Adipose and Muscle Tissue in Glucocorticoid-Induced Metabolic Disease

by

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Dedication

This dissertation is dedicated to my fiancé, Thomas C. Jacobs, who has been a true inspiration throughout my degree by working tirelessly in pursuit of his own career. He supported and encouraged me every step of the way and I could not imagine having to go through any of it without him.

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Abstract

Introduction: Glucocorticoids are steroid hormones induced by stress that are necessary for proper glucose homeostasis and tissue development. Glucocorticoids also have potent immunosuppressant properties and are commonly used to treat a variety of inflammatory conditions such as asthma, cancer and autoimmune disease in children and adults. Chronic elevations due to excess stress or chronic glucocorticoid treatment can lead to metabolic disease. Though glucocorticoid-induced metabolic disturbances such as insulin resistance, non-alcoholic fatty liver disease (NAFLD) and muscle wasting are well known, the impact of pre-existing obesity on metabolic syndrome and the specific transcriptional changes leading to these outcomes remain undefined. Additionally, the effects of childhood glucocorticoid exposure on adult metabolic health is largely unknown. The aim of this dissertation is to identify the underlying physiological and mechanistic processes that lead to poor metabolic health following chronically elevated glucocorticoids, and how obesity status affects glucocorticoid-induced metabolic disease, focusing on effects in adipose and muscle.

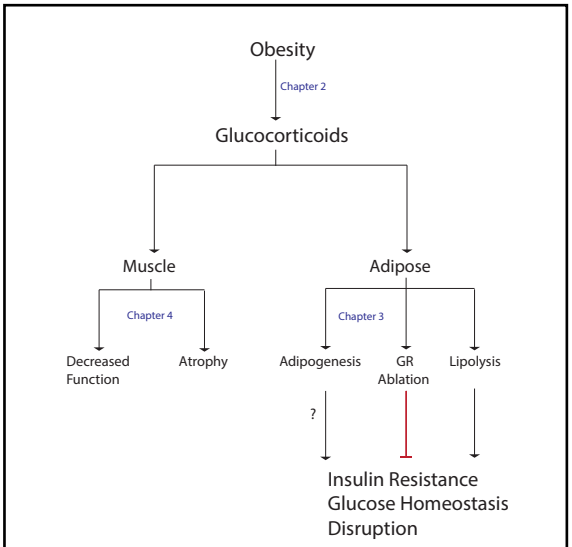

Figure A: Schematic of dissertation chapter topics.

Methods: To determine the impact of obesity on glucocorticoid associated metabolic disease, clinical measures and adipose tissue transcriptional changes were studied in lean and obese patients with Cushing's disease. To extend these findings, lean and obese mice were treated with dexamethasone and a variety of physiological and biochemical outcomes related to metabolic syndrome were assessed. To understand the contribution of the adipocyte glucocorticoid receptor (GR) in dexamethasone-induced metabolic disease, metabolic disease outcomes were measured in lean and obese GR knockout mice. Dexamethasone regulation of adipogenic genes was also assessed in adipocytes in order to determine potential GR targets during early adipogenesis, a process negatively associated with insulin resistance. Lastly, juvenile dexamethasone exposure studies were conducted in mice to determine how this affects body composition and glucose homeostasis in adulthood.

Results: Obesity resulted in synergistic elevations of glucocorticoid-induced lipolysis, insulin resistance, NAFLD and muscle atrophy. Ablation of the adipocyte GR attenuated these effects, with the exception of muscle loss. In regards to dexamethasone-induced adipogenesis, *Klf5* was induced early and in the absence of other stimuli, suggesting it may be a promising target for future study. Evidence of reduced glucose tolerance was observed in adult male and female mice following short-term juvenile exposure to dexamethasone. Reduced lean and fat mass was noted in dexamethasone-treated adult male mice, suggesting that male mice are more susceptible to adverse outcomes during this window of exposure.

Conclusion: Diet-induced obesity amplifies the adverse metabolic outcomes of glucocorticoids, such as insulin resistance and NAFLD, in humans and mice, as well as exacerbates muscle wasting in mice. These data implicate adipose as a key tissue in glucocorticoid-induced systemic insulin resistance and NAFLD, though it is unclear how obesity is leading to greater dexamethasone-induced muscle wasting, and support that lipolysis is the most likely mediator linking adipocyte glucocorticoid action to metabolic disease. Additionally, short-term juvenile dexamethasone exposure was shown to disrupt normal glucose regulation in adult mice and had negative impacts on lean mass in male mice. Further studies focus on the specific mechanisms linking glucocorticoid signaling in adipocytes to insulin resistance, NAFLD and muscle health, as well as how obesity exacerbates these outcomes. Future research is also warranted to fully elucidate the impact of childhood glucocorticoid exposure on metabolic health in adulthood. The findings presented here contribute to the greater body of research and could potentially be used to influence treatment strategies for those with elevated glucocorticoids.

Chapter 1: Introduction

Glucocorticoid function and regulation

Glucocorticoids, also known as corticosteroids, are a class of steroid hormones whose main function is to maintain adequate glucose levels to the brain and other organs during times of starvation and stress (Kuo et al. 2015). Glucocorticoids are necessary for proper physiological function and follows a circadian rhythm as well as through actions of the hypothalamus-pituitary-adrenal (HPA) axis (S. M. Smith and Vale 2006; So et al. 2009). Under normal physiological circumstances, glucocorticoids are highest in the morning with smaller peaks throughout the day and are lowest during sleep (Chan and Debono 2010). Outside of the normal circadian rhythm, a stressful event or prolonged fasting can also activate the HPA axis. Initially there is a stimulus to the hypothalamus causing the release of corticotropin releasing hormone (CRH) in the median eminence of the brain. CRH then binds and activates CRH-1 receptors on the anterior pituitary leading to the production and secretion of pro-hormone, proopiomelanocortin (POMC), that is then cleaved in to several hormones, including adrenocorticotropic hormone (ACTH). ACTH is released into the bloodstream and acts on the MC2R receptors, located in the zona fasciculata region of the adrenal cortex to promote the synthesis and release of glucocorticoids (Molina 2013). Under normal circumstances, glucocorticoids negatively feedback to the pituitary and hypothalamus to prevent excess glucocorticoid production.

Glucocorticoids are synthesized from cholesterol by way of multiple enzymatic conversion steps ultimately leading to the production of corticosterone, cortisone (also known as 11-deoxycortisol) or cortisol. Corticosterone is the active endogenous form of glucocorticoids in rodents; however, in humans, cortisol is the active endogenous form. Cortisone is inactive, but can be interconverted with cortisol via 11b-hydroxylase (11-ßHSD) enzymes 1 and 2, respectively (Paterson et al. 2005). These hydroxylase enzymes are key to the local tissue regulation of glucocorticoid activity. Once released into the bloodstream, glucocorticoids, being fat-soluble molecules, are able to diffuse into the lipid membranes of any cell and can act in virtually all tissues given the widespread expression of the glucocorticoid receptor (GR). The GR is the primary receptor for glucocorticoids; however, glucocorticoids are quite similar in their chemical structure to mineralocorticoids, another class of steroid hormones, and can activate the mineralocorticoid receptor (MR) as well. Therefore, tissues that are important for blood pressure regulation, such as those found in the kidneys, express 11-ßHSD2 thereby inactivating cortisol (via conversion to cortisone) in effort to suppress cortisol-MR binding. The majority of metabolic tissues express higher levels of 11-ßHSD1 to allow for cortisol-GR binding and activity (Paterson et al. 2005).

In order to elevate blood glucose when needed, glucocorticoids promote the breakdown of muscle and fat to provide substrates for gluconeogenesis via the transcriptional regulation of key genes. As mentioned above, glucocorticoids are able to diffuse into cell membranes; once in the cell, glucocorticoids bind the GR in the cytosol, which allows for the translocation of the ligand-receptor complex into the nucleus. In the nucleus, the glucocorticoid-GR complex activates target genes by binding to a specific motif, known as glucocorticoid regulatory elements (GREs),

that can be located within or around that gene (J.-C. Wang et al. 2004; Reddy et al. 2009; Jolma et al. 2015). Once bound, the GR can either suppress or promote transcription, depending on the gene. In major metabolic tissues, such as the liver, adipose tissue and muscle, the GR activates genes involved in gluconeogenesis, adipocyte lipolysis and muscle atrophy, respectively, in order to support glucose production via gluconeogenesis in the liver.

Induction of Adipocyte Lipolysis by Glucocorticoids

Adipocyte lipolysis is the breakdown of tri-, di- and monoglycerides into their constitutive elements, glycerol and free fatty acids, and chronic elevation of these metabolites can lead to insulin resistance and fatty liver disease. There are several enzymes regulating adipose tissue lipolysis including adipose triglyceride lipase (ATGL; encoded by *Pnpla2*), hormone-sensitive lipase (HSL; encoded by *Lipe*), monoacylglycerol lipase (MGL; encoded by *Mgll*) lipoprotein lipase (LPL; encoded by *Lpl*) and perilipin (encoded by *Plin*) (Zechner et al. 2012). Of these, *Lipe*/HSL and *Pnpla2*/ATGL have been suggested as potential GR targets in glucocorticoid activation of lipolysis. ATGL is primarily responsible for the breakdown of triglycerides into diglycerides via the removal of one fatty acid and is known as the rate limiting enzyme in lipolysis; however, some have proposed that at high levels it can hydrolyze all three acylglycerols (Jenkins et al. 2004; X. Yang et al. 2011). HSL is the primary enzyme in diglyceride hydrolysis. Both of these proteins require activation, CGI-58 is responsible for binding and activating ATGL, whereas PKA and AMPK are known to phosphorylate HSL on multiple serine sites leading to HSL activity.

Glucocorticoid-induced lipolysis is a well-known phenomenon that has been demonstrated *in vitro* and *in vivo* (Harvey et al. 2018; Xu et al. 2009; Djurhuus et al. 2004, 2002; Kršek et al. 2005). Physiologically, the substrates produced from lipolysis can aid in hepatic gluconeogenesis. Glycerol is a non-carbohydrate precursor that can be converted into glucose, and fatty acids can be converted into acetyl-CoA which can activate pyruvate carboxylase, one rate limiting enzyme in gluconeogenesis. In addition, acetyl-CoA can provide energy for the costly production of glucose (Williamson, Kreisberg, and Felts 1966). We and others have shown that glucocorticoids lead to increased ATGL expression, HSL expression or both along with elevations of other markers of lipolysis, such as non-esterified fatty acids (NEFA; free fatty acids), glycerol and circulating catecholamines (Harvey et al. 2018; Xu et al. 2009; Djurhuus et al. 2004, 2002; Kršek et al. 2005; Hochberg, Harvey, et al. 2015). However, the exact mechanisms leading to glucocorticoid activation of these enzymes, or their relative importance are yet to be fully understood. One group has proposed that *Pde3b*, encoding a phosphodiesterase responsible for inactivation of cAMP, a key player in early activation of lipolysis, is down regulated (Xu et al. 2009), while others suggest that upregulation of β-adrenergic receptors is what is leading to glucocorticoid-induced lipolysis (Lacasa, Agli, and Giudicelli 1988); however, these findings have not been confirmed by further study. Elevated lipolysis can lead to ectopic lipid storage, such as is seen in non-alcoholic fatty liver disease and is associated with insulin resistance (Boden et al. 1995; Seppälä-Lindroos et al. 2002).

