ significantly on all allostatic load parameters. This hypothesis was supported. However, because all allostatic load parameters could not be included in the same MANOVA model due to the small sample size, I can only report about group differences in the eight allostatic load parameters included in the final MANOVA model (HbA1c,
FPG, insulin, BMI, waist-to-hip ratio, DBP, triglycerides, and HDL cholesterol). Of the parameters included in the final model, five were metabolic parameters, two were anthropometric parameters, and one was a cardiovascular parameter.

The groups were significantly different on all allostatic load parameters included in the final model. The type 2 DM group had significantly higher HbA1c, FPG, insulin, BMI, waist-to-hip ratios, DBP, and triglyceride levels and significantly lower HDL cholesterol levels compared to the healthy group. Additionally, HbA1c, FPG, and insulin canonical coefficient values indicated that the two groups differed the most on these variable. HbA1c was most strongly associated with type 2 DM status followed by FPG, insulin, BMI and waist-hip ratio. DBP, HDL cholesterol, and triglycerides were the weakest predictors of type to DM status.

**Hypothesis 5: Effect of allostatic load on stress reactivity and stress recovery**

**5a. Main effects of allostatic load.** I hypothesized that higher total allostatic load scores would be associated with stress system response patterns. This hypothesis was not supported. Results from the cortisol, DHEA-S, and sAA stress response analyses did not show that that there was a significant main effect of total allostatic load.

The results from the cortisol reactivity score and cortisol recovery score analyses were in the opposite direction of what was hypothesized. Although only marginally statistically significant ($p = 0.06$), total allostatic load was positively related to cortisol reactivity such that increased allostatic load was associated with larger cortisol reactivity scores. Total allostatic load significantly predicted cortisol recovery such that higher allostatic load was related to higher recovery scores, which is the opposite direction of the hypothesis Total allostatic load did not significantly predict cortisol $AUC_i$ or $AUC_g$. 
Additionally, allostatic load did not significantly predict reactivity scores, recovery scores, AUCg or AUCi for DHEA-S or sAA.

5b. Allostatic load by type 2 diabetes status interaction. I hypothesized that the magnitude of the effect of allostatic load on stress reactivity and stress recovery would depend on type 2 DM status (i.e., there would be a significant group X allostatic load interaction). I hypothesized that allostatic load would be a stronger predictor of stress reactivity and stress recovery in the type 2 DM group compared to the healthy control group. This hypothesis was not supported. None of the stress biomarker models that included an allostatic load-by-group term revealed significant interaction effects.
CHAPTER FOUR: DISCUSSION

Overview

Type 2 DM is one of the most common and costly chronic diseases in the world, and contributes to the death of over 230,000 Americans annually (CDC, 2011). Thus, it is extremely important to identify factors that contribute to its pathogenesis and pathophysiology. The complex pathophysiology of type 2 DM has made it difficult to develop truly effective prevention and treatment interventions. In recent years, scientists have started to investigate the role of psychological factors, such as psychosocial stress, in the pathophysiology of type 2 DM (Nathan et al., 2009). Although data from observational studies provide suggestive evidence that psychosocial stress is a factor affecting type 2 DM onset and progression, mechanisms involved in the relationship are unclear. A better understanding of the stress physiological processes in type 2 DM may assist scientists in developing targeted, stress-specific interventions and also may help to identify individuals with type 2 DM who are more physiologically vulnerable to the negative health effects of psychosocial stress.

There has been a paucity of research examining stress physiological processes in individuals with type 2 DM. The majority of the research on stress physiology in type 2 DM has not examined stress physiology in the context of acute psychosocial stressors. Rather, they examined stress system physiology following pharmacological challenges (e.g., dexamethasone suppression test) or in non-stressor settings (e.g., cortisol diurnal rhythm, basal stress biomarker levels) (e.g., Cameron, Kronfol, Greden, & Carroll, 1984; Chiodini et al., 2006; Lederbogen et al., 201; Roy, Roy, & Brown, 1998). Findings from these studies suggest that individuals with type 2 DM have impaired stress system
functioning, but these findings do not provide information about how acute psychosocial stressors specifically affect stress system functioning (de Kloet, Joels, & Holsboer, 2005).

The goal of this dissertation was to address the gap in the literature by examining acute psychosocial stress and stress physiology in men with type 2 DM without major disease-related complications (e.g., retinopathy, uncontrolled hypertension, unstable angina, nephropathy). The primary aim was to investigate the effects of an acute psychosocial stressor on HPA-axis and SAM-axis function. A secondary aim of this dissertation was to explore the relationship between allostatic load, which is an index of chronic stress, and stress physiology. The present chapter examines findings from this dissertation study in greater detail. Specifically, this chapter will discuss the meaning of the findings and propose possible explanations for them; synthesize findings across the analyses; examine study imitations; and make suggestions for future research.

**Stress Physiology and Type 2 DM Findings**

**Stressor Confirmation**

To test the efficacy of the stressor used in this dissertation, I analyzed both subjective (self-reported stress levels) and objective indicators (SBP, DBP, heart rate, salivary cortisol) of stress reactivity. Results revealed that SBP, DBP, heart rate, cortisol, and self-reported stress levels significantly increased for participants in both groups following the TSST stressor task. These findings provide convincing evidence that the TSST task successfully induced both physiological and psychological responses in both groups of participants.
HPA-Axis Findings

Overall, the cortisol and DHEA-S findings did not demonstrate that type 2 DM is associated with dysregulated HPA-axis responses to an acute psychosocial stressor. As discussed previously in this dissertation, there have been few prior studies investigating the effects of acute psychosocial stress on HPA-axis function in humans with type 2 DM. Thus, the HPA-axis findings from this dissertation contribute important new information to the type 2 DM literature by demonstrating that men with type 2 DM without major disease-related complications have intact HPA-axis function.

Salivary cortisol. The findings from this study did not show that type 2 DM participants had dysregulated salivary cortisol responses to an acute psychosocial stressor. Rather, the type 2 DM participants had a robust cortisol response, which suggests that the HPA-axis has not been significantly altered by type 2 DM at this point in their disease progression. This finding is consistent with findings from the one other study to date that has specifically examined the cortisol response to an acute psychosocial stressor in humans (men and women) with type 2 DM (Faulenbach et al., 2012). Similar to the present study, Faulenbach and colleagues (2012) found that salivary cortisol levels, heart rate, and blood pressure increased significantly from baseline to post-stressor in men and women with type 2 DM without major disease-related complications. However, the researchers could not make conclusions about how the cortisol responses of individuals with type 2 DM differed from healthy individuals because the study did not include a healthy control group. Thus, the cortisol findings from this dissertation study provide valuable new information regarding the comparability of cortisol responses between individuals with type 2 DM and healthy controls. Taken together, findings from
this dissertation study and Faulenbach et al. (2012) provide suggestive evidence that individuals with type 2 DM without major disease-related complications have intact cortisol responses to acute psychosocial stress.

In the context of daily cortisol rhythms and cortisol negative feedback control, the findings from this dissertation conflict with findings from previous studies examining cortisol patterns in individuals with type 2 DM. More specifically, findings from previous studies that investigated the diurnal cortisol rhythm and negative feedback control in individuals with type 2 DM indicate that type 2 DM is associated with altered cortisol patterns (e.g., flattened diurnal rhythm, impaired negative feedback control, blunted cortisol awakening response) (e.g., Bruehl, Wolf, & Convit, 2009; Cameron et al., 1984; Chiodini et al., 2006; Hudson et al., 1984). However, the prior research did not examine cortisol patterns following acute psychosocial stress exposure, which could explain the discrepancy in the findings.

It is difficult to compare the findings from these studies to the current study because the methodologies used in the prior studies tested different physiological mechanisms and addressed different research questions. More specifically, findings on the diurnal cortisol rhythm and basal cortisol levels are reflective of HPA-axis function in the context of low levels of cortisol. The effects of low levels of cortisol (i.e., nighttime levels, non-stressed levels) are primarily mediated by mineralocorticoid receptors, whereas the effects of high levels of cortisol (i.e., morning levels and stress levels) are primarily mediated by glucocorticoid receptors (Sapolsky et al., 2000). Cortisol produces different physiological effects depending on the type of receptors it binds to in the periphery or CNS. Typically, cortisol only binds to glucocorticoid receptors when
mineralocorticoid receptors are saturated, which only occurs when there are high levels of circulating cortisol (e.g., during times of stress) (Sapolsky et al., 2000). Thus, studies examining diurnal rhythm patterns and basal cortisol levels may be tapping into different HPA-axis physiological mechanisms compared to studies examining cortisol patterns following acute psychosocial stress exposure. I did not examine daily cortisol patterns in these participants so future studies should examine daily cortisol patterns in conjunction with laboratory-based acute psychosocial stress exposure to better understand cortisol pattern alterations in individuals with type 2 DM.

It is plausible that the type 2 DM participants in the present study may have displayed alterations in diurnal cortisol patterns and negative feedback control. Furthermore, the participants in the previous studies may have displayed robust cortisol responses following acute psychosocial stress exposure. However, this suggestion is speculative and would require additional research. Future studies should perform a comprehensive assessment of cortisol patterns that includes: testing the effects of acute psychosocial stress exposure; assessing the cortisol diurnal rhythm and cortisol awakening response; and testing the negative feedback loop. Furthermore, it is possible that the alterations in cortisol diurnal rhythm and the negative feedback loop precede alterations in the cortisol response to acute psychosocial stress. Thus, prospective studies should be conducted to determine the temporal order of cortisol pattern alterations and type 2 DM.

Another explanation for the unexpected robust cortisol stress response results could be the clinical characteristics of type 2 DM sample (e.g., no major complications, short duration of disease). Previous studies have found differences in diurnal cortisol
patterns between individuals with type 2 DM with and without chronic complications (e.g., retinopathy, neuropathy, and nephropathy). Individuals with type 2 DM with chronic complications displayed the greatest degree of diurnal cortisol pattern alterations (e.g., Chiodini et al., 2006; Roy et al., 1998; Vermes, Steinmetz, Schoorl, van der Veen, & Tilders, 1985). Roy and colleagues (1998) also found that total daily cortisol output was positively associated with longer duration of diabetes. The participants in this dissertation study did not have major disease-related complications and had a relatively short disease duration ($M = 6.14$ years, $SD = 3.98$). Thus, it is plausible that participants in the present study were “too healthy” and that alterations in cortisol patterns occur later in the disease process. Studies should be conducted that compare diurnal cortisol patterns and cortisol stress response patterns across the type 2 DM disease continuum. (i.e., compare healthy, pre-diabetes, earlier stages of type 2 DM, later stages of type 2 DM).

Assessing cortisol responses across the continuum could provide valuable information about the time course of HPA-axis alterations and the temporal relationship between type 2 DM and HPA-axis alterations (i.e., Do the alterations come before or after type 2 DM onset?).

The results of the present study diverge from prior research using rodent models (mice and rats) to study the effects of acute stress on type 2 DM stress physiology. Studies using rodent models of diabetes have found that, in male rodents, type 2 DM is characterized by elevated basal levels of glucocorticoids, blunted glucocorticoid response to stressors, and delayed recovery from physical stressors (i.e., HPA-axis does not turn off following termination of the physical stressor) (e.g., Chan, Inouye, Vranic, & Matthews, 2002; Magariños & McEwen, 2000; Plotsky, Thrivikraman, Watts, & Hauger,
Winocur et al., 2005). A possible explanation for the conflicting results between the present study and the rodent studies is that the findings from studies using rodent models of type 2 DM may not be generalizable to participants in the present study. More specifically, the nature of the stressors used (e.g., type of stressor, time of stress testing), and the severity of the rodents’ disease may limit the extent to which the findings are applicable to the experiences of humans with type 2 DM (King, 2012).

For example, the findings from studies conducted by Chan and colleagues (2002) and Magariños and McEwen (2000) used male streptozotocin (STZ) treated mice and rats, which involves chemically inducing DM in the animals. In this model, β-cells are destroyed and endogenous insulin production is immediately reduced. Findings from studies using STZ models may not be generalizable to the type 2 DM participants from this dissertation study because the β-cells of participants in this study were still adequately producing insulin (i.e., participants were not taking insulin). The STZ model may be more applicable to research questions regarding HPA-axis functioning in type 2 diabetics taking insulin, which usually occurs latter on in the disease process (Fonseca, 2009).

Plotsky and colleagues (1992) and Winocur and colleagues (2005) used male Zucker diabetic fatty rats, which are an inbred strain of rats with a mutation in the leptin receptor. These rats become obese by 4 weeks of age, develop hyperinsulinemia by 8 weeks, and develop diabetes by 10 weeks (approximately 5 years of age in humans) (King, 2012; Sengupta, 2013). Although the Zucker diabetic fatty rat model more closely mimics type 2 DM disease progression in humans than the STZ model, the Zucker rats develop diabetes much earlier in their lifecycles compared to humans. An additional
component of these studies that limits the generalizability of the findings is that the HPA-axis testing was performed when the rats were young. More specifically, Winocur and colleagues (2005) tested the rats at 6 months of age (approximately 13 years in humans) and Plotsky and colleagues (2002) tested the rats at 10 weeks of age (approximately 6 years in humans). Thus, the rats were tested during childhood and adolescence, which does not parallel the typical type 2 DM disease progression in humans.

Although using non-human models of type 2 DM may limit the generalizability of the results, they provide a high degree of experimental control which increases the internal validity. More specifically, non-human animal models enable researchers to ensure that the disease severity and duration is uniform across all animals; whereas in the present study there was within group variability in disease duration and diabetes medications, as well as both within group and between group variability in age, BMI, and the presence of comorbid conditions. This variability is a limitation of the present study and may be a possible explanation for the discrepancy between cortisol findings from studies using rodent models and the present study.

There also may have been alterations in other physiological mechanisms that directly affect circulating cortisol levels, such as corticosteroid binding globulin (CBG), which were not assessed in the present study. Although the type 2 DM participants did not display diminished salivary free cortisol levels (i.e., bioavailable cortisol not bound to CBG or other plasma proteins), they may have displayed diminished CBG levels and total serum cortisol levels (i.e., measure of bound and unbound cortisol). CBG levels decrease in response to inflammation (Gagliardi, Ho, & Torpy, 2010) and insulin resistance (Fernandez-Real et al., 2002). Thus, individuals with type 2 DM likely have
lower CBG levels compared to healthy individuals, which leads to increased circulating free cortisol levels and decreased total cortisol levels. Future studies should examine the relationships between CBG and total cortisol levels and stress system function in type 2 DM.

**Salivary DHEA-S.** This study was the first to date to examine the effect of an acute psychosocial stressor on DHEA-S levels in individuals with type 2 DM. Findings from this study provide evidence that type 2 DM is associated with decreased levels of DHEA-S. The type 2 DM group displayed significantly lower DHEA-S levels across all test session time points compared to the healthy group, and this finding remained significant even after controlling for age. This finding is consistent with previous observational studies that found diminished DHEA(S) levels in men with type 2 DM in non-stressor settings (e.g., Barrett-Connor, Khaw, & Yen, 1986; Yamauchi et al., 1996).

Diminished DHEA-S levels may be a physiological mechanism linking psychosocial stress to type 2 DM disease progression and negative health outcomes later on. Scientists speculate that increased levels of DHEA(S) following stress exposure may play a role in down-regulating cortisol (Balazs, Schweizer, Frey, Rohner-Jeanrenaud, & Odermatt, 2008). Although there was no evidence of elevated post-stressor cortisol levels in the type 2 DM group in this study, it is possible that DHEA(S) levels will continue to decline as the disease progresses and participants age. Declining levels of DHEA(S) may lead to impaired stress recovery and increased post-stressor cortisol levels making individuals with type 2 DM more vulnerable psychosocial stress.

The DHEA-S recovery change score finding provides additional evidence that type 2 DM may be associated with impaired stress recovery secondary to diminished
DHEA-S levels. Although only marginally statistically significant ($p=0.07$), the type 2 DM group displayed larger DHEA-S recovery scores compared to the healthy group, which suggests that the type 2 DM group had a greater decrease in DHEA-S from stress to recovery. In other words, the DHEA-S levels in the healthy group remained elevated following the stressor, which may protect them from the negative effects of stress (Morgan et al., 2004). In contrast, DHEA-S levels declined following the stressor in the type 2 DM group, which may indicate that they have diminished stress buffering effects. This idea is speculative at this time and additional research is needed to further elucidate the stress buffering effects of DHEA(S), as well as DHEA(S) patterns in type 2 DM. It is important to note, however, that the DHEA-S recovery score finding is in the opposite direction as to what I hypothesized. I hypothesized that the type 2 DM group would display dampened DHEA-S recovery (i.e., higher post-stress levels of DHEA-S compared to healthy group). Because no previous studies have looked at DHEA-S response patterns to an acute psychosocial stressor, I based my hypothesis on the previously published data on cortisol and type 2 DM.

Future studies should investigate DHEA-S stress response patterns across the type 2 DM disease continuum to determine if individuals at later stages in the disease process have alterations in DHEA-S patterns compared to individuals earlier in the type 2 DM disease process. Additionally, prospective studies could provide information about the trajectory of the alterations in DHEA-S levels in individuals with type 2 DM.

**SAM-Axis**

This study was the first to examine the effects of an acute psychosocial stressor on SAM-axis function (i.e., sAA) in individuals with type 2 DM, and contributes novel
information to the existing literature about SAM-axis function in men with type 2 DM. In general, the findings from this study did not show that type 2 DM is associated with dysregulated SAM-axis responses to an acute psychosocial stressor. The type 2 DM participants had a strong sAA response that was not significantly different from the healthy participants, which suggests that the SAM-axis stress response has not been significantly altered by type 2 DM at this stage of the disease for these participants. However, there was a marginally significant group difference ($p = 0.06$) in sAA AUC$_i$ such that the type 2 DM group had greater AUC$_i$ compared to the healthy group. This finding may suggest that men with type 2 DM without major disease-related complications have increased SAM-axis stress responses compared to healthy controls. However, given that there were no other sAA stress response differences between the two groups, the relevance of this finding is unclear. It could be that increased sAA AUC$_i$ is an early indicator of SAM-axis dysfunction in individuals with type 2 DM without major-disease related complications. Prospective studies should be conducted to investigate if sAA AUC$_i$ is predictive of later SAM-axis dysfunction in individuals with type 2 DM.

Similar to the cortisol stress response pattern, it is possible that alternation in SAM-axis response to an acute psychosocial stressor may develop later on in the disease process. This explanation is supported by findings from studies that demonstrated that type 2 DM patients with autonomic neuropathy have dampened epinephrine release in response to exercise stress compared to type 2 DM patients without neuropathy who are similar to participants in the present study (Bottini et al., 1995). Furthermore, studies have found that lower catecholamine levels following exercise stress was predictive of future cerebrovascular or cardiovascular events (Endo et al., 2000), which provides
additional suggestive evidence that SAM-axis dysfunction is associated with type 2 DM complications. Additional research is needed to further clarify the nature of SAM-axis function following acute psychosocial stress exposure in individuals with type 2 DM. More specifically, studies should investigate if individuals with type 2 DM without major disease-related complications have different SAM-axis stress response patterns compared to individuals with major disease-related complications. Future research also should explore if certain complications (e.g., autonomic neuropathy) are more strongly associated with alterations in SAM-axis function.

**Allostatic Load Findings**

Findings from this dissertation provide evidence that men with type 2 DM have increased allostatic load. Men in the type 2 DM group had significantly higher total allostatic load compared to men in the healthy group. There also were significant group differences on the individual allostatic load parameters. Of the allostatic load parameters tested, the type 2 DM group had significantly higher HbA1c, FPG, insulin, BMI, waist-hip ratios, DBP and triglycerides and significantly lower HDL cholesterol. Taken together, these findings provide evidence that type 2 DM is associated with increased allostatic load, which suggests the type 2 DM is a chronic endogenous stressor. These findings are consistent with the previous research on the pathophysiology of type 2 DM, which has found that type 2 DM is associated with increased levels secondary mediators of allostatic load (Codario, 2011; Kannel, 2011).

Furthermore, HbA1c was most strongly associated with type 2 DM status followed by FPG, insulin, BMI and waist-hip ratio. DBP, HDL cholesterol, and triglycerides were the weakest predictors of type to DM status. This finding is not
surprising given that HbA1c, FPG, and insulin are directly related to type 2 DM disease pathophysiology. However, these findings may have clinical utility in that the 8 biomarkers included in the final allostatic load model may be the “biomarker signatures” associated with type 2 DM disease progression, and researchers and clinicians can use them predict type 2 DM disease trajectory (Gruenewald, Seeman, Ryff, Karlamangla, & Singer, 2006). Furthermore, the finding that HbA1c was the parameter most associated with type 2 DM status provides additional support for the use of HbA1c to monitor type 2 DM disease management. Future studies should examine allostatic load in larger samples of individuals with type 2 DM.

It is important to note that all 15 allostatic load parameters could not be included in the final model, which is a limitation of the sample size of the study. Although this study included a comprehensive assessment of allostatic load, not all parameters could be statistically tested in the same model because of the small sample size. That being said, the variables included in the final model were selected in a systematic manner using canonical discriminant analysis, which increases the likelihood that the variables in the final model are the most strongly related to type 2 DM. Future studies, should examine allostatic load in larger samples so all allostatic load parameters can be statistically tested. Researchers should aim to have the sample sizes of each group be considerably larger than the total number of allostatic load parameters (Stevens, 2009).

The allostatic load analyses also revealed interesting and relevant information about the health status of the sample. Results revealed that four participants should be reclassified based on their allostatic load parameters. More specifically, the discriminant analysis reclassified three type 2 DM participants as healthy and one healthy participant
as type 2 DM. In other words, three type 2 DM participants were more similar to the healthy group participants in terms of allostatic load, and one healthy participant was more similar to the type 2 DM participants. The allostatic load reclassification finding may suggest that some of the type 2 DM participants either were early on in the disease process or they had well-controlled diabetes. I preformed exploratory analyses (not reported here) and tested additional linear mixed models for each stress biomarker with the three type 2 DM participants reclassified as healthy. However, the reclassification did change the previous findings. In general, the allostatic load parameter finding provides additional support that the type 2 DM sample in this study was relatively healthy, and additional studies should examine type 2 DM across the disease continuum to determine if HPA-axis and SAM-axis alterations occur later on in the disease process.

Unexpectedly, the findings did not show that total allostatic load was related stress physiological processes. This could be due to the relatively healthy status of the entire study sample. More specifically, the total allostatic load score for the entire sample was relatively low \((M = 8, SD = 5.24; \text{type 2 DM}, M = 12.64, SD = 4.29; \text{healthy}, M = 4.75, SD = 2.53)\). The maximum possible allostatic load score is 30 so a mean of 8 suggests the overall sample was relatively healthy, which may have reduced the ability to detect associations between allostatic load and stress system function and allostatic load by group interactions.

**Limitations**

This study had limitations, and thus, the results should be interpreted in the context of these limitations. Many of the limitations were mentioned earlier in this chapter, but they are discussed in greater detail here. First, the size of sample, especially
the size of the type 2 DM group, was relatively small, which decreased statistical power. The sample size was particularly problematic when conducting the allostatic load parameter one-way MANOVA analysis because the number of allostatic load parameters was greater than the number of participants in the type 2 DM group. Future studies should aim to test HPA-axis and SAM-axis in larger samples of individual with type 2 DM.

Another limitation is that the diurnal rhythms of the stress biomarkers and negative feedback control were not assessed, which limited the ability to compare the results from this dissertation to those from previous studies on stress system function in type 2 DM. Additionally, the study used a cross-sectional design, which limits the ability to make assumptions about the temporal relationship between stress system alterations and type 2 DM disease onset. Future studies should incorporate a longitudinal component to determine if stress system function changes as individuals advance along the type 2 DM continuum (i.e., progress from healthy to pre-diabetes to early type 2 DM to advanced type 2 DM).

Although I excluded individuals who had medical disorders (e.g., depression, ADHD, cognitive impairments, severe mental illness) or took medications (e.g., corticosteroids, psychotropic medications) that were known to significantly affect either stress physiology or their abilities to perform the TSST task, I did not exclude participants for other factors that may have affected the results. For example, I did not experimentally match participants for age, BMI, diabetes medications (except insulin), hypertension, or dyslipidemia (high cholesterol). There was both within- and between-group variability on these variables, which may have confounded the results and may
have reduced the likelihood of detecting group differences in stress system 4 weeks prior. Future studies should try to match the groups on age, BMI, hypertension, and dyslipidemia.

Although not necessarily a limitation, the type 2 DM participants in this study were relatively healthy, which may have affected the ability to detect group differences. More specifically, they were free from major disease-related complications, were not taking insulin, and had average HbA1c levels less than 7%, which is below the levels that the ADA (2012) recommends. The results also revealed that three type 2 DM participants (21% of group) should be reclassified as healthy. Additionally, the majority of the type 2 DM participants were in the moderate or low risk categories for all allostatic load parameters except BMI.

