padj=0.04) and *BCAT1* (80% increase, p=0.048) were all significantly upregulated. Together these data support the hypothesis that protein catabolism and a reduction in protein synthesis also occur in adipose tissue in response to glucocorticoid exposure.

Genes Involved in Proximal Insulin Signaling are Unchanged in Adipose Tissue from Cushing's Disease Patients

As described in Figures 1B and 2F, we observed insulin resistance in concert with elevated glucocorticoid levels in both mice and humans. Several mechanisms have been proposed linking glucocorticoids to insulin sensitivity including elevated lipolysis. As shown in Figure 7A, There was a slightly higher expression of insulin pathway transcripts including *FOXO1*, insulin receptor (*INSR*), the insulin receptor substrates *IRS1* or *IRS2* and p85 regulatory subunit of phosphoinositide-3-kinase (*PIK3R1*), consistent with previous studies (Hazlehurst et al. 2013; Gathercole et al. 2007; Tomlinson et al. 2010), though in our hands none of these genes reached

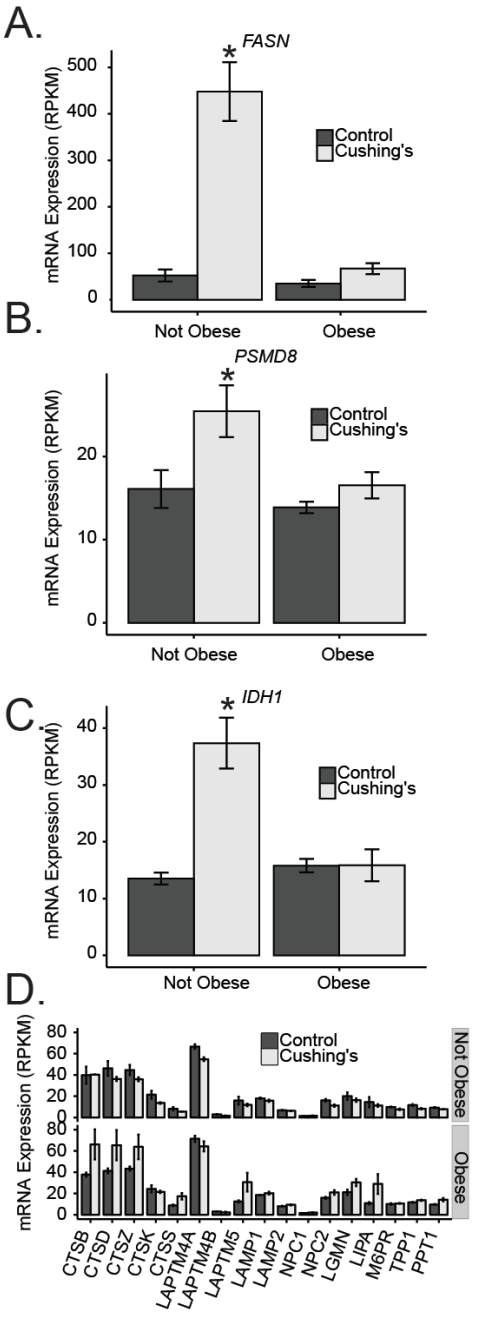

Figure 8: Obesity modifies transcript expression in Cushing's disease. FASN (A), PSMD8 (B), IDH1 (C), and lysosomal (D) transcripts in non-obese and obese Cushing's subjects. Asterisks indicate q<0.05.

statistical significance. The insulin pathway was generally expressed at significantly higher levels in the Cushing's disease patients compared to controls (KEGG pathway, net enrichment score 1.84, padj=0.006). These data do not support transcriptional downregulation of proximal insulin signaling genes as mediating insulin resistance in subcutaneous adipose tissue.