Glucocorticoid-Dependent Regulation of Muscle Atrophy

Glucocorticoids also promote catabolic effects in muscle. Muscle atrophy, also described as a decrease in muscle mass, is a consequence of excess glucocorticoids and when prolonged this can lead to muscle weakness and frailty (Blom et al. 2017; Barry and Gallagher 2003). It is

likely glucocorticoid-induced muscle atrophy occurs to provide substrates for hepatic gluconeogenesis, similar to what I described with lipolysis above. Indeed, proteolysis in the muscle leads to the generation of amino acids, substrates that feed into the gluconeogenic pathway. Glucocorticoids cause reduced muscle strength in children and adults, as well as enhanced leucine oxidation (Beaufrere et al. 1989; Blom et al. 2017; Barry and Gallagher 2003). Furthermore, there is evidence for glucocorticoid activation of muscle proteolysis (Price et al. 1994; Wing and Goldberg 1993; D. Dardevet et al. 1995) and reduced protein synthesis (Long, Wei, and Barrett 2001; Menconi et al. 2007). Mechanistically, glucocorticoids have been shown to upregulate genes involved in muscle atrophy, such as the atrogenes, *Fbxo32* and *Trim63*, encoding the proteins MuRF-1 and Atrogen-1 respectively (Hochberg, Harvey, et al. 2015) as well as the FoxO proteins (X Wang et al. 2017). With muscle health being pertinent to overall physical fitness, it is important to understand the impact of excess glucocorticoids in muscle.

Glucocorticoids Promote Adipogenesis

While glucocorticoid induce catabolic actions on muscle and adipose tissue resulting in the flux of amino and fatty acids to other tissues, they also play an essential role in the formation of new adipocytes, especially *in vitro* (Chapman, Knight, and Ringold 1985; Hartman et al. 2018). Adipogenesis is a complex process and while much is known regarding the genes involved, there is still much to unravel as to how these genes are regulated. PPARγ along with C/EBP proteins (α, β *and* δ) are key proteins in early adipogenesis and are termed the 'master regulators' of adipocyte differentiation. C/EBPδ and C/EBPβ are expressed first in the process and are responsible for the activation of PPARγ. PPARγ leads to C/EBPα expression and these two

proteins are involved in a positive feedback loop, see review (Lefterova and Lazar 2009). Upregulation of the aforementioned genes and proteins happens in the first several hours following the addition of adipogenic cocktail, which typically includes insulin, IBMX and dexamethasone. Though the requirement of glucocorticoids in the differentiation is evident, as removal of glucocorticoids or GR antagonism prevents lipid accumulation and differentiation (Chapman, Knight, and Ringold 1985; Hartman et al. 2018), the mechanisms governing this process remain unclear. Several gene targets have been proposed as potential GR targets during adipogenesis including *Cebpd, Nfil3, Dexras1 and Ccar1* (Y. Yang et al. 2017; Cha et al. 2013; Ou et al. 2014; Cao, Umek, and McKnight 1991) as well as multiple members of the Kruppel-like family proteins (Wu and Wang 2013). Adipogenesis is important in normal development and has shown to be beneficial for glucose homeostasis (S. Y. Kim et al. 2011); therefore, it is important to determine the exact role of glucocorticoids in this process.

The combined effects on lipolysis, proteolysis and adipogenesis cause a constellation of acute and chronic metabolic effects. The comorbidities and metabolic processes associated chronically elevated glucocorticoids, such as muscle wasting, insulin resistance, increased fat mass, lipolysis and NAFLD are well known; however, the underlying mechanisms that lead to these side effects have not been fully elucidated. Moreover, the effect of obesity on glucocorticoid-induced metabolic disease had never been evaluated.

Causes and physiological relevance of glucocorticoid excess

Systemic glucocorticoids levels are tightly regulated under normal conditions governed by endogenous signals including circadian rhythm and negative feedback, as mentioned above. However, there are a variety of conditions by which glucocorticoid regulation is disrupted and there is even evidence to suggest that disruption even at an early age can lead to impact a variety of psychological and metabolic outcomes later in life (Wilson et al. 2015; Chow et al. 2007; Van Dongen-Melman et al. 1995; Tangalakis et al. 1992; Khalife et al. 2013; De Blasio et al. 2007), indicating short-term disruption during key developmental windows can lead to long-term consequences. Glucocorticoid elevation can happen as a result of stress, this is evidenced by elevated cortisol levels individuals who feel helpless (Müller 2011) or exposed to multiple other types of stress such as public speaking and achievement tasks (Nater et al. 2005; Kirschbaum, Wust, and Hellhammer 1992), and animal stress exposure studies indicate chronic social stress leads to elevations in cortisol and possibly influences CRH binding (Jöhren, Flügge, and Fuchs 1994; Flügge 1995), which can lead to disruptions in normal circadian rhythm.

Conditions in which there is excessive glucocorticoid levels fall under the diagnosis of Cushing's syndrome. Cushing's syndrome is the umbrella term used to describe the symptoms arising from prolonged glucocorticoid elevations and encompasses a multitude of conditions including ectopic cortisol-producing tumors, Cushing's disease and prescribed therapeutics. Cushing's disease is a caused by a pituitary tumor that constitutively secretes ACTH, thereby overriding the negative feedback system and ultimately resulting in a constant overproduction of cortisol.

Glucocorticoids are frequently medically prescribed to treat many serious autoimmune and inflammatory conditions, such asthma, cancer, COPD, lupus and rheumatoid arthritis, as they are potent anti-inflammatory agents. Commonly prescribed glucocorticoids include prednisone, prednisolone, cortisone, corticosterone, hydrocortisone, betamethasone and dexamethasone, to name a few. The main distinction among these drugs is that some activate the glucocorticoid and mineralocorticoid receptors; whereas others are specific to the glucocorticoid receptor. The majority of data provided here are following dexamethasone treatment, a potent synthetic glucocorticoid specific to the glucocorticoid receptor potentially limiting the relevance of these findings. Though Cushing's disease is rare (Lindholm et al. 2001), Cushing's syndrome due to medication is quite common with an estimated 1-3% of developed populations being prescribed glucocorticoids at any given time (Overman, Yeh, and Deal 2013; Fardet, Petersen, and Nazareth 2011; Hsiao et al. 2010; Laugesen et al. 2017). Individuals with Cushing's syndrome are prone to insulin resistance, non-alcoholic fatty liver disease (NAFLD), muscle wasting and hypertension, as well as increased fat and abnormal distribution of fat mass. It is important to note that the majority of the research surrounding side effects from chronically elevated glucocorticoids has been assessed in mostly lean populations, as synthetic glucocorticoids were developed and approved in the mid-1900s. Today, there is a growing epidemic of obesity, with glucocorticoids still being widely prescribed.

Glucocorticoid Excess as a Public Health Issue

Considering the variety of conditions requiring corticosteroid treatment, combined with the prevalence of chronic stress, there is a sizable number of individuals, young and old, lean and obese that are exposed to elevated glucocorticoids. Epidemiologic studies reviewing prevalence

of chronic stress and glucocorticoid use demonstrate the impact of excess glucocorticoids on public health. For example, a national representative study in 2006 reported that an estimated 6.4% of working Americans meet the criteria for Major Depressive Disorder (R. R. C. Kessler et al. 2006) with depression being shown to lead to elevated cortisol levels throughout the day in humans (Dienes, Hazel, and Hammen 2103), and 7.8% of Americans suffering from Post-Traumatic Stress Disorder (R. Kessler et al. 1995). Stress is a known stimulus of HPA axis leading to increased cortisol production, as such glucocorticoids are commonly referred to as stress-induced hormones. Stress is an interesting psychological condition as it can be brought on by any number of things and effects people in different ways, mentally and physically (Gradus 2017); as a result, stress levels among the general population are difficult to measure. However, there are several reports that highlight the significance of elevated stress levels across developed countries by highlighting the prevalence common stress disorders such as of PTSD, anxiety and depression (Stansfeld and Candy 2006; Wiegner et al. 2015; R. Kessler et al. 1995).

Cushing's syndrome encompasses a variety of conditions leading excess glucocorticoids. Cushing's disease is among the conditions of Cushing's syndrome, and results from a pituitary adenoma that secretes ACTH, leading to elevated cortisol production. Cushing's disease is quite rare, with an estimated annual incidence of 1.2–2.4 per million across inhabitants of Spain and Denmark (Lindholm et al. 2001; Etxabe and Vazquez 1994) and an estimated annual incidence of 6.9–7.6 cases per million in the US (Broder et al. 2015). Cortisol-secreting adrenal tumors are even less common with an estimated 0.2–0.6 cases per million (Lindholm et al. 2001). As mentioned above, though endogenous forms of Cushing's syndrome are rare, Cushing's syndrome brought on by exogenous glucocorticoid administration is quite common with an

estimated of 1-3% of US, UK and Danish populations being prescribed some form of corticosteroid treatment at any given time (Overman, Yeh, and Deal 2013; Fardet, Petersen, and Nazareth 2011; Hsiao et al. 2010; Laugesen et al. 2017).

Given the high prevalence of corticosteroid treatment, there are a couple of important factors to consider. The first is the rationale for which individuals being prescribed these drugs. Glucocorticoids are effective and potent anti-inflammatory drugs, as such they are prescribed to treat many inflammatory and autoimmune diseases, as well as cancer. To date, they are the best available and approved drugs for this type of treatment (Becker 2013). In this context, glucocorticoids are life-saving, or at least life-extending, to many disease populations, and will continue to be prescribed to considerably sick individuals. The second consideration is for the special populations being administered glucocorticoids, which include pregnant women, children, elderly individuals and people with obesity. There is considerably less data on the effects of chronically elevated glucocorticoids in these populations, but again the use is widespread.

Prenatal Glucocorticoid Exposure and Fetal Outcomes

Glucocorticoids are often prescribed to pregnant women when there is a high risk of preterm birth, as glucocorticoids are vital to development of the fetus. Indeed, maternal glucocorticoid treatment has been shown to prevent a variety of adverse events in in preterm infants such as respiratory distress syndrome (R. Ballard and Ballard 1976), as well as reduced morbidity and mortality risk in premature infants and other benefits to the fetus, see review (Braun et al. 2013). Though it is difficult to find reports of overall prevalence, one study monitored prescriptions

among pregnant women across multiple facilities in the US over a four-year period and found that out of the 150 thousand plus recorded births, 3.9% of these women were given some form of glucocorticoids (Andrade et al. 2004). Outside of maternal exposure to exogenous glucocorticoids, added stress during pregnancy can increase the mother's cortisol levels, which has been observed in cases of severe morning sickness and lead to undernutrition (Ünsel, Benian, and Tamer Erel 2004; Kauppila, Jarvinen, and Haapalahti 1976).