Information gained from self-report measures assessing diabetes-related quality of life and diabetes-related distress (not reported here) provided further evidence that the type 2 DM participants in this study were relatively healthy. Supplementary descriptive analyses revealed that the type 2 DM participants reported having high levels of diabetes-related quality of life, which was assessed using the Diabetes Related Quality of Life – Brief Clinical Inventory ($M = 4.04, SD = 0.40$). This measure assesses diabetes-related self-care behaviors and satisfaction with diabetes treatment using a 5-point Likert scale with higher scores indicating higher quality of life (Burroughs, Desikan, Waterman, Gilin, & McGill, 2004). Furthermore, they reported low levels of diabetes-related distress, which was assessed using the Problem Areas in Diabetes Scale ($M = 13.48, SD = 10.98$). Higher scores suggest higher levels of diabetes-related distress, and scores below 40 on this scale are indicative of minimal diabetes-related distress (Welch,
Jacobson, & Polonsky, 1997). Additionally, I compared general quality of life between the type 2 DM and healthy participants using the Medical Outcome Studies SF-12 quality of life measure (Ware, Kosinski, & Keller, 1996). The type 2 DM group reported significantly lower physical and mental well-being compared to the healthy group. Thus, this sample of individuals with type 2 DM reported having relatively high diabetes-related quality of life, but reported having lower overall quality of life compared to the healthy participants. As discussed earlier, alterations in stress system responses to acute psychosocial stress may occur later in the disease process after complications develop and quality of life declines. This is an important empirical question and should be tested in future longitudinal and cross-sectional studies that examine type 2 DM and stress system function across the type 2 DM disease continuum.

**Overall Conclusions**

Overall, the findings from this dissertation contribute important new information to the type 2 DM scientific literature by demonstrating that men with type 2 DM without major disease-related complications (e.g., retinopathy, uncontrolled hypertension, unstable angina, nephropathy) have intact HPA-axis and SAM-axis responses to acute psychosocial stressors. A major strength of the present study was the assessment of multiple stress biomarkers, which made it is first study to assess both HPA-axis and SAM-axis responses to acute psychosocial stress in type 2 DM.

In general, the findings from this dissertation are encouraging because they suggest that the HPA-axis and SAM-axis stress responses have not been significantly altered by type 2 DM in individuals without major disease-related complications. Because the participants in this study were relatively healthy, findings from this study
may provide evidence for the importance of managing diabetes early in the disease process. The study also provided novel information about DHEA-S levels in response to an acute psychosocial stressor. The DHEA-S findings provide suggestive evidence that individuals with type 2 DM have lower levels of DHEA-S compared to healthy individuals, as well as diminished post-stressor DHEA-S levels. These findings may indicate individuals with type 2 DM have impaired stress recovery, which makes them more physiological vulnerable to acute psychosocial stressors. It also is possible that decreased DHEA-S levels are the earliest manifestations of HPA-axis dysfunction. Future research should focus on examining stress system functioning in individuals with type 2 DM across the type 2 DM disease continuum using both cross-sectional and longitudinal designs. Furthermore, studies should comprehensively assess stress system function and include assessments of diurnal patterns, negative feedback control, and acute stress responses of HPA-axis and SAM-axis stress biomarkers.

In addition to further exploring stress system function across the type 2 DM continuum, it is important to investigate factors other than alterations in stress system function that are involved in the relationship between psychosocial stress and type 2 DM. Additional factors include psychosocial factors (e.g., coping, social support, quality of life, depression) and health behaviors (e.g., diet, physical activity, smoking, treatment adherence). It is possible that certain health behaviors and psychosocial factors mediate the relationship between psychosocial stress and type 2 DM. In conclusion, additional research is needed in this area to gain a clearer understanding of the biobehavioral mechanisms involved in the relationship between psychosocial stress and type 2 DM onset and progression. A better understanding of mechanisms involved in this
relationship can assist scientists in developing targeted, stress-specific interventions and also help to identify individuals with type 2 DM who are most vulnerable to the negative health effects of psychosocial stress.
Table 1.  
Demographic characteristics for type 2 DM and healthy participants. Data are means ± SEM and % of sample.  

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n = 20)</th>
<th>Type 2 DM (n = 14)</th>
<th>Total Sample (N = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>37.25 (2.72)</td>
<td>55.7 (1.79)</td>
<td>4.85 (2.35)</td>
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<td><strong>Race</strong></td>
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<td></td>
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<tr>
<td>White/Caucasian</td>
<td>90%</td>
<td>100%</td>
<td>94%</td>
</tr>
<tr>
<td>Asian/Asian American</td>
<td>10%</td>
<td>0%</td>
<td>5.88%</td>
</tr>
<tr>
<td>Native American/Alaskan</td>
<td>0%</td>
<td>0%</td>
<td>2.9%</td>
</tr>
<tr>
<td><strong>Highest level of education</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High school</td>
<td>0%</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Some college</td>
<td>0%</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>College</td>
<td>40%</td>
<td>42.9%</td>
<td>41.2%</td>
</tr>
<tr>
<td>Postgraduate</td>
<td>60%</td>
<td>42.9%</td>
<td>52.9%</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Employed full-time</td>
<td>50%</td>
<td>71.4%</td>
<td>58.82%</td>
</tr>
<tr>
<td>Employed part-time</td>
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<td>0%</td>
<td>14.71%</td>
</tr>
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<td>Unemployed</td>
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<td>0%</td>
<td>2.9%</td>
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<td>28.6%</td>
<td>11.76%</td>
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<td>20%</td>
<td>0%</td>
<td>11.76%</td>
</tr>
<tr>
<td><strong>Income</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$0 - $9,999</td>
<td>5%</td>
<td>0%</td>
<td>2.9%</td>
</tr>
<tr>
<td>$15,000 - $19,999</td>
<td>15%</td>
<td>7.1%</td>
<td>11.8%</td>
</tr>
<tr>
<td>$20,000 - $34,999</td>
<td>30%</td>
<td>14.3%</td>
<td>23.5%</td>
</tr>
<tr>
<td>$35,000 - $49,999</td>
<td>15%</td>
<td>0%</td>
<td>8.8%</td>
</tr>
<tr>
<td>$50,000 - $74,999</td>
<td>10%</td>
<td>35.7%</td>
<td>20.6%</td>
</tr>
<tr>
<td>$75,000 - $99,999</td>
<td>20%</td>
<td>35.7%</td>
<td>26.5%</td>
</tr>
<tr>
<td>$100,000 - $199,999</td>
<td>5%</td>
<td>0%</td>
<td>2.9%</td>
</tr>
</tbody>
</table>
Table 2.  
Clinical characteristics for type 2 DM and healthy participants. Data are means ± SEM and % of sample.

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n = 20)</th>
<th>Type 2 DM (n = 14)</th>
<th>Total Sample (N = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CES-D total score</td>
<td>2.85 (0.52)</td>
<td>3.14 (0.83)</td>
<td>2.97 (0.45)</td>
</tr>
<tr>
<td>Years since DM diagnosis</td>
<td>-</td>
<td>6.14 (1.06)</td>
<td>-</td>
</tr>
<tr>
<td>Diagnosed chronic diseases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>15%</td>
<td>64.2%</td>
<td>35.3%</td>
</tr>
<tr>
<td>High cholesterol</td>
<td>5%</td>
<td>71.1%</td>
<td>32.4%</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>10%</td>
<td>14.3%</td>
<td>11.8%</td>
</tr>
<tr>
<td>Angina</td>
<td>0%</td>
<td>17.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Gout</td>
<td>0%</td>
<td>10%</td>
<td>5.9%</td>
</tr>
<tr>
<td>Hypertension medications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lisinopril</td>
<td>5%</td>
<td>28.6%</td>
<td>14.7%</td>
</tr>
<tr>
<td>Diltiazam</td>
<td>0%</td>
<td>17.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Altace</td>
<td>0%</td>
<td>17.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Benicar</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Losartan</td>
<td>5%</td>
<td>7.1%</td>
<td>5.9%</td>
</tr>
<tr>
<td>Diltiazam</td>
<td>0%</td>
<td>7.1%</td>
<td>2.9%</td>
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<tr>
<td>Cholesterol medications</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Simvastatin</td>
<td>0%</td>
<td>21.4%</td>
<td>8.8%</td>
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<tr>
<td>Atorvastatin</td>
<td>0%</td>
<td>28.5%</td>
<td>11.8%</td>
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<tr>
<td>Niaspan</td>
<td>0%</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Diabetes medications</td>
<td></td>
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<td></td>
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<tr>
<td>Metformin</td>
<td>-</td>
<td>71.4%</td>
<td>29.4%</td>
</tr>
<tr>
<td>Actos</td>
<td>-</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Byetta</td>
<td>-</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Januvia</td>
<td>-</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Glipizide</td>
<td>-</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Glucovance</td>
<td>-</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Prandin</td>
<td>-</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Amaryl</td>
<td>-</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Other medications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levothyroxine</td>
<td>10%</td>
<td>10%</td>
<td>11.6%</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>5%</td>
<td>0%</td>
<td>2.9%</td>
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<tr>
<td>Allopurinol</td>
<td>0%</td>
<td>7.1%</td>
<td>2.9%</td>
</tr>
<tr>
<td>Nexium</td>
<td>5%</td>
<td>0</td>
<td>2.9%</td>
</tr>
</tbody>
</table>
Table 3.
Stress salivary biomarker descriptive statistics. Data are means ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n=20)*</th>
<th>Type 2 DM (n=14)</th>
<th>Total Sample (N=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortisol (nmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.18 (0.74)</td>
<td>6.06 (0.88)</td>
<td>5.60 (0.56)</td>
</tr>
<tr>
<td>Stress reactivity</td>
<td>7.58 (1.99)</td>
<td>10.41 (2.38)</td>
<td>8.74 (1.52)</td>
</tr>
<tr>
<td>Stress recovery</td>
<td>6.60 (1.43)</td>
<td>9.97 (1.71)</td>
<td>8.00 (1.12)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;i&lt;/sub&gt;</td>
<td>8.20 (1.92)</td>
<td>10.73 (2.29)</td>
<td>9.24 (1.46)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;g&lt;/sub&gt;</td>
<td>14.80 (2.16)</td>
<td>18.29 (2.58)</td>
<td>16.24 (1.66)</td>
</tr>
<tr>
<td><strong>DHEA-S (ng/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4.85 (0.38)</td>
<td>3.78 (0.45)</td>
<td>4.41 (0.30)</td>
</tr>
<tr>
<td>Stress reactivity</td>
<td>-0.15 (0.38)</td>
<td>0.13 (0.46)</td>
<td>-0.03 (0.29)</td>
</tr>
<tr>
<td>Stress recovery</td>
<td>-0.004 (0.23)</td>
<td>0.66 (0.28)</td>
<td>0.27 (0.18)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;i&lt;/sub&gt;</td>
<td>-0.13 (0.30)</td>
<td>-0.05 (0.36)</td>
<td>-0.10 (9.23)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;g&lt;/sub&gt;</td>
<td>5.95 (0.46)</td>
<td>4.68 (0.55)</td>
<td>5.42 (0.36)</td>
</tr>
<tr>
<td><strong>sAA (UI/L)</strong>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>115.50 (22.17)</td>
<td>136.30 (19.03)</td>
<td>123.21 (14.26)</td>
</tr>
<tr>
<td>Stress reactivity</td>
<td>21.49 (13.38)</td>
<td>37.88 (15.59)</td>
<td>28.45 (10.09)</td>
</tr>
<tr>
<td>Stress recovery</td>
<td>6.56 (16.46)</td>
<td>13.38 (19.18)</td>
<td>9.45 (12.31)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;i&lt;/sub&gt;</td>
<td>17.17 (12.50)</td>
<td>29.80 (14.56)</td>
<td>22.53 (9.40)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;g&lt;/sub&gt;</td>
<td>178.27 (25.86)</td>
<td>174.18 (30.13)</td>
<td>176.54 (19.32)</td>
</tr>
</tbody>
</table>

*Note: 19 healthy participants were included in the sAA analyses.
Table 4.  
Allostatic load descriptive statistics. Data are means ± SEM and % of sample.

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n=20)</th>
<th>Type 2 DM (n=14)</th>
<th>Total Sample (N=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Allostatic Load Score</strong></td>
<td>4.75 (0.57)</td>
<td>12.64(1.15)</td>
<td>5.15 (0.88)</td>
</tr>
<tr>
<td><strong>Cardiovascular Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP mean (mmHg)</td>
<td>116.78(2.44)</td>
<td>126.83(3.53)</td>
<td>120.92(2.19)</td>
</tr>
<tr>
<td>SBP low risk</td>
<td>50%</td>
<td>21.43%</td>
<td>38.24</td>
</tr>
<tr>
<td>SBP moderate risk</td>
<td>50%</td>
<td>64.29%</td>
<td>55.88</td>
</tr>
<tr>
<td>SBP high risk</td>
<td>0%</td>
<td>14.29%</td>
<td>5.88</td>
</tr>
<tr>
<td>DBP mean (mmHg)</td>
<td>70.48(1.44)</td>
<td>78.47(2.17)</td>
<td>73.77(1.39)</td>
</tr>
<tr>
<td>DBP low risk</td>
<td>95%</td>
<td>50%</td>
<td>76.47</td>
</tr>
<tr>
<td>DBP moderate risk</td>
<td>5%</td>
<td>42.86%</td>
<td>20.59</td>
</tr>
<tr>
<td>DBP high risk</td>
<td>0%</td>
<td>7.14%</td>
<td>2.94</td>
</tr>
<tr>
<td><strong>Anthropometric Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²) mean</td>
<td>24.71(0.63)</td>
<td>34.59(1.74)</td>
<td>28.78 (1.16)</td>
</tr>
<tr>
<td>BMI low risk</td>
<td>45%</td>
<td>14.29%</td>
<td>32.35%</td>
</tr>
<tr>
<td>BMI moderate risk</td>
<td>50%</td>
<td>7.14%</td>
<td>32.35%</td>
</tr>
<tr>
<td>BMI high risk</td>
<td>5%</td>
<td>78.57%</td>
<td>35.29%</td>
</tr>
<tr>
<td>W-H mean</td>
<td>0.90(0.001)</td>
<td>1.00(0.02)</td>
<td>0.95 (0.01)</td>
</tr>
<tr>
<td>W-H low risk</td>
<td>80%</td>
<td>14.29%</td>
<td>52.94%</td>
</tr>
<tr>
<td>W-H moderate risk</td>
<td>20%</td>
<td>50%</td>
<td>32.35%</td>
</tr>
<tr>
<td><strong>Neuroendocrine Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol mean (nmol/L)</td>
<td>5.18(0.74)</td>
<td>6.06(0.88)</td>
<td>5.60 (0.56)</td>
</tr>
<tr>
<td>Cortisol low risk</td>
<td>75%</td>
<td>78.57%</td>
<td>76.47%</td>
</tr>
<tr>
<td>Cortisol moderate risk</td>
<td>10%</td>
<td>7.14%</td>
<td>8.82%</td>
</tr>
<tr>
<td>Cortisol high risk</td>
<td>15%</td>
<td>14.29%</td>
<td>14.71%</td>
</tr>
<tr>
<td>DHEA-S mean(ng/ml)</td>
<td>4.85(0.38)</td>
<td>3.78(0.45)</td>
<td>4.41(0.30)</td>
</tr>
<tr>
<td>DHEA-S low risk</td>
<td>75%</td>
<td>50%</td>
<td>73.53%</td>
</tr>
<tr>
<td>DHEA-S moderate risk</td>
<td>15%</td>
<td>42.86%</td>
<td>14.71%</td>
</tr>
<tr>
<td>DHEA-S high risk</td>
<td>10%</td>
<td>7.14%</td>
<td>11.76%</td>
</tr>
<tr>
<td>sAA mean (UI/L)*</td>
<td>115.50 (22.17)</td>
<td>136.30 (19.03)</td>
<td>123.21 (14.26)</td>
</tr>
<tr>
<td>sAA low risk</td>
<td>80%</td>
<td>85.71%</td>
<td>82.35%</td>
</tr>
<tr>
<td>sAA moderate risk*</td>
<td>10%</td>
<td>7.14%</td>
<td>8.82%</td>
</tr>
<tr>
<td>sAA moderate risk*</td>
<td>10%</td>
<td>7.14%</td>
<td>8.82%</td>
</tr>
</tbody>
</table>

*Note: 19 healthy participants were included in the sAA analysis*
Table 4 (continued).

Allostatic load descriptive statistics. Data are means ± SEM and % of sample.

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n=20)</th>
<th>Type 2 DM (n=14)</th>
<th>Total Sample (N=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metabolic Parameters</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HDL mean (mg/dl)</td>
<td>50.65 (2.75)</td>
<td>40.57(1.80)</td>
<td>46.5(1.96)</td>
</tr>
<tr>
<td>HDL low risk</td>
<td>25%</td>
<td>0%</td>
<td>14.71</td>
</tr>
<tr>
<td>HDL moderate risk</td>
<td>45%</td>
<td>50%</td>
<td>47.06</td>
</tr>
<tr>
<td>HDL high risk</td>
<td>30%</td>
<td>50%</td>
<td>38.24</td>
</tr>
<tr>
<td>LDL mean (mg/dl)</td>
<td>105.15(5.77)</td>
<td>86.14 (7.33)</td>
<td>97.32(4.76)</td>
</tr>
<tr>
<td>LDL low risk</td>
<td>85%</td>
<td>92.86%</td>
<td>88.24%</td>
</tr>
<tr>
<td>LDL moderate risk</td>
<td>10%</td>
<td>0%</td>
<td>5.88%</td>
</tr>
<tr>
<td>LDL high risk</td>
<td>5%</td>
<td>7.14%</td>
<td>5.88%</td>
</tr>
<tr>
<td>TRI mean (mg/dl)</td>
<td>85.45(6.60)</td>
<td>134.57(18.20)</td>
<td>105.68(9.28)</td>
</tr>
<tr>
<td>TRI low risk</td>
<td>100%</td>
<td>71.43%</td>
<td>88.24%</td>
</tr>
<tr>
<td>TRI moderate risk</td>
<td>0%</td>
<td>7.14%</td>
<td>2.94%</td>
</tr>
<tr>
<td>TRI high risk</td>
<td>0%</td>
<td>21.43%</td>
<td>8.82%</td>
</tr>
<tr>
<td>TC mean (mg/dl)</td>
<td>172.90 (5.92)</td>
<td>153.57(8.37)</td>
<td>164.94(5.10)</td>
</tr>
<tr>
<td>TC low risk</td>
<td>85.00%</td>
<td>92.86%</td>
<td>88.24%</td>
</tr>
<tr>
<td>TC moderate risk</td>
<td>15.00%</td>
<td>7.14%</td>
<td>11.76%</td>
</tr>
<tr>
<td>TC high risk</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Insulin mean (IU/ml)</td>
<td>4.15(0.7)</td>
<td>15.79(2.10)</td>
<td>8.94(1.37)</td>
</tr>
<tr>
<td>Insulin low risk</td>
<td>100%</td>
<td>35.71%</td>
<td>73.53%</td>
</tr>
<tr>
<td>Insulin moderate risk</td>
<td>0%</td>
<td>35.71%</td>
<td>14.71%</td>
</tr>
<tr>
<td>Insulin high risk</td>
<td>0%</td>
<td>28.57%</td>
<td>11.76%</td>
</tr>
<tr>
<td>FPG mean</td>
<td>88.55(1.84)</td>
<td>131.21(7.53)</td>
<td>106.12 (4.87)</td>
</tr>
<tr>
<td>FPG low risk</td>
<td>90.00%</td>
<td>14.29%</td>
<td>58.82%</td>
</tr>
<tr>
<td>FPG moderate risk</td>
<td>10.00%</td>
<td>35.71%</td>
<td>20.59%</td>
</tr>
<tr>
<td>FPG high risk</td>
<td>0%</td>
<td>50.00%</td>
<td>20.59%</td>
</tr>
<tr>
<td>HbA1c mean</td>
<td>5.27 (0.06)</td>
<td>6.61(0.17)</td>
<td>5.82 (0.14)</td>
</tr>
<tr>
<td>HbA1c low risk</td>
<td>90%</td>
<td>0%</td>
<td>52.94%</td>
</tr>
<tr>
<td>HbA1c moderate risk</td>
<td>10%</td>
<td>50%</td>
<td>26.47%</td>
</tr>
<tr>
<td>HbA1c high risk</td>
<td>0%</td>
<td>50%</td>
<td>20.59%</td>
</tr>
<tr>
<td><strong>Immune Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum CRP mean (mg/L)</td>
<td>1.79(0.42)</td>
<td>2.66(0.57)</td>
<td>2.15 (5.10)</td>
</tr>
<tr>
<td>CRP low risk</td>
<td>50%</td>
<td>21.43%</td>
<td>38.24%</td>
</tr>
<tr>
<td>CRP moderate risk</td>
<td>25%</td>
<td>42.86%</td>
<td>32.35%</td>
</tr>
<tr>
<td>CRP high risk</td>
<td>25%</td>
<td>35.71%</td>
<td>29.41%</td>
</tr>
</tbody>
</table>

*Note: 19 healthy participants were included in the sAA analysis*
Table 5.
Pearson product moment correlations between all allostatic load parameters.

<table>
<thead>
<tr>
<th></th>
<th>HbA1c</th>
<th>LDL</th>
<th>HDL</th>
<th>BMI</th>
<th>SBP</th>
<th>DBP</th>
<th>DHEA</th>
<th>FPG</th>
<th>CRP</th>
<th>TRI</th>
<th>W-H</th>
<th>INS</th>
<th>Cortisol</th>
<th>sAA</th>
<th>TC</th>
</tr>
</thead>
<tbody>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>-0.30</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>HDL</td>
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* p < 0.05
+ Allostatic load parameters included in the one-way MANOVA model.
Table 6.
*Canonical structure coefficients for each allostatic load CDA model.*

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<th></th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
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</table>

1 The variable in each model with the smallest canonical structure coefficient

+ The final model that was tested in the one-way MANOVA.
Table 7.
Results from the allostatic load parameter one-way MANOVA and univariate follow-up tests.

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<td>HbA1c</td>
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<td>INS</td>
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<td>BMI</td>
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<td>W-H</td>
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<td>DBP</td>
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<td>TRI</td>
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<tr>
<td>HDL</td>
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</table>

* p<0.001
Figure 1. Health screening session visit timeline
Figure 2. Test session visit timeline
Figure 3. Mean self-reported stress levels (± SEM) at baseline, stress, and recovery by type 2 DM ($n = 14$) and healthy ($n = 20$) participants.
Figure 4. Mean systolic blood pressure (mmHg) (± SEM) at baseline, stress, rest, and recovery by type 2 DM ($n = 14$) and healthy ($n = 20$) participants.
Figure 5. Mean diastolic blood pressure (mmHg) (+ SEM) at baseline, stress, rest, and recovery by type 2 DM (n = 14) and healthy (n = 20) participants.
Figure 6. Mean heart rate (bpm) (± SEM) at baseline, stress, rest, and recovery by type 2 DM (n = 14) and healthy (n = 20) participants.
Figure 7. Mean salivary cortisol levels (nmol/L) (± SEM) at baseline, stress, and recovery by type 2 DM (n = 14) and healthy (n = 20) participants.
Figure 8. Mean salivary DHEA-S levels (ng/ml) (± SEM) at baseline, stress, and recovery by type 2 DM (n = 14) and healthy (n = 20) participants.
Figure 9. Mean salivary alpha-amylase (sAA) (U/mL) (± SEM) at baseline, stress, and recovery by type 2 DM (n = 14) and healthy (n = 19) participants.
REFERENCES


Fonseca, V. A. (2009). Defining and Characterizing the Progression of Type 2 Diabetes. *Diabetes Care, 32*(suppl 2), S151–S156. doi:10.2337/dc09-S301


role of estrogens, corticosteroids, and behavioral coping styles.


Toshihiro, M., Saito, K., Takikawa, S., Takebe, N., Onoda, T., & Satoh, J. (2008). Psychosocial factors are independent risk factors for the development of Type 2 diabetes in Japanese workers with impaired fasting glucose and/or impaired glucose tolerance. *Diabetic*


doi:10.1016/S0303-7207(01)00455-5
Appendices
Appendix A. Institutional Review Board Approval Letter

1. Pennsylvania State University IRB

2. Mount Nittany Hospital
1. Pennsylvania State University IRB
Date: August 21, 2012

From: Julie A. James, Compliance Coordinator

To: Laura C. Klem

Subject: Results of Review of Proposal - Full (IRB #39698)

Approval Expiration Date: August 13, 2013

“Biobehavioral Effects of Psychosocial Stress in Cigarette Smokers with Type 2 Diabetes”

The Institutional Review Board (IRB) has reviewed and approved your proposal for use of human participants in your research. By accepting this decision, you agree to obtain prior approval from the IRB for any changes to your study. Unanticipated participant events that are encountered during the conduct of this research must be reported in a timely fashion.