Inflammatory Gene Expression

Several pathways involved in immune function were downregulated in adipose tissue from Cushing's disease patients. This is consistent with the effects of cortisol in suppressing immune function. Adipose tissue leukocyte infiltration both relies on an intact immune system and also responds to changes in adiposity (Lumeng and Saltiel 2011). Among immune genes, we detected reductions in several genes that form the class II major histocompatibility complex, notably *HLA-DPB2, HLA-DRA, HLA-DRB9* and *HLA-DQA1* (NS for all; Figure 7B). These genes normally present antigens for T-cell recruitment. Consistent with this, we observed reductions in the mRNA of *IL32*, a hormone secreted by Natural Killer and T lymphocytes (Dinarello and Kim 2006). We also observed a downregulation in transcripts that are interferon gamma dependent. Together, these data support the hypothesis that the decreased T-cell activation observed with cortisol signaling also impacts adipose tissue.

Modifying Effect of Obesity on Glucocorticoid Responsiveness

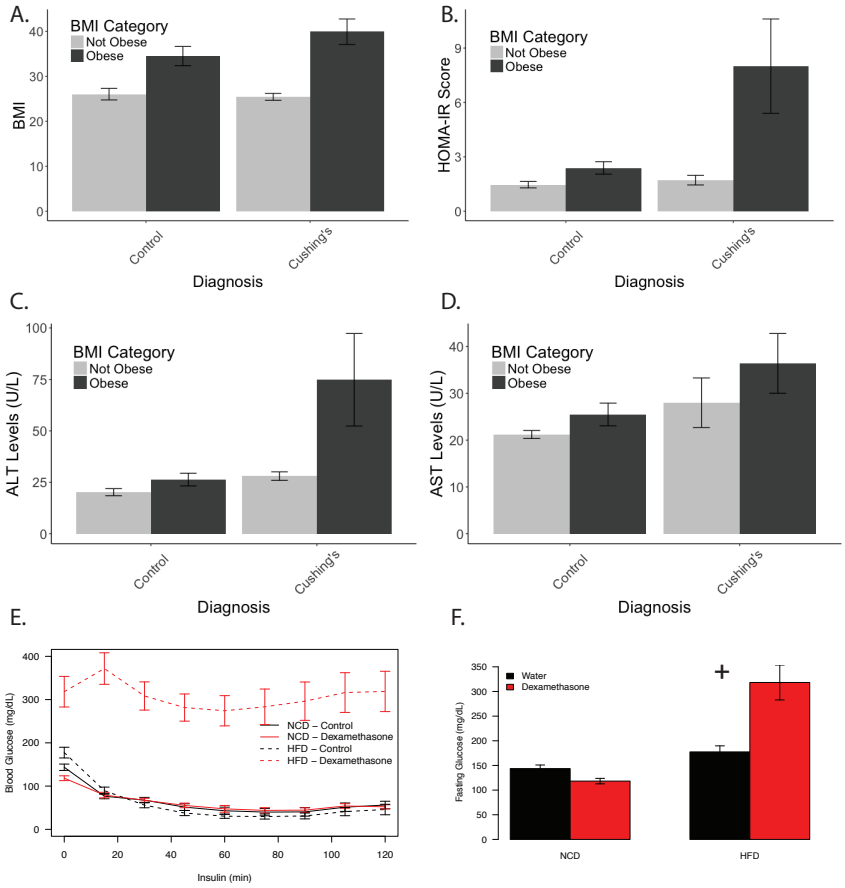

Figure 9: Adverse metabolic outcomes due to Cushing's are exacerbated in the presence of obesity. BMI (A) HOMA-IR score (B), ALT (C) and AST (D) levels in patients with Cushing's (n=5) and or non-secreting adenoma (n=13) after stratifying for obesity. Mouse blood glucose levels during ITT (E) and prior to insulin injection (basal; F). Insulin was given via IP injection at a concentration of 2.5 U/kg following five weeks of dexamethasone (NCD n=12; HFD n=12) or vehicle (NCD n=12; HFD n=12) treatment and 17 weeks of diet.

