

Age differences in Glucocorticoid Sensitivity of Inflammatory Cytokine Production Before and After stress

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1 Abstract

Acute stress results in local inflammation that is regulated by glucocorticoids (GCs), while chronic stress can result in systemic inflammation that is often accompanied by adverse health outcomes. Many studies have documented gender differences in the HPA axis and inflammatory stress responses contributing to different patterns of diseases susceptibilities between men and women. Women typically mount a stronger immune response to bacterial infection protecting them infectious diseases but may increase susceptibility to autoimmune disorders. Men are more susceptible to infectious diseases but are at lower risks for autoimmune disorders. Few studies have explored GC sensitivity as the primary mechanism regulating gender differences in HPA axis reactivity and inflammatory stress responses. In this study, we exposed healthy young and old male and female adults to a psychosocial stressor and studied in-vitro GC sensitivity as measured by Dexamethasone inhibition of lipopolysaccharide(LPS)-stimulated interleukin-6 (IL-6) production. We found that GC sensitivity is increased in young males compared to older males, which has been previously described. However there were no significant differences in GC sensitivities between older and younger females. Our data does not support our hypothesis that GC sensitivity is the main factor regulating the sex differences seen in neuroendocrine and inflammatory stress responses. However, the fact that GC sensitivity changes with age and cortisol in men might be a reflection of sex differences in the neuroendocrine-immune feedback loop. In women, this feedback mechanism may be less efficient contributing to a higher susceptibility to inflammatory diseases.

2 Introduction

Stress initiates an acute inflammatory response and activates the Hypothalamic-Pituitary-Adrenal (HPA) axis, which results in glucocorticoid (GC) secretion. Within the immune system, GCs can suppress cytokine production thereby terminating inflammation (Sapolsky, Romero et al. 2000). Conversely, inflammatory cytokines can stimulate the HPA axis and activate GC secretion (Mastorakos, Chrousos et al. 1993; Späth-Schwalbe, Born et al. 1994; Bethin, Vogt et al. 2000). This feedback between these two systems is important for maintenance of host homeostasis and protection against illness. Sex steroids may affect the responsiveness of this feedback mechanism by reducing or enhancing GC sensitivity, which could potentially explain why females are more susceptible to autoimmune disorders compared to males. Declining androgen and estrogen levels may contribute to increased susceptibility to chronic diseases among older men and women. The aim of this research project is to investigate age and sex differences in GC sensitivity pre and post stress. Knowing how sex steroids contribute to inter-gender and inter-individual differences in inflammatory responses is important for understanding the pathogenesis of diseases to provide better care for males and females with chronic inflammatory or infectious diseases such as atherosclerosis, HIV, AIDS, cancer, osteoporosis, dementia, cancer, and diabetes.

3 Background

A. Inflammation

Acute Inflammation

Inflammation is the body's first line of defense and a non-specific immune response against harmful stimuli such as bacterial or viral infections, damaged cells, or psychological

stress. This response occurs to isolate and prevent the spread of toxins while also initiating the healing process. Upon encountering an antigen, immune cells, such as macrophages and monocytes, release pro-inflammatory cytokines to initiate proliferation of more cytokines and recruitment of immune cells (Gomez, Boehmer et al. 2005). Some of these cytokines include interleukins (IL), interferons (IFN), and tumor necrosis factors (TNF). During stress, the sympathetic nervous system (SNS) is stimulated resulting in neurally-mediated release of adrenaline and norepinephrine into the bloodstream (Herman, Figueiredo et al. 2003). Noradrenaline binds to α and β -adrenergic receptors inducing a cellular cascade that releases transcription factor Nuclear Factor- κ B (NF- κ B) from inhibition so that it becomes active and able to translocate to the nucleus (Bierhaus, Wolf et al. 2003). At this point, NF- κ B can initiate transcription of immunologically relevant proteins such as cytokines. Cytokines are produced in a positive feedback loop, which enhances the immune response. In addition to stress, cytokines, oxidative damage, DNA damage, ultraviolet irradiation, and bacterial or viral antigens can also activate the NF- κ B pathway (Bierhaus, Wolf et al. 2003; Creely, McTernan et al. 2007; Verhelst, Carpentier et al. 2011; Hayden and Ghosh 2012).

Accumulation of these cytokines and immune cells results in inflammation, which is characterized by redness, swelling, heat, pain, and sometimes loss of function (Rhen and Cidlowski 2005). This acute localized inflammatory response ceases upon termination of the immune challenge, and thus the healing process begins mediated by anti-inflammatory cytokines such as interleukin-10 (IL-10). Glucocorticoids, a product of the HPA axis, can also help terminate the inflammatory response by inhibiting NF- κ B translocation into the nucleus (Scheinman, Cogswell et al. 1995). The HPA axis activates to prepare the body to appropriately

respond to and recover from physical, viral, or psychological threats. Acute inflammation is a healthy localized immune response following trauma or infection. If the antigen or stress persists for a prolonged period of time however, acute inflammation can become systemic (McEwen 1998).

Systemic Inflammation: High Grade & Low Grade

Systemic inflammation is a form of dysregulated inflammation and consists of high-grade and low-grade systemic inflammation. High-grade systemic inflammation can result from a whole body infection, which induces an imbalance favoring pro-inflammatory cytokine production (Elenkov, Iezzoni et al. 2005). Noninfectious agents such as burns, pancreatitis, ischemia, and hemorrhage can also be a cause (Bone, Balk et al. 1992). In response to an antigen, the body mounts an acute and localized inflammatory response, which happens in conjunction with a compensatory anti-inflammatory response. Only when the balance between these two responses is disrupted does this inflammatory response become harmful. In this case, the immune system cannot efficiently bring the body back to homeostasis leading to an increase in antigenic burden, accumulation and spread of pro-inflammatory cytokines, and prolonged systemic inflammation as opposed to acute local inflammation. As this process progresses, tissue damage occurs throughout the body eventually leading to organ dysfunction and failure (Singer, De Santis et al. 2004).

Significantly different from systemic high-grade inflammation is systemic low-grade inflammation, which develops as a consequence of aging (Howcroft, Campisi et al. 2013). The immune systems of the elderly respond to decades worth of antigenic exposure followed by

progressive activation of macrophages and inflammatory responses. Furthermore, baseline concentrations of inflammatory markers such as IL-6, IL-8 and C-reactive proteins (CRP) seem to increase with age, even among healthy older adults (Wei, Xu et al. 1992; Ferrucci, Harris et al. 1999; Harris, Ferrucci et al. 1999; Ershler and Keller 2000; Bruunsgaard and Pedersen 2003; Kiecolt-Glaser, Preacher et al. 2003). Higher baseline levels of inflammatory markers, especially IL-6, a potent pro-inflammatory cytokine, are strongly associated with an increased risk of mortality (Harris, Ferrucci et al. 1999). It is likely that repeated or prolonged episodes of systemic high grade inflammation or acute inflammation combined with a genetic predisposition may contribute to age-related diseases such as atherosclerosis, Alzheimer's, and dementia (Krabbe, Pedersen et al. 2004; Marchesi 2011). Atherosclerosis refers to the formation and built up of plaque, which is caused by an inflammatory response to lesions in the arteries by endothelial cells. In an attempt to repair the lesions, monocytes, macrophages, and cytokines accumulate in the area eventually forming plaques (Willerson and Ridker 2004). Low-grade inflammation also plays a role in the pathogenesis of insulin resistance, type I and II diabetes, obesity, cancer, and cardiovascular diseases (Black 2003; Pickup 2004; Creely, McTernan et al. 2007; Fernández-Real and Pickup 2008).

During acute inflammation, activation of the HPA axis resulting in GC production protects the body from systemic inflammatory states. However, age related insults to the pro-inflammatory or anti-inflammatory feedback, through mechanisms that are currently unclear, may contribute to systemic inflammatory states. Decreased production of sex steroids has been speculated to exert changes in endocrine and inflammatory responses and may also contribute to low grade inflammation (Blum, Müller et al. 2005; Pour, Grobbee et al. 2007). Clear patterns of

dysregulation exist between males and females thus contributing to a dimorphic pattern of disease susceptibilities.

B. Sexual Dimorphisms

Androgens in males and estrogens in females may influence the inflammatory response and outcome after acute injuries. There are also sex differences present in cytokine production with females demonstrating lower TNF and higher IL-10 in response to sepsis, while males demonstrating higher IL-6 responses (Schröder, Kahlke et al. 1998; Oberholzer, Keel et al. 2000). Another study illustrated that estradiol diminished LPS stimulated production of IL-1, IL-6, TNF, and NF- κ B binding activity (Deshpande, Khalili et al. 1997; Murphy, Guyre et al. 2010). This suggests a beneficial effect of estrogens on immune function in response to infection.

Approximately 78% of those affected by an autoimmune disease are women (Fairweather, Frisancho-Kiss et al. 2008). Animal studies consistently reveal that females have higher levels of immunoglobulins, stronger antibody responses to infection, and higher susceptibility to autoimmune diseases (Ahmed and Talal 1990; Fox 1992; Chao 1996; Rosmalen, Pigmans et al. 2001). In humans, the results are less clear; some studies reveal that estrogens suppressed immune responses rather than enhanced it (Kalland 1980; Luster, Hayes et al. 1984). While in other studies researchers found that human females typically express stronger innate, cellular, and humoral-mediated responses to infection compared to males (Chao 1996; Giron-Gonzalez, Moral et al. 2000; Marriott and Huet-Hudson 2006; Fairweather, Frisancho-Kiss et al. 2008; Klein 2012). This type of immunity can help terminate infections faster; however it may

also contribute to higher concentration of immunoglobulins and subsequently dysregulation of the inflammatory response.