The dated, IRB-approved informed consent/assents to be used when enrolling participants for this research can be accessed by navigating to and logging into PRAMS (www.prams.psu.edu). Once there, please click on the documents button in order to access said documents. Participants must receive a copy of the approved informed consent form to keep for their records.

If signed consent is obtained, the principal investigator is expected to maintain the original signed consent forms along with the IRB research records for this research at least three (3) years after termination of IRB approval. For projects that involve protected health information (PHI) and are regulated by HIPAA, records are to be maintained for six (6) years. The principal investigator must determine and adhere to additional requirements established by the FDA and any outside sponsors.

If this study will extend beyond the above noted approval expiration date, the principal investigator must submit a completed Continuing Progress Report to the Office for Research Protections (ORP) to request renewed approval for this research.

On behalf of the IRB and the University, thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of human participants.

Please Note: The ORP encourages you to subscribe to the ORP listserv for protocol and research-related information. Send a blank email to: L-ORP-Research-L-subscribe-request@lists.psu.edu

JAJ/pr

cc: Jan S. Ulbrecht
    Kimberly N. Walter
    Sheila G. West
2. Mount Nittany Hospital
June 7, 2013

Laura Cousino Klein, PhD
Department of Biobehavioral Health
219 BBH Building
University Park PA 16802

Dear Dr. Klein:

The Institutional Review Board met on June 7, 2013, and reviewed your request for approval of the use of the following protocol and consent form; recruiting flyer:

Biobehavioral Effects of Psychosocial Stress in Cigarette Smokers with Type 2 Diabetes

Please note that it will be your responsibility to notify the Board immediately of any adverse reactions with the use of this protocol and to provide an update at the time of the yearly review of all protocols by the Mount Nittany Medical Center’s Institutional Review Board.

It will be your responsibility to submit a request for an annual renewal at least one month prior to the expiration of the approval.

If you have any questions, please do not hesitate to contact me.

Sincerely,

Jeanne A. Lumadue, M.D., PhD
Chair, Institutional Review Board

JAL/dr
Appendix B. Recruitment Materials

1. Advertisements
2. Letter to Medical Offices
3. Penn State Listserv Text
4. Diabetes Registry Letter
5. Penn State Hershey Medical Group Recruitment Script
1. Advertisements
Do you have type 2 diabetes?

Male Type 2 Diabetics Needed for Research Study

The Biobehavioral Health studies Lab at Penn State is now recruiting volunteers for a Penn State study examining the biological and psychological response to a cognitive challenge.

You can receive up to $65 in compensation for your time.

You may be eligible if you:
- Are a man between the ages of 25 and 65
- Have type 2 diabetes
- Do not take insulin

Call 814-865-3319 or email diasmoke@psu.edu
Do you have type 2 diabetes?

The Biobehavioral Health Studies Lab at Penn State is now recruiting volunteers for a Penn State study on type 2 diabetes and smoking. This is not a smoking cessation study, and you will not be required to quit smoking to participate.

You may be eligible if you:

- Have type 2 diabetes mellitus
- Do not take insulin
- Do or do not smoke cigarettes
- Are a man between the ages of 25 and 65

Receive up to $65 in compensation for your time.

Contact the Biobehavioral Health Studies Lab at 814-865-3319 or DiaSmoke@psu.edu to learn more about the study.

This study is under the direction of Dr. Klein (Biobehavioral Health; 814-865-8813)
The Biobehavioral Health Studies Lab at Penn State is now recruiting volunteers for a Penn State study examining biological and psychological responses to a cognitive challenge.

You can receive up to $65 in compensation for your time.

You may be eligible if you:
- Are a man between the ages of 25 and 65
- Are or are not a cigarette smoker
- Do or do not have type 2 diabetes

Call 814-865-3319 or email diasmoke@psu.edu
Are you a male?

The Biobehavioral Health Studies Lab at Penn State is now recruiting volunteers for a Penn State study on smoking and diabetes. This is not a smoking cessation study, and you will not be required to quit smoking to participate.

You may be eligible if you:

- A male between age 25 & 65
- Do or do not smoke cigarettes daily
- Do or do not have type 2 diabetes mellitus

Receive up to $65 in compensation for your time.

Contact the Biobehavioral Health Studies Lab at 814-865-3319 or DiaSmoke@psu.edu to learn more about the study.

This study is under the direction of Dr. Klein (Biobehavioral Health; 814-865-8813)
Researchers seek men ages 25 to 65 for study

The Biobehavioral Health Studies Lab at Penn State is now recruiting volunteers for a Penn State study on smoking and diabetes. This is not a smoking cessation study, and you will not be required to quit smoking to participate. You may be eligible if you are a man between the ages of 25 and 65, are or are not a cigarette smoker, and do or do not have type 2 diabetes mellitus. You can receive up to $65 in compensation for your time. Contact the Biobehavioral Health Studies Lab at 814-865-3319 or DiaSmoke@psu.edu to learn more about the study. This study is under the direction of Dr. Klein (Biobehavioral Health; 814-865-8813)
The Biobehavioral Health Studies Lab at Penn State University is looking for males with type 2 diabetes to participate in a paid research study. Participants may earn up to $65 for completing the study. In order to be eligible you must be a male between the ages of 25 and 65 who has been diagnosed with type 2 diabetes. If you meet these requirements, you may be eligible to participate. If you would like more information about this paid study, please call 814-865-3319 or email diabetestudy@psu.edu. Once again, that’s 814-865-3319 or diabetestudy@psu.edu.
Penn State Research Volunteer Website

Researchers seek men for study on diabetes and smoking

Research Participant Criteria

To be eligible for this study, you must meet the following criteria:

- **Gender:** Male
- **Age:** 25 - 65 Years

Research Study Details

Biobehavioral Effects of Cognitive Challenge in Cigarette Smokers with Type 2 Diabetes

The Biobehavioral Health Studies Lab at Penn State is now recruiting volunteers for a Penn State study on smoking and diabetes. This is not a smoking cessation study, and you will not be required to quit smoking to participate. You may be eligible if you are a man between the ages of 25 and 65, are or are not a cigarette smoker, and do or do not have type 2 diabetes mellitus. You can receive up to $65 in compensation for your time. Contact the Biobehavioral Health Studies Lab at 814-865-3319 or DiaSmoke@psu.edu to learn more about the study. This study is under the direction of Dr. Klein (Biobehavioral Health; 814-865-8813)

Study Dates, Times and Location

**Start Date:** Jan 14, 2013 12:00 AM
**Study Available by Appointment?** Yes
**Location:** Penn State University Park
**Approximate Participant Study Length:** 4 Hours

This study involves 2 laboratory sessions. The first session is a health screening session and will last approximately 1 hour. The purpose of this session is to determine if you are eligible for the remaining parts of the study. If you remain eligible following the health screening session, you will be asked to come to the laboratory for 1 additional 3-hour test session. All laboratory sessions will take place at Penn State’s Clinical Research Center on the Penn State University Park campus. The health screening session will be scheduled between 7 and 10 am. The laboratory test session will begin at 2 PM if you are not a smoker and 1:45 PM if you are a smoker. The appointments will be scheduled on a day that is convenient for you.

**Compensation:** up to $65

Contact Information

Kim Walter
814-865-3319
DiaSmoke@psu.edu
Newspaper Advertisement

Research Study
Penn State University

Men Needed for Study

The Behavior/Health Studies Lab at Penn State is now recruiting volunteers for a Penn State study examining biological and psychological responses to cognitive challenge. You can receive up to $50 in compensation for your time.

You may be eligible if:
- You are a man between the ages of 25 and 65
- You are not a current smoker
- You do not have type 2 diabetes

Call 814-865-3435 or email dissminist@psu.edu
2. Letter to Medical Offices
Medical office address

Dear Practice Manager,

My colleagues from Penn State University at University Park and I currently are conducting a study on tobacco smoking and type 2 diabetes. This study has been reviewed and approved by the PSU Institutional Review Board for conducting research with human participants. I am writing to request your assistance in recruitment of participants for this study. The study involves up to 2 laboratory sessions at the Clinical Research Center at Penn State University Park. The first session is a health screening session and will last about 1 hour. If they remain eligible following the health screening, they will be asked to come to the laboratory for an additional 3-hour test session. Participants will be compensated for their time.

I have enclosed recruitment flyers. We are recruiting males ages 25 to 65 who do or do not have diabetes and who do or do not smoke cigarettes. It is our hope that you would be willing to post these flyers where they can be accessed by your patients. We do not require any of your time, resources, or personnel (other than posting the flyers).

I sincerely appreciate your consideration of assisting us with the recruitment for this study. If there is anything we can do to help you, or if you have any questions or concerns, please do not hesitate to contact me at any time.

Sincerely,

Laura Cousino Klein, Ph.D.
Associate Professor of Biobehavioral Health and of Human Development and Family Studies
Director, Biomarker Core Laboratory
219 Biobehavioral Health Building
The Pennsylvania State University
University Park, PA 16802
Phone: 814.865 8813
Email: kklein@psu.edu
3. Penn State Listserv Text
The Biobehavioral Health Studies Lab at Penn State is now recruiting volunteers for a Penn State study on smoking and diabetes. This is not a smoking cessation study, and you will not be required to quit smoking to participate. You may be eligible if you are a man between the ages of 25 and 65, are or are not a cigarette smoker, and do or do not have type 2 diabetes mellitus. You can receive up to $65 in compensation for your time. Contact the Biobehavioral Health Studies Lab at 814-865-3319 or DiaSmoke@psu.edu to learn more about the study. This study is under the direction of Dr. Klein (Biobehavioral Health; 814-865-8813)
4. Diabetes Registry Letter
[Date]

[Patient Name and Address]

Dear [Patient]:

I would like to tell you about an opportunity to participate in a Penn State University Park research study. The Principal Investigator for this study is Dr. Laura Cousino Klein (lcklein@psu.edu; 814-865-8813). The purpose of this study is to understand how cigarette smoking and type 2 diabetes mellitus influence the biological and psychological responses to a cognitive challenge. We will use several measures to assess this research question including blood markers, saliva markers, blood pressure, and questionnaires.

The study involves up to 2 laboratory sessions at the Clinical Research Center at Penn State University Park. The first session is a health screening session and will last about 1 hour. If you remain eligible following the health screening, you will be asked to come to the laboratory in the afternoon for an additional 3-hour test session.

For your time, you can be compensated up to $65 in cash: we will compensate you (1) $10 in cash for completing the health screening session, (2) $35 in cash for the laboratory test session, and (3) a $20 cash bonus for completing the entire study. Free parking is provided every time you come to campus for a study session.

You may be eligible to participate if you are a male between the ages of 25 and 65 who has type 2 diabetes, but does not take insulin (all other diabetes medications are allowed). To participate, you may or may not be a cigarette smoker. If you believe you qualify for this study and would like to learn more, contact the study coordinator, Kim Walter, at 814-865-3319 or email diastmoke@psu.edu. Please know that contacting her does not commit you to anything, and your response to this letter will in no way affect the way you are treated in our office. Your decision to be in this research is voluntary and your choice to participate or not participate will not affect your overall care.

Sincerely,

[Name of Doctor]

IRB Protocol #39698

“This Research has been approved by the Institutional Review Board, under federal regulations, at Penn State University Park”
5. Penn State Hershey Medical Group Recruitment Script
DiaSmoke Study
Script for University Physician doctors

Hello, my name is Dr. __________ and I’m calling from Penn State University Physicians. I would like to tell you about an opportunity to participate in a Penn State University Park research study. The purpose of this study is to understand how cigarette smoking and type 2 diabetes mellitus influence the biological and psychological responses to a cognitive challenge. The study involves up to 2 laboratory sessions at the Clinical Research Center at Penn State University Park. The first session is a health screening session and will last about 1 hour. If you remain eligible following the health screening, you will be asked to come to the laboratory in the afternoon for an additional 3-hour test session. The Principal Investigator for this study is Dr. Laura Cousino Klein.

For your time, you can be compensated up to $65 in cash: You will receive $10 in cash for completing the health screening session and $35 in cash for the laboratory test session, and a $20 cash bonus for completing the entire study. Free parking is provided every time you come to campus for a study session.

Are you interested in learning more about the study to see if you would like to participate? Please know that this does not commit you to anything, and your response in no way affects the way you are treated in our office. Your decision to be in this research is voluntary and your choice to participate or not participate will not affect your overall care.

(If no, thank him for his time)

OK. Thank you for your time. Have a nice day.

(If yes)

Great! I will give your phone number and email address to Kim Walter, who is the study coordinator.
Appendix C. Telephone Screening Interview
TELEPHONE SCREENING FORM
DIABETES & SMOKING

Screening Date: ________________

Interviewer’s Name: ________________

Name: ____________________________ Home Phone: ________________

E-mail: ____________________________ Cell Phone: ________________

Occupation: ________________________

Hello, this is ________________, calling from Penn State University. I am calling regarding an ongoing research project on smoking and diabetes that you expressed an interest in participating. Do you have 15 minutes for me to tell you about the study?

If no, Is there a better time that I could call you back?

The purpose of this study is to understand how cigarette smoking and type 2 diabetes mellitus influence the biological and psychological response to a cognitive challenge. We will use many measures to assess this research question including blood markers, blood pressure, and questionnaires.

This study involves 2 laboratory sessions. The first session is a health screening session and will last approximately 1 hour. The purpose of this session is to determine if you are eligible for the remaining parts of the study. If you remain eligible following the health screening session, you will be asked to come to the laboratory for 1 additional 3-hour test session. All laboratory sessions will take place at Penn State’s Clinical Research Center on the Penn State campus. The health screening session will be scheduled between 7 and 10 am. The laboratory test session will begin at 2 PM if you are not a smoker and 1:45 PM if you are a smoker. The appointments will be scheduled on a day that is convenient for you.

You will be asked to fast overnight before the health screening session. At the health screening session, you will complete consent forms, a set of questionnaires about your health, and have measurements taken (e.g., height, weight, hip circumference, waist circumference, blood pressure, carbon monoxide via expired air). Ms. Walter will contact you after the health screening session about your eligibility for the laboratory test session.

During the laboratory test sessions, you will complete several questionnaires about your health, feelings, and behaviors. You also will participate in a “speech and math assessment.” You also will provide saliva samples 4 times during the laboratory test session. This procedure is non-invasive and includes rolling a small piece of cotton in your mouth until it is completely wet. Your blood will be collected 2 times by a nurse during the laboratory test session. In addition to the saliva and blood samples, we will measure your blood pressure and heart rate responses several times throughout the laboratory test session.

For your time, you can be compensated up to $305, we will compensate you $10 for completing the health screening session, $35 for the laboratory test session, and a $20 bonus for completing the entire study.
1. Do you think that you would like to participate in this study? □ Yes □ No
   "If no, thank them for their time"

2. Do you believe that your home and work schedule will fit with the time commitments? □ Yes □ No
   "If no, thank them for their time"

Great, I am going to quickly verify your potential eligibility for this study by asking you a few questions.

3. How old are you and what is your date of birth?
   a. Age: _______________
   b. Date of birth _______________
   "If not 25-65, then not eligible"

4. What is your estimated height and weight?
   Height _______________ Weight _______________ BMI _______________

5. Have you been diagnosed with type 2 diabetes by a qualified healthcare professional (medical doctor, nurse practitioner, or physician's assistant)? □ Yes □ No
   "If no, skip to question 8"
   "If no, participant will not be eligible for the type 2 diabetes groups"

6. When were you diagnosed with type 2 diabetes?
   Date of diagnosis _______________

7. Are you currently taking any diabetes medications? (specify) □ Yes □ No
   If yes, name of medications: __________________________________________
   __________________________________________
   __________________________________________

8. Have you taken any oral steroids within the last 6 weeks? □ Yes □ No
   "If yes, participant will be excluded"

9. Are you taking any other medications? Describe the medications and their purpose
   "The responses for item 9 will be reviewed by Dr. Klein and/or the study co-investigators to determine eligibility"
10. Do you currently use tobacco products other than cigarettes such as chewing tobacco, snuff, pipes, cigars?
   □ Yes □ No
   << If yes, participant will be excluded from the study >>

11. Are you a current smoker    Yes □ No □
   << If no, skip to question 15 >>
   << If no, participant is not eligible for the smoking groups >>

12. On average, how many cigarettes do you smoke per day? ________________
   << If less than 10, participant will be excluded from study >>.

13. How long have you been smoking cigarettes? _____ years _____ months
   << If least than 2 years, participant will be excluded >>.

14. How soon after waking up do you usually smoke your first cigarette? _____ hours _____ minutes

15. Are you currently using nicotine replacement products (e.g., patch, gum)?   Yes □ No □
   << If yes, participant will be excluded >>

16. Have you quit using other tobacco products such as chewing tobacco, snuff, pipes, cigars within the past 5 years?   Yes □ No □
   << Skip question 16 if a current other tobacco product user (question 10 = yes) >>
   << If yes, participant will be excluded >>

17. Have you quit smoking within the past 5 years?   Yes □ No □
   << Skip question 17 if a current smoker (question 11 = yes) >>
   << If no, skip to question 18 >>

18. Are you allergic or sensitive to latex?   Yes □ No □
10. Do you have or have you had any of the following medical conditions:

* The responses for item 19 will be reviewed by Dr. Klein and/or the study co-investigators to determine eligibility

☐ Yes ☐ No heart attack
☐ Yes ☐ No angina
☐ Yes ☐ No retinopathy
☐ Yes ☐ No impaired vision not correctable with eye glasses or contact lenses
☐ Yes ☐ No neuropathy
☐ Yes ☐ No stroke
☐ Yes ☐ No TIA (mini stroke)
☐ Yes ☐ No high blood pressure
☐ Yes ☐ No high cholesterol
☐ Yes ☐ No renal or kidney disease
☐ Yes ☐ No rheumatoid arthritis
☐ Yes ☐ No blood clotting disorder
☐ Yes ☐ No liver disease or cirrhosis
☐ Yes ☐ No any condition that requires the use of steroids
☐ Yes ☐ No gout (requiring treatment)
☐ Yes ☐ No anemia (or sickle cell anemia)
☐ Yes ☐ No lung disease (such as bronchitis, emphysema, asthma)
☐ Yes ☐ No cancer within the last 10 years
☐ Yes ☐ No thyroid disease (bring copy of TSH results if in last 8 months)
☐ Yes ☐ No problems with immune system (hepatitis, AIDS, lupus)
☐ Yes ☐ No any other medical condition not specified in this list__________________________

20. Do you have an irregular heart beat or any other heart condition? Yes ☐ No ☐

(GSRC physician should be consulted)

21. Have you had any major surgery in the last 6 months? Yes ☐ No ☐

If yes, what was the surgery? __________________

22. Are you willing to fast overnight, at least 7 hours, prior to your health screening session? Yes ☐ No ☐

<<If no, participant will be excluded>>

23. Have you been told you are a “difficult stick” for taking blood? Yes ☐ No ☐

24. Have you donated blood or plasma in the past 2 months? Yes ☐ No ☐

If yes, how long has it been since you donated? __________________

<<If yes, participant will be excluded>>

24. Would you abstain from donating blood during the study? Yes ☐ No ☐

<<If no, participant will be excluded>>

25. Do you have a medical device implanted in your body? Yes ☐ No ☐

26. Are you a US Citizen? Yes ☐ No ☐

If no, what type of Visa do you have? __________________
**CES-D**

Now I have some questions about your feelings during the past week. For each of the following statement, please tell me if you felt that way: Rarely or none of the time; some of the time; much of the time; most or all of the time.

<table>
<thead>
<tr>
<th></th>
<th>Rarely or none of the time</th>
<th>Some of the time</th>
<th>Much of the time</th>
<th>Most or all of the time</th>
<th>R</th>
<th>E</th>
<th>F</th>
<th>DK</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. I was bothered by things that usually don’t bother me.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. I did not feel like eating; my appetite was poor.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. I felt that I could not shake off the blues even with help from my family and friends.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. I felt that I was just as good as other people.</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. I had trouble keeping my mind on what I was doing.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. I felt depressed.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. I felt that everything I did was an effort.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. I felt hopeful about the future.</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. I thought my life had been a failure.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. I felt fearful.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. My sleep was restless.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. I was happy.</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. It seemed that I talked less than usual.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. I felt lonely.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15. People were unfriendly.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16. I enjoyed life.</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17. I had crying spells.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18. I felt sad.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. I felt that people disliked me.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20. I could not get going.</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TOTAL SCORE:_______ <16**

<< If total score is greater than 16, participant will be excluded >>
CONCLUSION OF TELEPHONE HEALTH HISTORY

For this current study, we cannot include people who have certain health conditions or are taking certain medications. Therefore, I will have one of study investigators review the information you gave me during this telephone call. After that, someone from our research staff will get back in touch with you. In order to contact you, I will need some additional information:

What is your full name? ____________________________________________

Home Address:

Street: __________________________________________________________

City, State: _____________________________________________________

Zip Code: ______________________________________________________

Email address: __________________________

Home phone: _________________

Day/work phone: _________________

Best time and place to reach you:

Morning (8-12): _________________

Afternoon (1-5): _________________

Evening (7-9): _________________

Thank you for your time.

__________________________________________

PLEASE ASK THIS BEFORE HANGING UP:

May we keep your name and contact information (phone, e-mail) in our research volunteer database in order to contact you about your possible interest in participating in other studies at the University, both with our research group and with other groups with whom we collaborate?

____ yes, contact me about other studies for which I might be eligible ______ no, do not contact me about other studies

Experimenter: Be sure to remove this page after subject is scheduled for lab session(s).
Appendix D. Telephone Screening Ineligibility Letter
DATE

Ms./Mr. ____________
Participant’s address
Participant’s address

Dear Ms./Mr. XXX:

Thank you for taking time to respond to our telephone screening for our study on smoking and diabetes. Because of the nature of our research project, we have a selective screening process to ensure that participants are similar to one another on many different health-related variables. After reviewing your screening information, it has been determined that you are not eligible to participate in our study. If you have any questions about this letter, please do not hesitate to contact us at 814-865-3319.

Thank you again for your interest in our research.

Sincerely,

Kimberly Walter
Doctoral Candidate in Biobehavioral Health

cc: Dr. Laura Klein (814)865-8813
Appendix E. Reminder Letters

1. Health Screening Session

2. Test Session
1. Health Screening Session
DATE

Mr. XXX,  
Participant's address  
Participant's address  

Dear Mr. XXX:

Thank you for volunteering for our study. Your health screening session appointment has been scheduled for DATE at TIME. The session will take place on the Penn State University campus in Noll Lab’s Clinical Research Center (CRC). The following are general instructions for your appointment:

1. Please wear a short sleeved shirt so that we can easily draw a blood sample from your arm.

2. Please do not eat anything after midnight the night before your visit because we will need to do a fasting blood draw.

3. Please do not drink alcohol for 48 hours before your visit.

4. Please do not exercise for 12 hours before your visit.

5. Please do not donate blood or plasma for two weeks prior to your scheduled visit or else your lab session will need to be rescheduled.

6. Because of our clinical schedule, we require a 24-hour notice for cancellation. Please call 814-865-3319 and leave a message for Kimberly Walter if you must reschedule or cancel your appointment.

7. Attached is a map with directions to your appointment at the CRC. Parking will be available for you at no charge. Please park in the area designated “Clinical Research Subjects”. Please walk to the front of the building facing North Atherton and take the elevator to the 2nd floor. Someone from our research team will be waiting on the 2nd floor for you.

Please do not hesitate to contact us at 814-865-3319 should you have any further questions.

Sincerely,

Kimberly Walter  
Doctoral Candidate in Biobehavioral Health

cc: Dr. Laura Klein (814) 865-8813
2. Test Session
DATE

Ms./Mr. _______
Participant’s address
Participant’s address

Dear Mr. XXX:

Thank you for volunteering for our study. Your laboratory test session has been scheduled for DATE at 2 pm. The session will take place on the Penn State University campus in Noll Lab’s Clinical Research Center (CRC). The following are general instructions for your appointment:

1. Please wear a short sleeved shirt so that we can easily draw a blood sample from your arm.

2. Please do not donate blood or plasma for two weeks prior to your scheduled visit or else your lab session will need to be rescheduled.

3. Because of our clinical schedule, we require a 24-hour notice for cancellation. Please call 814-865-3319 and leave a message for Kimberly Walter if you must reschedule or cancel your appointment.

4. Attached is a map with directions to your appointment at the CRC. Parking will be available for you at no charge. Please park in the area designated “Clinical Research Subjects”. Please walk to the front of the building facing North Atherton and someone from our research team will be waiting to escort you to your appointment.

Please do not hesitate to contact us at 814-865-3319 should you have any further questions.