Though essential for at risk births, prenatal glucocorticoid exposure has been reported to have negative consequences on the fetus such as increased blood pressure (Tangalakis et al. 1992), psychological abnormalities (Khalife et al. 2013) and has been shown to alter global DNA methylation in fetus that persists to further generations in guinea pigs (Crudo et al. 2012); though the implications for DNA methylation findings are not fully understood at this time. Metabolically speaking, maternal exposure is associated with impaired beta cell development and insulin content (Blondeau et al. 2001) and as well as glucose homeostasis disturbances (De Blasio et al. 2007; de Vries et al. 2007) in animals. Furthermore, one study in Denmark reported a 20% increase in Type 1 diabetes and a 51% increase in Type 2 diabetes in human offspring from prenatally-exposed mothers when compared to unexposed mothers (Greene et al. 2013).

Glucocorticoid Exposure in and Children

Glucocorticoid treatment is common among children with juvenile inflammatory arthritis (JIA) (Hansen et al. 2014), asthma (Arabkhazaeli et al. 2016) and cancer (Pufall et al. 2015). Though childhood cancer and JIA are considered rare in the US, with an estimated 10,270 new diagnoses

in 2017 for cancer (Siegel, Miller, and Ahmedin 2017) and an estimated 11.7 cases per 100,000/yr for JIA (Peterson et al. 1996), asthma is more common with an estimated childhood prevalence of 8.3% (CDC 2016). Previous studies have also indicated potential increased risk of adult obesity in children exposed to glucocorticoids (Aljebab, Choonara, and Conroy 2017), though other studies have not found this to be the case (Belle et al. 2018). These conflicting reports are likely due to all the confounding variables associated with childhood cancer aside from glucocorticoid treatment. In Chapter 4, obesity and other metabolic outcomes will be assessed in adult mice following juvenile dexamethasone treatment to expand on these human findings. Other assessments of body composition changes due to childhood glucocorticoid exposure include those investigating outcomes in bone and muscle. There are data that show children on chronic glucocorticoid treatment have lower bone mineral density and are at a higher risk for fractures compared to healthy controls (Hansen et al. 2014). Interestingly, glucocorticoids have been shown to improve muscle strength and function in children with muscular dystrophy, though the mechanism for this is unclear, it is suggested that this is partially due to the anti-inflammatory effects of the drug (Petnikota et al. 2016); therefore, it is unclear whether the same would be true of healthy children. Analogous to prenatal treatment, glucocorticoid treatment in children often leads to hypertension (Kamdem et al. 2008; Magiakou 1997); however, in the case of pediatric Cushing's patients this was reversed in as little as three months following tumor removal. Moreover, hyperglycemia and insulin resistance are common occurrence following childhood glucocorticoid exposure (Aljebab, Choonara, and Conroy 2017; Chow et al. 2013). Glucose homeostasis will also be assessed in Chapter 4 to determine if this finding is altered in adult mice following juvenile dexamethasone exposure.

Prevalence and Implications of Excess Glucocorticoids in the Elderly

Given the evidence for glucocorticoid-induced muscle atrophy, the contribution to metabolic disease and the immunosuppressive nature of these drugs, elevated glucocorticoids may have the largest influence on geriatric health. Elderly individuals are commonly prescribed glucocorticoids for issues such as COPD (Fardet, Petersen, and Nazareth 2011) with close to roughly 10% of COPD-diagnosed elderly individuals in the US (Akinbami LJ 2011), glucocorticoids are also commonly prescribed for other issues common to elderly individuals such as inflammatory bowel disease (Parian and Ha 2015). To put this into perspective, the prevalence for glucocorticoid use are highest for elderly populations with an estimated 2.7% of elderly women and 3.5% of elderly men in the US reported use of glucocorticoids, and an alarming 10% of the elderly population of Denmark reported use (Overman, Yeh, and Deal 2013; Laugesen et al. 2017). Not surprisingly, several studies of glucocorticoid use show evidence of association with osteoporosis, hypertension and diabetes in this population (S. Y. Kim et al. 2011; Ben Dhaou et al. 2012; Baldwin and Apel 2013). The added risk of osteoporosis and fractures (Van Staa et al. 2000) is arguably of the highest concern with this population being prone to falls and are known to have increased risk for sarcopenia (Dutta and Suppl 1997). Furthermore, as I have previously shown, glucocorticoid-induced muscle loss is greater in obesity and there is an increase of sarcopenic obesity in the elderly population (Johnson Stoklossa et al. 2017) potentially putting these individuals at an even greater risk of overall frailty.

Prevalence of Obesity and Glucocorticoid Use

As previously mentioned, obesity is a major problem in the US, and is becoming a worldwide epidemic. Most recent reports of estimate that 13% of adults have obesity and 39% of adults being overweight (WHO 2016); in addition, it is reported that over one-third of the geriatric population (ages 65 and over) has obesity (CDC 2012). Furthermore, childhood obesity is rising rapidly with a reported 18.5% of children ages 2-19 having obesity (Hales et al. 2017). Given the prevalence of obesity, combined with that of glucocorticoid use in developed countries across all ages, it is likely that many individuals are exposed to both. This may be exacerbated by local activation of cortisol in obesity (Livingstone et al. 2016). The combination of obesity and excess glucocorticoids is understudied; however, given my findings presented in Chapter 2, it appears that glucocorticoids in the presence of obesity is quite detrimental to metabolic health in both mice and humans. In support of this, Riddell and colleagues have reported that providing HFD and glucocorticoids in concert to young and adult rats, prior to the onset of obesity also resulted in hyperglycemia, insulin resistance and NAFLD (D'souza et al. 2012; Beaudry et al. 2013; Shpilberg et al. 2012). Future studies should investigate the effect of genetic obesity in concert with excess glucocorticoids to determine whether these findings are more attributable to diet or obesity status.

The literature on glucocorticoid exposure in the populations presented above is likely influenced by multiple confounders including reason for treatment. However, it is apparent that there is a sufficient number of individuals exposed to excess glucocorticoids and much evidence to suggest that these drugs have a significant impact on overall health. Therefore, excess glucocorticoids may pose a threat to public health, warranting further exploration with controlled study design to

account for the actual effects of glucocorticoids in individuals of all ages. We will explore the effects of obesity on glucocorticoid-induced metabolic disease in humans and mice in Chapter 2.

This dissertation describes my work to evaluate the underlying mechanisms that contribute to the observed glucocorticoid-induced metabolic dysfunction, as well as the effect of obesity on these outcomes. Here I evaluate chronically elevated glucocorticoids in humans, cell and rodent models examining the direct and indirect effects of glucocorticoids on metabolism. Specifically, in Chapter 2, using novel mouse models of Cushing's, I highlight the metabolic consequences of elevated glucocorticoids and make the case that glucocorticoid-induced lipolysis, insulin resistance and NAFLD are exacerbated in the presence of obesity. In Chapter 3, I evaluate the role of glucocorticoid signaling in adipocytes including the induction of adipogenesis, as well as the contribution of adipocyte GR to glucocorticoid-induced metabolic disease. In Chapter 4, I describe studies related to the involvement of glucocorticoids on muscle atrophy and glucose homeostasis in a novel juvenile mouse model and in adult mice, as well as the modifying effects of obesity on muscle degradation in adult mice. Taken together, these data have provided novel insights into the underlying metabolic processes and molecular mechanisms leading to glucocorticoid induced insulin resistance and fatty liver disease, as well as the modifying effects of obesity.

Chapter 2-Glucocorticoid-induced metabolic disturbances are exacerbated in obesity

Introduction

The data described here are partially adapted from (Harvey et al. 2018; Hochberg, Harvey, et al. 2015). Glucocorticoids are important for proper glucose homeostasis during stress or fasting, but can lead to symptoms similar to those seen in metabolic syndrome if elevated for prolonged durations. Cushing's syndrome encompasses a variety of conditions which manifest in response to chronically elevated levels of glucocorticoids, including exogenous corticosteroid treatment as well as endogenous overproduction of cortisol, and is often associated with changes in adipose mass and distribution, non-alcoholic fatty liver disease (NAFLD) and impaired glucose tolerance (Paredes and Ribeiro 2014). Cushing's disease, or persistently high circulating levels of cortisol secondary to a pituitary adenoma, leads to significant truncal obesity and diabetes (Cushing 1932). Obesity and diabetes are major factors in morbidity and mortality in Cushing's disease (Ntali, Grossman, and Karavitaki 2015). Cushing's disease is very rare, with an incidence of 1.2–2.4 per million (Lindholm et al. 2001), but iatrogenic Cushing's syndrome, caused by chronic glucocorticoid treatment, is very common and leads to similar clinical manifestations. It is estimated that at any given time 1-3% of the US, UK and Danish populations are prescribed exogenous corticosteroids, which may increase their risk for developing the associated metabolic complications (Overman, Yeh, and Deal 2013; Fardet, Petersen, and Nazareth 2011; Hsiao et al. 2010; Laugesen et al. 2017).

Numerous studies have shown that glucocorticoids have profound effects on adipose tissue metabolism, including promotion of adipocyte differentiation (Hauner, Schmid, and Pfeiffer 1987) and induction of lipolysis and lipogenesis (Samra et al. 1998; Campbell et al. 2011; Divertie, Jensen, and Miles 1991; Kršek et al. 2006). Glucocorticoids, through binding to the glucocorticoid receptor, exert transcriptional induction and repression of numerous genes (Reddy et al. 2009; Surjit et al. 2011). Despite the widespread chronic glucocorticoid exposure, there have been no human *in vivo* studies on global gene expression changes in adipose tissue in response to long-term exposure to glucocorticoids.

Similar to Cushing's, obesity is accompanied by a multitude of metabolic disturbances, such as insulin resistance and NAFLD, and is a worldwide epidemic (The GBD 2015 Obesity Collaborators 2017). Comparing the high rates of medically prescribed corticosteroids with the prevalence of overweight and obesity in developed countries, the combination of obesity and glucocorticoid excess may be present in many individuals. Given the similar co-morbidities associated with obesity and chronically elevated glucocorticoids, we hypothesized that the combinations of these two conditions would lead to worse metabolic outcomes than either of them alone. This is supported by studies in rats showing that corticosterone and high-fat diets combine to cause worsened insulin resistance and non-alcoholic fatty liver disease (Beaudry et al. 2013; Shpilberg et al. 2012).

To study the effect of excess endogenous glucocorticoids on adipose tissue, we used RNA sequencing of adipose tissue biopsies from Cushing's disease patients and controls with non-secreting adenomas. We found a distinctive pattern of changes in many transcripts that are highly

associated with Cushing's disease. Many of these genes explain previously observed metabolic effects of excess glucocorticoids described *in vitro*, in both animal models and in humans. These include enhanced fatty acid and triglyceride biosynthesis, protein degradation, activation of glycolysis and reductions in immune responses. We were able to confirm many of these findings by treating mice with dexamethasone for several weeks.