Androgens may suppress expression of pro-inflammatory cytokines, humoral and cell-mediated immune responses, and enhance expression of anti-inflammatory cytokines (Fox 1992; Malkin, Pugh et al. 2004). This type of immunity can also lead to a dysregulated inflammatory response due to a shift favoring anti-inflammation. Acute inflammation is part of a healthy response to harmful stimuli; consequently, suppression of this inflammatory response can increase susceptibility to infections but protects against immunocompromised states.

Dihydrotestosterone (DHT) treatment to human endothelial cells suppressed LPS stimulated IL-6 and TNF- α expression thus reducing inflammation (Norata, Tibolla et al. 2006). Testosterone replacement therapy in type-2 diabetic men was also associated with decreases in IL-1, IL-6, and TNF- α production by antigen presenting cells (APC) (Corrales, Almeida et al. 2006). Both of these studies suggest that androgens have an immunosuppressive effect that may better protect against autoimmune disorders, but increase susceptibility to infectious diseases such as dysentery, gonorrhoea, meningitis, pneumonia, lymphoma and leukemia (Klein 2000).

Some sex differences in inflammatory responses might also be caused by X-chromosome inactivation and X-linked genes (Klein 2012). There are as many as 1,100 genes on the X chromosome that can influence immune function (Fish 2008). X-chromosome inactivation among females results in expression of X-linked gene mutations in approximately 50% of cells. Since males do not undergo X-chromosome inactivation, 100% of X linked gene mutations are expressed (Pennell, Galligan et al. 2012). This suggests that males are more susceptible to X-linked gene mutation diseases. In contrast, the additional X chromosome in females may increase

susceptibility to certain diseases such as systemic lupus erythematosus (SLE), a serious autoimmune disorder in which the body's immune system begins to attack its own healthy tissue. Normal XX females and XXY males with Klinefelter syndrome are at an increased risk for SLE compared to XY males (Scofield, Bruner et al. 2008). The extra X chromosome increased susceptibility to SLE as many candidate SLE genes have been identified on the X chromosome (Sawalha, Webb et al. 2008).

Androgens, estrogens, and X linked genes modulate several aspects of immunity and inflammation, which contribute to different diseases susceptibilities between males and females. Investigation of the causes of this dimorphic disease pattern is crucial to better prevention and treatment. Despite the wealth of data documenting the suppressive effects of androgens or potentiating effects of estrogens, there exists an equal amount of data that suggests otherwise (Kalland 1980; Luster, Hayes et al. 1984; Klein, Bird et al. 2000; Ito, Bebo et al. 2001; Straub 2007). Thus other factors such as glucocorticoids, may contribute to the sex differences seen in diseases susceptibilities.

C. HPA Axis Regulatory Effects on Inflammation

Glucocorticoids, the most common being cortisol in humans, are produced by the adrenal glands and released into the bloodstream upon activation of the HPA axis. The HPA axis is characterized by initial hypothalamic secretion of Corticotrophin Releasing Factor (CRF) to the pituitary gland, followed by secretion of adrenocorticotrophic hormones (ACTH) from the pituitary gland into the bloodstream, and subsequently GC secretion from the adrenal glands (Kirschbaum and Hellhammer 2000; Dedovic, Duchesne et al. 2009). Glucocorticoids have many

roles within the body some of which can affect metabolic, immune, and cardiovascular functions (McEwen 1998). With respect to immune function, GCs help mediate recovery by exerting an immunosuppressive effect on pro-inflammatory cytokine production to reduce inflammatory markers (Mukaida, Morita et al. 1994; Wilckens and De Rijk 1997; Sapolsky, Romero et al. 2000). Upon stress, initial activation of the SNS elicits an acute immune response preparing the body to fight off infections that may occur during the perceived threat. Activation of the HPA axis follows after to restore homeostasis and terminate inflammation.

Activation of the HPA axis can also occur from immune challenges (Buckingham, Loxley et al. 1996). Cytokines such as IL-6, IL-1, and TNF- α has been shown to stimulate the HPA axis in humans and was found to activate the HPA axis independent of CRF in mice (Mastorakos, Chrousos et al. 1993; Späth-Schwalbe, Born et al. 1994; Bethin, Vogt et al. 2000). IL-6 specifically may modulate HPA axis activation via sensitization of the adrenal cortex to ACTH (Žarković, Ignjatović et al. 2008). These studies show that while GCs play an important role regulating cytokine response after an immune challenge, cytokines can also modulate GC production via activation of the HPA axis. This may function as part of the anti-inflammatory response to terminate inflammation. This interaction between the immune and neuroendocrine system reveals an important feedback loop essential for maintaining host homeostasis.

Accumulation of cytokines results in acute inflammation. Without GCs to assist in down-regulating cytokine activity, the immune system can remain constitutively active even after termination or in absence of an immune challenge. While acute stress can enhance immune responses by acutely initiating inflammation, chronic stress may result in dysregulation of the inflammatory responses (Dhabhar 2000; Elenkov, Iezzoni et al. 2005; Robles, Glaser et al.

2005). Within a healthy individual, GCs bind to GC-receptors in the hypothalamus and pituitary gland to suppress further CRH and ACTH production thereby controlling cortisol production. This negative feedback loop within the HPA axis prevents over signaling of GCs and controls the stress response.

A study conducted by Kunz-Ebrecht et al. revealed that cortisol high responders typically exhibited lower plasma IL-6 concentration but reported experiencing more stress and impaired mental health compared to cortisol non-responders (Kunz-Ebrecht, Mohamed-Ali et al. 2003). While high cortisol sensitivity can down regulate cytokine expression, it seems to have some adverse effects on stress perception and outcomes. Accordingly, plasma IL-6 concentrations were highest for low cortisol responders. The results of this study in addition to others strongly suggests that excessive GC signaling, due to increased GC bioavailability or GC sensitivity, can suppress immune cell activities (Mukaida, Morita et al. 1994; Wilckens and De Rijk 1997; Kunz-Ebrecht, Mohamed-Ali et al. 2003). This is also seen among individuals who suffer from excessive cortisol secretion, also known as Cushing's syndrome. These individuals are immunocompromised and consequently more vulnerable to infections (Kronfol, Starkman et al. 1996; Sutton, Parks et al. 2011). In contrast, insufficient GC signaling, due to decreased GC bioavailability or GC sensitivity, generates an overly reactive immune system (Raison and Miller 2003). Within a physiologically appropriate concentration however, GCs are important for initiating and terminating an effective stress and inflammatory response. Dysregulation of the HPA axis may be associated with insufficient or excessive GC signaling resulting in an dysregulated inflammatory response and an increased risk of illness and death (McEwen 1998).

D. Glucocorticoid Sensitivity

Glucocorticoid sensitivity, the degree to which the body's cells are responsive to GCs, may be an important factor mediating the interaction between the immune and neuroendocrine systems. Chronically stressed individuals such as spousal dementia caretakers exhibit higher concentrations of IL-6 compared to non-caregivers. However, since GCs can suppress cytokine production, how can these highly stressed individuals have such high concentrations of IL-6? This phenomenon suggests that repeated chronic stress may reduce GC sensitivity such that an individual's immune system remains insensitive to its own endogenously produced GCs. Sheldon Cohen et al. proposed a model that describes this phenomenon in which chronic stress induces glucocorticoid receptor resistance (GCR) and consequently a failure to down-regulate the inflammatory response (Cohen, Janicki-Deverts et al. 2012).

The effects of GCs on inflammation can only occur if GC binds to a GC receptor, which are present on many immune cells as well as other cell types in the body. Glucocorticoid Receptor Resistance refers to a decrease in immune cell sensitivity to GCs caused by either a mutation in the GC-binding domain, GC receptor gene, or reduced receptor number (Chrousos, Vingerhoeds et al. 1982; Malchoff, Brufsky et al. 1993). This resistance leads to a failure to terminate the inflammatory response (Cole 2008; Cole, Mendoza et al. 2009; Cohen, Janicki-Deverts et al. 2012). Cohen observed that individuals who recently experienced long term stress exhibited GCR and were more susceptible to a cold compared to those who did not recently experience a major stressor (Cohen, Janicki-Deverts et al. 2012). Individuals who are GC resistant and developed a cold following rhinovirus exposure also exhibited higher LPS stimulated leukocyte production of IL-6 and TNF-a in response to dexamethasone challenge

(Cohen, Janicki-Deverts et al. 2012). This indicates that leukocytes were non-responsive to dexamethasone such that inflammation remained. These results suggest that GC sensitivity may affect immune cell sensitivity to HPA axis regulation resulting in susceptibility to illness.

E. The Impact of Gender on Glucocorticoid Sensitivity, HPA axis, and Inflammatory Response

Gender, menstrual cycle phase, and oral contraceptive (OC) use affect the HPA axis response to stress and subsequently the inflammatory response (Kirschbaum, Kudielka et al. 1999; Roca, Schmidt et al. 2003). Animals models have consistently shown that ACTH and glucocorticoids responses to stress are greater in females compared to males (Kitay 1961; Kant, Lenox et al. 1983; Lesniewska, Miskowiak et al. 1990). However, in human studies, the results are less clear cut. Kirschbaum et al. discovered that men typically show a higher ACTH response compared to women regardless of menstrual cycle phase in response to a psychosocial stress test (Trier Social Stress Test; TSST) (Kirschbaum, Kudielka et al. 1999). Additionally free cortisol responses were similar between men and women in the luteal phase, both of whom displayed higher levels compared to women in the follicular phase or using oral contraceptives (OC) (Kirschbaum, Kudielka et al. 1999). Since women in the follicular luteal phase have lower levels of estrogen compared to women in the follicular phase, this suggests that estrogen may not enhance the HPA axis response. However, other studies have documented a potentiating effect of estrogen on HPA axis reactivity (Kirschbaum, Schommer et al. 1996; Deshpande, Khalili et al. 1997; Ito, Bebo et al. 2001; Puder, Freda et al. 2001).