Sincerely,

Kimberly Walter
Doctoral Candidate in Biobehavioral Health

cc: Dr. Laura Klein (814) 865-8813
Appendix F. Informed Consent Form
Title of Project: Biobehavioral Effects of Cognitive Challenge in Cigarette Smokers with Type 2 Diabetes

Principal Investigator: Laura Cousino Klein, Ph.D
Department of Biobehavioral Health
315 East HHD Building
The Pennsylvania State University
University Park, PA 16802
Email: lsk18@psu.edu
Phone: 814.865.3813

Other Investigator(s): Kimberly Walter, M.S., Sheila West, Ph.D.

1. Purpose of the study:

The purpose of this study is to understand how cigarette smoking and type 2 diabetes mellitus influence the biological and psychological responses to a cognitive challenge. We will use many measures to assess this research question including blood markers, saliva markers, blood pressure, and questionnaires.

Because the validity of the results of the study could be affected if certain details of this study are fully divulged to you prior to your participation, the purpose of the study cannot be completely explained to you at this time. You will have an opportunity to receive a complete explanation of the study’s purpose following completion of the laboratory test session.

2. Procedures to be followed:

To participate, you must be between 25 and 65 years old. You also may be normal weight or overweight, have hypertension, or have mild elevations in cholesterol levels. Additionally, you may not donate blood or plasma during the 4 weeks prior to the study and up until 4 weeks after your participation has ended.

Health Screening Session
The health screening session will be scheduled following the initial telephone screening. The session will be scheduled between 7 and 10 am at the Clinical Research Center (CRC) at The Pennsylvania State University. The health screening session visit will last approximately 1 hour. You will be asked to fast after midnight the night before the session and not to exercise for 12 hours before the session. You also will be asked not to drink alcohol for 48 hours before the session. At the screening visit, you will complete consent forms, a set of questionnaires about your health, and have measurements taken (e.g., height, weight, blood pressure, carbon monoxide via expired air). If your blood pressure is >160/100, you will have 15 mLs (about 1 tablespoon) of blood drawn to determine the amount of cholesterol levels in your blood and your general health. You will be compensated $10 for your time and informed that a research team member will contact you about your eligibility for the laboratory test session. A clinician will review your results and approve your participation in the study. The clinician also may ask you to complete a 12-lead EKG at the CRC.

Research Study
If you are eligible to participate in this study, you will be asked to come to the CRC for 1, 3-hour laboratory test session. The laboratory test session will be scheduled on a day that is convenient for you. The laboratory test session will be scheduled at 2 pm if you are not a smoker and at 1:45 pm if you are a smoker. Completing this study will require a total of about 4 hours (including the 1 hour screening visit) at the CRC.
Laboratory Test Session Procedures:

1. You will be asked to arrive at the CRC at 2:00 pm if you are not a smoker and at 1:45 pm if you are a smoker.
2. Your height and weight will be measured.
3. You carbon monoxide will be measured by blowing air in a small device.
4. You will provide a saliva sample by rolling a small cotton swab in your mouth for 2 minutes.
5. If you are a smoker, you will be escorted outside to smoke a final cigarette.
6. A blood pressure cuff will be placed on your arm, and 2 blood pressure readings will be taken using an automated blood pressure monitor.
7. A heart rate monitor chest strap transmitter and a wrist receiver will be placed on you to assess your heart rate throughout the lab session.
8. You will sit quietly in a chair for 15 minutes and will complete questionnaires that ask about your feelings and behaviors.
9. Your blood pressure and heart rate will be recorded throughout the lab session.
10. You will provide a second saliva sample.
11. A CRC nurse will perform a blood draw by inserting a small needle into a vein in your arm. About 20 mls of blood will be collected (less than 2 tablespoons).
12. You will participate in a “speech and math assessment” that will last approximately 30 minutes. Following a preparation period, you will be asked questions by a small panel of people who have been trained to determine your suitability for a job. Then you will be asked to perform subtraction to assess how quickly and accurately you are able to do this. Each task will be explained to you in more detail before you do them. Please understand that if you have any questions you may ask them at any time, and that you may stop the tasks at any time during the session.
13. Next, you will sit quietly in a chair for 15 minutes and complete another set of questionnaires.
14. You will provide a third saliva sample and a CRC nurse will collect a second blood sample (about 20 ml.
15. You will sit quietly in a chair 30 minutes, and complete a final set of questionnaires.
16. You will provide a final saliva sample.
17. You will have your hip circumference and waist circumference measured.
18. At the end of the laboratory test session, you will be compensated $35 for your time, and you will be given a $20 bonus for completing both the health screening session and the laboratory test session. You also will be debriefed about the study.
3. Discomforts and risks:

**Blood Sampling**
The risks of taking blood samples include some local pain and/or discomfort where the needle is inserted, bruising, and inflammation of the vein. There is also a slight risk of developing a blood clot at the blood draw site. As with any procedure involving taking blood, infection may occur (less than 1 in 10,000). Well-trained and experienced nurses will take your blood. All precautions will be taken to avoid infection.

Blood sampling can also cause light-headedness and dizziness. If this occurs, the symptoms will be alleviated by having you lie flat with your feet raised. Should you feel this way, we will stop the experiment and you will be given a drink of water. We ask that you remain in the laboratory until we have checked your blood pressure and we are sure that you feel OK.

**Saliva Sampling**
There are no known risks associated with collection of saliva. For people who tend to have a dry mouth and produce little saliva, there can be some discomfort in rolling the cotton cloth over the tongue. You will be provided with water to drink to help alleviate this concern.

**Blood Pressure**
There is a possibility for red blotching or mild bruising appearing on the skin above and below the location of the blood pressure cuff. Studies indicate that bruising is rare (occurring in less than 1/5 of 1% of patients) and it is typically not uncomfortable.

**Questionnaires**
You will be asked to complete several questionnaires throughout your 2 visits to the CRC. These questionnaires are kept confidential. Only your Subject ID will be attached to the questionnaires. No identifiable information will be used. Answering some of the questions could cause one to become uncomfortable. You will have the option to not answer any questions that you do not wish to answer without penalty.

**Speech and Math Assessment**
During the 30 minute speech and math assessment, potential risks include feeling nervous or uncomfortable during this task. You may also feel tired or stressed from having to complete the requested tasks. The tasks may cause a rise in blood pressure and heart rate. You will be monitored during the tasks and if any unsafe blood pressure is noted (not anticipated), the procedure will be discontinued. If you have any questions you may ask them at any time, and you may stop the tasks at any time during the session without penalty.

4. Benefits:

There is no immediate benefit to you from participating in this study. You will be informed of any information collected in this study that may affect your health (medical and psychological). If we find that your cholesterol screening laboratory blood test results are abnormal, you will be contacted by a nurse who will go over the results with you and recommend that you discuss them with your family physician.

There may be benefits to society from the research being conducted. It is hoped that results from this study will increase understanding of cigarette smoking and may provide information to help design better medications, interventions, and strategies to help people who want to quit smoking be successful.
5. Duration/time of the procedures and study:

If you complete this study, it will require approximately a total of 4 hours of your time. The health screening session will last approximately 1 hour and the laboratory test session will last approximately 3 hours.

6. Alternative procedures that could be utilized:

This study is designed to help us understand how cigarette smoking and type 2 diabetes mellitus influence the biological and psychological response to a cognitive challenge. There are no alternative procedures that can be used to measure blood markers, blood pressure, and self-reported behaviors and well-being on questionnaires.

7. Statement of confidentiality:

Your participation in this research is confidential. All records are coded with a unique ID number and no names are used. Records containing names or other identifying information are kept under lock at the Biobehavioral Health Studies Lab. Only Dr. Klein and Ms. Walter will have access to your identity and to information that can be associated with your identity.

Your confidentiality will be kept to the degree permitted by the technology used. No guarantees can be made regarding the interception of data sent via the Internet by any third parties.

The Pennsylvania State University’s Office for Research Protections, the Institutional Review Board, and the Office for Human Research Protections in the Department of Health and Human Services may review records related to this research study.

All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records. In the event of publication of this research, no personal identifying information will be disclosed. Your blood and saliva specimens and questionnaires will be coded with your unique ID number. Questionnaires will be stored indefinitely with identifiers removed. Blood and saliva specimens will be kept up to 5 years after the research has been completed unless you consent to future use as indicated on the last page of this consent form.

8. Right to ask questions: Please contact the principal investigator, Dr. Laura Klein at (814) 883-8624 or the co-investigator, Kimberly Walter at (774) 696-4348 with questions, complaints or concerns about the research. You can also call these numbers if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact The Pennsylvania State University’s Office for Research Protections (ORP) at (814) 863-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

9. Payment for participation:

You will be compensated up to $65 for participating in this study. Compensation is as follows:

1. You will receive $10 at the end of the health screening session.
2. You will receive $35 at the end of the laboratory test session.
3. If you complete both CRC visits, you will receive a $20 bonus at the end of the laboratory test session.

In the event that laboratory test session is ended early by the investigator or you, you will be compensated for your participation at a pro-rated rate of $10/hr.
10. Voluntary participation:

Your decision to be in this research is voluntary. You can stop at any time. You do not have to answer any questions you do not want to answer. Refusal to take part in or withdrawing from this study will involve no penalty or loss of benefits that you would receive otherwise.

If at any time during any of study sessions the investigator or CRC clinicians believe that continuing the study puts your health at risk; then the investigator can terminate the session and withdraw you from the study.

11. Injury Clause

In the unlikely event you become injured as a result of your participation in this study, medical care is available. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

12. Abnormal Test Results

In the event that abnormal lab test results are obtained during the health screening session, you will be informed as soon as the unexpected result(s) is/are received and reviewed (within one week of your screening visit). You will be instructed to contact your private physician for further assessment. The lab test results from screening will be made available to your private physician at your request and with your permission.

You must be 18 years of age or older to take part in this research study. If you agree to take part in this research study and the information outlined above, please sign your name and indicate the date below.

You will be given a copy of this signed and dated consent form for your records.

Participant Signature ___________________________ Date ____________

Person Obtaining Consent ________________________ Date ____________
13. Storage of Leftover Blood Samples and Saliva Samples for Future Research Studies

In addition to the main part of the research study, there is an optional part of the study. You can participate in the main part of the research without agreeing to take part in this optional part.

As part of this study, we are obtaining blood and saliva from you. If you agree, the research team would like to store leftover samples of your blood and saliva that are collected so that your blood and saliva can be studied in the future after this study is over. These future studies may provide additional information that will be helpful in understanding smoking and diabetes, but it is unlikely that these studies will have a direct benefit to you. Neither your doctor nor you will receive results of these future research tests, nor will the results be put in your health record. If you have any questions, you should contact the principal investigator, Dr. Laura Klein at (814) 865-8813.

Your leftover samples will be labeled with a code number and stored in Dr. Laura Klein’s locked laboratory. If you consent to the collection of samples of your blood and saliva for future research, the period for the use of the samples is unknown. If you agree to allow your blood and saliva to be kept for future research, you will be free to change your mind at any time. You should contact Dr. Laura Klein at (814) 865-8813 and let her know you wish to withdraw your permission for your blood and saliva to be used for future research. If you do this, any unused blood and saliva will be destroyed and not used for future research studies.

You should initial below to indicate your preferences regarding the optional storage of your leftover blood and saliva for future research studies.

a. Your samples may be stored and used for future research studies to learn about, prevent, treat, or cure smoking, diabetes, and other health problems.

    _____ Yes    _____ No

b. Your samples may be shared with other investigator/groups without any identifying information.

    _____ Yes    _____ No

Participant: If you have read the information in this form and agree to and give your permission for your participation in this optional part of the research please print your name and sign below.

________________________  ______________________  ______________  __________________________
Signature of Participant    Date     Time         Printed Name

Person Explaining the Research: Your signature below means that you have explained the optional part of the research to the participant/participant representative and have answered any questions he/she has about the research.

________________________  ______________________  ______________  __________________________
Signature of person who explained this optional research

Page 6 of 6
Appendix G. Health Screening Session Procedures
Health Screening Session Procedures

1. Participants arrive at the CRC following an overnight fast
2. Participants provide written informed consent
3. Participants complete questionnaire Set [Demographic form; SES ladder; medication use form; Brief COPE Scale; Interpersonal Social Support Evaluation; Cognitive Failures Questionnaire; Problem Areas in Diabetes Scale]
4. Participant’s height, weight, carbon monoxide (CO), systolic blood pressure, diastolic blood pressure, and heart rate measured.
5. CRC clinician will conduct a clinical evaluation
6. A 12-lead EKG may be ordered at the GCRC clinician’s discretion based on the screening values of blood pressure
7. A CRC clinician will collect 15 ml of blood via a venipuncture.
8. Participants will be compensated $10 for their time and informed that a research team member will contact them via telephone about the laboratory session.
Appendix H. Health Screening Session Questionnaires

1. Demographic Form
2. Tobacco Use Form
3. Medication Use Form
4. Brief COPE Scale
5. Interpersonal Social Support Evaluation
6. Cognitive Failures Questionnaire
7. Problem Areas in Diabetes scale (Type 2 DM Only)
1. Demographic Form
Demographics Questionnaire

Participant ID: 

1. What is your current age? 

2. Are you male or female? 
   - Male 
   - Female 

3. What is your occupational status? 
   - Employed full time 
   - Employed part time 
   - Unemployed, looking for work 
   - Unemployed, not looking for work 
   - Retired 
   - Homemaker 
   - Disabled 
   - Other, please specify below: 

4. What is your marital status? 
   - Married 
   - Living as married 
   - Separated 
   - Divorced 
   - Widowed 
   - Single, never been married 
   - Dating (One Person/Exclusive) 

5. What is the highest grade or level of schooling you completed? 
   - Less than 8 years 
   - 8 through 11 years 
   - 12 years or completed high school 
   - Post-high school training other than college (vocational or technical) 
   - Some college 
   - College graduate 
   - Postgraduate 

6. Are you Hispanic or Latino? 
   - Yes 
   - No 

7. Which one or more of the following would you say is your race? 
   - Mark all that apply 
   - Black / African American 
   - Asian / Asian American 
   - Native Hawaiian / Pacific Islander 
   - White / Caucasian 
   - Native American / Alaska Native 

8. How many children under the age of 18 live in your household? 

9. Thinking about members of your family living in this household, what is your combined annual income, meaning the total pre-tax income from all sources earned in the past year? 
   - $0 to $9,999 
   - $10,000 to $14,999 
   - $15,000 to $19,999 
   - $20,000 to $34,999 
   - $35,000 to $49,999 
   - $50,000 to $74,999 
   - $75,000 to $99,999 
   - $100,000 to $199,999 
   - $200,000 or more 

10. Do you rent or own your home? 
    - Rent 
    - Own 
    - Occupy without paying monetary rent
11. Think of this ladder as representing where people stand in our society. At the top of the ladder are the people who are the best off - those who have the most money, the most education, and the most respected jobs. At the bottom are the people who are the worst off - who have the least money, least education, and the least respected jobs or no job. The higher up you are on this ladder, the closer you are to the people at the very top. The lower you are, the closer you are to the people at the very bottom.

Where would you place yourself on this ladder?

Please place a large "X" on the rung where you think you stand at this time in your like, relative to other people in our society.
2. Tobacco Use Form
Tobacco Use History Questionnaire

Diabetes and Smoking Study

Participant ID:

1. Have you smoked at least 100 cigarettes in your entire life?
   - Yes
   - No

2. How old were you when you first started smoking cigarettes FAIRLY REGULARLY?
   - [ ] [ ] [ ] Years old

3. Do you now smoke cigarettes every day, some days, or not at all?
   - Every day
   - Some days
   - Not at all

4. On the average, about how many cigarettes do you now smoke each day?
   - [ ] [ ] Each day

5. Is your usual cigarette brand menthol or non-menthol?
   - Menthol
   - Non-menthol
   - No usual type

6. What is the total number of years you have smoked EVERY DAY? Do not include any time you stayed off cigarettes for 6 months or longer.
   - [ ] [ ] Years

7. During the PAST 12 MONTHS, have you TRIED to QUIT smoking COMPLETELY?
   - Yes
   - No

8. Have you EVER TRIED to QUIT smoking COMPLETELY?
   - Yes
   - No

9. Would you say that it was more than 3 times?
   - Yes
   - No

10. Thinking back to when you tried to QUIT smoking in the PAST 12 MONTHS: Did you use a telephone help line or quit line?
    - Yes
    - No

11. In the PAST 12 MONTHS have you SEEN a medical doctor?
    - Yes
    - No

12. During the PAST 12 MONTHS, did any medical doctor ADVISE you to stop smoking?
    - Yes
    - No

13. Has a medical doctor EVER ADVISED you to stop smoking?
    - Yes
    - No
<table>
<thead>
<tr>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
</table>
| 14. In the PAST 12 MONTHS have you SEEN a dentist?                      | □ Yes  
□ No                                                                 |
| 15. During the PAST 12 MONTHS, did any dentist ADVISE you to stop smoking? | □ Yes  
□ No                                                                 |
| 16. Has a dentist EVER ADVISED you to stop smoking?                     | □ Yes  
□ No                                                                 |
| 17. Are you seriously considering quitting smoking within the next 6 months? | □ Yes  
□ No                                                                 |
| 18. Are you planning to quit within the next 30 days?                   | □ Yes  
□ No                                                                 |
| 19. Overall, on a scale from 1 to 10 where 1 is NOT AT ALL interested and 10 is EXTREMELY interested, how interested are you in quitting smoking? | □□□□□□□□□□ (Scale of 1 – 10) |
| 20. If you did try to quit smoking altogether in the next 6 months, how LIKELY do you think you would be to succeed --- not at all, a little likely, somewhat likely or very likely? | □ Not at all  
□ A little likely  
□ Somewhat likely  
□ Very likely |
| 21. Have you EVER used any of the following EVEN ONE TIME? (Check all that apply) | □ Chewing tobacco such as Redman, Levi Garrett, or Beechnut  
□ Snuff such as Skoal, Skoal Bandits, or Copenhagen |
| 22. Do you NOW use chewing tobacco or snuff every day, some days or not at all? | □ Every day  
□ Some days  
□ Not at all |
| 23. On how many of the past 30 days did you use chewing tobacco or snuff? | □□□□□□□□□□ Days |
| 24. How soon after you wake up do you typically FIRST use chewing tobacco? | □□□□□□□□□□ Hours  □□□□□□□□□□ Minutes |
3. Medication Use Form
# DAILY MEDICATION USE

Please list all medications you take on a regular basis. For each medication or supplement, please list the frequency (e.g., how often) and the dose (e.g., how much you take each time). This information can be found on the prescription label.

## ADULT PRESCRIPTION MEDICATIONS ONLY:

<table>
<thead>
<tr>
<th>MEDICATION NAME</th>
<th>FREQUENCY</th>
<th>DOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example: Advair® or Fluticasone/Salmeterol Inhaler</td>
<td>2 puffs/day</td>
<td>250/50 mcg/puff</td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

## ADULT OVER-THE-COUNTER MEDICATIONS OR SUPPLEMENTS (obtained without a prescription)

<table>
<thead>
<tr>
<th>MEDICATION OR SUPPLEMENT NAME</th>
<th>FREQUENCY</th>
<th>DOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example: Multivitamin</td>
<td>1 tablet/day</td>
<td>n/a</td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4. Brief COPE Scale
Brief COPE

These items deal with ways you cope with the stress in your life. There are many ways to try to deal with problems. Obviously, different people deal with things in different ways, but I'm interested in how you deal with problems. Each item says something about a particular way of coping. I want to know to what extent you do what the item says. How much or how frequently. Don't answer on the basis of whether it seems to work or not—just whether or not you do it. Use these response choices. Try to rate each item separately in your mind from the others. Make your answers as true FOR YOU as you can.

1 = I haven't been doing this at all
2 = I've been doing this a little bit
3 = I've been doing this a medium amount
4 = I've been doing this a lot

1. I turn to work or other activities to take my mind off things.
2. I concentrate my efforts on doing something about the situation I'm in.
3. I say to myself "this isn't real".
4. I use alcohol or other drugs to make myself feel better.
5. I get emotional support from others.
6. I give up trying to deal with it.
7. I take action to try to make the situation better.
8. I refuse to believe that it has happened.
9. I say things to let my unpleasant feelings escape.
10. I get help and advice from other people.
11. I use alcohol or other drugs to help me get through it.
12. I try to see it in a different light, to make it seem more positive.
13. I criticize myself.
14. I try to come up with a strategy about what to do.
15. I get comfort and understanding from someone.
16. I give up the attempt to cope.
17. I look for something good in what is happening.
18. I make jokes about it.

19. I do something to think about it less, such as going to movies, watching TV, reading, daydreaming, sleeping, or shopping.

20. I accept the reality of the fact that it has happened.

21. I express my negative feelings.

22. I try to find comfort in my religion or spiritual beliefs.

23. I try to get advice or help from other people about what to do.

24. I try learning to live with it.

25. I think hard about what steps to take.

26. I blame myself for things that happened.

27. I pray or meditate.

28. I make fun of the situation.
5. Interpersonal Social Support Evaluation
IEL-12

Instructions: This scale is made up of a list of statements each of which may or may not be true about you. For each statement circle "definitely true" if you are sure it is true about you and "probably true" if you think it is true but are not absolutely certain. Similarly, you should circle "definitely false" if you are sure the statement is false and "probably false" if you think it is false but are not absolutely certain.

1. If I wanted to go on a trip for a day (for example, to the country or mountains), I would have a hard time finding someone to go with me.
   1. definitely false  2. probably false  3. probably true  4. definitely true

2. I feel that there is no one I can share my most private worries and fears with.
   1. definitely false  2. probably false  3. probably true  4. definitely true

3. If I were sick, I could easily find someone to help me with my daily chores.
   1. definitely false  2. probably false  3. probably true  4. definitely true

4. There is someone I can turn to for advice about handling problems with my family.
   1. definitely false  2. probably false  3. probably true  4. definitely true

5. If I decide one afternoon that I would like to go to a movie that evening, I could easily find someone to go with me.
   1. definitely false  2. probably false  3. probably true  4. definitely true

6. When I need suggestions on how to deal with a personal problem, I know someone I can turn to.
   1. definitely false  2. probably false  3. probably true  4. definitely true

7. I don't often get invited to do things with others.
   1. definitely false  2. probably false  3. probably true  4. definitely true

8. If I had to go out of town for a few weeks, it would be difficult to find someone who would look after my house or apartment (the plants, pets, garden, etc.).
   1. definitely false  2. probably false  3. probably true  4. definitely true
9. If I wanted to have lunch with someone, I could easily find someone to join me.

1. definitely false  2. probably false  3. probably true  4. definitely true

10. If I was stranded 10 miles from home, there is someone I could call who could come and get me.

1. definitely false  2. probably false  3. probably true  4. definitely true

11. If a family crisis arose, it would be difficult to find someone who could give me good advice about how to handle it.

1. definitely false  2. probably false  3. probably true  4. definitely true

12. If I needed some help in moving to a new house or apartment, I would have a hard time finding someone to help me.

1. definitely false  2. probably false  3. probably true  4. definitely true
6. Cognitive Failures Questionnaire
### The Cognitive Failures Questionnaire

The following questions are about minor mistakes which everyone makes from time to time, but some of which happen more often than others. We want to know how often these things have happened to you in the past 6 months. Please circle the appropriate number.

<table>
<thead>
<tr>
<th></th>
<th>Very often</th>
<th>Quite often</th>
<th>Occasionally</th>
<th>Very rarely</th>
<th>Never</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Do you read something and find you haven’t been thinking about it and must read it again?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2. Do you find you forget why you went from one part of the house to the other?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3. Do you fail to notice signs posts on the road?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4. Do you find you confuse right and left when giving directions?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5. Do you bump into people?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>6. Do you find you forget whether you’ve turned off a light or a fire or locked the door?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>7. Do you fail to listen to people’s names when you are meeting them?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>8. Do you say something and realize afterwards that it might be taken as insulting?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9. Do you fail to hear people speaking to you when you are doing something else?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10. Do you lose your temper and regret it?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>11. Do you leave important letters unanswered for days?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>12. Do you find you forget which way to turn on a road you know well but rarely use?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>13. Do you fail to see what you want in a supermarket (although it’s there)?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>14. Do you find yourself suddenly wondering whether you’ve used a word correctly?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Question</td>
<td>Very often</td>
<td>Quite often</td>
<td>Occasionally</td>
<td>Very rarely</td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------------------------------------------</td>
<td>------------</td>
<td>-------------</td>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>15.</td>
<td>Do you have trouble making up your mind?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>16.</td>
<td>Do you find you forget appointments?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>17.</td>
<td>Do you forget where you put something like a newspaper or a book?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>18.</td>
<td>Do you find you accidentally throw away the thing you want and keep what you meant to throw away – as in the example of throwing away the matchbox and putting the used match in your pocket?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>19.</td>
<td>Do you daydream when you ought to be listening to something?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>20.</td>
<td>Do you find you forget people’s names?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>21.</td>
<td>Do you start doing one thing at home and get distracted into doing something else (unintentionally)?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>22.</td>
<td>Do you find you can’t quite remember something although it’s “on the tip of your tongue”?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>23.</td>
<td>Do you find you forget what you came to the shops to buy?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>24.</td>
<td>Do you drop things?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>25.</td>
<td>Do you find you can’t think of anything to say?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

References

7. Problem Areas in Diabetes scale (Type 2 DM Only)
INSTRUCTIONS: Which of the following diabetes issues are currently a problem for you? Circle the number that gives the best answer for you. Please provide an answer for each question.