To investigate the modifying effects of obesity on glucocorticoid-induced metabolic disease, we stratified the patients based on their BMI and found exacerbated effects of joint obesity and Cushing's disease. To evaluate this further, I developed a novel mouse model system with diet-induced obesity followed by glucocorticoid exposure. Using this system, I measured a variety of metabolic outcomes. There is an array of physiological changes that occur as a result of elevated glucocorticoids including increased fat mass (Hochberg, Harvey, et al. 2015; Abad et al. 2001; Geer et al. 2010), NAFLD (Shpilberg et al. 2012) and increased lipolysis (Djurhuus et al. 2004; Kršek et al. 2005; Djurhuus et al. 2002), all of which have been associated with decreased insulin sensitivity (Zhang et al. 2015; Boden et al. 1995; Seppälä-Lindroos et al. 2002).

Recent tissue-specific knockouts of glucocorticoid signaling mediators had implicated adipose tissue as a central node linking glucocorticoid action and lipolysis to systemic insulin resistance and NAFLD (Mueller et al. 2017; Shen et al. 2017; S. A. Morgan et al. 2014; Y. Wang et al. 2014). Here I present the finding that chronically elevated glucocorticoids, via dexamethasone treatment, in the presence of diet-induced obesity have synergistic effects on lipolysis, insulin resistance and fatty liver disease. Obese dexamethasone-treated mice have reduced fat mass compared to all other groups, yet have hyperglycemia and severe insulin resistance. Therefore, I

hypothesized that glucocorticoid-induced adipocyte lipolysis drives insulin resistance in obese animals.

Methods

Patient Recruitment

The study was approved by the institutional review board of the University of Michigan Medical System. Written informed consent was obtained from all patients. Patients were recruited consecutively from those undergoing transsphenoidal adenomectomy at the University of Michigan for Cushing's disease or non-functioning pituitary adenoma over a 12-month period. Exclusion criteria were age <18, current hormone treatment including glucocorticoids, malignancy, inflammatory disease, diabetes type 1 and established pituitary hormone deficiencies. For each patient, a data sheet was completed including, age, sex, anthropometric measurements, diagnosis of hypertension, diabetes, results of blood tests and medications. Fasting blood samples were assayed for glucose (Siemens Advia 1800) and insulin (Life Technologies) as instructed by the manufacturers. Patient recruitment and tissue collection was co-ordinated by my collaborator Dr. Irit Hochberg.

Subcutaneous Fat Biopsy

During the course of pituitary surgery, a routine subcutaneous fat graft for sealing the surgical field is taken immediately after anesthesia, but before glucocorticoid treatment. Approximately 500 mg of this fat graft was used in this study with ~100 mg fresh adipose tissue was utilized for

ex vivo lipolysis assay and ~200 mg snap frozen in liquid nitrogen and stored at -80 °C for RNA preparation and ceramide analysis.

Human Transcriptomic Analysis

Total RNA was extracted from adipose tissue using the RNEasy kit (Qiagen) and its quality was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies). At the University of Michigan DNA Sequencing Core, cDNA libraries from polyA mRNA were prepared using TruSeq cDNA synthesis kit and sequenced using a HiSeq 2000 (Illumina). Samples were run on 2 lanes of a HiSeq 2000 (Illumina) generating 8,612,682 to 16,469,501 single-ended 50 bp reads per sample. These were aligned to the human genome (Enembl GRCh37.74, Genbank Assembly ID GCA_000001405.14) using TopHat version 2.0.10 (D. Kim et al. 2013), Bowtie 2 version 2.1.0 (Langmead and Salzberg 2012) and Samtools version 0.1.18. Reads were mapped to known genes using HTseq (Anders, Pyl, and Huber 2014). Gene expression was analyzed using DESeq2 version 1.2.10 (Love, Huber, and Anders 2014). These subjects corresponded to the patients described in Table 2, with the exception of subjects 29 and 31 (both Cushing's disease patients), which had clinical data but no RNAseq data. RNAseq analyses were performed by my collaborator Dr. Quynh Tran.

Animal Procedures

Twenty-four C57BL/6 adult male mice were purchased from The Jackson Laboratory (stock #000664) at nine weeks of age. Following a one-week acclimation period, mice were either

treated with 1 mg/kg/day of dexamethasone (Sigma-Aldrich) in their drinking water (N=12) or used as controls (N=12). All animals were on a light dark cycle of 12/12 h and housed at 22°C. Following a week of acclimation, mice were placed on diets or treated with dexamethasone as described in the figure legends. Mice were treated with vehicle (water) or approximately 1mg/kg/d of water-soluble dexamethasone (Sigma-Aldrich; catalog #2915), a synthetic glucocorticoid, dissolved in their drinking water for 12 weeks. Additional cohorts of mice used in these experiments either remained on a standard diet (normal chow diet; NCD; 5L0D LabDiet; 13% fat; 57% carbohydrate; 30% protein) or were provided a high fat diet (45% fat from lard; 35% carbohydrate mix of starch, maltodextrin and sucrose; 20% protein from casein; cat# D12451) for either 8 or 12 weeks followed by dexamethasone treatment. Mice were group housed with four mice per cage and food consumption was measured weekly by weight reductions per cage and calculated to reflect estimated intake of each mouse per day in a given cage. Mice remained on their respective diets for the duration of the study. All mice were provided with access to food and water *ad libitum* throughout the study, unless otherwise noted.

Water intake was measured weekly to determine the concentrations of dexamethasone consumed per cage. Average concentration per mouse was estimated by accounting for number of mice in the cage. For the longer, six-week dexamethasone treatments, 16 HFD-fed, dexamethasone-treated mice appeared ill and were euthanized and thus removed from all analyses once symptoms were noticed. Symptoms included lethargy, weight loss and evidence of pancreatitis in some of the mice. Animal body weight and composition was determined weekly using a digital scale and EchoMRI 2100, respectively. Body weight quickly stabilized following removal from the CLAMS in both groups. At the end of treatment, all cohorts of mice were

fasted for 16 h beginning a ZT10, dexamethasone water was not removed during this time, and euthanized by cervical dislocation after isoflurane anesthesia at ZT3 of the following day. Immediately following euthanasia, mice were dissected and the right inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) depots were carefully removed and weighed. Adipose tissues, along with a section of the left lateral lobe of the liver were snap frozen in liquid nitrogen for later analysis. Small pieces of tissues were fixed in 10% phosphate-buffered formalin for histology. Animal procedures were approved by the University of Tennessee Health Science Center and University of Michigan Institutional Animal Care and Use Committees.

Determination of Serum Dexamethasone

Serum from 16-hour fasted lean and obese mice following six weeks of dexamethasone treatment was acquired prior to euthanizing at the end of the study and sent to the University of Michigan Pharmacokinetic and Mass Spectrometry Core for LC-MS analysis of dexamethasone concentration. Dexamethasone standard was used to make a calibration curve from 2.5 to 100 ng/mL. A separate weighing of dexamethasone was used to make quality control standards at 3 and 30 ng/mL. Quality control standards were run in triplicate before and during sample analysis. For each calibration standard and quality control standard, 10 μL of blank plasma, 10 μL of calibration or QC standard, and 40 μL of internal standard were mixed in a 96-well plate. Each analytical sample was prepared by mixing 10 μL mouse plasma, 10 μL acetonitrile and 40 μL internal standard into a well of a 96-well plate. Some samples were below 10 μL in volume. In these cases, the volume collected was diluted to 10 μL and prepared in the same manner as the

other samples. The plate was mixed at 1000 rpm for 5 min, then centrifuged at 3500 rpm for 10 min. Four microliters of supernatant were injected for analysis onto a Waters Xevo TQD triple quadrupole UPLC mass spectrometer for analysis.

Insulin Tolerance Tests and Hyperinsulinemic Euglycemic Clamp Experiments

Insulin responsiveness was assessed via an insulin tolerance test (ITT). Following a six-hour fast beginning at ZT1, mice were given an intraperitoneal (IP) injection of insulin (Humulin R, Lilly) as described in figure legends. Blood was collected from the tail at basal and 15-minute intervals post-injection and glucose was determined using a One Touch Ultra Glucometer (Lifescan).

For the hyperinsulinemic euglycemic clamp experiments, done by the University of Michigan Metabolism, Bariatric Surgery and Behavior Core under the direction of Dr. Nathan Qi and Melanie Schmitt, C57BL/6J adult (70d) male mice were fed HFD for eight weeks and treated with dexamethasone in their drinking water for three weeks or regular drinking water. Animals were anesthetized with an IP injection of sodium pentobarbital (50–60 mg/kg). Indwelling catheters were inserted into the right jugular vein and the right carotid artery, respectively. The free ends of catheters were tunneled subcutaneously and exteriorized at the back of the neck via a stainless-steel tubing connector (coated with medical silicone) that was fixed subcutaneously upon closure of the incision. Animals with healthy appearance, normal activity, and weight regain to or above 90% of their pre-surgery levels were used for the study. Experiments were carried out in conscious and unrestrained animals using techniques described previously

(McGuinness et al. 2009; Ayala et al. 2006; Halseth et al. 1999). Briefly, the primed (1.0 uCi)-continuous infusion (0.05 uCi/min and increased to 0.1 μCi/min at t = 0) of [3-^{3}H] glucose (50 μCi/ml in saline) was started at t = -120min. After a five-hour fast, the insulin clamp was initiated at t = 0, with a prime-continuous infusion (40 mU/kg bolus, followed by 8.0 mU/kg/min) of human insulin (Novo Nordisk). Euglycemia (120~130 mg/dL) was maintained during the clamp by measuring blood glucose every 10 min and infusing 50% glucose at variable rates, accordingly. Blood samples were collected from the right carotid artery at t = 80, 90, 100, and 120 min for determination of glucose specific activity. Blood insulin concentrations were determined from samples taken at t = -10 and 120 min. A bolus injection of [1-^{14}C]-2-deoxyglucose ([^{14}C]2DG; PerkinElmer) (10 μCi) was given at t = 120 min. Blood samples were taken at 2, 5, 10, 15, and 25 min after the injection for determination of plasma [^{14}C]2DG radioactivity. At the end of the experiment, animals were anesthetized with an intravenous injection of sodium pentobarbital and tissues were collected and immediately frozen in liquid nitrogen for later analysis of tissue [1-^{14}C]-2-deoxyglucose phosphate ([^{14}C]2DGP) radioactivity. Blood glucose was measured using an Accu-Chek glucometer (Roche, Germany). Plasma insulin was measured using the Linco rat/mouse insulin ELISA kits. For determination of plasma radioactivity of [3-^{3}H]glucose and [1-^{14}C]2DG, plasma samples were deproteinized with $ZnSO_4$ and $Ba(OH)_2$ and counted using a Liquid Scintillation Counter (Beckman Coulter LS6500 Multi-purpose Scintillation Counter). Glucose turnover rate, hepatic glucose production and tissue glucose uptake were calculated as described elsewhere (Kraegen et al. 1985; Ayala et al. 2006; Halseth et al. 1999).