Thus the impact of estrogens on the HPA axis response remains inconclusive; however it is likely that the impact of sex steroids on GC sensitivity may play a bigger role in modulating stress response differences. In a study that examined GC sensitivity between males and females, Rohleder et al. found that in response to TSST, GC sensitivity increased in males one hour after stressor, but decreased in females in the luteal phase (Rohleder, Schommer et al. 2001). This GC sensitivity profile was accompanied by reduced cytokine production in males but increased in females one hour after TSST (Rohleder, Schommer et al. 2001). However, it is still unclear exactly how estrogen may affect GC sensitivity since no one has yet to examine differences in GC sensitivity between post and pre-menopausal women.

Testosterone may have an inhibitory effect on HPA axis activity via suppression of CRH-induced cortisol to TSST (Rubinow, Roca et al. 2005). Men in a testosterone replacement condition exhibited reduced cortisol response to CRH injections but increased ACTH compared to men in the gonadal suppression condition. It is possible that testosterone suppression of CRH-induced cortisol lessened the restriction normally placed on ACTH production by cortisol in the presence of high cortisol. Consequently, higher ACTH levels are observed. The negative feedback loop normally inhibiting further production of ACTH is deactivated in the presence of low cortisol concentrations.

In another study, Rohleder et al. investigated HPA axis response and GC sensitivity of elderly men compared to testosterone treated elderly men and young controls (Rohleder, Kudielka et al. 2002). They found that free cortisol responses to TSST did not differ significantly between the three groups, however GC sensitivity increased after TSST in young controls and testosterone treated elderly men. With age, testosterone levels diminish and as does GC

sensitivity, however this is partially restored by testosterone treatment in elderly men. Despite androgen's suppressive effects on the HPA axis, it appears that it also contributes to increased GC sensitivity. This suggests that young men require less cortisol to reduce inflammation, which may in fact be a mechanism to compensate for the suppressive effects of testosterone on cortisol output. This response also facilitates timely termination of cytokines thereby protecting males from systemic inflammation.

4 Summary

Sex based differences in regulation of inflammation and HPA axis activity contribute to the differences in susceptibility to infectious and autoimmune diseases between males and females (Gaillard and Spinedi 1998; Verthelyi 2001; McCormick and Mathews 2007; Pennell, Galligan et al. 2012). Men respond to infection or stress with greater GC sensitivity in conjunction with a stronger anti-inflammatory response. This type of endocrine and immune response profile may protect against autoimmunity, however predisposes males to infections. In contrast, women typically respond with an enhanced cortisol response, reduced GC sensitivity, and increased antibody production. This response protects against acute infections however under conditions of prologue stress, may predispose women to dysregulations in inflammatory responses resulting in autoimmune disorders.

5 Aims

We set out in the present study to test for differences in GC sensitivity between older and younger men and women as a possible explanation for the differences in inflammatory and neuroendocrine responses to stress. Not much research has been done concerning differences in

GC sensitivity between young and postmenopausal women especially. By examining these differences, we hope to shed light on the modulatory effects of estrogen on the inflammatory and neuroendocrine response to stress as past studies on human female participants reveal conflicting data. We had three specific aims:

1. To determine whether acute psychosocial stress can induce an endocrine and inflammatory response as indicated by past studies and how these responses may differ between age and gender.
2. To test for differences in GC sensitivity between age and gender.
3. To determine if differences in GC sensitivity could explain the differences seen in the endocrine and inflammatory response to TSST between age and gender.

6 Materials & Methods

Subjects

42 healthy men and women between the ages of 18 to 65 participated in this study. The study sample consisted of 17 men and 25 women. Within the men group, 8 were between 18 to 45 years and 9 between 55 and 65 years old. Within the women group, 8 were in their luteal phase and in the young group, while 17 were older and postmenopausal. Before entering the study, all participants underwent a thorough medical examination regarding past and current health problems. Eligibility was determined and assessed in a telephone interview. Participants were excluded if diabetic, pregnant, have allergies, autoimmune diseases, psychiatric disorders, or were currently taking corticosteroid or psychotropic medications.

Stress Induction Paradigm

Each participant was exposed to the Trier Social Stress Test (TSST) on two consecutive days. Participants reported to the lab between 13:00 and 18:30h. After catheter insertion and a 45

minute rest period, first blood and saliva was collected. Participants were informed about the TSST procedure before exposure to the TSST, which consisted of a 15 minute public speaking and mental arithmetic task in front of an a two person panel (one male and female) and camera. It has consistently induced endocrine and cardiovascular responses in 70 to 80% of all participants [56]. Blood samples were drawn and collected in EDTA tubes at six various time points: 1 minute before TSST and 1, 10, 30, 60, and 120 minutes after TSST.

Blood Collection and Measurement of Plasma IL-6 Assay

Blood was drawn 1 min before TSST, 30 and 120 minutes after TSST and collected in EDTA tubes. Blood was centrifuged immediately and plasma supernatant extracted and stored at -80°C. Plasma IL-6 concentrations were determined using a commercial ELISA kit. The limit of detection for IL-6 was 0.09 pg/ml with inter- and intra-assay CVs of 9.2% and 5.3% respectively. The specific protocol is described in greater detail in Rohleder et al. (2009) (Rohleder, Marin et al. 2009). The plasma IL-6 response was computed as delta scores (Plasma IL-6 concentration at 120 min post-TSST minus IL-6 Plasma concentration pre-TSST).

Glucocorticoid Sensitivity Assay

Blood was collected in heparinized tubes and diluted with 10:1 saline. Diluted blood (400ul) was added to 50 ul of lipopolysaccharide (LPS) and 50ul of 5 different increasing concentrations of dexamethasone (DEX) in a 24 well plate for each time point. The final concentration on the plate was 30 ng/ml LPS and 0, 10⁻¹⁰, 10⁻⁹, 10⁻⁸, 10⁻⁷ and 10⁻⁶ mol/liter DEX. Blood was incubated for 18 hours at 37°C and 5% CO₂ in a humidified atmosphere. 18

hours later, plates were centrifuged for 10 minutes at 2000 x g and 4°C and plasma supernatant extracted and stored at -80°C until assayed.

Biochemical Analysis

Free cortisol in saliva was measured using a time-resolved immunoassay with fluorometric detection as described in Dressendorfer et al. (1992) (Dressendorfer, Kirschbaum et al. 1992). LPS induced IL-6 production was measured in supernatants using a commercial ELISA kit (BD Pharmingen, San Diego, CA). 96-well plates were coated with capture antibody (monoclonal anti-TNF- α or anti-IL6) and incubated overnight. The next morning, plates were washed and blocked for one hour at room temperature with 200ul of assay buffer, which is a mixture of Fetal Bovine Serum (FBS) and 1x PBS. 100 ul of the standard or plasma sample was added to each well (diluted 1:600 for IL-6) and incubated at room temperature for 2 hours. After 2 hours, the plates are washed and 100 ul of working detector (consisting of detection antibody and streptavidin HRP enzyme at 1:250 dilution factor for IL6) was added following another 1 hour incubation at room temperature. After incubation, 100 ul of substrate solution (tetramethylbenzidine and hydrogen peroxide) was added to each well and incubated for 25 minutes at room temperature in the dark, followed by 50 ul of stop solution (H₂SO₄). The plates are read by an ELISA reader at 450 nm.

Statistical Analysis

As an index for GC sensitivity, we calculated inhibitory concentration at 50% (IC₅₀) of each individual dose-response curve for DEX inhibition of LPS-induced IL-6 production. The IC₅₀ reflects the concentration of DEX necessary to terminate cytokine production by half. IL-6

percent concentrations were also calculated to determine the percent inhibition of LPS-stimulated IL-6 production due to each concentration of DEX. This calculation also gives us a dose response curve of LPS-stimulated IL-6 production. These two measures of GC sensitivity were used to determine sex and age differences in GC sensitivity.

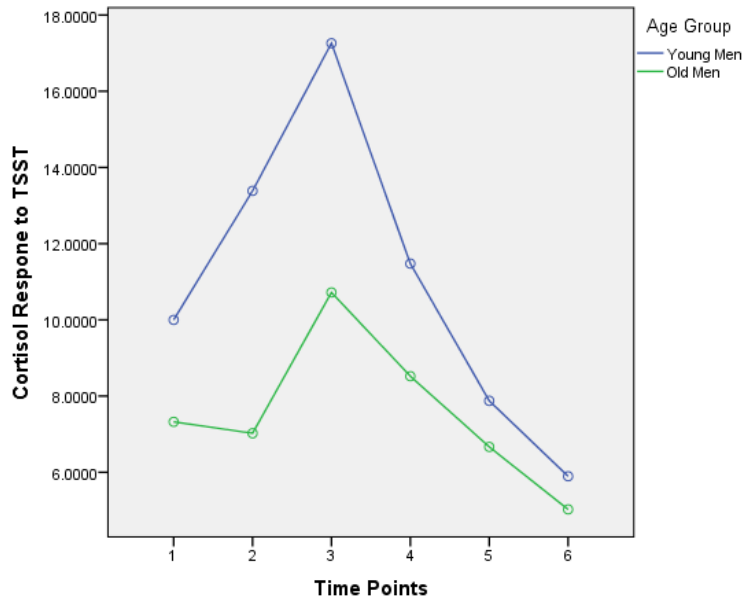
Repeated measures ANOVAs were conducted testing for sex and age differences in TSST-induced cortisol responses and TSST-induced plasma IL-6 responses. Repeated measures ANOVAs were also conducted separately for men and women testing for stress responses and age differences in dose response curves of LPS-stimulated IL-6 production and IC50's. A final set of repeated measures ANOVAs were conducted separately for men and women testing for sex and age differences in measures of GC sensitivity and free cortisol or plasma IL-6 responses to TSST.