<table>
<thead>
<tr>
<th>Issue</th>
<th>Not a problem</th>
<th>Minor problem</th>
<th>Moderate problem</th>
<th>Somewhat serious problem</th>
<th>Serious problem</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Not having clear and concrete goals for your diabetes care?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>2. Feeling discouraged with your diabetes treatment plan?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>3. Feeling scared when you think about living with diabetes?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4. Uncomfortable social situations related to your diabetes care</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>(e.g., people telling you what to eat)?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Feelings of deprivation regarding food and meals?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>6. Feeling depressed when you think about living with diabetes?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>7. Not knowing if your mood or feelings are related to your diabetes</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>8. Feeling overwhelmed by your diabetes?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>9. Worrying about low blood sugar reactions?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>10. Feeling angry when you think about living with diabetes?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>11. Feeling constantly concerned about food and eating?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>12. Worrying about the future and the possibility of serious</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>complications?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. Feelings of guilt or anxiety when you get off track with your</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>diabetes management?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. Not &quot;accepting&quot; your diabetes?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>15. Feeling unsatisfied with your diabetes physician?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>16. Feeling that diabetes is taking up too much of your mental and</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>physical energy every day?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17. Feeling alone with your diabetes?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>18. Feeling that your friends and family are not supportive of your</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>diabetes management efforts?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. Coping with complications of diabetes?</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>20. Feeling “burned out” by the constant effort needed to manage</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>diabetes?</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Appendix I. Test Session Procedures
I. Laboratory Session Procedures (stressor/non-stressor lab sessions)

1. Participant arrival at CRC at 2:00 pm;
2. Carbon monoxide measured via expired air
3. Participant provides saliva sample #1 for later assaying of cotinine
4. Acclimation period (5 min): Dinamap BP cuff placed on dominant arm and 2 BP readings taken. If the mean of the two blood pressure readings is below 160/100 mmHg, the participant will be allowed to continue with the session. If the mean is greater than 160/100, the participant will be excluded from completing the remainder of the study session.
5. A heart rate (HR) monitor chest strap transmitter will be placed around the participant's torso and a wrist receiver will be placed on his wrist to assess your heart rate throughout the lab session.
6. Questionnaire Set #1 (10 min) [Positive and Negative Affect Scale; Mood Assessment Scale; Mindfulness Attention Scale]
7. Baseline period (10 min): BP taken at 2-min intervals; HR taken continuously
8. Saliva sample #2 (5 min): Baseline saliva sample for salivary free cortisol and salivary alpha amylase
9. GCRC nurse will perform blood draw #1 via venipuncture. (10 min)
10. Trier Social Stressor Test (TSST) (30 min) (see TSST procedures upload)
11. BP taken at 2-min intervals and HR taken continuously during the TSST task;
12. Rest period (15 min): BP taken at 2-min intervals and HR taken continuously;
    Questionnaire Set #2 [Post-task appraisal questionnaire; Mood Assessment Scale; Positive and Negative Affect Scale]
13. Saliva sample #3 (2 min)
14. CRC nurse will perform blood draw #2 via venipuncture
15. Recovery period (30 min): BP 5-min intervals and HR sampled continuously;
    Questionnaire #3 [Perceived Stress Scale; Mood Assessment Scale; Positive and Negative Affect Scale; Diabetes Quality of Life Scale Brief Clinical Inventory (type 2 DM only); Five Facet Mindfulness Questionnaire; SF-12V2; trait anxiety]
16. Saliva sample #4 (5 min): Recovery saliva sample for salivary free cortisol and salivary alpha amylase
17. Participant will be debriefed, given $35 for the session, and a $20 bonus for completing both sessions.
Appendix J. Test Session Questionnaire Set #1

1. Positive and Negative Affect Scale

2. Mood Assessment Scale

3. Mindfulness Attention Awareness Scale
1. Positive and Negative Affect Scale
The PANAS

This scale consists of a number of words that describe different feelings and emotions. Read each item and then mark the appropriate answer (1 to 5) in the space next to that word. Indicate to what extent you feel this way right now, that is, at the present moment.

<table>
<thead>
<tr>
<th>1 very slightly or not at all</th>
<th>2 a little</th>
<th>3 moderately</th>
<th>4 quite a bit</th>
<th>5 extremely</th>
</tr>
</thead>
<tbody>
<tr>
<td>_____ interested</td>
<td>_____ irritable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_____ distressed</td>
<td>_____ alert</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_____ excited</td>
<td>_____ ashamed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_____ upset</td>
<td>_____ inspired</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_____ strong</td>
<td>_____ nervous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_____ guilty</td>
<td>_____ determined</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_____ scared</td>
<td>_____ attentive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_____ hostile</td>
<td>_____ jittery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_____ enthusiastic</td>
<td>_____ active</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>_____ proud</td>
<td>_____ afraid</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2. Mood Assessment Scale
# MOOD ASSESSMENT SCALE

**ID #**
**TIME**
**Session #**
**Date**

Please indicate below how you are feeling at this moment. Answer quickly & honestly.

<table>
<thead>
<tr>
<th></th>
<th>Not at all</th>
<th>Very much</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nervous, tense, worried</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>2. Happy</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>3. Thirsty</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>4. Frustrated</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>5. Impatient</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>6. Tired, low energy</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>7. Nauseous or queasy</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>8. Angry</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>9. Sad</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>10. Hungry</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>11. Restless</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>12. Focused, attentive</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>13. Irritable</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>14. Curious, interested</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>15. Disorganized</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>16. Relaxed, comfortable</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>17. Stressed</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>18. Light-headed</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
</tbody>
</table>
3. Mindfulness Attention Awareness Scale
## Day-to-Day Experiences

Instructions: Below is a collection of statements about your everyday experience. Using the 1-6 scale below, please indicate how frequently or infrequently you currently have each experience. Please answer according to what *really reflects* your experience rather than what you think your experience should be. Please treat each item separately from every other item.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Almost Always</td>
<td>Very Frequently</td>
<td>Somewhat Frequently</td>
<td>Somewhat Infrequently</td>
<td>Very Infrequently</td>
<td>Almost Never</td>
</tr>
</tbody>
</table>

I could be experiencing some emotion and not be conscious of it until some time later.  
1  2  3  4  5  6

I break or spill things because of carelessness, not paying attention, or thinking of something else.  
1  2  3  4  5  6

I find it difficult to stay focused on what’s happening in the present.  
1  2  3  4  5  6

I tend to walk quickly to get where I’m going without paying attention to what I experience along the way.  
1  2  3  4  5  6

I tend not to notice feelings of physical tension or discomfort until they really grab my attention.  
1  2  3  4  5  6

I forget a person’s name almost as soon as I’ve been told it for the first time.  
1  2  3  4  5  6

It seems I am “running on automatic,” without much awareness of what I’m doing.  
1  2  3  4  5  6

I rush through activities without being really attentive to them.  
1  2  3  4  5  6

I get so focused on the goal I want to achieve that I lose touch with what I’m doing right now to get there.  
1  2  3  4  5  6

I do jobs or tasks automatically, without being aware of what I’m doing.  
1  2  3  4  5  6

I find myself listening to someone with one ear, doing something else at the same time.  
1  2  3  4  5  6
<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Almost Always</td>
<td>Very Frequently</td>
<td>Somewhat Frequently</td>
<td>Somewhat Infrequently</td>
<td>Very Infrequently</td>
<td>Almost Never</td>
</tr>
<tr>
<td>I drive places on ‘automatic pilot’ and then wonder why I went there.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>I find myself preoccupied with the future or the past.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>I find myself doing things without paying attention.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>I snack without being aware that I’m eating.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>
Appendix K. Trier Social Stress Test Procedures
Trier Social Stress Test Procedures (Stressor Task)

1. Primary experimenter turns on job interview speech instruction video that will describe the speech task to the participant. The primary experimenter then leaves the room.

2. After 10 minutes, the 2-person evaluation panel enters the room and the lead evaluator introduces herself and the second evaluator.

3. The lead evaluator then instructs the participant that it is time to give the 5-minute job interview speech and that the evaluators will listen to the speech and evaluate their performance and the content of the speech, including the quality of the participant’s presentation and ability to communicate his/her points effectively. The lead evaluator also will inform the participant that he/she will be videotaped through a camera so that their speech and non-verbal behavior can be analyzed later by us and Dr. Klein. The participant will be told that the videotape will be evaluated for poise, articulation, style, and your communication abilities.

4. If participant stops talking during the 5-minute speech, the lead evaluator will say: “Your time is not up, please continue speaking.” If participant asks: “How much time is left?”, the lead evaluator will say: “I will tell you when to stop speaking. Please continue with your speech.” If participant states: “I have nothing else to say”, evaluator responds: “Your time is not up, please continue speaking. You may repeat things you’ve already said if necessary.” If participant is moving their hands or gesturing, say: “You need to keep your hands still. Your blood pressure is being monitored.”

5. At end of 5-minute time period, the lead evaluator will inform that participant that they are done and then will turn on the video with the “Math By 7’s” task instructions. After the instructions are complete, the lead evaluator will inform the participant that it is time to start the math task. The participant is instructed to count backwards by 7’s starting with the number 9095. When participant makes a mistake, the lead evaluator will say: “No, that is incorrect. The last correct number was X.” or “That is incorrect, begin counting from X.” If participant closes eyes or looks away, the lead evaluator will say “Don’t close your eyes.” If participant gestures or moves their hands, the lead evaluator will say “You need to keep your hands still. Your blood pressure is being monitored.”

6. After 2 minutes of counting the lead evaluator will say “You’re running out of time; you really need to speed it up.”

7. After 4 minutes of counting the lead evaluator will instruct the participant to stop.

8. The lead evaluator then will turn on the video with the “Math By 13’s” task instructions.

9. After the instructions are complete, the lead evaluator will inform the participant that it is time to start the math task. The participant is instructed to count backwards by 13’s starting with the number 6233. When participant makes a mistake, the lead evaluator will say: “No, that is incorrect. The last correct number was X.” or “That is incorrect, begin counting from X.” If participant closes eyes or looks away, the lead evaluator will say “Don’t close your eyes.” If participant gestures or moves their hands, the lead evaluator will say “You need to keep your hands still. Your blood pressure is being monitored.”

10. After 2 minutes of counting the lead evaluator will say “You’re running out of time; you really need to speed it up.”

11. After 4 minutes of counting the lead evaluator will instruct the participant to stop.
Appendix L. Test Session Questionnaire Set # 2

1. Post-Task Appraisal Questionnaire

2. Positive and Negative Affect Scale

3. Mood Assessment Scale
1. Post-Task Appraisal Questionnaire
Post Task Appraisal

Directions: For each of the following questions, please circle the answer on the scale below the questions which best describes how you feel about the task you just completed.

1. How stressful did you find the task to be?

   1  2  3  4  5
   Not at all Stressful Moderately Stressful Very Stressful

2. How well do you think you were able to cope with the task?

   1  2  3  4  5
   Not Well Moderately Well Very Well

3. How demanding did you find the task to be?

   1  2  3  4  5
   Not at all Demanding Moderately Demanding Very Demanding

4. How threatening did you find the task to be?

   1  2  3  4  5
   Not at all Threatening Moderately Threatening Very Threatening

5. How well did you perform the task?

   1  2  3  4  5
   Not Well Moderately Well Very Well
2. Positive and Negative Affect Scale
The PANAS

This scale consists of a number of words that describe different feelings and emotions. Read each item and then mark the appropriate answer (1 to 5) in the space next to that word. Indicate to what extent you feel this way right now, that is, at the present moment.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>very slightly or not at all</td>
<td>a little</td>
<td>moderately</td>
<td>quite a bit</td>
<td>extremely</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>interested</th>
<th>irritable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>distressed</td>
<td>alert</td>
</tr>
<tr>
<td></td>
<td>excited</td>
<td>ashamed</td>
</tr>
<tr>
<td></td>
<td>upset</td>
<td>inspired</td>
</tr>
<tr>
<td></td>
<td>strong</td>
<td>nervous</td>
</tr>
<tr>
<td></td>
<td>guilty</td>
<td>determined</td>
</tr>
<tr>
<td></td>
<td>scared</td>
<td>attentive</td>
</tr>
<tr>
<td></td>
<td>hostile</td>
<td>jittery</td>
</tr>
<tr>
<td></td>
<td>enthusiastic</td>
<td>active</td>
</tr>
<tr>
<td></td>
<td>proud</td>
<td>afraid</td>
</tr>
</tbody>
</table>
3. Mood Assessment Scale
## MOOD ASSESSMENT SCALE

<table>
<thead>
<tr>
<th>ID #</th>
<th>TIME</th>
<th>SESSION #</th>
<th>DATE</th>
</tr>
</thead>
</table>

Please indicate below how you are feeling at this moment. Answer quickly & honestly.

<table>
<thead>
<tr>
<th></th>
<th>Not at all</th>
<th>Very much</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nervous, tense, worried</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>2. Happy</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>3. Thirsty</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>4. Frustrated</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>5. Impatient</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>6. Tired, low energy</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>7. Nauseous or queasy</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>8. Angry</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>9. Sad</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>10. Hungry</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>11. Restless</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>12. Focused, attentive</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>13. Irritable</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>14. Curious, Interested</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>15. Disorganized</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>16. Relaxed, comfortable</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>17. Stressed</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
<tr>
<td>18. Light-headed</td>
<td>1 2 3 4 5 6 7</td>
<td></td>
</tr>
</tbody>
</table>
Appendix M. Test Session Questionnaire Set #3

1. Positive and Negative Affect Scale
2. Mood Assessment Scale
3. Perceived Stress Scale
4. Diabetes Quality of Life Scale Brief Clinical Inventory
5. Medical Outcomes Study SF-12V2
6. Spielberger Trait Anxiety Scale
7. Five Facet Mindfulness Questionnaire
1. Positive and Negative Affect Scale
The PANAS

This scale consists of a number of words that describe different feelings and emotions. Read each item and then mark the appropriate answer (1 to 5) in the space next to that word. Indicate to what extent you feel this way right now, that is, at the present moment.

1 very slightly or not at all
2 a little
3 moderately
4 quite a bit
5 extremely

_____ interested
_____ irritable
_____ distressed
_____ alert
_____ excited
_____ ashamed
_____ upset
_____ inspired
_____ strong
_____ nervous
_____ guilty
_____ determined
_____ scared
_____ attentive
_____ hostile
_____ jittery
_____ enthusiastic
_____ active
_____ proud
_____ afraid
2. Mood Assessment Scale
MOOD ASSESSMENT SCALE

ID # ___________________ TIME ___________________
Session # ___________ Date ___________

Please indicate below how you are feeling at this moment. Answer quickly & honestly.

<table>
<thead>
<tr>
<th></th>
<th>Not at all</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nervous, tense, worried</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>2. Happy</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>3. Thirsty</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>4. Frustrated</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>5. Impatient</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>6. Tired, low energy</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>7. Nauseous or queasy</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8. Angry</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>9. Sad</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>10. Hungry</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>11. Restless</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>12. Focused, attentive</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>13. Irritable</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>14. Curious, interested</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>15. Disorganized</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>16. Relaxed, comfortable</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>17. Stressed</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>18. Light-headed</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>
3. Perceived Stress Scale
### Perceived Stress Scale

**Participant ID:**

<table>
<thead>
<tr>
<th></th>
<th>Never</th>
<th>Almost Never</th>
<th>Sometimes</th>
<th>Fairly Often</th>
<th>Very Often</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. In the last month, how often have you been upset because of something that happened unexpectedly?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. In the last month, how often have you felt that you were unable to control the important things in your life?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. In the last month, how often have you felt nervous and “stressed”?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. In the last month, how often have you felt confident about your ability to handle your personal problems?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. In the last month, how often have you felt that things were going your way?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. In the last month, how often have you found that you could not cope with all the things that you had to do?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. In the last month, how often have you been able to control irritations in your life?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. In the last month, how often have you felt that you were on top of things?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. In the last month, how often have you been angered because of things that were outside of your control?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The questions in this scale ask you about your feelings and thoughts during the last month. In each case, you will be asked to place an “X” in the box indicating how often you felt or thought a certain way.
4. Diabetes Quality of Life Scale Brief Clinical Inventory (Type 2 DM Only)
Diabetes Quality of Life Brief Clinical Inventory

1. How satisfied are you with your current diabetes treatment?

<table>
<thead>
<tr>
<th>Very Satisfied</th>
<th>Moderately Satisfied</th>
<th>Neither</th>
<th>Moderately Dissatisfied</th>
<th>Very Dissatisfied</th>
</tr>
</thead>
</table>

2. How satisfied are you with the amount of time it takes to manage your diabetes?

<table>
<thead>
<tr>
<th>Very Satisfied</th>
<th>Moderately Satisfied</th>
<th>Neither</th>
<th>Moderately Dissatisfied</th>
<th>Very Dissatisfied</th>
</tr>
</thead>
</table>

3. How often do you find that you eat something you shouldn’t rather than tell someone that you have diabetes?

<table>
<thead>
<tr>
<th>Never</th>
<th>Very Seldom</th>
<th>Sometimes</th>
<th>Often</th>
<th>All the time</th>
</tr>
</thead>
</table>

4. How often do you worry about whether you will miss work?

<table>
<thead>
<tr>
<th>Never</th>
<th>Very Seldom</th>
<th>Sometimes</th>
<th>Often</th>
<th>All the time</th>
</tr>
</thead>
</table>

5. How satisfied are you with the time it takes to determine your sugar level?

<table>
<thead>
<tr>
<th>Very Satisfied</th>
<th>Moderately Satisfied</th>
<th>Neither</th>
<th>Moderately Dissatisfied</th>
<th>Very Dissatisfied</th>
</tr>
</thead>
</table>

6. How satisfied are you with the time you spend exercising?

<table>
<thead>
<tr>
<th>Very Satisfied</th>
<th>Moderately Satisfied</th>
<th>Neither</th>
<th>Moderately Dissatisfied</th>
<th>Very Dissatisfied</th>
</tr>
</thead>
</table>

7. How often do you have a bad night’s sleep because of diabetes?

<table>
<thead>
<tr>
<th>Never</th>
<th>Very Seldom</th>
<th>Sometimes</th>
<th>Often</th>
<th>All the time</th>
</tr>
</thead>
</table>

8. How satisfied are you with your sex life?

<table>
<thead>
<tr>
<th>Very Satisfied</th>
<th>Moderately Satisfied</th>
<th>Neither</th>
<th>Moderately Dissatisfied</th>
<th>Very Dissatisfied</th>
</tr>
</thead>
</table>
5. Medical Outcomes Study SF-12V2
SF-12v2™ Health Survey

This survey asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities.

Answer every question by selecting the answer as indicated. If you are unsure about how to answer a question, please give the best answer you can.

1. In general, would you say your health is:
   - Excellent
   - Very good
   - Good
   - Fair
   - Poor

2. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?
   - Yes, limited a lot
   - Yes, limited a little
   - No, not limited at all

   a. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf
   b. Climbing several flights of stairs

3. During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of your physical health?
   - All of the time
   - Most of the time
   - Some of the time
   - A little of the time
   - None of the time

   a. Accomplished less than you would like
   b. Were limited in the kind of work or other activities

4. During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?
   - All of the time
   - Most of the time
   - Some of the time
   - A little of the time
   - None of the time

   a. Accomplished less than you would like
   b. Did work or activities less carefully than usual
6. Spielberger Trait Anxiety Scale
Self-Evaluation TRAIT Questionnaire
Developed by Charles D. Spielberger
STAI Form Y-2

Directions: A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number 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.

1 = NOT AT ALL  2 = SOMewhat  3 = MODERATELY SO  4 = VERY MUCH SO

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
7. Five Facet Mindfulness Questionnaire
Five Facet Mindfulness Questionnaire

Description:

This instrument is based on a factor analytic study of five independently developed mindfulness questionnaires. The analysis yielded five factors that appear to represent elements of mindfulness as it is currently conceptualized. The five facets are observing, describing, acting with awareness, non-judging of inner experience, and non-reactivity to inner experience. More information is available in:

Please rate each of the following statements using the scale provided. Write the number in the blank that best describes your own opinion of what is generally true for you.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>never or very rarely true</td>
<td>rarely true</td>
<td>sometimes true</td>
<td>often true</td>
<td>very often or always true</td>
</tr>
</tbody>
</table>

1. When I’m walking, I deliberately notice the sensations of my body moving.
2. I’m good at finding words to describe my feelings.
3. I criticize myself for having irrational or inappropriate emotions.
4. I perceive my feelings and emotions without having to react to them.
5. When I do things, my mind wanders off and I’m easily distracted.
6. When I take a shower or bath, I stay alert to the sensations of water on my body.
7. I can easily put my beliefs, opinions, and expectations into words.
8. I don’t pay attention to what I’m doing because I’m daydreaming, worrying, or otherwise distracted.
9. I watch my feelings without getting lost in them.
10. I tell myself I shouldn’t be feeling the way I’m feeling.
11. I notice how foods and drinks affect my thoughts, bodily sensations, and emotions.
12. It’s hard for me to find the words to describe what I’m thinking.
13. I am easily distracted.
14. I believe some of my thoughts are abnormal or bad and I shouldn’t think that way.
15. I pay attention to sensations, such as the wind in my hair or sun on my face.
16. I have trouble thinking of the right words to express how I feel about things.
17. I make judgments about whether my thoughts are good or bad.
18. I find it difficult to stay focused on what’s happening in the present.
19. When I have distressing thoughts or images, I “step back” and am aware of the thought or image without getting taken over by it.
20. I pay attention to sounds, such as clocks ticking, birds chirping, or cars passing.
21. In difficult situations, I can pause without immediately reacting.
22. When I have a sensation in my body, it’s difficult for me to describe it because I can’t find the right words.
23. It seems I am “running on automatic” without much awareness of what I’m doing.
24. When I have distressing thoughts or images, I feel calm soon after.
25. I tell myself that I shouldn’t be thinking the way I’m thinking.
26. I notice the smells and aromas of things.
27. Even when I’m feeling terribly upset, I can find a way to put it into words.
28. I rush through activities without being really attentive to them.
29. When I have distressing thoughts or images I am able just to notice them without reacting.
30. I think some of my emotions are bad or inappropriate and I shouldn’t feel them.
31. I notice visual elements in art or nature, such as colors, shapes, textures, or patterns of light and shadow.
32. My natural tendency is to put my experiences into words.
33. When I have distressing thoughts or images, I just notice them and let them go.
34. I do jobs or tasks automatically without being aware of what I’m doing.
35. When I have distressing thoughts or images, I judge myself as good or bad, depending what the thought/image is about.
36. I pay attention to how my emotions affect my thoughts and behavior.
37. I can usually describe how I feel at the moment in considerable detail.
38. I find myself doing things without paying attention.
39. I disapprove of myself when I have irrational ideas.
Appendix N. Debriefing Statement
Post-Study Debriefing Statement

Experimenter: Upon completion of the laboratory test session, read the following statement to the participant.

The purpose of the study you just completed was not fully divulged to you prior to your participation because your responses to the “speech and math” task could have been affected. In particular, we were interested in knowing how your body responds to different challenges; such as giving a speech in front of a video camera that is being taped for later evaluation. During this study, we were looking at changes in your blood pressure, heart rate, immune system, and hormone system in response to the “speech and math” task. Many people find the “speech and math” task to be very challenging, and as a result we are able to measure the body’s changes in response to the task. Some people show large responses to having to give a speech, while other people do not respond very much at all. Because we were only interested in how your body responded while you were giving the speech, we did not need to videotape your speech. As a result, your speech will not be shown to a panel of psychologists, and it will not be evaluated for content at a later time.

If you feel a need to speak to a professional concerning any uncomfortable feelings from your participation in this research, you may contact the Principal Investigator, Dr. Laura Klein. Her contact information can be found on the first page of the consent form you signed for this study.

Do you have any questions about your participation today?

Thank you for your time and for volunteering for our study.
Appendix O. Blood Processing Protocol
DiaSmoke Study
Blood Sample Processing

**Introduction:** Participants will have their blood draw 2 times during the lab test session visit: 1) end of baseline period; 2) end of rest period.

**Note:** You are required to wear closed toed shoes, long pants, gloves, and a lab coat when handling biological samples.