Serum Glycerol and Fatty Acid Determination

Following 12 weeks of dexamethasone treatment, 22-week-old *ad libitum* chow fed C57BL/6J male mice were anesthetized with isoflurane and blood was collected into heparin-coated capillary tubes via retro orbital bleed both prior to and 15 minutes following intraperitoneal injection of 10mg/kg isoproterenol (Sigma-Aldrich; catalog #I6504-1G) in Dulbecco's phosphate-buffered saline (Thermo Fisher; catalog #BW17512F1). Serum from these mice, as well as from a cohort of 28-week old mice on either HFD or chow, six-weeks post-dexamethasone treatment was collected following an overnight fast beginning at ZT10. For the human samples 25 mg pieces of adipose tissue were pre-incubated for 15 minutes in KRBH buffer (sigma) at 37 °C and then incubated for 1 hour at 37 °C in 300 ml KRBH in duplicate. Glycerol was assessed via Serum Triglyceride Determination Kit (Sigma-Aldrich; catalog #TR0100-1KT) and fatty acids were quantified using the HR Series NEFA-HR(2) kit (Wako Diagnostics; catalog #276-76491), in accordance with manufacturer's guidelines.

Cell culture

3T3-L1 fibroblasts (pre-adipocytes; ATCC; authenticated via STRS analysis) were cultured in 10% newborn calf serum, Dulbecco's Modification of Eagle's Medium (DMEM; 4.5 g/L D-glucose; Fisher Scientific; catalog #11965118) with penicillin, streptomycin and glutamine (PSG) until confluence. Cells were switched to a differentiation cocktail at two days post confluence (250nM dexamethasone, 500 uM 3-isobutyl-1-methylxanthine and 1ug/mL insulin in 10% fetal bovine serum, in 4.5g/L glucose DMEM with PSG) for four days (Chiang S, Chang L 2006). Media was replaced with differentiation medium containing only insulin for an additional

three days. For the following three days, cells remained in media with no additional treatment. Cells used for these experiments were not cultured beyond 22 passages. To assess markers of lipolysis, cells remained in media and were treated with ethanol (vehicle) or 250nM dexamethasone for five days before lysing, with dexamethasone media being refreshed on day three and extracted on day five.

Assessment of Triglyceride Content in Cells and Tissue

3T3-L1 cells were grown and treated as described above. At the end of the treatment period, cells were lysed in homogenization buffer (50 mM Tris pH 8, 5 mM EDTA, 30 mM Mannitol, protease inhibitor) and subjected to three freeze thaw cycles with liquid nitrogen, thawed at room temperature. Frozen liver tissue was homogenized using a TissueLyser II (Qiagen). Lipids were extracted using a chloroform:methanol (2:1) extraction. Triglyceride content was assessed using the Serum Triglyceride Determination Kit and absorbance was detected as described in (Lu et al. 2014).

mRNA Extraction and Analysis

Cells and tissues were lysed in TRIzol using the TissueLyser II, as decribed above, and RNA was extracted using a PureLink RNA kit (Life Technologies; catalog #12183025). cDNA was synthesized from 0.5-1ug of RNA using the High Capacity Reverse Transcription Kit (Life Technologies; catalog #4368813). Primers, cDNA and Power SYBR Green PCR Master Mix (Life Technologies; catalog #4368708) were combined in accordance with the manufacturer's

guidelines and quantitative real-time PCR (qPCR) was performed as previously described (Lu et al. 2014) using the QuantStudio 5 (Thermo Fisher Scientific). mRNA expression levels of all genes were normalized to *Actb* for adipose tissue and *Gapdh* for muscle tissue after confirming that these mRNAs are unaffected by dexamethasone or HFD treatment. Data were analyzed using the ΔΔCt method after evaluation of several reference genes. qPCR primer sequences are listed in Table 1. Statistical tests were performed as described below based on tests of normality and homoscedasticity, then p-values were adjusted for multiple comparisons based on the number of genes tested for each tissue across this manuscript.

Protein Extraction and Analysis

Cells and tissues were lysed in RIPA buffer (50 mM Tris, pH 7.4, 0.25% sodium deoxycholate, 1% NP40, 150 mM sodium chloride, 1 mM EDTA, 100 uM sodium orthovanadate, 5 mM sodium fluoride, 10 mM sodium pyrophosphate and 1x protease inhibitor), centrifuged at 14,000rpm for 10 minutes at 4°C. Lysates were heated with loading buffer at 85-95°C and proteins were separated by SDS-PAGE (Life Technologies) and transferred onto nitrocellulose membranes overnight at room temperature. Membranes were blotted at room temperature using anti-adipose triglyceride lipase antibodies (ATGL; molecular weight 54; Cell Signaling Technologies; catalog #30A4, RRID:AB_2167953) and antibodies against hormone-sensitive lipase (HSL; molecular weight 81; Cell Signaling Technologies; catalog #4107, RRID:AB_2296900) and its PKA phosphorylation sites on serine 563 and 660 (Cell Signaling Technologies; catalog #4139, RRID:AB_2135495 and #4126, RRID:AB_490997, respectively). Antibody complexes were detected by anti-mouse and anti-rabbit fluorescent conjugated

antibodies (Invitrogen) and visualized using an Odyssey CLx image scanner. Blots were quantified using Image Studio software version 5.2 (LiCOR) and normalized to Revert Total Protein Stain (LiCOR; catalog #926-11011).

Histology

Tissues were fixed in 10% phosphate-buffered formalin for 24 hours and then stored in 70% ethanol until further processing. Tissues were dehydrated, embedded in paraffin and sent to the University of Michigan Comprehensive Cancer Center Tissue Core where they were processed and stained with hematoxylin and eosin (H&E) to assess cell morphology. Slides were imaged using the 10x objective of an Olympus iX18 inverted microscope and cellSense software.

Statistics

For the human data, descriptive statistics including means and standard errors were determined for clinical measurements. All statistical tests were performed using the R package (version 3.0.2, (R Core Team 2013)). Normality assumption was checked via Shapiro-Wilk test. Wilcoxon rank sum tests were used when data were not normally distributed. Welch's *t*-test was performed if the equal variance assumption was rejected by Levene's test (car package version 2.0-19), otherwise a Student's *t*-test was used. Longitudinal measurements such as body weight, food intake, body composition and insulin tolerance tests were analyzed via mixed linear models and a χ^2 test between models with and without dexamethasone treatment as a covariate. This used the lme4 package, version 1.1-7 (Bates et al. 2014). To correct for multiple hypotheses, p-

values were adjusted by the method of Benjamini and Hochberg (Benjamini and Hochberg 1995). Statistical significance in this study was defined as a p/q-value of less than 0.05. For RNAseq, the DESeq2 algorithm excludes genes with very high variance to improve statistical power (Love, Huber, and Anders 2014). The analysis we focused on in this manuscript was without adjustment for BMI or age and is presented in Supplementary Table 1 of (Hochberg, Harvey, et al. 2015). All data are presented as mean +/- standard error of the mean.

We used Gene Set Enrichment Analysis (GSEA v2.0.13 (Subramanian et al. 2005; Clark and Ma'ayan 2011)) to determine whether our rank-ordered gene list for the comparison of Cushing's disease versus control patients is enriched in genes from gene ontology, KEGG, transcription factor or microRNA target gene sets (MSigDB version 4.0). The gene list was ranked based on *t*-statistics and the statistical significance of the enrichment score was determined by performing 1000 phenotype permutations. Other settings for GSEA were left to the software defaults. GSEA results are summarized in Table 3.

For the mouse findings, all data are presented as mean +/- standard error of the mean. For animal studies, two-way ANOVA analyses were performed to test for significance of diet and dexamethasone treatment, as well as their interaction. Pairwise comparisons, normality and equal variance were tested using Shapiro-Wilk and Levene's tests, respectively. Pending those results, a Mann-Whitney, Welch's or Student's *t*-test were used. P-values below p=0.05 were considered significant. All statistical tests were performed using the R software package version 3.30. All code and raw data from this study are available through the Gene Expression Omnibus

(GSE66446) and at http://bridgeslab.github.io/CushingAcromegalyStudy (Hochberg, Tran, et al. 2015).

Results

Patient Characteristics

Clinical and metabolic measurements were obtained for five Cushing's disease patients and 11 control subjects, who were admitted with non-secreting adenomas. Patient characteristics are shown in Table 2. Cushing's disease patients were in general younger and had smaller tumors than the patients with non-secreting adenomas. In the Cushing's disease cohort there was a non-significant elevation in body weight (p=0.47), body mass index (BMI) (p=0.27) and abdominal circumference (p=0.07, Figure 1A), consistent with Cushing's disease patients having elevated fat mass and truncal obesity (Lamberts and Birkenhäger 1976).

Table 1: Primer sequences used for qPCR analyses.

Gene	Forward 5'-3' Sequence	Reverse 5'-3' Sequence
Acaca	GCTAAACCAGCACTCCCGAT	GTATCTGAGCTGACGGAGGC
Aco1	AACACCAGCAATCCATCCGT	GGTGACCACTCCACTTCCAG
Acsl1	GCCTCACTGCCCTTTTCTGA	GCAGAATTCATCTGTGCCATCC
Acss2	CGTTCTGTGGAGGAGCCAC	GGCATGCGGTTTTCCAGTAA
Actb	ATGTGGATCAGCAAGCAGGA	AAGGGTGTAAAACGCAGCTCA
Agpat2	CGTGTATGGCCTTCGCTTTG	TCCATGAGACCCATCATGTCC
Dgat2	AACACGCCCAAGAAAGGTGG	GTAGTCTCGGAAGTAGCGCC
Dhcr7	ATGGCTTCGAAATCCCAGCA	GAACCAGTCCACTTCCCAGG
Dhcr24	AGCTCCAGGACATCATCCCT	TACAGCTTGCGTAGCGTCTC
Fasn	GGAGGTGGTGATAGCCGGTA	TGGGTAATCCATAGAGCCCAG
Gapdh	CACTTGAAGGGTGGAGCCAA	ACCCATCACAAACATGGGGG

Gpam	AGCAAGTCCTGCGCTATCAT	CTCGTGTGGGTGATTGTGAC
Gpd1	GTGAGACGACCATCGGCTG	TTGGGTGTCTGCATCAGGT
Idh1	CTCAGAGCTCTCTTGGACCGA	CATCTCCTTGCATCTCCACCA
Ldhb	AAAGGCTACACCAACTGGGC	GCCGTACATTCCCTTCACCA
Mdh1	GGAACCCCAGAGGGAGAGTT	TGGGGAGGCCTTCAACAAAC
Me1	GGACCCGCATCTCAACAAG	TCGAAGTCAGAGTTCAGTCGT
Psmd1	TGCCAATCATGGTGGTGACA	ACACATCCTGACGTGCAGTT
Psmd8	ACGAGTGGAACCGGAAGAAC	CCGTGGTTGGCAGGAAATTG
Rplp0	GAAACTGCTGCCTCACATCCG	GCTGGCACAGTGACCTCACAC
Rplp13a	GCGGATGAATACCAACCCCT	CCTGGCCTCTCTTGGTCTTG
Scd1	CACTCGCCTACACCAACGG	GAACTGGAGATCTCTTGGAGC

We detected a non-significant elevation in HOMA-IR score (2.6 Fold, p=0.67 by Wilcoxon test, Figure 1B), driven largely by increases in fasting insulin levels (p=0.30). Three out of the five Cushing's disease patients had diabetes, while only 1 of the 11 controls had diabetes (p=0.03 via χ^2 test). These data are consistent with disrupted glucose homeostasis often seen in patients with Cushing's syndrome. We observed significant elevations in both ALT and AST in serum from Cushing's disease patients, indicative of liver disease (Figure 1C). To evaluate lipolysis in explants from these patients we measured glycerol release from isolated subcutaneous adipose tissue and found a 3.1-fold elevation in glycerol release from these tissues (p=0.049 via Student's *t*-test; Figure 1D). These data support previous studies which implicate elevated lipolysis (Kršek et al. 2006) and higher rates of non-alcoholic fatty liver disease in Cushing's disease patients (Rockall et al. 2003).