7 Results

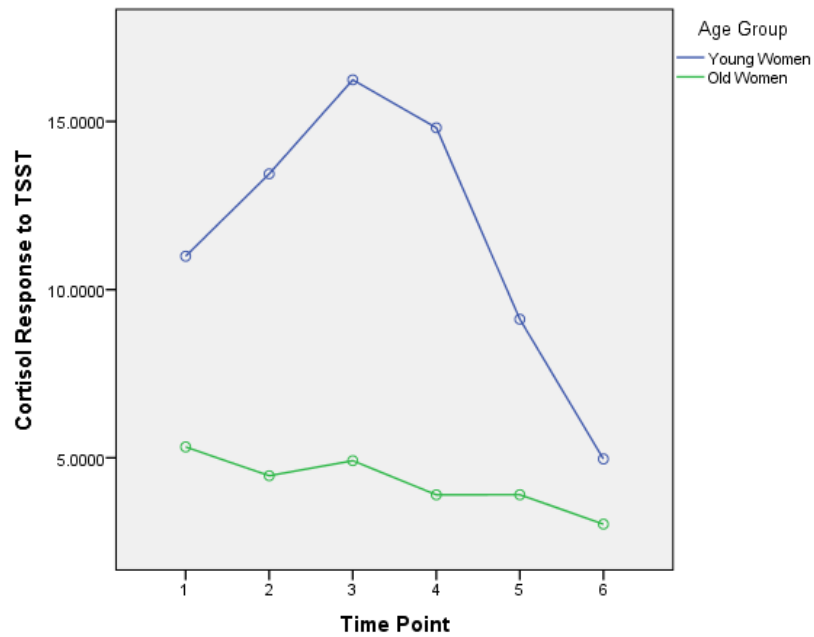
Stress responses of cortisol and IL-6

To test if TSST exposure induced significant increases in cortisol, we computed two repeated measures ANOVAs separately for male and female participants. For both men and women, a significant time effect was found indicating stress-induced activation of the HPA axis (males: $F=9.64$; $p<0.001$; females: $F=4.50$; $p=0.001$). Additionally, we found a significant age group by time interaction for women, which indicates different cortisol stress responses between old and young women ($F=2.76$; $p=0.022$). Figure 1B shows that older women do not show a cortisol stress response. There was not a significant difference in cortisol stress responses for between old and young men ($F=1.73$; $p=0.15$; see Figure 1A).

To test if exposure to the TSST induced significant increases in plasma IL-6, we computed two repeated measures ANOVAs separately for male and female participants. For both men and women, a significant time effect was found indicating stress-induced activation of the inflammatory response (males: $F=7.78$; $p=0.002$; females: $F=14.78$; $p<0.001$). However in both genders, there were no significant differences between old and young participants (males: $F=0.119$; $p=0.89$; females: $F=.315$; $p=0.73$; data not shown).



1A



1B.

Figure 1: Cortisol Responses to TSST (a) between young and old men and (b) between old and young women in nmol/L. Time point 1 represents 1 min pre TSST, Time points 2, 3, 4, 5 and 6 represent 1, 10, 30, 60, and 120 min post TSST respectively. There is a significant time by age interaction as indicated by the almost non-existent cortisol responses of older women compared to younger women. No such significant effect was seen between older and younger men.

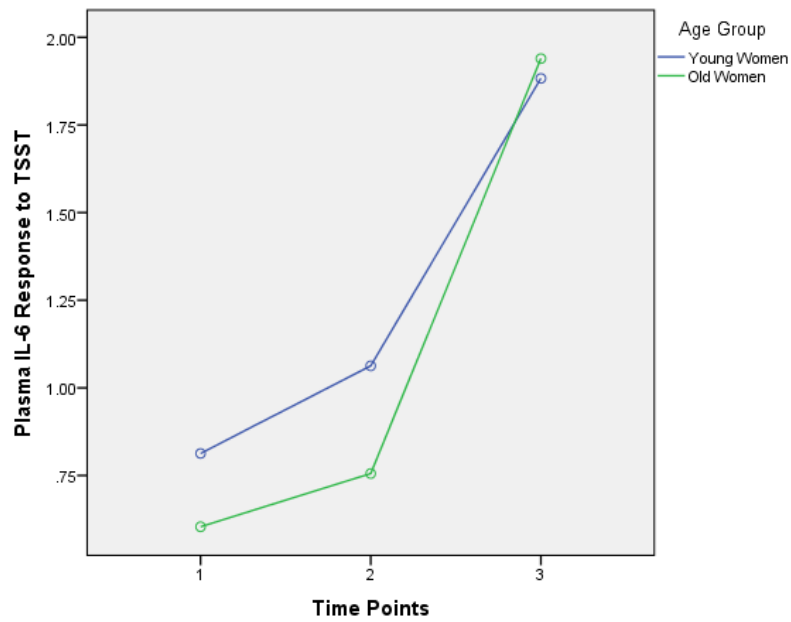
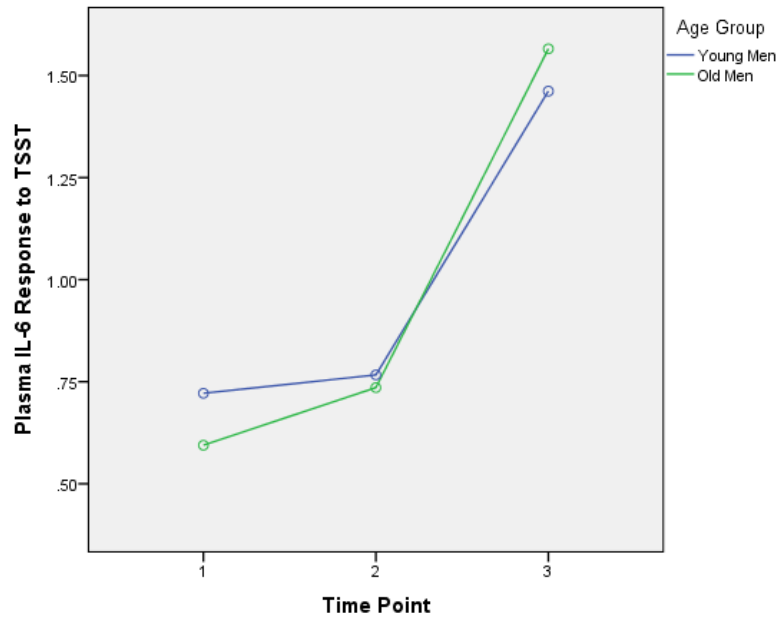


Figure 2: Plasma IL-6 Responses to TSST (a) between young and old men and (b) between young and old women in pg/mL. Time point 1 represents 1 min pre TSST, Time points 2 and 3 represent 30 and 60 mins post TSST respectively. There is no difference in plasma IL-6 between old and young participants.

Sex and age differences in GC sensitivity and stress responses of GC sensitivity

To examine sex and age differences in GC sensitivity, two different sets of analyses were used. First, repeated measures ANOVAs will be computed separately for men and women testing for stress responses and age differences in dose response curves of LPS-stimulated IL-6 production. Another set of repeated measures ANOVAs will be computed separately for men and women testing stress responses and age differences in IC₅₀'s.

Repeated measures ANOVAs computed separately for men and women revealed a strong DEX effect showing that IL-6 production was inhibited by co-incubation with glucocorticoids (DEX effect: men: $F=239.91$; $p<0.001$; women: $F=197.62$; $p<0.001$), data not shown.

In men, we found a marginally significant time by DEX interaction ($F=1.68$; $p=0.09$) indicating some changes in GC sensitivity response to stress (see Figure 2). However, there was not a significant time by DEX interaction for women, which indicates that GC sensitivity did not respond to stress ($F=.467$, $p=.91$). There was no significant three-way interaction between time, DEX, and age group in either of the genders (men: $F=0.73$; $p=0.69$; women: $F=0.96$; $p=0.48$), which indicates that there were no age-group differences in GC sensitivity responses to stress, refer to Figure 3A & 3B.

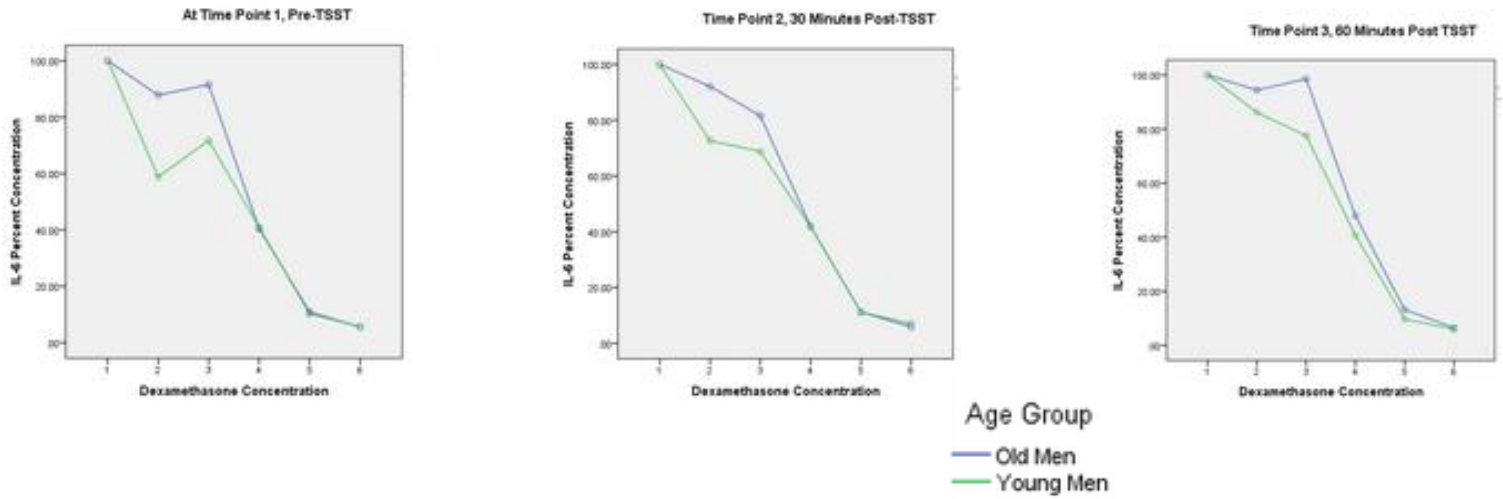


Figure 3A: No significant three way interaction between time, DEX, and age group in men. Dexamethasone 1, 2, 3, 4, 5, and 6 represent 0 , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} mol/liter DEX respectively. The blue line indicates old men, the green line young men.