**A. Pre-GCRC preparation**

1. Label cryovials prior to the session with a BLACK SHARPIE
2. Cryovials will be used to store serum
3. Each subject will have 12 cryovials (6 per blood draw x 2 blood draws).
4. Serum from each blood draw will be aliquoted into 6 cryovials
5. After aliquoting, cryovials will be stored in cryovial boxes by biomarker type (e.g, cortisol, CBG, CRP, Extra 1, Extra 2, Extra 3, Extra 4, Extra 5, Extra 6).
6. Cryovial box labeling information
   - Each biomarker (i.e., aliquot) will have a separate box.

```
Klein DiaSmoke Study  | Serum Cortisol
ID: 101 – 1xx        | Box #1
Thaw 1
Thaw 2
Thaw 3
```

7. Cryovial labeling example for participant ID 101:

<table>
<thead>
<tr>
<th>Cortisol Example:</th>
<th>DS-101</th>
</tr>
</thead>
<tbody>
<tr>
<td>CORT1</td>
<td>Study  = DS</td>
</tr>
<tr>
<td></td>
<td>Participant ID # = 101</td>
</tr>
<tr>
<td></td>
<td>Biomarker = CORT</td>
</tr>
<tr>
<td></td>
<td>Draw 1 = 1</td>
</tr>
<tr>
<td>DS-101 CORT2</td>
<td>Study  = DS</td>
</tr>
<tr>
<td></td>
<td>Participant ID # = 101</td>
</tr>
<tr>
<td></td>
<td>Biomarker = CORT</td>
</tr>
<tr>
<td></td>
<td>Draw 2 = 2</td>
</tr>
</tbody>
</table>
CBG Example:

\[
\begin{align*}
\text{DS-101} & \quad \text{CBG1} \rightarrow \quad \text{Study} = \text{DS} \\
& \quad \text{Participant ID #} = 101 \\
& \quad \text{Biomarker} = \text{CBG} \\
& \quad \text{Draw 1} = 1 \\
\text{DS-101} & \quad \text{CBG2} \rightarrow \quad \text{Study} = \text{DS} \\
& \quad \text{Participant ID #} = 101 \\
& \quad \text{Biomarker} = \text{CBG} \\
& \quad \text{Draw 2} = 2 \\
\end{align*}
\]

8. Kim will write the study visit date on each cryovial

B. Red top tube equipment needed:
1. Large grey centrifuge located in GCRC sample processing room
2. Gloves
3. Cryovial box labeled with study name, PI name, date, biomarker
4. Cryovials labeled with test session ID number
5. Blood processing sheet (in sample processing binder)
6. 200 ul pipetter
7. Pipette tips
8. Cryovial rack
9. Test tube rack
10. Stop watch/clock
11. Weigh boat
12. Transfer pipette

C. Initial processing

**NOTE:** Make sure you carefully handle tubes to prevent cell lysis.
1. Put on gloves
2. Get red top tube, green top heparin tube, and time of blood draw from the nurse
3. Ask nurse if green top tube was gently inverted 5 times. If it was not, then do so now.
4. Place red top tube and green top tubes in test tube rack.
5. Record time of blood draw (from experimenter or nurse) on the blood processing log
6. Get a 10 ml red top tube and fill with water. This will be used as the balance tube. The volume of water in the balance tube should match the amount of blood in the red top tube. There are extra balance tubes in the racks next to the large gray centrifuge.
7. The red top tube needs to sit for 30 minutes before centrifuging. The green top tube can sit for up to 4 hours before testing blood glucose.

8. Set 30 minute timer and start timer.

D. Green Top Tube – Do while red top tube is sitting.
   - The whole blood in the green top tube will be used to measure blood glucose levels using a glucose meter

1. Get supplies
   - TRUEresult glucose meter – located in soft black case in blood supply Tupperware.
   - TRUEresult test strips – located in glucose meter case
   - Weigh boat
   - Gloves
   - Transfer pipettes
   - green top heparin tube with blood

2. Put on gloves
3. Check dates on test strip vial. Do not use test strips if 4 months after opening vial (see date on top of vial) or if strips are expired (see EXP label on vial)
4. Using a transfer pipette, pipette blood from the green top heparin tube and pipette one drop onto the weigh boat. Place transfer pipette in sharps container. Place the green top tube back in the test tube rack. DO NOT DISCARD GREEN TOP TUBE UNTIL A RESULT IS DISPLAYED ON METER!
5. Remove 1 test strip from the vial and close vial immediately. Use strips quickly after removal from vial
6. With the meter off, insert test strip contact end (blocks facing ups) into test port. Meter will turn on automatically

7. With test strip still in meter, touch edge of sample tip to blood drop and allow blood to be drawn into strip. Remove test strip sample tip from the sample
drop immediately after the meter deeps and dashes appear across the meter display.

![Caution!](image)

8. After the test is finished, result is displayed and the test release button flashes. Record result in sample processing log
9. Discard the test strip. Hold meter with strip pointing down over the biohazard container. Press the strip release button (large button in center of the meter above where you insert the test strip). Meter will automatically turn off.

E. Red top tube processing

**NOTE:** Make sure you carefully handle tube to prevent cell lysis.

**NOTE:** Make sure you check the centrifuge settings – 15 minutes and 3.0 RPM. Other studies may change the settings so it is important to check prior to starting the centrifuge.

1. After 30 minutes, red top tube is ready to be centrifuged.
2. Place the red top tube with blood and matching balance tube with water in the large gray centrifuge (located on counter without the sink). Tubes should be placed in buckets directly across from one another (i.e., the exact same location in both buckets).
3. Close lid to the centrifuge and centrifuge blood for 15 minutes at @ 3000 rpm (3.0 on centrifuge).
4. Set timer for 15 minutes and start timer
5. While blood is being centrifuged, set up the 9 cryovials for that blood draw in the cryovial rack
6. Once centrifuge stops:
   - Remove tubes (red top tube and balance tube) and place red top tube in test tube rack.
   - Do not jiggle or drop the freshly centrifuged red top tube (if you do, it will have to be re-centrifuged)
   - If you notice red mixed in with the serum, you should re-centrifuge the tubes for 15 minutes. Make sure to note this on the log sheet.
7. Carefully pipette the appropriate amount of serum into each cryovial (e.g., 100 uL in cortisol cryovial). Make sure you ONLY pipette the serum. The serum will be the clearer, pinkish liquid on top (the red blood cells are sitting at the bottom of the tube).

Order of Pipetting

NOTE: You will use different pippeters depending on the volume being pipetted

NOTE: You may need to adjust the pippeter to match the volume need for each cryovial

NOTE: Record actual serum volumes pipetted on the sample processing sheet in the log book

A. Yellow top 200 uL pipetter for cortisol and CRP
   - Cortisol = 100 uL
   - CRP = 100 uL

B. Blue top 1000 uL pipette for CBG
   - CBG = 500 uL

C. Transfer pipette for 6 extra cryovials
   - Pipette no more than 1.8 ml (1800 uL) into the 6 extra cryovials (look at mark on cryovial). Do not overfill the cryovials!
   - Extra #1
   - Extra #3
   - Extra #4
   - Extra #5
   - Extra #6

9. Recap red top tube and discard tube and pipette tip into sharps container
10. Recap cryovials with serum with the screw tops
11. Record volumes in study sample log book
12. Place unused cryovials in the plastic bag with unused cryovials from other participants
13. Bring cryovials to first floor and place in the top shelf of the freezer with Dr. Penny Kris-Etherton’s name on it. Place in appropriate cryovial sample boxes (See cryovial layout sheet in sample processing binder for an example of how to place cryovials in box).
Appendix P. Biomarker Assay Information

1. Salivary Free Cortisol
2. Salivary DHEA-S
3. Salivary Alpha-Amylase
4. Serum C-Reactive Protein
5. Quest Diagnostics Procedures
1. Salivary Free Cortisol
High Sensitivity

SALIVARY CORTISOL

ENZYME IMMUNOASSAY KIT

For Research Use Only

Item No. 1-3002, (Simple) 96-Well Kit:
1-3102-5, (5-Pack) 480 Wells

Rev. March 2011

Must use IFU that is shipped with product.
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HS SALIVARY CORTISOL EIA KIT

Intended Use
The Salimaster™ cortisol kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary cortisol. It is intended only for research use in humans and non-human animals.

Please read the complete kit insert before performing this assay. Failure to follow instructions and recommendations for sample collection and sample handling may result in false values.

For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

Introduction
Cortisol (hydrocortisone, Compound P) is the major glucocorticoid produced in the adrenal cortex. It is secreted in response to stress, with levels peaking in the early morning and dropping to lowest values at night. Levels are independently regulated by circadian rhythms and are affected by additional stressors, such as exercise or physical activity.

In blood, cortisol is bound to a protein, cortisol-binding globulin (CBG). The vast majority of cortisol is bound to CBG in saliva samples. The relatively small unbound cortisol has a half-life of approximately 10 minutes. Measuring cortisol levels in saliva has been proposed as a non-invasive and convenient method to assess stress levels.

Stress consistently report high concentrations in saliva and salivary cortisol, indicating that salivary cortisol levels reliably estimate serum cortisol levels.

Test Principle
A microtitre plate is coated with monoclonal antibodies to cortisol. Cortisol in standards and samples compete with cortisol, linked to horseradish peroxidase for the antibody-binding sites. After incubation, unbound components are washed away. Bound cortisol peroxidase is measured by the reaction of the peroxidase enzyme on the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. The concentration of cortisol is inversely proportional to the amount of cortisol present. (13)

pH Indicator
A pH indicator in the assay diluent alerts the user to sample with high or low pH values. Acidic samples will turn the diluent yellow-green. Alkaline samples will turn the diluent purple. Deep yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Corrected values from samples with a pH > 7.5 or < 9.0 may be artificially inflated or lowered. (14)

Storage
All components of the kit are stable at 2-8°C until the kit's expiration date.

Safety Precautions:
• Liquid solute solutions are 3 M, which is a hazardous acid. This solution is corrosive. Use with care.
• See “Material Safety Information” at the end of the procedure.

Materials Needed But Not Supplied
• Precision pipettes to deliver 15 and 25 µL
• Precision micropipettes to deliver 50 µL and 200 µL
• Vortex
• Plate rotator with 0.08-0.17 inch orbit (if unavailable, top plate to mix)
• Plate washer with a 450 mm filter
• Log-linear graph paper or computer software for data reduction
• Deionized water
• Reagent reservoirs
• One disposable tube capable of holding 34 mL
• Pipette tips
• Sterile pipettes to deliver up to 34 mL
Materials Supplied with Single Kit

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity/Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microtitre Plate</td>
<td>1-96-well</td>
</tr>
<tr>
<td>Cardiol Standard</td>
<td>5 vials/200 mL each</td>
</tr>
<tr>
<td>Cardiol Controls</td>
<td>2 vials/500 mL each</td>
</tr>
<tr>
<td>Wash Buffer Concentrate</td>
<td>1 bottle/100 mL</td>
</tr>
<tr>
<td>Assay Diluent</td>
<td>1 bottle/60 mL</td>
</tr>
<tr>
<td>Cardiol Enzyme Conjugate Concentrate</td>
<td>1 vial/5 mL</td>
</tr>
<tr>
<td>TMB Substrate Solution Non-water ready-to-use</td>
<td>1 bottle/25 mL</td>
</tr>
<tr>
<td>5 M Step Solution</td>
<td>1 bottle/12.5 mL</td>
</tr>
<tr>
<td>Non-Specific Binding (NSB) Wells</td>
<td>1 strip</td>
</tr>
</tbody>
</table>

Must use IFU that is shipped with product.

Specimen Collection

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Severe hemorrhage normally present in dietary products can react with anti-cardiac antibodies and cause false results. Antibody and high-fat foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, sample must be thoroughly washed before sample is collected.

Diners may collect whole saliva by tilting the head forward, allowing the saliva to pool on the floor of the mouth, then pouring the saliva through a short straw into a polypropylene vial. Samples from adults and from children aged 6 and above may also be collected using the Salimetrics Oral Swab (SOS), Item No. 5001. Samples from children under the age of 6 may be collected with the Salimetrics Children’s Swab (CCS), Item No. 5001.06. The Salimetrics Infant’s Swab (IS), Item No. 5001.08, is available for use with children under the age of 6 months.

Samples visibly contaminated with blood should be re-collected. We recommend that samples be screened for possible blood contamination (15.4) using a suitable screening test such as the Salimetrics Blood Contamination ELA Kit (Item No. 1-1102-3-1102-5). Do not use dietricts, which result in false positive values due to salivary enzymes.

It is important to record the time and date of specimen collection when samples are obtained due to the normal variation in control levels.

Sample Handling and Preparation

After collection, it is important to keep samples cold in order to avoid bacterial growth in the specimen. Refract the samples within 30 minutes, and record them before -20°C within 8 hours after collection. (Samples may be stored at -20°C or lower for long-term storage.)

Do not add sodium azide to saliva samples as a preservative, as it may cause interference in the assay.

Focusing saliva samples will precipitate the nucleus. On day of assay, test complete, verify, and control at 1,500 x g (3,000 rpm) for 15 minutes. Samples Must use IFU that is shipped with product.

Reagent Preparation

- Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is necessary for the 24 mL of shov декабря diluted in Step 5 (conjugate dilution) to come to room temperature.
- Bring microtitre plates to room temperature before use. It is important to keep the zip-lock pouch with the plates closed until removed to room temperature as humidity may have an effect on the coated wells.
- Prepare IX wash buffer by diluting wash buffer concentrate 10-fold with room temperature deionized water (500 mL of IX wash buffer to 900 mL of deionized H2O). Dilute only enough for current day’s use, and discard any leftover reagents. (If precipitates have formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)

Procedure

Step 1: Determine your plate layout. Here is a suggested layout:

<table>
<thead>
<tr>
<th>Plate</th>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
<th>Column 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.00 mL</td>
<td>0.05 mL</td>
<td>0.05 mL</td>
<td>0.05 mL</td>
</tr>
<tr>
<td>B</td>
<td>0.05 mL</td>
<td>0.00 mL</td>
<td>0.05 mL</td>
<td>0.05 mL</td>
</tr>
<tr>
<td>C</td>
<td>0.33 mL</td>
<td>0.33 mL</td>
<td>0.33 mL</td>
<td>0.33 mL</td>
</tr>
<tr>
<td>D</td>
<td>0.11 mL</td>
<td>0.11 mL</td>
<td>0.11 mL</td>
<td>0.11 mL</td>
</tr>
<tr>
<td>E</td>
<td>0.03 mL</td>
<td>0.03 mL</td>
<td>0.03 mL</td>
<td>0.03 mL</td>
</tr>
<tr>
<td>F</td>
<td>0.01 mL</td>
<td>0.01 mL</td>
<td>0.01 mL</td>
<td>0.01 mL</td>
</tr>
<tr>
<td>G</td>
<td>Zero</td>
<td>Zero</td>
<td>Zero</td>
<td>Zero</td>
</tr>
<tr>
<td>H</td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
</tr>
</tbody>
</table>

Must use IFU that is shipped with product.
Step 2: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. If you choose to place non-specific binding wells in 3-4, 2, remove steps 1 and 2 from the strip holder and keep off the bottom wells. Place the strips back into the strip holder leaving H-1, 2 blank. Break off NSB wells from the strip of NSB included in the foil pouch. Place in H-1. 2. Alternatively, NSB may be placed wherever you choose on the plate. Repeat the step to fill the appropriate wells and discard. Store at 4°C.

Caution: Extra NSB wells should not be used for determination of standards, controls, or unknowns.

1. Do not insert wells from one plate into a different plate.

Step 3: Pipette 10 ml of assay diluent into a disposable tube. Set aside for Step 5.

Step 4:
- Pipette 25 ml of standards, controls, and unknowns into appropriate wells. Standards, controls, and unknowns should be assessed in duplicate.
- Pipette 25 ml of assay diluent into 2 wells to serve as the zero well.
- Pipette 25 ml of assay diluent into each NSB well.
- Pipette 25 ml of assay diluent into each NSB well.

Step 5: Make a 1:1600 dilution of the conjugate by adding 15 ml of the conjugate to the 1 ml of assay diluent prepared in Step 3. (Scale down proportionally if not using the entire plate.) Immediately mix the diluted conjugate solution and pipette 200 ml into each well using a multichannel pipette. Make note of any wells with dark yellow or purple pH indicator changes (see description of pH indicator, p. 5).

Step 6: Mix plate on rotator for 5 minutes at 500 rpm (or wrap to mix) and incubate at room temperature for an additional 5 minutes.

Step 7: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently swirling wash buffer into each well with a squat bottle, or by pipetting 300 ml of wash buffer into each well, and then discarding the liquid by inverting the plate over a sink. After each wash, the plate should be thoroughly blotted on paper towels before being turned upright. Using a plate washer following is still recommended after the last wash, just before the addition of the TMB.

Step 8: Add 500 µl of TMB solution to each well with a multichannel pipette.

Step 9: Mix on a plate mixer for 5 minutes at 500 rpm (or wrap to mix) and incubate the plate in the dark at room temperature for an additional 25 minutes.

Step 10: Add 50 µl of stop solution with a multichannel pipette.

Step 11:
- Mix on a plate mixer for 3 minutes at 500 rpm (or wrap to mix).
- Wipe off bottom of plate with a water-moistened lint-free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding stop solution. (Correction at 490 to 650 nm micro absorbance.)

Quality Control

The Salmonella’s high and low colony control cultures should be run with each assay. The control range established at Salmonella are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in technique and instrumentation.

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the average OD of the zero, standards, controls, and unknowns.
3. Calculate the percent bound (B/B₀) for each standard, control, and unknown by dividing the average OD (B) by the average OD for the zero (B₀).
4. Determine the concentrations of the controls and unknowns by interpolation using the standard curve. We recommend using a log-logistic or log-normal curve.
5. If a dilution of the sample is used, multiply the results by the dilution factor. Samples with absorbances greater than 0.5 (OD of 0.77 and 0.73 should be diluted with assay diluent and retest for accuracy.

When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and each set of strips.

Typical Results

The following chart and graph are for illustration only and should not be used to calculate results from any assay.

<table>
<thead>
<tr>
<th>Well</th>
<th>Sample</th>
<th>Average OD</th>
<th>B</th>
<th>B/B₀</th>
<th>Control (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁, A₂</td>
<td>S₁</td>
<td>0.054</td>
<td>0.071</td>
<td>0.048</td>
<td>3.000</td>
</tr>
<tr>
<td>B₁, B₂</td>
<td>S₂</td>
<td>0.236</td>
<td>0.213</td>
<td>0.145</td>
<td>1.000</td>
</tr>
<tr>
<td>C₁, C₂</td>
<td>S₃</td>
<td>0.524</td>
<td>0.501</td>
<td>0.340</td>
<td>0.233</td>
</tr>
<tr>
<td>D₁, D₂</td>
<td>S₄</td>
<td>0.897</td>
<td>0.874</td>
<td>0.593</td>
<td>0.111</td>
</tr>
<tr>
<td>E₁, E₂</td>
<td>S₅</td>
<td>1.191</td>
<td>1.196</td>
<td>0.812</td>
<td>0.257</td>
</tr>
<tr>
<td>F₁, F₂</td>
<td>S₆</td>
<td>1.279</td>
<td>1.356</td>
<td>0.921</td>
<td>0.012</td>
</tr>
<tr>
<td>G₁, G₂</td>
<td>Bo</td>
<td>1.495</td>
<td>1.475</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>H₁, H₂</td>
<td>NSB</td>
<td>0.023</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Must use IFU that is shipped with product.
**Hazardous Ingredients**

Liquid step solution is caustic; use with care. We recommend the procedures listed below for all kit reagents. Specific kit component MSDS sheets are available from Salimetrics upon request.

**Handling**

Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard absorbent materials while wearing protective clothing. Follow local regulations for disposal.

**Emergency Exposure Measures**

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing, give oxygen and call a physician.

---

**H5 Salivary Cortisol EIA Kit Performance Characteristics**

**Correlation with Serum**

The correlation between serum and saliva cortisol was determined by assaying 49 matched samples using the Diagnostic Systems Laboratories serum Cortisol EIA and the Salimetrics H5 Salivary Cortisol EIA.

The correlation between saliva and serum was highly significant, r (47) = 0.91, p < 0.0001.

**Sensitivity**

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of replicates at the 0 pug/dL level. The minimal concentration of cortisol that can be distinguished from 0 is < 0.003 pug/dL.

**Linearity of Dilutions**

Two saliva samples were diluted with assay diluent and assayed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution Factor</th>
<th>Expected (pg/dL)</th>
<th>Observed (pg/dL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>2.175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>1.081</td>
<td>1.067</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>0.544</td>
<td>0.502</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8</td>
<td>0.772</td>
<td>0.733</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.16</td>
<td>0.116</td>
<td>0.109</td>
<td></td>
<td>90.1</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.508</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>0.754</td>
<td>0.747</td>
<td></td>
<td>97.7</td>
</tr>
<tr>
<td>1.4</td>
<td>0.327</td>
<td>0.318</td>
<td></td>
<td>92.5</td>
</tr>
<tr>
<td>1.8</td>
<td>0.064</td>
<td>0.038</td>
<td></td>
<td>60.6</td>
</tr>
<tr>
<td>1.16</td>
<td>0.032</td>
<td>0.031</td>
<td></td>
<td>98.9</td>
</tr>
</tbody>
</table>

*The above information is believed to be accurate but is not all-inclusive. This information should be used only as a guide. Salimetrics will not be liable for accidents or damage resulting from misuse of product.*

Must use IFU that is shipped with product.
Recovery

Six saliva samples containing different levels of endogenous cortisol were spiked with known quantities of cortisol and assayed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous (ng/ml)</th>
<th>Added (ng/ml)</th>
<th>Expected (ng/ml)</th>
<th>Observed (ng/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.088</td>
<td>2.069</td>
<td>2.958</td>
<td>2.176</td>
<td>104.3</td>
</tr>
<tr>
<td>2</td>
<td>0.077</td>
<td>0.290</td>
<td>0.377</td>
<td>0.280</td>
<td>100.8</td>
</tr>
<tr>
<td>3</td>
<td>0.062</td>
<td>0.011</td>
<td>0.073</td>
<td>0.071</td>
<td>92.3</td>
</tr>
<tr>
<td>4</td>
<td>0.066</td>
<td>2.560</td>
<td>2.666</td>
<td>2.722</td>
<td>106.1</td>
</tr>
<tr>
<td>5</td>
<td>0.010</td>
<td>0.300</td>
<td>0.310</td>
<td>0.038</td>
<td>95.6</td>
</tr>
<tr>
<td>6</td>
<td>0.086</td>
<td>0.011</td>
<td>0.097</td>
<td>0.094</td>
<td>96.0</td>
</tr>
</tbody>
</table>

Precision

1. The intra-assay precision was determined from the mean of 14 (low) and 18 (high) replicates each.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Mean (ng/ml)</th>
<th>Standard Deviation (ng/ml)</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>14</td>
<td>1.009</td>
<td>0.022</td>
<td>2.25</td>
</tr>
<tr>
<td>Level 2</td>
<td>14</td>
<td>0.067</td>
<td>0.064</td>
<td>3.65</td>
</tr>
</tbody>
</table>

2. The inter-assay precision was determined from the mean of average duplication for 12 separate runs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Mean (ng/ml)</th>
<th>Standard Deviation (ng/ml)</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>12</td>
<td>1.028</td>
<td>0.038</td>
<td>3.75</td>
</tr>
<tr>
<td>Level 2</td>
<td>12</td>
<td>0.101</td>
<td>0.036</td>
<td>3.41</td>
</tr>
</tbody>
</table>

Specificity of Antiserum

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spiked Concentration (μg/ml)</th>
<th>% Crossover-reactivity in H5 Salivary Cortical FAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisolone</td>
<td>100</td>
<td>0.585</td>
</tr>
<tr>
<td>Prednisone</td>
<td>1000</td>
<td>ND</td>
</tr>
<tr>
<td>Cortisol</td>
<td>1000</td>
<td>0.013</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>500</td>
<td>0.138</td>
</tr>
<tr>
<td>21-Deoxycortisol</td>
<td>1000</td>
<td>0.041</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>1000</td>
<td>ND</td>
</tr>
<tr>
<td>Desoxycorticosterone</td>
<td>1000</td>
<td>18.2</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1000</td>
<td>0.615</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>10,000</td>
<td>0.214</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1000</td>
<td>0.615</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>Estra-1,3,5(Δ3)</td>
<td>10,000</td>
<td>ND</td>
</tr>
<tr>
<td>Testosterone</td>
<td>10,000</td>
<td>0.606</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>10,000</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not detected (-<0.004)

Must use IFU that is shipped with product.
Salivary Cortisol Expected Ranges

Each laboratory should establish its own ranges of expected values. The following values have been reported for salivary cortisol.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Overall Range (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children, neonate</td>
<td>275</td>
<td>ND - 4.47</td>
</tr>
<tr>
<td>Children, age 0 months</td>
<td>185</td>
<td>ND - 7.94</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>AM Range (pmol/L)</th>
<th>PM Range (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children, ages 2-5.5</td>
<td>112</td>
<td>0.034 - 0.468</td>
<td>0.833 - 3.607</td>
</tr>
<tr>
<td>Children, ages 6-11</td>
<td>285</td>
<td>0.084 - 0.828</td>
<td>ND - 3.215</td>
</tr>
<tr>
<td>Adolescents, ages 12-18</td>
<td>498</td>
<td>0.221 - 1.873</td>
<td>ND - 5.299</td>
</tr>
<tr>
<td>Adult females, ages 21-39</td>
<td>20</td>
<td>0.112 - 1.764</td>
<td>ND - 3.998</td>
</tr>
<tr>
<td>Adult males, ages 21-39</td>
<td>51</td>
<td>0.122 - 1.551</td>
<td>ND - 4.181</td>
</tr>
<tr>
<td>Adult females, ages 30-70</td>
<td>72</td>
<td>0.112 - 1.515</td>
<td>ND - 2.755</td>
</tr>
<tr>
<td>Adult males, ages 30-70</td>
<td>72</td>
<td>0.112 - 1.515</td>
<td>ND - 3.150</td>
</tr>
<tr>
<td>All subjects</td>
<td>182</td>
<td>0.094 - 1.551</td>
<td>ND - 2.734</td>
</tr>
</tbody>
</table>

ND = Not detected

Expected ranges for neonates to 5.5 years were derived using the Isotopix Salivary Cortisol Assay kit.