In our small cohort of Cushing's disease subjects, we examined whether some of the dramatic transcriptional changes were modified by the obesity status of the patients (based on a BMI cutoff of 30). We were surprised to note that many genes that had strongly elevated transcripts in non-obese Cushing's disease patients had blunted effects in obese Cushing's disease patients. Some examples of this include *FASN* (padj<0.001), *PSMD8* (padj=0.034) and *IDH1* (padj<0.001 ;Figure 8A-C). Among genes that were more strongly induced in obese patients, most of these are involved in lysosomal function, including the cathepsins (*CTSB*, *CTSD*, *CTSZ*), *LAPTM5* and

LIPA (NS; Figure 8D). Although the small number of obese and non-obese Cushing's patients

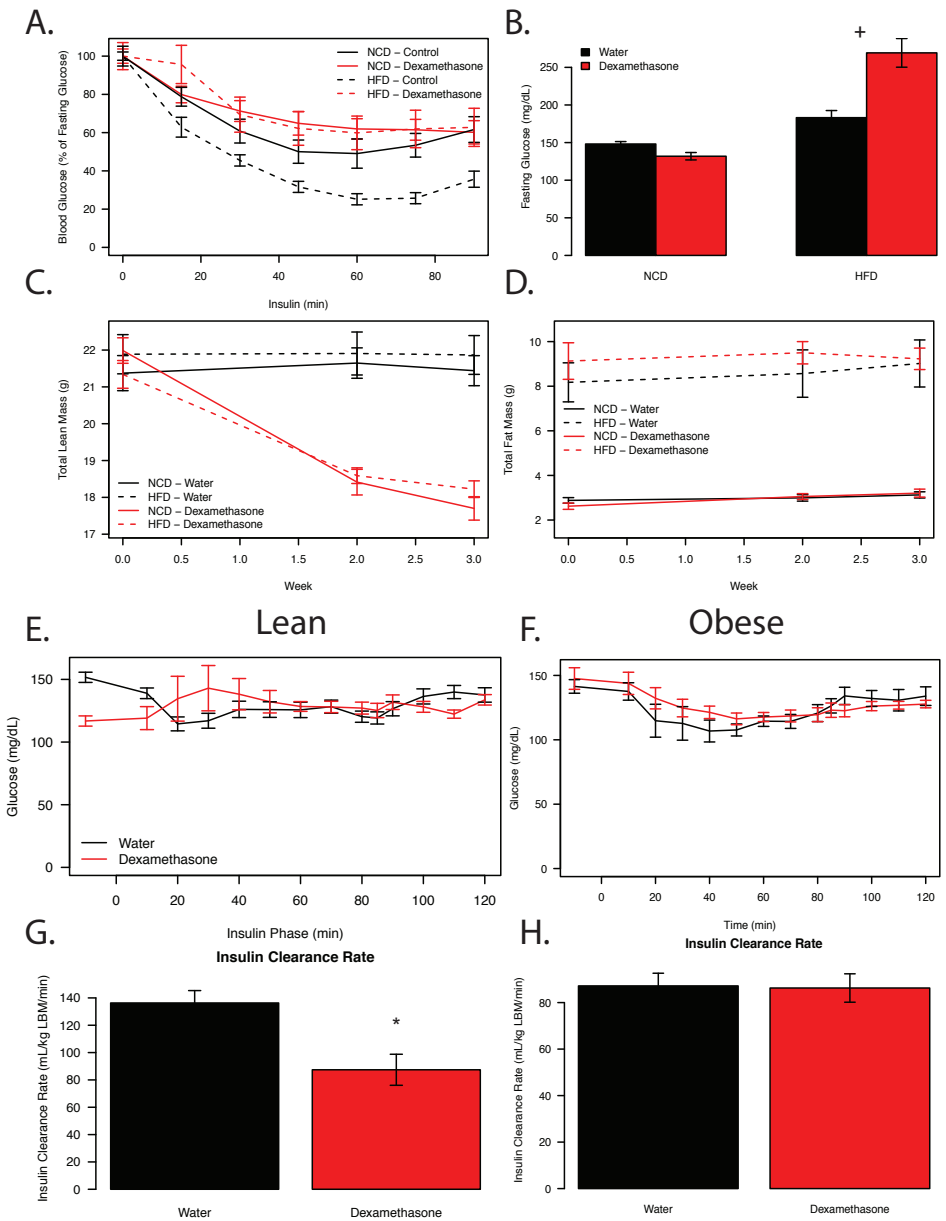

Figure 10: Shorter duration of dexamethasone treatment leads to hyperglycemia.
ITT (A) and baseline blood glucose (B) were measured in another cohort of mice following two weeks of dexamethasone (NCD n=10; HFD n=14) or vehicle (NCD n=13; HFD n=11) treatment and 10 weeks of diet. Insulin was given via IP injection at a dose of 0.75 U/kg (NCD) or 1.5 U/kg (HFD). Lean (D) and fat mass (E) were measured weekly via EchoMRI for the duration of the study. One week following the ITTs, blood glucose levels (F), insulin clearance rates (G) were measured during a hyperinsulinemic euglycemic clamp in the obese mice only. For clamp experiments, insulin was infused at 8 mU/kg/min following a prime continuous infusion of 40mU/kg bolus. Mice were fasted for five-hours prior experiments. Crosses indicate a significant interaction between diet and treatment. Asterisks indicate a statistically significant treatment effect for the pairwise comparison.