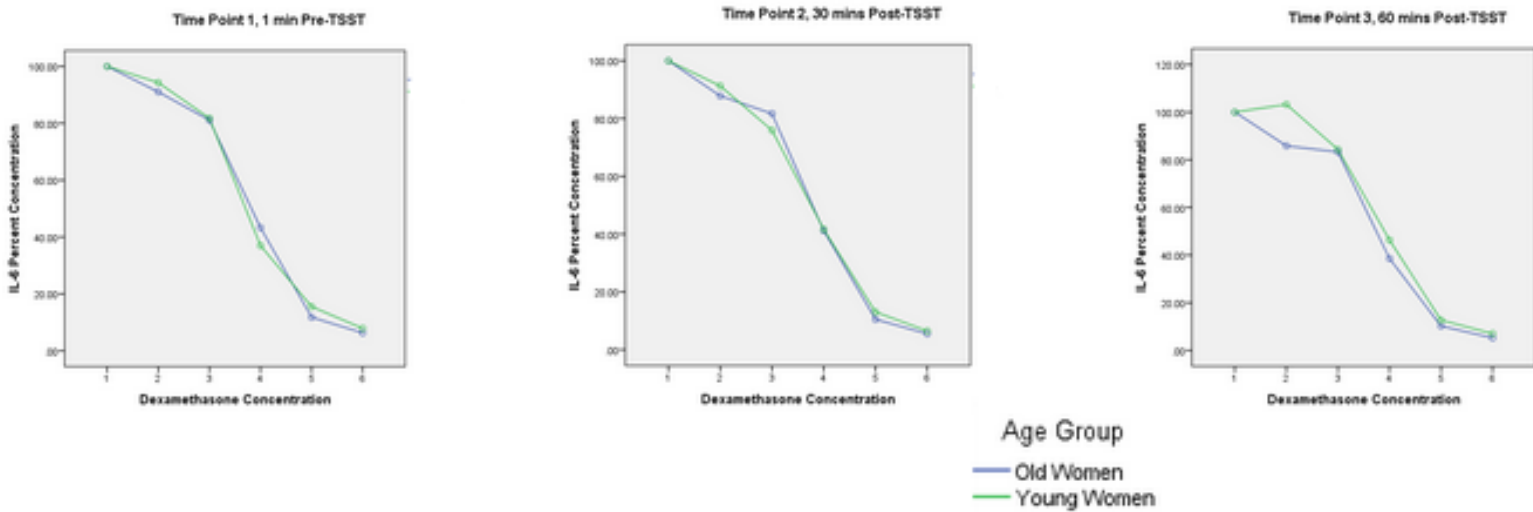
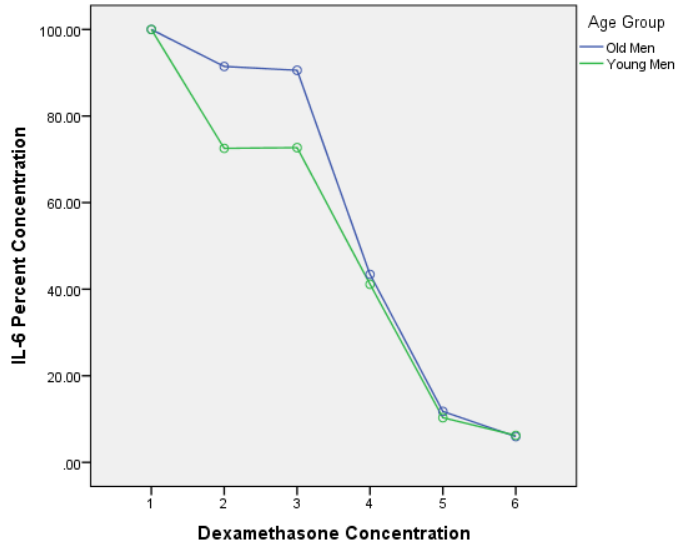
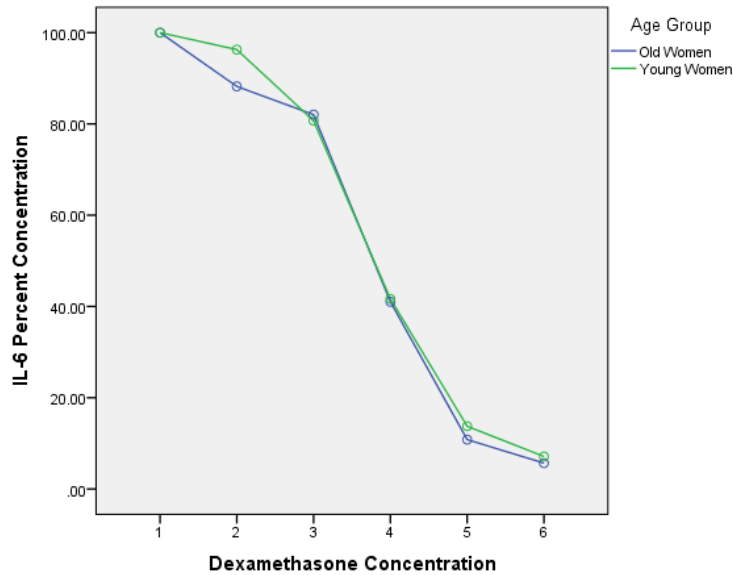


Figure 3B: No significant three way interaction between time, DEX, and age group in women. Dexamethasone 1, 2, 3, 4, 5, and 6 represent 0 , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} mol/liter DEX respectively. The blue line indicates old women, the green line young women.

A significant DEX by age group interaction was seen in men ($F=3.14$; $p=0.014$), which indicates differences in GC sensitivity between old and young men. As you can see by referring to Figure 4a, older men have a flatter curve suggesting lower GC sensitivity. No such differences were found between old and young women ($F=0.32$; $p=0.90$), Figure 4b.



4A



4B

Figure 4: DEX inhibition of LPS-stimulated production of IL-6 before and after TSST in (a) men and (b) women in pg/mL. Dexamethasone 1, 2, 3, 4, 5, and 6 represent 0 , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} mol/liter DEX respectively. The blue line indicates old, the green line young.

To test whether stress HPA axis and inflammatory stress responses could correlate with measures of glucocorticoid sensitivity, we first computed a set of Pearson correlations between indicators of cortisol and IL-6 stress responses and IC50s of DEX inhibition of LPS-induced IL-6 production. We found no correlation between TSST-induced cortisol responses and IC50's or between TSST-induced plasma IL-6 responses and IC50's (data not shown).

Finally, we conducted repeated measures ANOVAs with indicators of HPA axis and inflammatory stress responses as covariates to test sex and age group differences of dose response curves of LPS-stimulated IL-6 production. No relationship was found for plasma IL-6 stress responses (all F 's < 1). We found a significant three-way interaction between the factors time, DEX concentration, and stress-induced increases in free cortisol for male participants ($F=3.49$; $p=0.046$), which indicates a relationship between the HPA axis stress response and GC sensitivity changes over time. No such effect was seen in women. To better understand this effect and represent it graphically, we repeated analyses using a median split variable splitting the sample into low vs. high cortisol responders. A repeated measures ANOVAs using this median split as a between-subjects factors revealed a significant three-way interaction between the factors time, DEX concentration, and high vs. low free cortisol responders ($F=2.11$; $p=0.038$). Results are shown in Figure 5A and 5B.