Table 2: Clinical characteristics of Cushing's disease and control patients. Data represents mean +/- standard error.

	Cushing's disease (n=5)	Controls (n=11)	p-value
Height (cm)	166 ± 4.3	169 ± 2.4	0.47
Tumor size (cm)	0.95 ± 0.3	1.96 ± 0.14	0.01
Age (years)	39.8 ± 4.5	63.4 ± 2.7	0.0003

Table 3: Summarized gene set enrichment analysis of pathways. Selected pathway enriched in subcutaneous adipose tissue from Cushing's disease patients via GSEA analysis. NES is the net enrichment score, asterisk indicates q<0.25. For a complete list see Supplementary Tables 2-3 of (Hochberg, Harvey, et al. 2015).

Pathway	Dataset	NES
M_PHASE_OF_MITOTIC_CELL_CYCLE	Gene Ontology	2.60*
KEGG_CITRATE_CYCLE_TCA_CYCLE	KEGG	2.41*
KEGG_BIOSYNTHESIS_OF_UNSATURATED_FATTY_ACIDS	KEGG	2.41*
REACTOME_TRIGLYCERIDE_BIOSYNTHESIS	Reactome	2.24*
PYRUVATE_METABOLISM	Gene Ontology	2.24*
KEGG_VALINE_LEUCONE_AND_ISOLEUCINE_DEGRADATION	KEGG	2.16*
STEROID_BIOSYNTHETIC_PROCESS	Gene Ontology	2.11*
KEGG_STARCH_AND_SUCROSE_METABOLISM	KEGG	2.08*
PROTEASOME_COMPLEX	Gene Ontology	1.78*

KEGG_ALLOGRAFT_REJECTION	KEGG	-1.87*
KEGG_BASAL_CELL_CARCINOMA	KEGG	-1.86*
KEGG_RIBOSOME	KEGG	-2.33*

Dexamethasone Treatment of Mice as a Model of Cushing's Syndrome

To validate the gene expression changes observed in human subjects, we treated C67BL/6J mice with dexamethasone, a synthetic glucocorticoid in their drinking water to mimic the systemic effects of cortisol overproduction. Dexamethasone-treated mice had an initial catabolic phase in which their body weight was rapidly reduced (Figure 2A) and at the end of the study this resulted in a 19.34% decrease in body weight ($p<0.001$), an effect that was primarily in due to a reduction in lean body mass, which will be discussed in Chapter 4. After

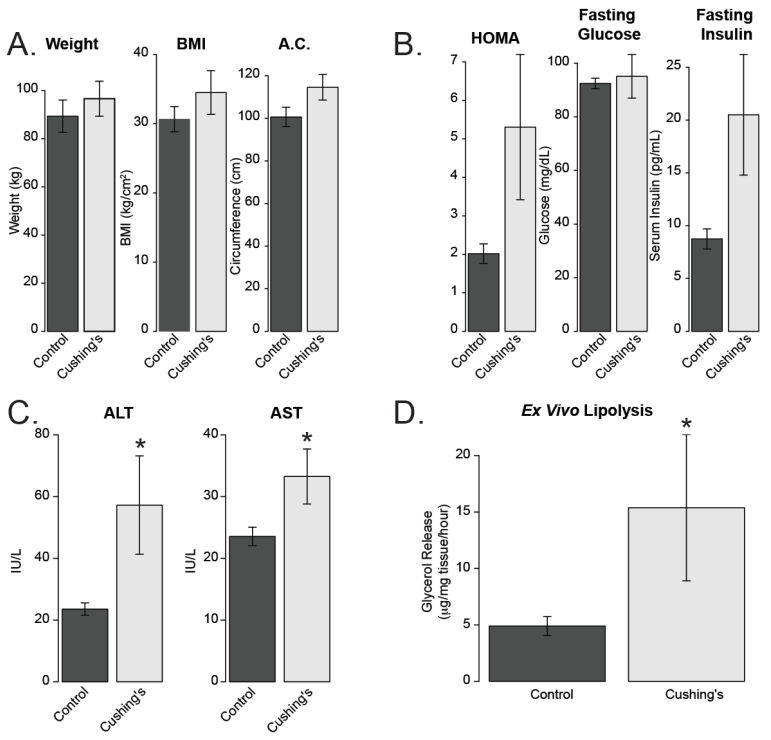

Figure 1: Metabolic characteristics of Cushing's disease patients. A) Morphometric data from control (non-secreting adenoma) and Cushing's disease subjects. A.C indicates abdominal circumference. B) HOMA-IR score, fasting insulin and fasting blood glucose from subjects. C) Liver enzymes from subjects D) Glycerol release from isolated subcutaneous adipose tissue. Asterisks indicates $p<0.05$.

approximately five weeks, we observed significant elevations in total fat mass (40.38% increase; p=0.008) and percent adiposity (71.59% increase; p<0.001) in the dexamethasone treated mice (Figure 2B-D), as well as significantly elevated inguinal white adipose tissue (iWAT; p=0.041) following 12 weeks of treatment when normalized to total body weight (Figure 2D). We did not detect a difference in food intake between the groups throughout the study that would account for the difference in fat mass (Figure 2E). To evaluate insulin sensitivity, we performed insulin tolerance tests (ITT) on these mice after 12 weeks of dexamethasone treatment and found that while they had reduced fasting glucose at this stage, they were resistant to insulin-induced reductions in blood glucose (Figure 2F).

Transcriptomic Analysis of Human Adipose Tissue from Cushing's Patients

To determine which genes and pathways are altered in adipose tissue in the human Cushing's disease subjects, we analyzed the transcriptome from subcutaneous adipose

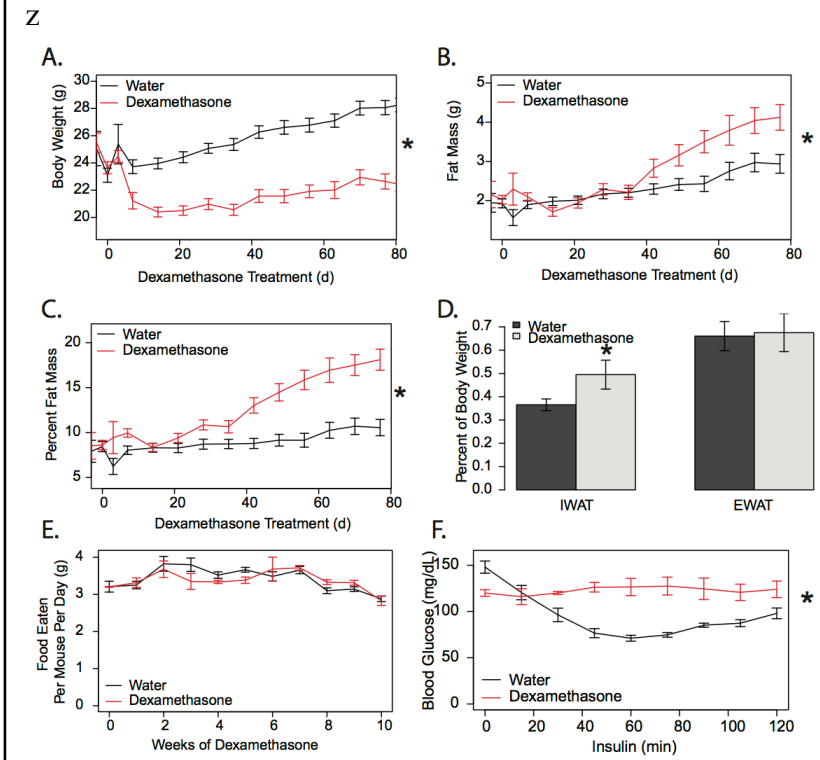

Figure 2: Dexamethasone treatment results in increased fat mass in mice and severe insulin resistance. Weekly body weight (A), fat mass (B), percent fat mass (C) inguinal (IWAT) and epididymal (EWAT) fat pad weights (D), average food consumption per mouse per day E) and ITT (F) from control dexamethasone treated mice. ITT was administered following a 6 hour fast, insulin (1 mU/g). Asterisks indicate p<0.05.

tissue mRNA from the five Cushing's disease patients and 11 controls. We identified 473 genes that had significantly different expression in Cushing's disease patients, of these 192 genes were expressed at a lower level and 281 at a higher level in the adipose tissue from the disease patients. These transcripts form a signature identifying transcriptional differences in adipose tissue in response to long-term exposure to glucocorticoids (Figure 3A).

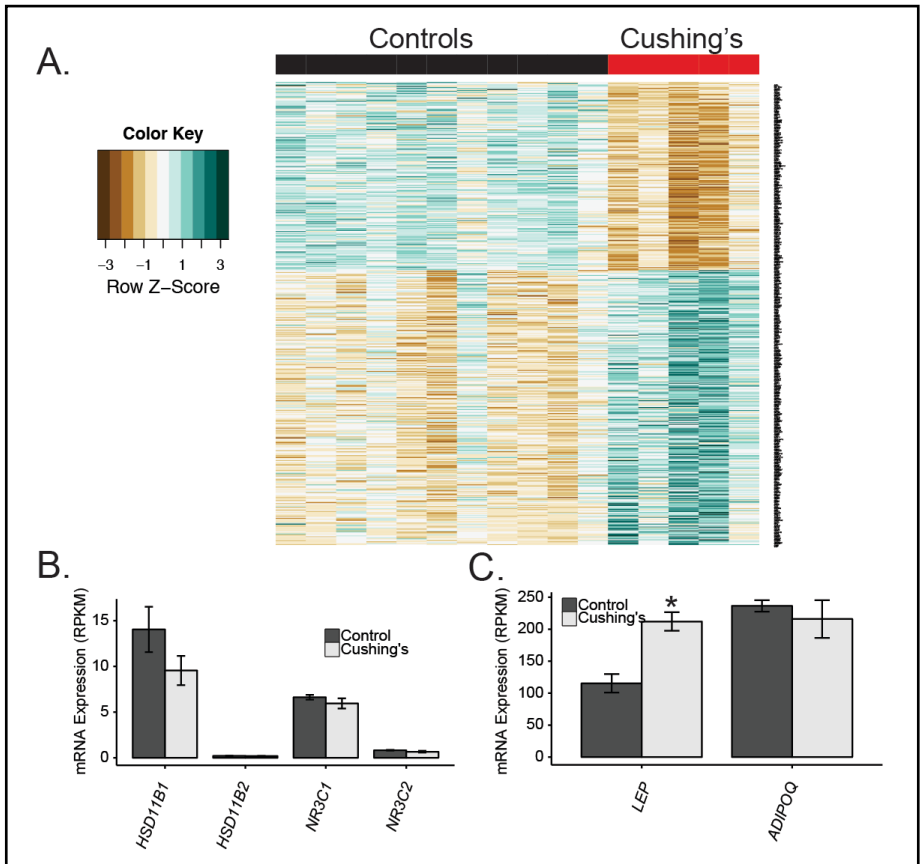

Figure 3: Differentially expressed transcripts in subcutaneous adipose tissue from Cushing's disease subjects. A) Heatmap of genes with significant differential expression. The bar on the top indicates control subjects (non-secreting adenoma; black) and Cushing's subjects (red). B) Genes involved in cortisol signaling. C) Leptin and Adiponectin mRNA levels. Asterisks indicate q<0.05.