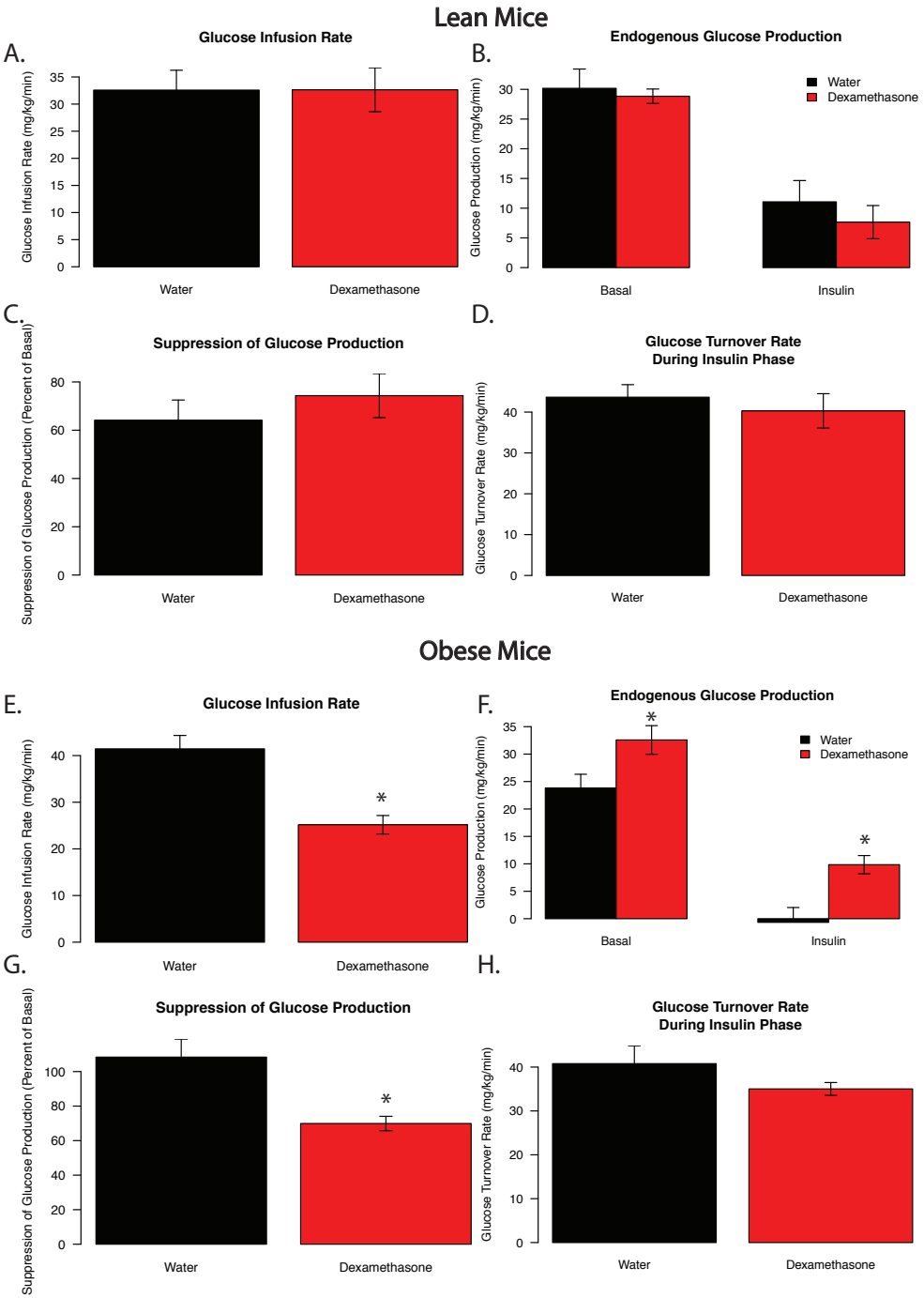

Figure 11: Reductions in glucose handling are exacerbated when elevated glucocorticoids and obesity are combined.

Mouse glucose infusion rate (GIR) endogenous glucose production (EGP), suppression of glucose production and glucose turnover rate for lean (A-D) and obese (E-H) during euglycemic clamp following three weeks of dexamethasone or vehicle and 11 weeks of NFD or HFD (n>10 for each group). For clamp experiments, insulin was infused at 4mU/kg/min for lean and 8 mU/kg/min following a prime continuous infusion of 40mU/kg bolus for obese mice. All mice were fasted for 5-6 hours prior to experiments. Crosses indicate a significant interaction between diet and treatment. Asterisks indicate a statistically significant treatment effect for the pairwise comparison.

in our study makes these provocative observations preliminary, it is suggestive of an

underappreciated role of lysosomes in obese patients with elevated cortisol levels. This led us to evaluate how obesity modifies glucocorticoid-induced metabolic disease.

Dexamethasone-Induced Insulin Resistance is Worsened in the Presence of Obesity

Based on the modifying effect of obesity on mRNA expression, we speculated that the glucocorticoid responses may vary according to obesity status. We re-analyzed the data from the