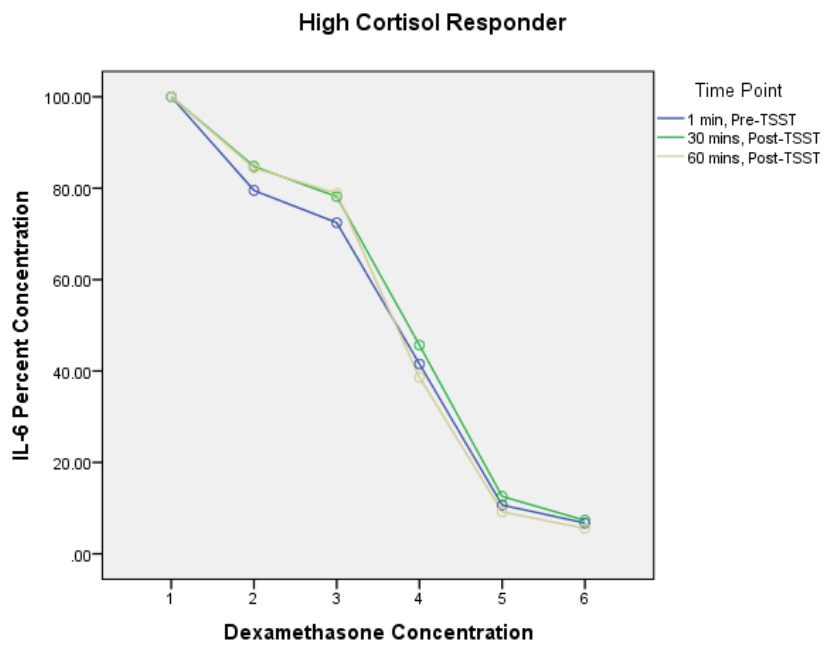
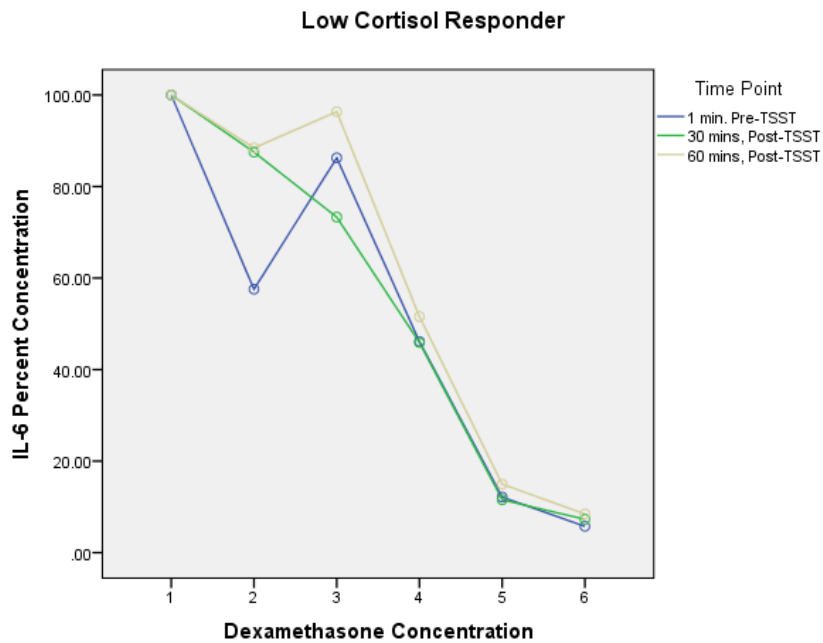


Figure 5A: Males who are low cortisol responders exhibit a higher GC sensitivity compared to high cortisol responders. A significant three-way interaction between the time, DEX concentration, and stress-induced increases in free cortisol for male participants. Dexamethasone 1, 2, 3, 4, 5, and 6 represent 0, 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} mol/liter DEX respectively.

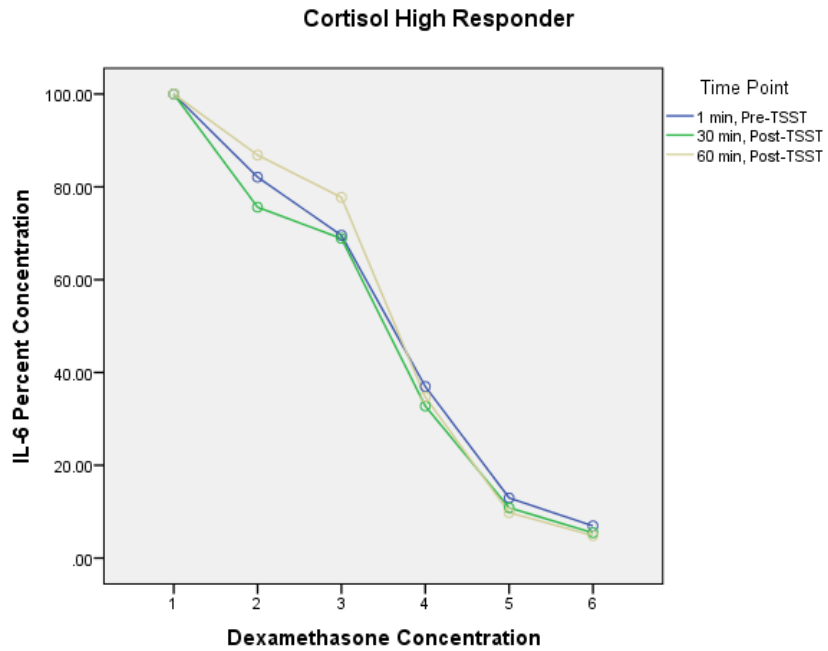
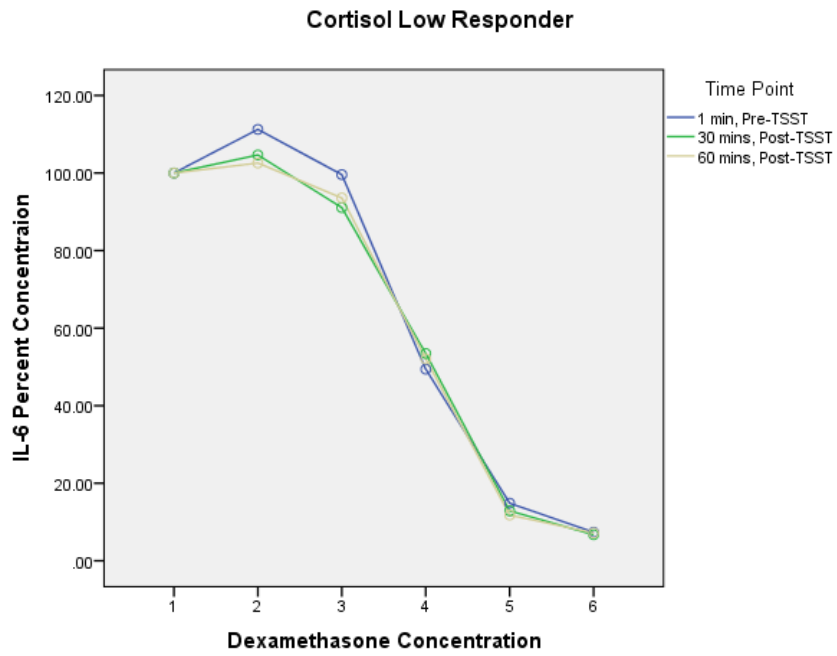


Figure 5B: No Significant three-way interaction between the time, DEX concentration, and stress-induced increases in free cortisol for female participants. Dexamethasone 1, 2, 3, 4, 5, and 6 represent 0, 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} mol/liter DEX respectively.

8 Discussion

Summary of Results

In this study, we wanted to investigate whether or not there were differences in GC sensitivity between old and young men and women and how this difference in GC sensitivity, if any, could play a role mediating the differences in inflammatory and neuroendocrine responses. We confirmed that the laboratory TSST stressor successfully induced a free cortisol and inflammatory response in both men and women. Older women however showed a blunted free cortisol response compared to younger women, while no significant differences between young and old men were seen. This provides evidence in support of the idea that estrogen may be an enhancer of HPA axis activity since older women have lower levels of estrogen compared to younger women. However, there were no significant sex and age difference in plasma IL-6 levels. We speculated that GC sensitivity could explain why older women may not have a strong cortisol stress response, but can express a similar inflammatory response profile to younger women.

We found that using the dose-response curves of LPS-stimulated IL-6 production, co-incubation with dexamethasone significantly inhibited IL-6 production for both men and women. In men only, we saw a marginally significant time by DEX interaction and a significant DEX by age group interaction. The former suggests that there are differences in GC sensitivity before and after stress. The latter suggest that GC sensitivities are different between older and younger men, with older men showing reduced GC sensitivity. This also confirms a past study conducted by Rohleder et al. showing reduced GC sensitivities among older men that can be partly restored with testosterone treatment (Rohleder, Kudielka et al. 2002). Women exhibited no such

differences. Unfortunately, there was no significant three way interaction between time, DEX and age in either gender groups. This ultimately means that GC sensitivity did not change over time between old and young men and women. Testing for age and sex differences in GC sensitivity using IC50's did not reveal any significant differences in either gender groups.

There was a three-way interaction between time, DEX, and TSST-induced cortisol response in male but not female participants. To better understand this interaction, we split within gender, high vs. low cortisol responders. Individuals in the low free cortisol responder groups are participants who exhibited a cortisol response that was less than the median gender cortisol response. This means that in response to the same TSST stressor, low cortisol responders produced less cortisol. There was a significant difference in GC sensitivity between male high and low free cortisol responders, with low free cortisol responders exhibiting higher GC sensitivity before and 30 minutes after TSST.

Implications

Unfortunately, no differences in GC sensitivity were found between older and younger women, which suggest that there may be another factor affecting the inflammatory and endocrine stress response profiles. The fact that there were no differences in TSST-induced cortisol and TSST-induced plasma IL-6 responses between older and younger men is also puzzling. However it provides evidence in support of the idea that there may be another factor that interacts with GC sensitivity and/or sex steroids to bring about differences in immunity. Certain studies have linked dietary pattern and obesity to higher incidences of HPA axis deregulations, impaired immune function, and changes to gene expression profiles, which all probably modulate the risk of

chronic diseases (Boynton, Neuhouser et al. 2007; Garcia-Prieto, Tebar et al. 2007; Bouchard-Mercier, Paradis et al. 2013). Additionally, since all young women participants were recruited in the luteal phase of the menstrual cycle, differences in GC sensitivity may have been masked due to the absence of women in the follicular phase, who with higher levels of estrogen, may be more different in GC sensitivity compared to postmenopausal women.

This study confirms that younger men may require less cortisol to reduce inflammation due to an enhanced GC sensitivity. Our data also indicates that GC sensitivity changes are related with age and free cortisol responses in men only. To recap, when divided into low vs. high cortisol responder groups, men within the low cortisol responder group showed higher GC sensitivity before and 30 minutes after TSST. No such effect was seen among women. Perhaps this higher GC sensitivity is a mechanism compensating for low cortisol response in men. It may also be possible that testosterone in men exerts a stronger influence on the responsiveness of the neuroendocrine and immune feedback mechanism. As mentioned in the background section, the feedback between these two systems is important for host homeostasis and protection against illness. Perhaps this feedback mechanism is less robust in females, which offers an explanation for why females are more susceptible to autoimmune conditions. It would interesting to see if androgens levels would have an effect on low vs high cortisol responders and GC sensitivity. Androgens have been shown to elicit a suppressive effect on neuroendocrine and inflammatory responses separately, but may exert some unknown effect on feedback loop between these two systems.