To identify conserved pathways underlying these changes, a gene set enrichment analysis was performed on these data. As summarized in Table 3, we detected enrichment of genes in several categories involved in metabolism, including higher expression of gene sets involved in lipid biosynthesis, glucose metabolism, activation of amino acid degradation, protein degradation, and reductions in protein synthesis. We also observed reduced

expression of transcripts involved in immune function that will be discussed in subsequent sections.

We next evaluated the transcript levels of the glucocorticoid receptor (*NR3C1*) and the mineralocorticoid receptor (*NR3C2*) genes and observed no significant downregulation of these receptors at the mRNA level in Cushing's patients (Figure 3B). Another potential mechanism for negative feedback of glucocorticoid signaling is through the enzymatic activities of 11ß-HSD1/2 which control the local concentrations of cortisol in adipose tissues. We observed a non-significant reduction in *HSD11B1* mRNA levels (24% reduced, padj=0.49), potentially desensitizing adipose tissue to cortisol by reducing the conversion of cortisone to cortisol. Induction of leptin by glucocorticoids has been previously reported in human adipocytes (Halleux et al. 1998) and in human adipose tissue *in vivo* (Papaspyrou-Rao et al. 1997). We observed 1.8-fold higher level of Leptin (*LEP*) expression (padj=0.014) and non-significantly higher resistin (*RETN)* expression but no significant changes in adiponectin mRNA levels (*ADIPOQ*, padj=0.94; Figure 3C).

Lipogenesis Genes are Upregulated in Response to Elevated Glucocorticoids

Increased subcutaneous fat mass is a hallmark of Cushing's syndrome and could potentially be mediated through activation of adipogenesis or lipogenesis. Our transcriptomic data support the hypothesis that lipogenesis is activated in these tissues via transcriptional activation of genes involved in fatty acid synthesis and triglyceride synthesis. Key fatty acid synthesis transcripts were significantly elevated including *ACACA* (padj=0.035), *FASN* (padj=0.003)=, *ACSL1*

(padj=0.025), and *ELOVL5* (padj=0.016; Figure 4A). Desaturation of fatty acids is an essential aspect of *de novo* fatty acid synthesis, we observed elevations in all fatty acid desaturases *SCD* (padj<0.001), *FADS1* (not significant, NS), *FADS2* (NS) and *HSD17B12* (NS; Figure 4B) as well as triglyceride synthesis genes *GPAM* (padj=0.004), *DGAT1* (NS), *DGAT2* (padj=0.01),

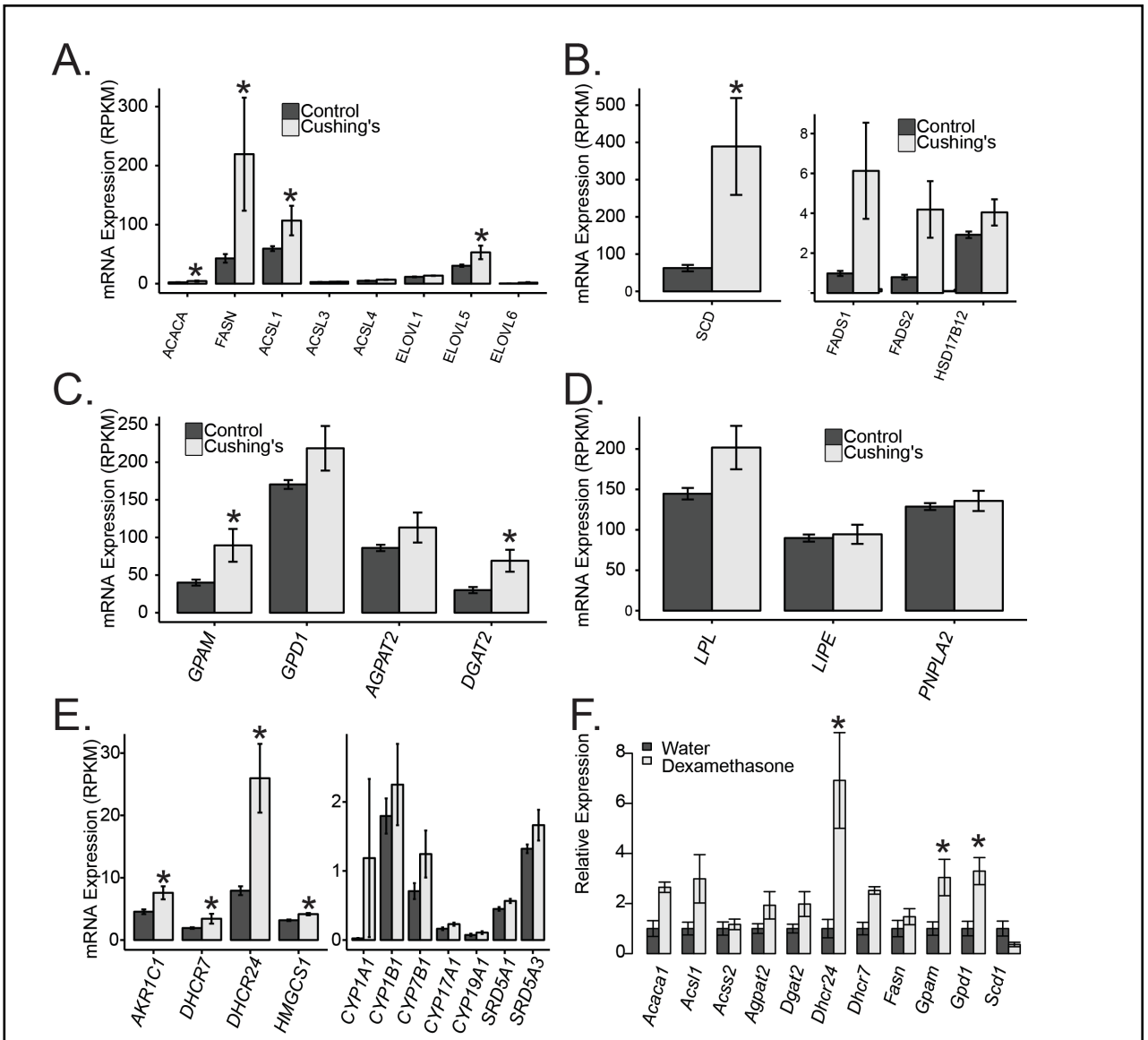

Figure 4: Elevated glucocorticoids result in elevated fatty acid and triglyceride synthesis genes. A) Fatty acid synthesis genes in Cushing's disease and control patients. B) Fatty acid desaturases in Cushing's disease patients. C) Triglyceride synthesis genes. D) Lipolysis genes. E) Steroid biogenesis genes. F) Evaluation of lipogenic genes in mouse subcutaneous adipose tissue. Asterisks for human transcripts indicate padj<0.05; mice p<0.05.

AGPAT2/3 (NS), and *GPD1* (NS) in subcutaneous adipose tissue from Cushing's disease patients (Figure 4C).

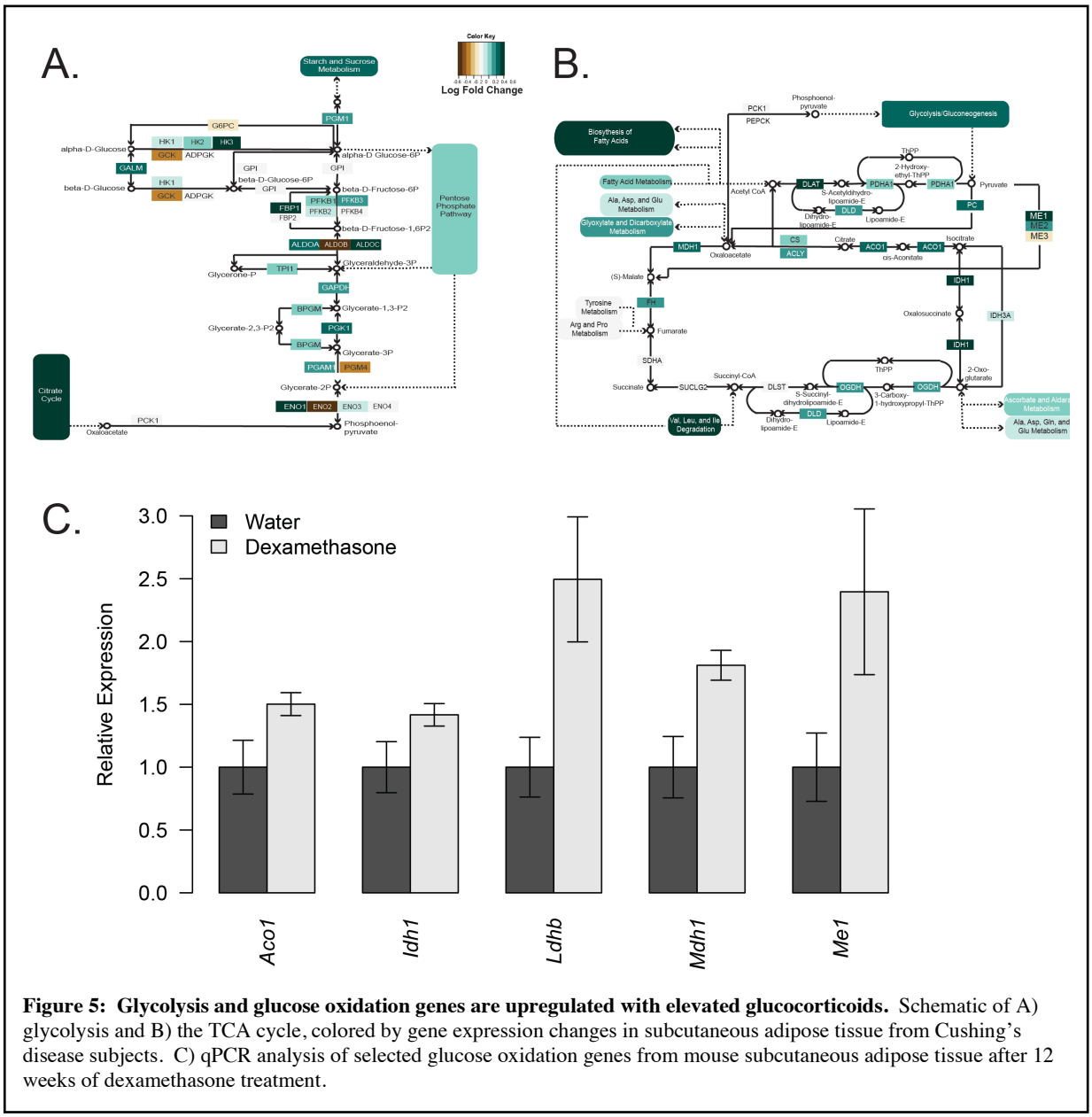

Figure 5: Glycolysis and glucose oxidation genes are upregulated with elevated glucocorticoids. Schematic of A) glycolysis and B) the TCA cycle, colored by gene expression changes in subcutaneous adipose tissue from Cushing's disease subjects. C) qPCR analysis of selected glucose oxidation genes from mouse subcutaneous adipose tissue after 12 weeks of dexamethasone treatment.