Expected ranges for 6 to 18 years were derived using the Isotopix Salivary Cortisol Assay kit. Adult ranges were derived using the Colorectal Assay kit.

References


Must use IFU that is shipped with product.

Seller’s Limited Warranty

"Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be post received within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller’s satisfaction to be defective. All claims should be submitted in writing. This warranty does not cover any damages due to accident, misuse, negligence, or abnormal use. Liability in all cases will be limited to the purchased cost of the kit.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use, or operation of Seller’s product or out of the breach of any express or implied warranties."

Must use IFU that is shipped with product.
2. Salivary DHEA-S
DRG® DHEA-S CLIA (CLA-4652)

Revised 8 Dec. 2006 rm (Vers. 1.1) USA: RUO

1 INTENDED USE
For the direct quantitative determination of DHEAS in human serum by chemiluminescence immunoassay (CLIA). For in vitro use only.

2 PRINCIPLE OF THE TEST
The principle of the following chemiluminescence immunoassay (CLIA) test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, control and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the luminescence substrate solution is added. The relative luminescence units (RLUs) are measured on a microtiter plate luminometer. The RLU values are inversely proportional to the concentration of DHEAS in the sample. A set of calibrators are used to plot a standard curve from which the amount of DHEAS in patient samples and controls can be directly read.

3 CLINICAL APPLICATIONS
DHEAS is one of the most abundant circulating steroid. It is produced by the adrenal and gonads. As a result, the determination of the level of DHEAS in serum is important in the evaluation of the functional state of these glands. It is also a precursor of testosterone and estrone. Besides the adrenals, in females, the ovaries have been shown to be an important source of DHEAS.

It has been reported that there is a fluctuation day by day of DHEAS in women during the ovulatory cycle. The principal production of testosterone in females is from the conversion of other related androgens especially DHEAS. An abnormal testosterone levels in women should be accompanied by the estimation of serum DHEAS. The use of serum testosterone determination in conjunction with Elisa DHEAS can be used to determine if the source of excess androgen production is ovarian or adrenal.

4 PROCEDURAL CAUTIONS AND WARNINGS
1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials or serum pools should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. A calibrator curve must be established for every run.
7. The kit control should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the control do not reflect established ranges.

9. The luminescence substrate solutions (A and B) are sensitive to light and should be stored in the original dark bottle away from direct sunlight.

10. The assay buffer is sensitive to light and should be stored in the original dark bottle away from direct sunlight.

11. When dispensing the substrate, do not use pipettes in which these liquids will come into contact with any metal parts.

12. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.

13. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.

14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

5 LIMITATIONS

1. All the reagents within the kit are calibrated for the direct determination of DHEAS in human serum. The kit is not calibrated for the determination of DHEAS in saliva, plasma or other specimens of human or animal origin.

2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.

3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.

4. Only calibrator A may be used to dilute any high serum samples. Only the urine diluent may be used to dilute any high urine samples. The use of any other reagents may lead to false results.

5. The results obtained with this kit should never be used as the sole basis for a clinical diagnosis. For example, the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should include all aspects of a patient’s background including the frequency of exposure to animals/products if false results are suspected.

6 SAFETY CAUTIONS AND WARNINGS

Human serum that may be used in the preparation of the standards and controls has been tested and found to be non-reactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. However, no test method can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen.

7 SPECIMENT COLLECTION AND STORAGE

Approximately 0.1 ml of serum is required per duplicate determination. Collect 4-5 ml of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.
Revised 8 Dec. 2006 rm (Vers. 1.1)

8 CHEMICAL HAZARDS
Avoid direct contact with reagents. In case of contact, wash with plenty of water.

9 SPECIMEN PRETREATMENT
This assay is a direct system; no specimen pretreatment is necessary.

10 REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED
1. Precision pipettes to dispense 20, 50, 100, 150 and 300 \( \mu l \)
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microwell plate luminometer

11 REAGENTS PROVIDED AND PREPARATION

1. Rabbit Anti-DHEAS Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.
   Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resealable pouch with desiccant.
   Storage: Refrigerate at 2-8°C
   Stability: 12 months or as indicated on label.

2. DHEAS-Horse Radish Peroxidase (HRP) Conjugate Concentrate - Requires Preparation.
   Contents: DHEAS-HRP conjugate in a protein-based buffer with a non-mercury preservative.
   Volume: 300 \( \mu l \) vial
   Storage: Refrigerate at 2-8°C
   Stability: 12 months or as indicated on label.
   Preparation: Dilute 1:50 in assay buffer before use (eg. 40 \( \mu l \) of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 240 \( \mu l \) of HRP in 12 ml of assay buffer. Discard any that is left over.

3. DHEAS Calibrators - Ready To Use.
   Contents: Seven vials containing DHEAS in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of DHEAS.
   *Listed below are approximate concentrations, please refer to vial labels for exact concentrations.
DRG® DHEA-S CLIA (CLA-4652)

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<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Concentration</th>
<th>Volume/Vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator A</td>
<td>0 µg/dL</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>Calibrator B</td>
<td>0.02 µg/dL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Calibrator C</td>
<td>0.1 µg/dL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Calibrator D</td>
<td>0.5 µg/dL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Calibrator E</td>
<td>2.5 µg/dL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Calibrator F</td>
<td>10 µg/dL</td>
<td>0.5 mL</td>
</tr>
</tbody>
</table>

Storage: Refrigerate at 2-8°C
Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

4. Control - Ready To Use.
Contents: One vial containing DHEAS in a protein serum-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of DHEAS. Refer to vial label for expected value and acceptable range.
Volume: 0.5 mL/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months in unopened vial or as indicated on label. Once opened, the control should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. Wash Buffer Concentrate - Requires Preparation.
Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 mL/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 mL of the wash buffer concentrate in 450 mL of water.

6. Assay Buffer - Ready To Use.
Contents: One vial containing a protein-based buffer with a non-mercury preservative.
Volume: 15 mL/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

Contents: One bottle containing luminol enhancer.
Volume: 1 mL/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: See below.

8. Chemiluminescence Substrate Reagent B - Requires Preparation.
Contents: One vial containing peroxide solution.

DRG International, Inc. USA Fax: (908) 233 0758 e-mail: corp@drg-international.com
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Volume: 1 ml/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: See below.

9. Chemiluminescence Substrate Reagent C - Requires Preparation.
Contents: One vial containing buffer with a non-mercury preservative.
Volume: 15 ml/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: See below.

Preparation of Working Substrate Solution:
Mix 1 part of the chemiluminescence substrate reagent A with 1 part of reagent B and dilute this mixture 1:5 with reagent C. This gives the ready to use substrate solution. Prepare fresh for each use.
If the whole plate is to be used prepare working substrate solution as follows:
Combine 1 ml of reagent A with 1 ml of reagent B. To the 2 ml of this mixture add 10 ml of reagent C.
Total volume=12 ml of working substrate solution.
Stability: Working substrate solution is stable for 24 hours at room temperature.

12 ASSAY PROCEDURE
Specimen Pretreatment: None.
Important Notes
1. All reagents must reach room temperature before use.
2. Once the procedure has been started, all steps should be completed without interruption to ensure equal elapsed time for each pipetting step.
3. The washing procedure influences the precision markedly; it is essential to ensure the washing is effective and thorough.

Procedure
1. Remove the required number of microwell strips. Re-seal the bag and return any unused strips to the refrigerator.
2. Pipette 25 µl of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
3. Pipette 100 µl of the conjugate working solution into each well
   (We recommend using a multichannel pipette).
4. Incubate on a plate shaker (approximately 200 rpm) for 30 minutes at room temperature.
5. Wash the wells 5 times with 300 µl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).
6. Pipette 100 µl of chemiluminescence working substrate solution into each well
   (We recommend using a multichannel pipette).
7. Incubate for 10-15 minutes at room temperature, without shaking.
8. Measure the RLUs in each well on a microplate luminometer within 20 minutes after addition of the substrate.

13 CALCULATIONS
1. Calculate the mean RLU of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean RLUs on the Y-axis and the calibrator concentrations on the X-axis. If immunosay software is being used, a 4-parameter curve is recommended.
3. Calculate the mean RLU of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample reads more than 10 µg/ml then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

TYPICAL TABULATED DATA**

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>RLU 1 x 10^3</th>
<th>RLU 2 x 10^3</th>
<th>Mean RLU x 10^3</th>
<th>RLU/RLUMAX (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 0 µg/ml</td>
<td>2120</td>
<td>2021</td>
<td>2070</td>
<td>100</td>
</tr>
<tr>
<td>B. 0.02 µg/ml</td>
<td>1856</td>
<td>1619</td>
<td>1737</td>
<td>83.9</td>
</tr>
<tr>
<td>C. 0.1 µg/ml</td>
<td>1076</td>
<td>1075</td>
<td>1076</td>
<td>51.9</td>
</tr>
<tr>
<td>D. 0.5 µg/ml</td>
<td>446</td>
<td>407</td>
<td>426</td>
<td>20.6</td>
</tr>
<tr>
<td>E. 2.5 µg/ml</td>
<td>72</td>
<td>69</td>
<td>71</td>
<td>3.4</td>
</tr>
<tr>
<td>F. 10 µg/ml</td>
<td>8</td>
<td>4</td>
<td>6</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**- It is recommended to use the RLU/RLUMAX values for comparative purposes since luminometers vary considerably between manufacturers. Results from different luminometers will show quite different RLU values, however, the RLU/RLUMAX values remain consistent.
TYPICAL CALIBRATOR CURVE
Sample curve only. Do not use to calculate results.

14 PERFORMANCE CHARACTERISTICS

14.1 SENSITIVITY
The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean RLU of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the DHEAS CLIA kit is 0.02 μg/ml.

14.2 SPECIFICITY (CROSS REACTIVITY)
The following compounds were tested for cross-reactivity with the Direct DHEAS CLIA kit with DHEAS cross-reacting at 100%.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>%Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEAS</td>
<td>100</td>
</tr>
<tr>
<td>Andosterone</td>
<td>16.0</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.7</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.9</td>
</tr>
<tr>
<td>DeoxyDHEAS</td>
<td>5.6</td>
</tr>
<tr>
<td>Prednisone</td>
<td>5.6</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1.6</td>
</tr>
</tbody>
</table>

No cross reaction was detected with Cortisol, Estradiol, Estrone, Estrone sulphate or pregnenolone.

14.3 INTRA-ASSAY PRECISION
Three samples were assayed sixteen times each on the same calibrator curve. The results (in μg/ml) are tabulated below:
DRG$^5$ DHEA-S CLIA (CLA-4652)

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<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.7</td>
<td>0.03</td>
<td>4.4</td>
</tr>
<tr>
<td>2</td>
<td>2.43</td>
<td>0.11</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>5.84</td>
<td>0.25</td>
<td>4.2</td>
</tr>
</tbody>
</table>

14.4 INTER-ASSAY PRECISION

Three samples were assayed ten times over a period of two weeks. The results (in μg/ml) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.61</td>
<td>0.045</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>2.37</td>
<td>0.16</td>
<td>6.7</td>
</tr>
<tr>
<td>3</td>
<td>6.55</td>
<td>0.33</td>
<td>5.1</td>
</tr>
</tbody>
</table>

14.5 RECOVERY

Samples were spiked by adding different DHEAS standards at 1:1 volume to two patient serum samples. The results (in μg/ml) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obs.Result</th>
<th>Exp.Result</th>
<th>Recovery%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Unspiked</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ 0.1 μg/ml standard</td>
<td>0.5</td>
<td>0.5</td>
<td>100.0</td>
</tr>
<tr>
<td>+ 0.5 μg/ml standard</td>
<td>0.8</td>
<td>0.7</td>
<td>114.2</td>
</tr>
<tr>
<td>+ 2.5 μg/ml standard</td>
<td>1.9</td>
<td>1.7</td>
<td>111.8</td>
</tr>
<tr>
<td>2 Unspiked</td>
<td>0.77</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ 2 μg/dL standard</td>
<td>0.52</td>
<td>0.44</td>
<td>117.0</td>
</tr>
<tr>
<td>+ 5 μg/dL standard</td>
<td>1.58</td>
<td>1.64</td>
<td>96.6</td>
</tr>
<tr>
<td>+ 10 μg/dL standard</td>
<td>5.44</td>
<td>5.39</td>
<td>100.9</td>
</tr>
</tbody>
</table>
14.6 LINEARITY

Three patient serum samples were diluted with calibrator A. The results (in μg/ml) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obs.Result</th>
<th>Exp.Result</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.22</td>
<td>-</td>
<td>115.3</td>
</tr>
<tr>
<td>1/2</td>
<td>1.28</td>
<td>1.11</td>
<td>111.4</td>
</tr>
<tr>
<td>1/4</td>
<td>0.62</td>
<td>0.56</td>
<td>111.7</td>
</tr>
<tr>
<td>1/8</td>
<td>0.31</td>
<td>0.28</td>
<td>111.7</td>
</tr>
<tr>
<td>2</td>
<td>2.30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1/2</td>
<td>1.16</td>
<td>1.15</td>
<td>100.9</td>
</tr>
<tr>
<td>1/4</td>
<td>0.55</td>
<td>0.58</td>
<td>95.7</td>
</tr>
<tr>
<td>1/8</td>
<td>0.29</td>
<td>0.29</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>9.44</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1/2</td>
<td>4.48</td>
<td>4.72</td>
<td>94.9</td>
</tr>
<tr>
<td>1/4</td>
<td>2.34</td>
<td>2.36</td>
<td>99.2</td>
</tr>
<tr>
<td>1/8</td>
<td>1.17</td>
<td>1.18</td>
<td>99.2</td>
</tr>
</tbody>
</table>

15 EXPECTED NORMAL VALUES, SERUM

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Range (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>0.39 – 4.65</td>
</tr>
<tr>
<td>Females</td>
<td>0.46 – 2.75</td>
</tr>
<tr>
<td>Postmenopausal Females</td>
<td>0.48 – 2.08</td>
</tr>
</tbody>
</table>

16 REFERENCES


DRG International, Inc. USA Fax: (908) 233 0758 e-mail: corp@drg-international.com
3. Salivary Alpha-Amylase
SALIVARY α-AMYLASE ASSAY KIT
Catalog No. 1-1902, (Single) 96-Well Kit, 1-1902-5, (5-Pack) 480 Wells

For Research Use Only.

Intended Use
The Salimetrics™ salivary α-amylase assay kit is specifically designed and validated for the kinetic measurement of salivary α-amylase activity. It is not intended for diagnostic use. It is intended only for research use in humans and some animals. Please read the complete kit insert before performing this assay. For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

Introduction
Technical advances that make the assessment of biomarkers in saliva possible have enabled researchers to non-invasively study biosocial processes related to stress in mammals. Much of the attention has focused on the activity of the lumbar hypothalamic-pituitary-adrenal (HPA) axis as indexed by individual differences and intra-individual change in salivary cortisol. Recent studies have indicated that the newly emerging focus of this endeavor on salivary cortisol may not enable researchers to adequately operationalize the psychobiology of the stress response. (1) Physiologists have known for decades that the stress response has at least two principal components. One involves corticotropin-releasing hormone, activation of the HPA axis, and the secretion of glucocorticoids (e.g., cortisol) into circulation. The second involves activation of the locus coeruleus/autonomic (sympathetic) nervous system and the release of catecholamines (e.g., norepinephrine) into the blood stream. (2) Theorists argue that, to advance our understanding of how biological, social, and behavioral processes interact to determine risk versus resilience, the next generation of studies will need to employ analytical models that operationalize both the behavioral and biological states of the equations using multi-method and trait measurement approaches. (3) Unfortunately, our ability to do so has been restricted because, in contrast to the highly sensitive, accurate, and valid measurement of HPA products in saliva (i.e., cortisol, dehydroepiandrosterone), the non-invasive measurement of autonomic (sympathetic) nervous system activity in saliva (i.e., catecholamines) has been problematic. (4)

In an attempt to overcome this problem, we conducted an extensive computerized literature search for potential surrogate markers of autonomic (sympathetic) nervous system activity that could be measured accurately in saliva. α-Amalase, the most abundant salivary enzyme in humans, has been identified as a biomarker that appears to fill this role. Best known for its function as a digestive enzyme that breaks down dietary starch, α-amylase has also been studied for its ability to bind to oral bacteria and to tooth enamel. It is believed to play a key role in the establishment and maintenance of the oral microbiome to form dental plaque. (4.5) Secretion of α-amylase from the salivary glands is controlled by autonomic nervous signals, and a substantial literature reveals that salivary α-amylase is a correlate of sympathetic activity under conditions of stress. Studies show that levels of salivary α-amylase increase under a variety of physically (i.e., exercise, heat and cold) and psychologically (i.e., written examinations) stressful conditions (6) in human subjects. Interestingly, studies show that cortisol levels often do not correlate with α-amylase levels (7). In contrast, salivary α-amylase is a sensor of the HPA axis and is highly correlated with NE changes as a response to stress. (8) However, more recent studies call this relationship into question. (7) The literature does show that stress-related increases in salivary α-amylase can be inhibited by the adrenergic blocker propranolol (9,10) and also that beta-adrenergic agonists are capable of stimulating α-amylase release without increasing salivary flow. (10,11) This link suggests that the same stimuli that increase autonomic (sympathetic) arousal may activate sympathetic input to the salivary glands. A recent review of salivary α-amylase concluded that, although only modest correlations have been found between salivary alpha-amylase and other sympathetic markers (NE, cardiovascular parameters), the response patterns of salivary α-amylase to both physical and psychological stressors do seem to correspond to the response patterns of the sympathetic nervous system. The salivary alpha-amylase response to stress is complex, however, and it appears to also involve the parasympathetic system to a lesser degree. (7) Another recent article has emphasized the contribution of the parasympathetic system to salivary alpha-amylase secretion, pointing out in particular that autonomic reflex activity from the oral cavity, which can increase the parasympathetic signaling to the salivary glands, may have the potential to obscure the effects of central SNS activity. (12)

Although further work is necessary to understand better the underlying physiological factors that influence salivary alpha-amylase secretion, studies have already shown that salivary α-amylase measurements may be employed as a non-invasive measure of sympathetic nervous system activation and are related to a variety of behavioral, social, health, and cognitive phenomena in human subjects. (13,24)

Test Principle
The method utilizes a chromogenic substrate, 2-chloro-p-nitrophenol linked with malononitrile. (25) The enzymatic action of α-amylase on this substrate yields 2-chloro-p-nitrophenol, which can be spectrophotometrically measured at 405 nm. The amount of amylase activity present in the sample is directly proportional to the increase in absorbance at 405 nm. For ease of use, the reaction is run in a 96-well microtiter plate with controls provided.

Precautions
1. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in false values.
2. See ‘Material Safety Data’ at the end of procedure.
3. Do not mix components from different lots of kit.
4. When using a multichannel pipette, α-amylase substrate solution should be added to duplicate wells at the same time, using the dispensing mode to avoid introducing bubbles into the wells.
5. Routine calibration of pipettes is critical for the best possible assay performance, and accurate timing is critical for correct assay results.
6. Cigarette use can be associated with lower alpha-amylase scores returned by this assay because acid substances in cigarette smoke are capable of changing the function and/or structure of the alpha-amylase enzyme.
7. Caffeine and other exogenous substances with sympathomimetic properties may be associated with higher alpha-amylase levels.
8. Time since eating, and the use of alpha-amylase inhibitors are also likely to be associated with alpha-amylase levels.
9. Controls should be assayed once on each day of testing. Volume supplied in the kit is sufficient for testing on four different days.
10. Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month.
11. Protect the α-amylase substrate reagent from exposure to direct sunlight.

Storage
All components of this kit are stable at 2 - 8°C until the kit’s expiration date.

Reagents
1. α-Amylase Substrate: 45 mL of a ready-to-use liquid preparation of 2-chloro-p-nitrophenol linked with malononitrile. Sodium azide, at 0.01%, is added as a preservative. Reagent warming trough is also provided.
2. α-Amylase Controls: One vial containing 100 µL of a high level of α-amylase activity and one vial containing 100 µL of a low level of α-amylase activity in saliva-like matrix. Controls come pre-diluted. Do not dilute.
3. α-Amylase Diluent: 30 mL of a phosphate buffered solution containing a non-mercury preservative.

Materials Supplied
1. 96-well microtiter plate
2. Reagent warming trough

Revision Date: 3-20-12

Must Use IFU that is shipped with product.
Materials Needed But Not Supplied

- Precision pipette to deliver* 8 µL
- Precision multichannel pipette to deliver* 320 µL
- Vortex
- Plate washer with a 405 µm filter
- Computer software for data reduction
- Microcentrifuge tubes for sample dilutions
- Pipette tips
- Timer
- Microtiter plate 37°C incubator rotor (Needed for heating of substrate.
We do not recommend heating the substrate in a 37°C incubator not specifically designed for microtiter plates.)

*without employing “blow-out” mechanism

Specimen Collection

Collecting whole saliva samples from adults and children may be done by using one of the Salimetrics Oral Swabs (SOS, SCS, SSIS), Item nos. 5001.02, 5001.06, 5001.08, or by unstimulated parotid saliva. Collection protocols are available on request. Do not add sodium azide to saliva samples as a preservative. Samples visibly contaminated with blood should be reprocessed.

Notes: The technique used to collect saliva (various swabs, passive drool), the collection point duration, and the oral fluid type (whole saliva vs. specific glandular saliva) all have an effect on estimates of salivary amylase activity. Recent studies have suggested that consistency in collection methods is important in order to avoid introducing unsystematic error into study data. (26, 37)

Typically, amylase concentrations in saliva from the parotid glands in the elderly are higher than those found in pooled whole saliva from the floor of the mouth. We find that saliva collected by placing a swab underneath the tongue on the floor of the mouth yields results similar to those from whole saliva collected by passive drool. We recommend that this technique be used for studies measuring amylase along with other analytes. Alternatively, if measuring a-amylase alone, the SOS may be used to collect samples of parotid saliva by placing it next to the cheek opposite the 2nd upper molar, where the duct from the parotid gland opens into the mouth. Unstimulated flow from the parotid glands is lower than from the submandibular glands in the floor of the mouth, if collecting parotid saliva, we recommend extending the collection time period in order to ensure the collection of sufficient amounts of saliva.

Although one study has reported that response patterns of sAA during the Trier Social Stress Task were consistent regardless of whether the amylase concentration (U/mL) or the amylase output (U/min) was examined, (33) there is still a concern that the effects of saliva flow rate on levels of salivary amylase may lead to problems in the interpretation of data. (34, 35). Salimetrics currently advises that researchers should note the time period needed to collect the desired amount of saliva, in order to estimate the flow rate (ml/min). For example, the usual volume of saliva exchanged in the oral cavity during speech, breathing, and swallowing is 0.2–0.4 ml/min. These data may be used for comparison in the data analysis.

If an acceptable device from the Salimetrics Oral Swab family (SOS, SCS, SSIS) is used to collect saliva for determination of sAA levels, the volume of saliva collected by the swab can be determined by weighing the device along with the storage tube and after collection. (An approximate value of 1.0 g may be assumed for the density of the saliva.) If the length of time the swab is in the mouth is also recorded, the flow rate can then be estimated. The device must be removed from the mouth before it reaches its capacity, however, after that point the estimate of flow rate will not be accurate. (33) This can be especially critical for smaller devices, such as those described in the SSIS swab, which can reach saturation fairly quickly. A preliminary study may be necessary to determine the optimum collection period and it may be difficult to find a collection period that will work for all participants.

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Avoid or high sugar foods can compromise assay performance by acting as a substrate, lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected. Record the time and date of specimen collection. After collection it is important to keep samples cold, in order to avoid bacterial growth in the specimen. Refrigerate samples within 30 minutes, and freeze at or below -20°C within 4 hours after collection. (Samples may be stored at -20°C or lower for long-term storage.)