9 Conclusions

Previous research has documented gender differences between the HPA axis and inflammatory response to stress separately to explain the discrepancy in disease susceptibilities between men and women. Furthermore the data concerning the effects of estrogen remains elusive, suggesting that there must be another factor mediating the differences seen in HPA and inflammatory responses. Few studies have looked into GC sensitivity as a possible mechanism for explaining differences in diseases susceptibilities. However, our data indicates that GCs may not be the main factor responsible for differences in disease patterns. Instead, sex differences in the neuroendocrine and immune feedback loop may contribute to differences in immunity seen between men and women. Further research is needed to investigate how androgens and estrogens exert their effects on the communication between the neuroendocrine and immune systems.

Works Cited

- Ahmed, S. A. and N. Talal (1990). "Sex hormones and the immune system—part 2. Animal data." Baillière's Clinical Rheumatology **4**(1): 13-31.
- Bethin, K. E., S. K. Vogt, et al. (2000). "Interleukin-6 is an essential, corticotropin-releasing hormone-independent stimulator of the adrenal axis during immune system activation." Proceedings of the National Academy of Sciences **97**(16): 9317-9322.
- Bierhaus, A., J. Wolf, et al. (2003). "A mechanism converting psychosocial stress into mononuclear cell activation." Proceedings of the National Academy of Sciences **100**(4): 1920-1925.
- Black, P. H. (2003). "The inflammatory response is an integral part of the stress response: Implications for atherosclerosis, insulin resistance, type II diabetes and metabolic syndrome X." Brain, Behavior, and Immunity **17**(5): 350-364.
- Blum, C. A., B. Müller, et al. (2005). "Low-Grade Inflammation and Estimates of Insulin Resistance during the Menstrual Cycle in Lean and Overweight Women." The Journal of Clinical Endocrinology & Metabolism **90**(6): 3230-3235.
- Bone, R. C., R. A. Balk, et al. (1992). "Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. the accp/sccm consensus conference committee. american college of chest physicians/society of critical care medicine." CHEST Journal **101**(6): 1644-1655.
- Bouchard-Mercier, A., A.-M. Paradis, et al. (2013). "Associations between dietary patterns and gene expression profiles of healthy men and women: a cross-sectional study." Nutrition Journal **12**(1): 24.
- Boynton, A., M. L. Neuhouser, et al. (2007). "Associations between healthy eating patterns and immune function or inflammation in overweight or obese postmenopausal women." Am J Clin Nutr **86**(5): 1445-1455.
- Brunsgaard, H. and B. K. Pedersen (2003). "Age-related inflammatory cytokines and disease." Immunol Allergy Clin North Am **23**(1): 15-39.
- Buckingham, J. C., H. D. Loxley, et al. (1996). "Activation of the HPA axis by immune insults: roles and interactions of cytokines, eicosanoids, glucocorticoids." Pharmacol Biochem Behav **54**(1): 285-298.
- Chao, T. C. (1996). "Female sex hormones and the immune system." Changgeng Yi Xue Za Zhi **19**(1): 95-106.
- Chrousos, G. P., A. Vingerhoeds, et al. (1982). "Primary cortisol resistance in man. A glucocorticoid receptor-mediated disease." The Journal of Clinical Investigation **69**(6): 1261-1269.
- Cohen, S., D. Janicki-Deverts, et al. (2012). "Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk." Proceedings of the National Academy of Sciences.

- Cole, S. W. (2008). "Social regulation of leukocyte homeostasis: The role of glucocorticoid sensitivity." Brain, Behavior, and Immunity **22**(7): 1049-1055.
- Cole, S. W., S. P. Mendoza, et al. (2009). "Social Stress Desensitizes Lymphocytes to Regulation by Endogenous Glucocorticoids: Insights from In Vivo Cell Trafficking Dynamics in Rhesus Macaques." Psychosomatic Medicine **71**(6): 591-597.
- Corrales, J. J., M. Almeida, et al. (2006). "Androgen-replacement therapy depresses the ex vivo production of inflammatory cytokines by circulating antigen-presenting cells in aging type-2 diabetic men with partial androgen deficiency." Journal of Endocrinology **189**(3): 595-604.
- Creely, S. J., P. G. McTernan, et al. (2007). "Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes." American Journal of Physiology - Endocrinology and Metabolism **292**(3): E740-E747.
- Dedovic, K., A. Duchesne, et al. (2009). "The brain and the stress axis: the neural correlates of cortisol regulation in response to stress." Neuroimage **47**(3): 864-871.
- Deshpande, R., H. Khalili, et al. (1997). "Estradiol Down-Regulates LPS-Induced Cytokine Production and NFkB Activation in Murine Macrophages." American Journal of Reproductive Immunology **38**(1): 46-54.
- Deshpande, R., H. Khalili, et al. (1997). "Estradiol down-regulates LPS-induced cytokine production and NFkB activation in murine macrophages." Am J Reprod Immunol **38**(1): 46-54.
- Dhabhar, F. S. (2000). "Acute stress enhances while chronic stress suppresses skin immunity: the role of stress hormones and leukocyte trafficking." Annals of the New York Academy of Sciences **917**(1): 876-893.
- Dressendorfer, R. A., C. Kirschbaum, et al. (1992). "Synthesis of a cortisol-biotin conjugate and evaluation as a tracer in an immunoassay for salivary cortisol measurement." J Steroid Biochem Mol Biol **43**(7): 683-692.
- Elenkov, I. J., D. G. Iezzoni, et al. (2005). "Cytokine dysregulation, inflammation and well-being." Neuroimmunomodulation **12**(5): 255-269.
- Ershler, W. B. and E. T. Keller (2000). "Age-Associated Increased Interleukin-6 Gene Expression, Late-Life Diseases, and Frailty." Annual Review of Medicine **51**(1): 245-270.
- Fairweather, D., S. Frisancho-Kiss, et al. (2008). "Sex differences in autoimmune disease from a pathological perspective." Am J Pathol **173**(3): 600-609.
- Fernández-Real, J. M. and J. C. Pickup (2008). "Innate immunity, insulin resistance and type 2 diabetes." Trends in Endocrinology & Metabolism **19**(1): 10-16.
- Ferrucci, L., T. B. Harris, et al. (1999). "Serum IL-6 level and the development of disability in older persons." Journal of the American Geriatrics Society **47**(6): 639-646.
- Fish, E. N. (2008). "The X-files in immunity: sex-based differences predispose immune responses." Nat Rev Immunol **8**(9): 737-744.

- Fox, H. S. (1992). "Androgen treatment prevents diabetes in nonobese diabetic mice." J Exp Med **175**(5): 1409-1412.
- Gaillard, R. C. and E. Spinedi (1998). "Sex- and stress-steroids interactions and the immune system: evidence for a neuroendocrine-immunological sexual dimorphism." Domestic Animal Endocrinology **15**(5): 345-352.
- Garcia-Prieto, M. D., F. J. Tebar, et al. (2007). "Cortisol secretory pattern and glucocorticoid feedback sensitivity in women from a Mediterranean area: relationship with anthropometric characteristics, dietary intake and plasma fatty acid profile." Clin Endocrinol (Oxf) **66**(2): 185-191.
- Giron-Gonzalez, J. A., F. J. Moral, et al. (2000). "Consistent production of a higher TH1:TH2 cytokine ratio by stimulated T cells in men compared with women." Eur J Endocrinol **143**(1): 31-36.
- Gomez, C. R., E. D. Boehmer, et al. (2005). "The aging innate immune system." Curr Opin Immunol **17**(5): 457-462.
- Harris, T. B., L. Ferrucci, et al. (1999). "Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly." The American journal of medicine **106**(5): 506-512.
- Hayden, M. S. and S. Ghosh (2012). "NF-kappaB, the first quarter-century: remarkable progress and outstanding questions." Genes Dev **26**(3): 203-234.
- Herman, J. P., H. Figueiredo, et al. (2003). "Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness." Frontiers in Neuroendocrinology **24**(3): 151-180.
- Howcroft, T. K., J. Campisi, et al. (2013). "The role of inflammation in age-related disease." Aging (Albany NY) **5**(1): 84.
- Ito, A., B. F. Bebo, Jr., et al. (2001). "Estrogen treatment down-regulates TNF-alpha production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice." J Immunol **167**(1): 542-552.
- Kalland, T. (1980). "Reduced natural killer activity in female mice after neonatal exposure to diethylstilbestrol." The Journal of Immunology **124**(3): 1297-1300.
- Kant, G. J., R. H. Lenox, et al. (1983). "Comparison of stress response in male and female rats: pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone." Psychoneuroendocrinology **8**(4): 421-428.
- Kiecolt-Glaser, J. K., K. J. Preacher, et al. (2003). "Chronic stress and age-related increases in the proinflammatory cytokine IL-6." Proceedings of the National Academy of Sciences **100**(15): 9090-9095.
- Kirschbaum, C. and D. H. Hellhammer (2000). "Salivary cortisol." Encyclopedia of stress **3**(379-383).