Despite increased lipid deposition and elevations of lipogenesis genes in Cushing's disease patients' adipose tissue, there have been several studies linking elevated glucocorticoids to increased lipolysis, potentially as a way to stimulate hepatic gluconeogenesis. In our patients,

we observed an increase in glycerol release in *ex vivo* explants of subcutaneous adipose tissue (Figure 1D), indicating elevated lipolysis. Among genes that may liberate fatty acids from triglycerides, Lipoprotein Lipase (*LPL*) was induced 1.45 fold (padj=0.055) in the Cushing's disease subjects, but neither Hormone Sensitive Lipase (*LIPE*) nor Adipose Triglyceride Lipase (*PNPLA2*) were significantly changed at the transcriptional level (Figure 4D). Additionally, we detected an elevation of Perilipin 4 (*PLIN4*) which is one of the proteins that coat intracellular lipid storage droplets and is important for lipolysis (induced 1.45-fold, padj=0.056, see Supplementary Table 1 of (Hochberg, Harvey, et al. 2015)).

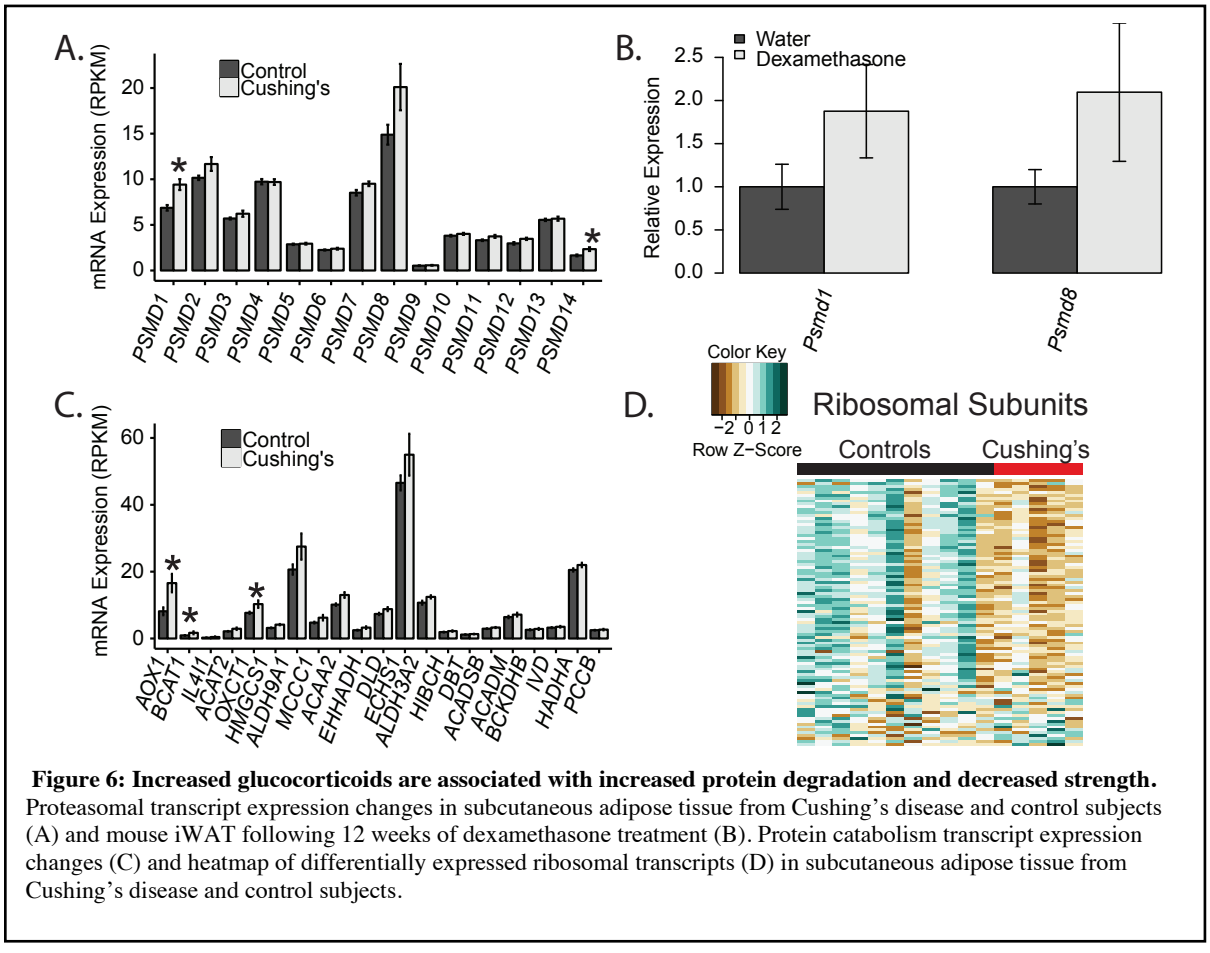

Figure 6: Increased glucocorticoids are associated with increased protein degradation and decreased strength. Proteasomal transcript expression changes in subcutaneous adipose tissue from Cushing's disease and control subjects (A) and mouse iWAT following 12 weeks of dexamethasone treatment (B). Protein catabolism transcript expression changes (C) and heatmap of differentially expressed ribosomal transcripts (D) in subcutaneous adipose tissue from Cushing's disease and control subjects.

Several genes that regulate steroid biogenesis were elevated in adipose tissue from Cushing's disease patients as described in Figure 4E. These include several cytochrome P450 family members, steroid reductases (*SRD5A1*, *SRD5A3;* NS), Aldo-keto reductase family 1 member C1 (*AKR1C1;* padj=0.012), 7- and 24-dehydrocholesterol reductases (*DHCR7,* padj=0.026; *DHCR24,* padj<0.001) and HMG-CoA synthase (*HMGCS1;* padj=0.002).

To examine whether lipogenesis genes are activated in the dexamethasone treated mice, we tested several lipogenic genes in subcutaneous adipose tissue, and observed general elevations in the lipid synthesis pathway *Fasn* (NS), *Gpam* (padj=0.047), *Gpd1*(padj=0.047), *Acss2* (NS), *Acs1*(NS), *Dgat* (NS), *Agpat2* (NS), *Dhcr7* (NS), *Dhcr24* (padj=0.047) and *Acaca1* (NS, padj=0.07; Figure 4F). In contrast to the human samples, did not observe an elevation in the mouse isoform of *SCD*, but saw instead a non-significant reduction in *Scd1* mRNA (padj=0.260).

Genes Controlling Glucose Oxidation Are Elevated in Cushing's Disease Patients

Several glucose metabolism genes, and specifically glycolysis and TCA cycle genes were expressed at higher levels in Cushing's disease patients (Figure 5). Strongly induced genes included *HK3* (NS), *FBP1* (NS), *ALDOC* (padj<0.001), *ENO1* (padj=0.004), *IDH1* (padj=0.036), *ME1* (padj<0.001) and *DLAT* (NS). Consistent with human adipose tissue findings, elevations in *Idh1* and *Me1* were also noted in mouse adipose tissue, along with other transcripts involved in glucose oxidation such as *Aco1, Ldhb* and *Mdh1;* however, these did not reach statistical significance (padj>0.05; Figure 5C).

The major glycogen synthesis transcripts were also induced, including *GYS2* (padj=0.004), *UGP2* (padj=0.01) and *GBE1* (padj=0.027). This agrees with biochemical studies which implicate glucocorticoid treatment in elevated hepatic and adipose tissue glycogenesis (Engel and Scott 1951; Baqué et al. 1996; Segal and Gonzalez Lopez 1963). The relevance of this effect in adipose tissue has not yet been explored.

Genes That Regulate Protein Catabolism are Upregulated in Adipose Tissue from Glucocorticoid Exposed Subjects

We found two major pathways of protein homeostasis altered in response to glucocorticoids.

In adipose tissue from Cushing's disease patients, we observed inductions of both the proteasomal pathways (via KEGG, net enrichment score 1.76, padj=0.01; Figure 6A), with Psmd1 and Psmd8 also observed to be elevated in mouse adipose tissue following dexamethasone treatment (Figure 6B), and genes involved in amino acid catabolism (Figure 6C) and a general downregulation of ribosomal genes (Figure 6D). Among the amino acid catabolism genes, *AOX1* (96% increase, padj=0.03), *OXCT1* (40% increase,

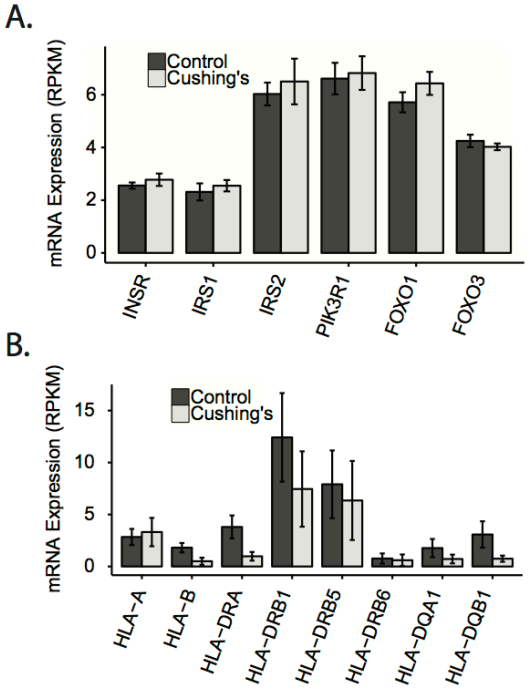

Figure 7: Expression of insulin signaling transcripts, ceramides and inflammatory transcripts in control vs. Cushing's disease subjects. A) Insulin signaling transcript expression levels. B) MHC complex transcript expression levels.