Freezing saliva samples will precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 g (g) (3000 rpm) for 15 minutes. Avoid multiple freeze-thaw cycles. However, if samples have been frozen, centrifugation again prior to assaying. Samples should be at room temperature before adding to assay plate. Pipette 25 µL sample into appropriate wells. Particular care must be made with this reaction, leading to inaccurate results.

Procedure

Bring all reagents to room temperature. It is recommended that samples be processed one strip at a time.

Step 1: Determine your plate layout. Here is a suggested layout.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>A</td>
<td>CaI</td>
<td>5 - 7</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>B</td>
<td>CaI</td>
<td>5 - 8</td>
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<td></td>
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<td></td>
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<tr>
<td>C</td>
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<td></td>
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<td>D</td>
<td>S - 2</td>
<td>5 - 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>S - 3</td>
<td>5 - 11</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>F</td>
<td>S - 4</td>
<td>5 - 12</td>
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<td></td>
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<td></td>
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<td>S - 6</td>
<td>5 - 14</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Step 2: Keep the desired number of strips in the strip holder and return the remaining strips back to the bag.

Step 3: Set your plate reader to incubate at 37°C, and to read in center measurements kinetic mode initially at one minute. Then, again two minutes later. Change the 405 µm filter with no reference filter. For plate readers without these options, incubation can take place in a plate incubator/rotator with manual movement of the plate into and out of the plate reader for 1 minute and 3 minute readings. Kit validation was performed under these conditions.

Step 4: Saliva samples are to be diluted with the a-amylase diastase provided. Prepare a 1:10 dilution of the saliva by pipetting 10 µL of saliva into 90 µL a-amylase diastase. Mix well. Further dilute by pipetting 10 µL of the 1:10 dilution into 190 µL a-amylase diastase (1:20). Final dilution is 1:280. The remainder of the 1:10 dilution may be set aside in case a different final dilution is necessary.

Step 5: Heat the a-amylase substrate solution to 37°C in the trough provided, using a preheated microtiter plate incubator. Be sure reagent is thoroughly warmed and mixed before use. (A minimum warm-up time of 30 minutes, from room temperature, is recommended.) Note: We do not recommend heating the substrate in a 37°C incubator not specifically designed for microtiter plates.

Step 6: For accurate timing, test only one strip at a time. Add 5 µL of controls (pre-diluted) and/or diluted saliva samples to individual wells. We strongly recommend reverse pipetting to avoid introducing any bubbles into the well.

Step 7: Add 320 µL of pre-labeled (37°C) a-amylase substrate solution to each well simultaneously using a multichannel pipette. Discard pipette tips to avoid reagent contamination. Do not return any of the a-amylase substrate solution left in the tips to the bulk tray once you have dispensed it into the wells. This could contaminate the bulk tray contents and affect any subsequent testing. Any well containing bubbles at the time of reading must be repeated.

Step 8: If reading kinetically in 37°C plate reader, immediately place plate in reader and start reader. Otherwise, follow these steps:

- Start timer immediately and mix (300-600 RPM) at 37°C.
- Read OD at exactly 1 minute and return to mixing at 37°C. Save 1 minute OD readings.
- Read OD at exactly 3 minutes. Save 3 minute OD readings.

Step 9 (all methods): Subtract the one minute readings from the three minute reading and multiply by the conversion factor (see below). The conversion factor takes the 1:200 sample dilution into account for the predilutions and controls. It is convenient to set up an Excel spreadsheet to subtract the ODs and multiply by the conversion factor. Results are expressed in U/mL.

Must Use IFU that is shipped with product.
Limitations
 Samples that exceed 400 U/mL (linearity limit) should be rerun at a dilution of 1:400. Results should be multiplied by 4. Values too low to read at a 1:200 dilution can be rerun at a dilution of 1:100. Results should be divided by 2.

Calibration
 This procedure is standardized using the millimolar absorptivity of 2-chloro-p-nitrophenol under the test conditions described.

Calculations

\[ \Delta \text{Abs\/min} \times \text{TV} \times \text{DF} = \text{U/mL of } \alpha\text{-amylase activity in sample} \]

\[ \text{MMA} \times 5 \times LP \]

Where:
\( \Delta \text{Abs\/min} \) = Absorbance difference per minute
\( \text{TV} \) = Total assay volume (0.338 mL)
\( \text{DF} \) = Dilution factor
\( \text{MMA} \) = Millimolar absorptivity of 2-chloro-p-nitrophenol (12.9)
\( 5 \) = Sample volume (0.008 mL)
\( LP \) = Light path = 0.97 (specific to plate received with kit)

\[ \Delta \text{Abs} / 2 \times 0.328 \times 200 = \Delta \text{Abs} \times 328 = \text{U/mL } \alpha\text{-amylase activity} \]

12.9 x 0.008 x 0.97

Example: If change in absorbance (OD change over 2 minutes) was 0.3, then 0.3 x 129 = 95.4 U/mL

* If using a Tecan plate reader and data capture by Assaymap software, multiply by 0.0358.

NOTE: Multiply value by 0.01657 to convert to SI Units (mKatal).

Quality Control
 The Salimetrics' high and low salivary \( \alpha \)-amylase controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Example Salivary \( \alpha \)-Amylase Values

Adult range (n=75) mean = 92.4 U/mL

Absolute range = 3.3 - 425 U/mL

*To be used as a guide for research purposes only. Each laboratory should establish its own range.

\( \alpha \)-Amylase Assay Performance Characteristics

A. Recovery

Known quantities of amylase were added to five saliva samples containing different levels of endogenous amylase.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous (U/mL)</th>
<th>Added (U/mL)</th>
<th>Expected (U/mL)</th>
<th>Observed (U/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.18</td>
<td>85.10</td>
<td>107.28</td>
<td>105.62</td>
<td>99.3</td>
</tr>
<tr>
<td>2</td>
<td>135.57</td>
<td>79.69</td>
<td>215.26</td>
<td>234.43</td>
<td>111.6</td>
</tr>
<tr>
<td>3</td>
<td>101.38</td>
<td>50.61</td>
<td>152.00</td>
<td>159.57</td>
<td>98.5</td>
</tr>
<tr>
<td>4</td>
<td>22.93</td>
<td>8.72</td>
<td>31.65</td>
<td>39.98</td>
<td>127.7</td>
</tr>
<tr>
<td>5</td>
<td>47.01</td>
<td>8.14</td>
<td>55.15</td>
<td>59.44</td>
<td>87.4</td>
</tr>
</tbody>
</table>

B. Sensitivity

The lower limit of sensitivity is governed by the change in absorbance. A change in absorbance less than 0.01 will not result in a reliable value. Samples should be rerun at a higher concentration.

C. Precision

The intra-assay precision was determined from the mean of 10 replicates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (U/mL)</th>
<th>Standard Deviation (U/mL)</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>10</td>
<td>4.46</td>
<td>11.8</td>
</tr>
<tr>
<td>L</td>
<td>20</td>
<td>10.8</td>
<td>7.2</td>
</tr>
</tbody>
</table>

D. Linearity of Dilution:

Two saliva samples were diluted with \( \alpha \)-amylase deficient and assayed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution Factor</th>
<th>Expected (U/mL)</th>
<th>Observed (U/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2</td>
<td>204.8</td>
<td>193.5</td>
<td>94.5</td>
</tr>
<tr>
<td>1</td>
<td>1.4</td>
<td>102.4</td>
<td>97.6</td>
<td>95.3</td>
</tr>
<tr>
<td>1</td>
<td>1.6</td>
<td>51.12</td>
<td>49.9</td>
<td>98.3</td>
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<tr>
<td>2</td>
<td>1.8</td>
<td>125.32</td>
<td>123.13</td>
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<td>627.82</td>
<td>647.97</td>
<td>105.2</td>
</tr>
<tr>
<td>2</td>
<td>2.2</td>
<td>215.91</td>
<td>224.64</td>
<td>105.0</td>
</tr>
<tr>
<td>2</td>
<td>2.4</td>
<td>71.97</td>
<td>78.68</td>
<td>108.5</td>
</tr>
</tbody>
</table>

Material Safety Data

Hazardous Ingredients

The substrate reagent contains potassium thiocyanate. Potassium thiocyanate is harmful if swallowed, inhaled, or if skin contact results in ingestion. May produce irritant fumes if exposed to bleach.

The substrate reagent contains 0.1% sodium azide as a preservative. Do not ingest. On contact with acid, sodium azide forms toxic hydrazoic acid. Explosive metal nitrates may form in copper or lead plumbing. Disposal requires large volumes of water to prevent the build-up of azide.

We recommend the procedures listed below for all kit reagents. Specific kit component MSD sheets are available from Salimetrics upon request.

Handling

Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing, give oxygen and call a physician.

*The above information is believed to be accurate but is not all inclusive. This information should only be used as a guide. Salimetrics shall not be liable for accidents or damage resulting from contact with reagents.

References


**Seller's Limited Warranty**

“Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment. Seller shall, at its option, either repair or replace the product that is proved to Seller’s satisfaction to be defective. All claims should be submitted in written form. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use. Liability, in all cases, will be limited to the purchased cost of the kit.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller’s product or out of the breach of any express or implied warranties.”
4. Serum C-Reactive Protein
Enzyme Immunoassays for the Quantitative High Sensitive Measurement of C-Reactive Protein in Human Serum and Plasma.

1 PRINCIPLE OF THE CRP ELISA
Microtiter strips coated with anti-CRP antibody are incubated with diluted standard sera and specimen samples. During this incubation step CRP is bound specifically to the wells. After removal of the unbound serum proteins by a washing procedure, the antigen-antibody complex in each well is detected with specific peroxidase-conjugated antibodies. After removal of the unbound conjugate, the strips are incubated with a chromogen solution containing tetramethylbenzidine and hydrogen peroxide: a blue colour develops in proportion to the amount of immunocomplex bound to the wells of the strips. The enzymatic reaction is stopped by the addition of 0.5M H₂SO₄ and the absorbance values at 450 nm are determined.
A standard curve is obtained by plotting the absorbance values versus the corresponding standard values. The concentration of CRP in specimen samples is determined by interpolation from the standard curve.

2 REAGENTS
1. Coated Microtiter Strips - MTP
   12 x 8-well strips coated with monoclonal antibodies to human CRP.
2. Standard Sera - CAL N - 5 vials,
   each containing 1/10 prediluted CRP standard solutions (0.2 mL):
   N having following values:
   CAL 0: 0 µg/mL; CAL 0.4: 0.4 µg/mL; CAL 1: 1 µg/mL; CAL 5: 5 µg/mL; CAL 10: 10 µg/mL.
   Contains 0.09 % NaN₃.
   Calibrated against the NIBSC 1st International Standard, 85/506.
3. Conjugate – CONJ - 1 vial,
   containing peroxidase conjugated monoclonal anti-human CRP antibodies (12 mL).
   Contains antimicrobial agents and an inert red dye.
4. Specimen Dilution Buffer – DIL 5x - 1 vial,
   containing 40 mL dilution buffer 5x concentrated.
   Contains 0.09 % NaN₃, and antimicrobial agents and an inert green dye.
5. Washing Solution – WASH 20x - 1 vial
   containing 50 mL 20 x concentrated phosphate buffered washing solution.
6. Chromogen Solution – CHROM - 1 vial, containing 15 mL of a solution containing H₂O₂ and tetramethylbenzidin.

7. Stopping Solution – STOP - 1 vial, containing 12 mL of 0.5M H₂SO₄

3 MATERIALS REQUIRED BUT NOT SUPPLIED
1. Precision micropipettes and standard laboratory pipettes.
2. Clean standard laboratory volumetric glassware.
3. Clean glass or plastic tubes for the dilution of the samples.
4. A microtiter plate reader capable of measuring absorbencies at 450 nm

4 WARNINGS AND PRECAUTIONS FOR USERS
1. Human blood components used in the preparation of the standard sera have been tested and found to be nonreactive for hepatitis B surface antigen and HIV 1. Since no known method can ever offer complete assurance that products derived from human blood will not transmit hepatitis or other viral infections, it is recommended to handle these standard sera in the same way as potentially infectious material. Dispose specimen samples and all materials used to perform this test as if they contain infectious agents.
2. Do not mix reagents or coated microtiter strips from kits with different lot numbers.
3. Some kit components contain sodium azide as a preservative. In order to prevent the formation of potentially explosive metal azides in laboratory plumbing, flush drains thoroughly after disposal of these solutions.

5 STORAGE CONDITIONS
2 °C to 8 °C
1. Store the microtiter strips in their original package with the desiccant until all the strips have been used.
2. Never use any kit components beyond the expiration date.

6 SPECIMEN COLLECTION AND PREPARATION
Human serum and plasma may be used in this assay.
Remove serum from clot as soon as possible to avoid haemolysis. Lipemic and/or haemolysed samples can cause false results.
Transfer the serum to a clean storage tube.
Specimens may be stored at 2 °C – 8 °C for a few days, or they can be stored frozen for a longer period of time. Avoid repeated freezing and thawing.
7  ASSAY PROCEDURE

7.1  General Remarks
1. Use a separate disposable tip for each sample transfer to avoid cross-contamination.
2. All reagents must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
3. Once the assay has been started, all steps should be completed without interruption.
4. Absorbance is a function of the incubation time and temperature. Therefore the size of the assay run should be limited. It is suggested to run no more than 20 specimen samples with one set of Reference Standards in duplicate.
5. If an ELISA Washer is used, adaptation of the washing step might be necessary to obtain optimal results.

7.2  Reconstitution of the Reagents

Washing Solution:
Dilute 50 mL of concentrated Washing Solution (5) to 1000 mL with distilled water. Reconstituted solution can be stored at least 1 month, store at 2 °C – 8 °C.

At higher temperatures, the concentrated Washing solution (5) may appear cloudy, without affecting its performance. Upon dilution, the solution will be clear.

Specimen Dilution Buffer::
Dilute 40 mL of the concentrated Specimen Dilution Buffer: to 200 mL with distilled water. Reconstituted solution can be stored at least 3 months or as long as solution remains clear. Store at 2 °C – 8 °C.

7.3  Assay Procedure
1. The 10x prediluted standard sera (2) are diluted 1:100 as follows:
   Pipette 10 μL of each calibrator into separate glass or plastic dilution tubes.
   Add 990 μL of diluted Specimen Dilution Buffer and mix carefully.
2. The specimen samples are diluted 1:1000 in two consecutive steps:
   Pipette 10 μL of each specimen sample into separate glass or plastic dilution tubes and add 990 μL of diluted Specimen Dilution Buffer. Mix thoroughly.
   Add 450 μL of diluted Specimen Dilution Buffer to 50 μL of these 100x prediluted samples. Mix thoroughly.
   Warning: do not store the diluted samples for more than 8 hours.
3. Pipette 100 μL of the diluted calibrators and samples into each of a pair of adjacent wells (1).
4. Incubate the covered microtiter strips for 30 ± 2 min at room temperature.
5. Wash the microtiter strips three times with Washing Solution.
   This can either be performed with a suitable microtiter plate washer or by briskly shaking out the contents of the strips and immersing them in washing solution. During the third step, the washing solution is left in the strips for 2-3 min. Change washing solution for each cycle. Finally empty the microtiter strips and remove excess fluid by blotting the inverted strips on absorbent paper.
6. Add 100 μL of Conjugate Solution (3) and incubate the covered microtiter strips for 30 ± 2 min at room temperature.

DRG International, Inc., USA Fax: (908) 233 0758 e-mail: corp@drg-international.com
7. Repeat the washing procedure as described in step 5.
8. Add 100 μL of Chromogen (6) Solution to each well.
9. Incubate for 10 ± 2 min at room temperature. Avoid light exposure during this step.
10. Add 50 μL of Stopping Solution (7) to each well.
11. Determine the absorbance of each well at 450 nm within 30 min following the addition of acid.

8 RESULTS
The average absorbance value of each calibrator is plotted against the corresponding CRP-value and the best calibration curve (e.g., log/linear) is constructed.
Use the average absorbance of each specimen sample obtained in the hsCRP ELISA to determine the corresponding value by simple interpolation from the curve.
Depending on the experience and/or availability of computer capability, other methods of data reduction may be used.

9 TROUBLE SHOOTING
In case of high background signal, the washing was insufficient. Repeat the test with more vigorous washing (increased number of cycles, soak time).

10 REFERENCES
2. KANDA T. C-reactive protein (CRP) in the cardiovascular system, Rinsho Byori. 2001 Apr. 49(4), 395-401.
3. URSELLA, M, MAZZONE, G, PORTALE, A, TESTA, G, PIGNATARO, M, COVINO, P, FENICI, G, B.
5. RIDKER PM. High-sensitivity C-reactive protein and cardiovascular risk: rationale for screening and primary prevention, Am J Cardiol. 2003 Aug 21, 92(4B), 17K-22K.
5. Quest Diagnostics Procedures
**HbA1c**
The HbA1c determination is based on the turbidimetric inhibition immunoassay (TINIA) for hemolyzed whole blood.

Glycohemoglobin (HbA1c) in the sample reacts with anti-HbA1c antibody to form soluble antigen-antibody complexes. Since the specific HbA1c antibody site is present only once on the HbA1c molecule, complex formation does not take place. The polyhapten react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex which can be determined turbidimetrically.

**Hemoglobin**
Liberated hemoglobin in the hemolyzed sample is converted to a derivative having a characteristic absorption spectrum which is measured bichromatically during the preincubation phase (sample + R1) of the above immunological reaction. A separate Hb reagent is consequently not necessary.

The final result is expressed as percent HbA1c and is calculated from the HbA1c/Hb ratio as follows:

\[
\text{HbA1c (\%)} = (\text{HbA1c/Hb}) \times 91.5 + 2.15
\]

<table>
<thead>
<tr>
<th>Inst Type</th>
<th>Analyte</th>
<th>Reagent</th>
<th>Units</th>
<th>QC Lot</th>
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<th>Inst Name</th>
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<th>Achieved Mean</th>
<th>SD</th>
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<td>Integra</td>
<td>GLYCOHB, HBA1C</td>
<td>ROCHE</td>
<td>PERCENT</td>
<td>33871</td>
<td>Pittsburgh</td>
<td>INT_397593</td>
<td>405</td>
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<td>1.2656</td>
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<td>Integra</td>
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<td>9.7600</td>
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**Insulin**

2. **ANALYTICAL PRINCIPLE**
The Siemens Immulite 2000 Insulin assay is a chemiluminescent immunometric “sandwich” assay. The solid phase (bead) is coated with monoclonal murine anti-insulin antibody. The liquid phase consists of alkaline phosphatase conjugated to polyclonal sheep anti-insulin antibody and alkaline phosphatase conjugated to monoclonal murine anti-insulin antibody.

The sample and the reagent are incubated together with the coated bead for 60 minutes. During this time, insulin in the sample forms the antibody sandwich complex with the monoclonal murine anti-insulin antibody on the bead, enzyme conjugated polyclonal sheep anti-insulin antibody and enzyme conjugated monoclonal murine anti-insulin antibody in the reagent. Unbound patient sample and enzyme conjugate are then removed by centrifugal washes. Finally, chemiluminescent substrate is added to the reaction tube containing the bead and the signal is generated in proportion to the bound enzyme.
<table>
<thead>
<tr>
<th>Inst Type</th>
<th>Analyte</th>
<th>Reagent</th>
<th>Units</th>
<th>QC Lot</th>
<th>Lab Name</th>
<th>N</th>
<th>Achvd Mean</th>
<th>SD</th>
<th>CV</th>
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</thead>
<tbody>
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<td>Immulite</td>
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<td>DIAGNOSTIC PRODUCTS</td>
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</tbody>
</table>

**Total Cholesterol**

2. **ANALYTICAL PRINCIPLE**

Cholesterol esters in serum are hydrolyzed by cholesterol esterase (CHE). The free cholesterol produced is oxidized by cholesterol oxidase (CHO) to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide (H₂O₂), which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromophore. The red quinoneimine dye formed is measured spectrophotometrically at 540/600 nm against an increase in absorbance.

CHE: Cholesterol esters → Cholesterol + Fatty Acids
CHO: Cholesterol + O₂ → Cholest-4-en-3-one + H₂O₂
PEROXIDASE: 2 H₂O₂ + 4 aminoantipyrine + phenol → Red dye + 4 H₂O₂

14.1 **Precision**

<table>
<thead>
<tr>
<th></th>
<th>Intra-assay</th>
<th></th>
<th>Inter-assay</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean, mg/dL</td>
<td>SD</td>
<td>CV%</td>
<td>Mean, mg/dL</td>
</tr>
<tr>
<td></td>
<td>103.60</td>
<td>1.23</td>
<td>1.19</td>
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<td>3.82</td>
<td>1.02</td>
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**HDL Cholesterol**

2. **ANALYTICAL PRINCIPLE**

In the presence of magnesium ions, dextran sulfate selectively forms water soluble complexes with LDL, VLDL, and chylomicrons, which are resistant to PEG-modified enzymes. The cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approx. 40%). HDL-cholesterol esters + H₂O HDL-Cholesterol + RCOOH (Fatty acids) Esterase: The hydrolysis of cholesterol esters to produce free cholesterol and fatty acids is catalyzed by the enzyme cholesterol esterase. HDL - Cholesterol + O₂ PEG cholesterol oxidase Δ4-cholestenone + H₂O₂: In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide.
2 H₂O₂ + 4-amino-antipyrine + HSDA** + H⁺ + H₂O peroxidase → purple blue pigment + 5 H₂O

**HSDA = Sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline

In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-amino-antipyrine and HSDA to form a purple blue dye. The color intensity of this dye is directly proportional to the cholesterol concentration and is measured photometrically.

### Precision

<table>
<thead>
<tr>
<th>HDL Cholesterol: Data from Initial Validation for BPT</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-assay</strong></td>
<td><strong>Inter-assay</strong></td>
</tr>
<tr>
<td>Mean, mg/dL</td>
<td>SD</td>
</tr>
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<td>0.78</td>
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</table>

### Triglyceride

The Beckman Coulter/Olympus Triglyceride procedure is based on a series of coupled enzymatic reactions. The method is an endpoint method that is not blanked for free glycerol, i.e., it measures free glycerol as well as glycerol released from triglycerides. In most specimens free glycerol is less than 10% of the glycerol released from triglycerides. The triglycerides in the sample are hydrolyzed by a combination of microbial lipases to give glycerol and fatty acids. The glycerol is phosphorylated by adenosine triphosphate (ATP) in the presence of glycerol kinase (GK) to produce glycerol-3-phosphate. The glycerol-3-phosphate is oxidized by molecular oxygen in the presence of GPO (glycerol phosphate oxidase) to produce hydrogen peroxide (H₂O₂) and dihydroxyacetone phosphate. The formed H₂O₂ reacts with 4-aminophenazone and N,N-bis(4-sulfoethyl)-3,5-dimethylaniline, disodium salt (MADB) in the presence of peroxidase (POD) to produce a chromophore, which is read at 660/800 nm. The increase in absorbance at 660/800 nm is proportional to the triglyceride concentration (plus free glycerol) in the sample.
Triglycerides + 3H₂O $\xrightarrow{\text{Lipase}}$ Glycerol + 3 Fatty Acids

Glycerol + ATP $\xrightarrow{\text{GK, Mg}^{2+}}$ Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O₂ $\xrightarrow{\text{GPO}}$ H₂O₂ + Dihydroxyacetone phosphate

2 H₂O₂ + MADB + 4AAP $\xrightarrow{\text{Peroxidase}}$ Blue Dye + OH⁻ + H₂O

Note: Glycerol in reaction step 2 is both endogenous glycerol, plus glycerol from step 1.

### 14.1 Precision

<table>
<thead>
<tr>
<th>Triglycerides : Data from Initial Validation for BPT</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean, mg/dL</td>
<td>SD</td>
<td>CV%</td>
</tr>
<tr>
<td>------------</td>
<td>------------</td>
<td>-------------</td>
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<tr>
<td>85.74</td>
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<tr>
<td>213.54</td>
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<tr>
<td>486.65</td>
<td>5.49</td>
<td>1.13%</td>
</tr>
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</table>
Kimberly N. Walter  
Vita

Education

Ph.D.  Biobehavioral Health, 2014  
The Pennsylvania State University; University Park, Pennsylvania

M.S.  Biobehavioral Health, 2010  
The Pennsylvania State University; University Park, Pennsylvania

B.S.N.  Nursing (with distinction), 2006  
The Pennsylvania State University; University Park, Pennsylvania

B.A.  Psychology (with honors, summa cum laude), 2002  
University of Virginia; Charlottesville, Virginia

Awards

Outstanding Graduate Student Teaching Award  2012  
Department of Biobehavioral Health  
The Pennsylvania State University

Hintz Graduate Education Enhancement Fellowship  2010-2011  
Department of Biobehavioral Health  
The Pennsylvania State University

Fund for Excellence in Graduate Recruitment  2008-2009  
Department of Biobehavioral Health  
The Pennsylvania State University

Selected Publications