- Kirschbaum, C., B. M. Kudielka, et al. (1999). "Impact of Gender, Menstrual Cycle Phase, and Oral Contraceptives on the Activity of the Hypothalamus-Pituitary-Adrenal Axis." Psychosomatic Medicine **61**(2): 154-162.
- Kirschbaum, C., N. Schommer, et al. (1996). "Short-term estradiol treatment enhances pituitary-adrenal axis and sympathetic responses to psychosocial stress in healthy young men." J Clin Endocrinol Metab **81**(10): 3639-3643.
- Kitay, J. I. (1961). "Sex Differences In Adrenal Cortical Secretion In The Rat." Endocrinology **68**(5): 818-824.
- Klein, S. (2000). "The effects of hormones on sex differences in infection: from genes to behavior." Neuroscience & Biobehavioral Reviews **24**(6): 627-638.
- Klein, S. L. (2012). "Immune cells have sex and so should journal articles." Endocrinology **153**(6): 2544-2550.
- Klein, S. L., B. H. Bird, et al. (2000). "Sex differences in Seoul virus infection are not related to adult sex steroid concentrations in Norway rats." J Virol **74**(17): 8213-8217.
- Krabbe, K. S., M. Pedersen, et al. (2004). "Inflammatory mediators in the elderly." Exp Gerontol **39**(5): 687-699.
- Kronfol, Z., M. Starkman, et al. (1996). "Immune regulation in Cushing's syndrome: Relationship to hypothalamic-pituitary-adrenal axis hormones." Psychoneuroendocrinology **21**(7): 599-608.
- Kunz-Ebrecht, S. R., V. Mohamed-Ali, et al. (2003). "Cortisol responses to mild psychological stress are inversely associated with proinflammatory cytokines." Brain, Behavior, and Immunity **17**(5): 373-383.
- Lesniewska, B., B. Miskowiak, et al. (1990). "Sex differences in adrenocortical structure and function. XXVII. The effect of ether stress on ACTH and corticosterone in intact, gonadectomized, and testosterone- or estradiol-replaced rats." Res Exp Med (Berl) **190**(2): 95-103.
- Luster, M. I., H. T. Hayes, et al. (1984). "Estrogen immunosuppression is regulated through estrogenic responses in the thymus." J Immunol **133**(1): 110-116.
- Malchoff, D. M., A. Brufsky, et al. (1993). "A mutation of the glucocorticoid receptor in primary cortisol resistance." J Clin Invest **91**(5): 1918-1925.
- Malkin, C. J., P. J. Pugh, et al. (2004). "The Effect of Testosterone Replacement on Endogenous Inflammatory Cytokines and Lipid Profiles in Hypogonadal Men." The Journal of Clinical Endocrinology & Metabolism **89**(7): 3313-3318.
- Marchesi, V. T. (2011). "Alzheimer's dementia begins as a disease of small blood vessels, damaged by oxidative-induced inflammation and dysregulated amyloid metabolism: implications for early detection and therapy." The FASEB Journal **25**(1): 5-13.
- Marriott, I. and Y. Huet-Hudson (2006). "Sexual dimorphism in innate immune responses to infectious organisms." Immunologic Research **34**(3): 177-192.

- Mastorakos, G., G. P. Chrousos, et al. (1993). "Recombinant interleukin-6 activates the hypothalamic-pituitary-adrenal axis in humans." Journal of Clinical Endocrinology & Metabolism **77**(6): 1690-1694.
- McCormick, C. M. and I. Z. Mathews (2007). "HPA function in adolescence: Role of sex hormones in its regulation and the enduring consequences of exposure to stressors." Pharmacology Biochemistry and Behavior **86**(2): 220-233.
- McEwen, B. S. (1998). "Protective and Damaging Effects of Stress Mediators." New England Journal of Medicine **338**(3): 171-179.
- Mukaida, N., M. Morita, et al. (1994). "Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression." Journal of Biological Chemistry **269**(18): 13289-13295.
- Murphy, A. J., P. M. Guyre, et al. (2010). "Estradiol suppresses NF-kappa B activation through coordinated regulation of let-7a and miR-125b in primary human macrophages." J Immunol **184**(9): 5029-5037.
- Norata, G. D., G. Tibolla, et al. (2006). "Dihydrotestosterone Decreases Tumor Necrosis Factor- α and Lipopolysaccharide-Induced Inflammatory Response in Human Endothelial Cells." The Journal of Clinical Endocrinology & Metabolism **91**(2): 546-554.
- Oberholzer, A., M. Keel, et al. (2000). "Incidence of Septic Complications and Multiple Organ Failure in Severely Injured Patients Is Sex Specific." Journal of Trauma and Acute Care Surgery **48**(5): 932-937.
- Pennell, L. M., C. L. Galligan, et al. (2012). "Sex affects immunity." Journal of Autoimmunity **38**(2-3): J282-J291.
- Pickup, J. C. (2004). "Inflammation and Activated Innate Immunity in the Pathogenesis of Type 2 Diabetes." Diabetes Care **27**(3): 813-823.
- Pour, H. R. N., D. E. Grobbee, et al. (2007). "Association of endogenous sex hormone with C-reactive protein levels in middle-aged and elderly men." Clinical Endocrinology **66**(3): 394-398.
- Puder, J. J., P. U. Freda, et al. (2001). "Estrogen Modulates the Hypothalamic-Pituitary-Adrenal and Inflammatory Cytokine Responses to Endotoxin in Women." Journal of Clinical Endocrinology & Metabolism **86**(6): 2403-2408.
- Raison, C. L. and A. H. Miller (2003). "When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders." Am J Psychiatry **160**(9): 1554-1565.
- Rhen, T. and J. A. Cidlowski (2005). "Antiinflammatory Action of Glucocorticoids — New Mechanisms for Old Drugs." New England Journal of Medicine **353**(16): 1711-1723.
- Robles, T. F., R. Glaser, et al. (2005). "Out of Balance: A New Look at Chronic Stress, Depression, and Immunity." Current Directions in Psychological Science **14**(2): 111-115.

- Roca, C. A., P. J. Schmidt, et al. (2003). "Differential Menstrual Cycle Regulation of Hypothalamic-Pituitary-Adrenal Axis in Women with Premenstrual Syndrome and Controls." Journal of Clinical Endocrinology & Metabolism **88**(7): 3057-3063.
- Rohleder, N., B. M. Kudielka, et al. (2002). "Age and sex steroid-related changes in glucocorticoid sensitivity of pro-inflammatory cytokine production after psychosocial stress." J Neuroimmunol **126**(1-2): 69-77.
- Rohleder, N., T. J. Marin, et al. (2009). "Biologic Cost of Caring for a Cancer Patient: Dysregulation of Pro- and Anti-Inflammatory Signaling Pathways." Journal of Clinical Oncology **27**(18): 2909-2915.
- Rohleder, N., N. C. Schommer, et al. (2001). "Sex differences in glucocorticoid sensitivity of proinflammatory cytokine production after psychosocial stress." Psychosom Med **63**(6): 966-972.
- Rosmalen, J. G., M. J. Pigmans, et al. (2001). "Sex steroids influence pancreatic islet hypertrophy and subsequent autoimmune infiltration in nonobese diabetic (NOD) and NODscid mice." Lab Invest **81**(2): 231-239.
- Rubinow, D. R., C. A. Roca, et al. (2005). "Testosterone suppression of CRH-stimulated cortisol in men." Neuropsychopharmacology **30**(10): 1906-1912.
- Sapolsky, R. M., L. M. Romero, et al. (2000). "How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions." Endocrine Reviews **21**(1): 55-89.
- Sawalha, A. H., R. Webb, et al. (2008). "Common variants within MECP2 confer risk of systemic lupus erythematosus." PLoS One **3**(3): e1727.
- Scheinman, R. I., P. C. Cogswell, et al. (1995). "Role of transcriptional activation of I κ B α in mediation of immunosuppression by glucocorticoids." Science **270**(5234): 283-286.
- Schröder, J., V. Kahlke, et al. (1998). "Gender differences in human sepsis." Archives of Surgery **133**(11): 1200-1205.
- Scofield, R. H., G. R. Bruner, et al. (2008). "Klinefelter's syndrome (47,XXY) in male systemic lupus erythematosus patients: support for the notion of a gene-dose effect from the X chromosome." Arthritis Rheum **58**(8): 2511-2517.
- Singer, M., V. De Santis, et al. (2004). "Multiorgan failure is an adaptive, endocrine-mediated, metabolic response to overwhelming systemic inflammation." Lancet **364**(9433): 545-548.
- Späth-Schwalbe, E., J. Born, et al. (1994). "Interleukin-6 stimulates the hypothalamus-pituitary-adrenocortical axis in man." Journal of Clinical Endocrinology & Metabolism **79**(4): 1212-1214.
- Straub, R. H. (2007). "The Complex Role of Estrogens in Inflammation." Endocrine Reviews **28**(5): 521-574.

- Sutton, B. J., G. E. Parks, et al. (2011). "Cushing's syndrome and nocardiosis associated with a pulmonary carcinoid tumor: report of a case and review of the literature." Diagn Cytopathol **39**(5): 359-362.
- Verhelst, K., I. Carpentier, et al. (2011). "Regulation of TNF-induced NF- κ B activation by different cytoplasmic ubiquitination events." Cytokine & growth factor reviews **22**(5): 277-286.
- Verthelyi, D. (2001). "Sex hormones as immunomodulators in health and disease." International Immunopharmacology **1**(6): 983-993.
- Wei, J., H. Xu, et al. (1992). "Increase of plasma IL-6 concentration with age in healthy subjects." Life sciences **51**(25): 1953-1956.
- Wilckens, T. and R. De Rijk (1997). "Glucocorticoids and immune function: unknown dimensions and new frontiers." Immunology Today **18**(9): 418-424.
- Willerson, J. T. and P. M. Ridker (2004). "Inflammation as a Cardiovascular Risk Factor." Circulation **109**(21 suppl 1): II-2-II-10.
- Žarković, M., S. Ignjatović, et al. (2008). "Cortisol response to ACTH stimulation correlates with blood interleukin 6 concentration in healthy humans." European Journal of Endocrinology **159**(5): 649-652.