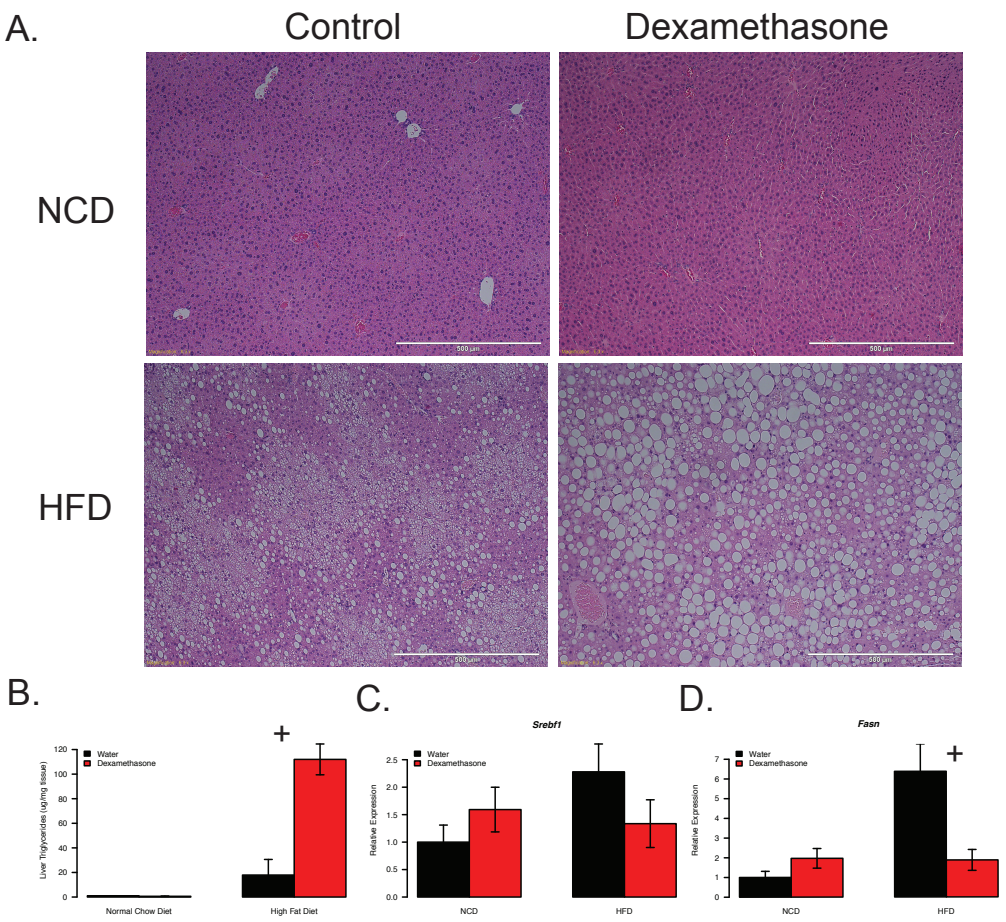

Figure 12: Increased glucocorticoids lead to greater severity of hepatic steatosis in obese mice.
Mouse Hematoxylin and Eosin stained liver sections (A) hepatic triglyceride levels (B) and qPCR of hepatic *de novo* lipogenic transcripts (C, D). Mice were euthanized at 28 weeks of age following six weeks of dexamethasone (NCD n=7; HFD n=5) or vehicle (NCD n=6; HFD n=9) treatment and 18 weeks of diet. Liver stains are representative samples from each group. (+) indicate a significant interaction between diet and treatment.

above patients, stratifying the Cushingoid and control groups by BMI, classifying these participants as either "Not obese" (BMI < 30) or "Obese" (BMI ≥ 30). We found no significant differences in BMI in the control groups compared to the Cushing's group (Figure 9A); however, a near-significant interaction between obesity status and Cushing's diagnosis on for HOMA-IR score ($p= 0.057$; Figure 9B) was observed. Furthermore, we observed only a modest (17%) increase in HOMA-IR score when comparing non-obese subjects with and without Cushing's disease, yet a but a 3.4-fold increase in the obese patients. Obesity and chronic elevations in glucocorticoids are associated with NAFLD (Rockall et al. 2003; Wanless and Lentz 1990). We observed increases in plasma ALT, a liver enzyme associated with liver disease, in obese Cushing's patients (38% increase in non-obese subjects versus a 2.8 fold increase in obese subjects, $p=0.13$ for the interaction of disease and obesity status; Figure 9C). Similar, though less robust effects were observed for AST (Figure 9D). These data support the hypothesis that both transcriptional and clinical parameters of patients with Cushing's disease are modified by obesity.

To further investigate the effect of obesity status on insulin sensitivity in the presence of elevated glucocorticoids we performed an insulin tolerance test (ITT) on lean (NCD) and diet-induced obese (HFD) mice that were untreated (water group) or treated with dexamethasone in their water. HFD-fed, dexamethasone-treated mice were significantly more resistant to insulin-stimulated glucose disposal when compared to all other groups (Figure 9E). Additionally, HFD dexamethasone-treated mice exhibited dramatic fasting hyperglycemia, with a significant interaction between diet and drug ($p=0.00009$; Figure 9F). While HFD animals had a 24% increase in fasting glucose when compared to NCD animals, in the presence of dexamethasone,

HFD-fed animals had a 122% increase in fasting glucose relative to NCD controls not treated with dexamethasone. In the lean, NCD-fed animals, dexamethasone caused an 18% decrease in fasting glucose.

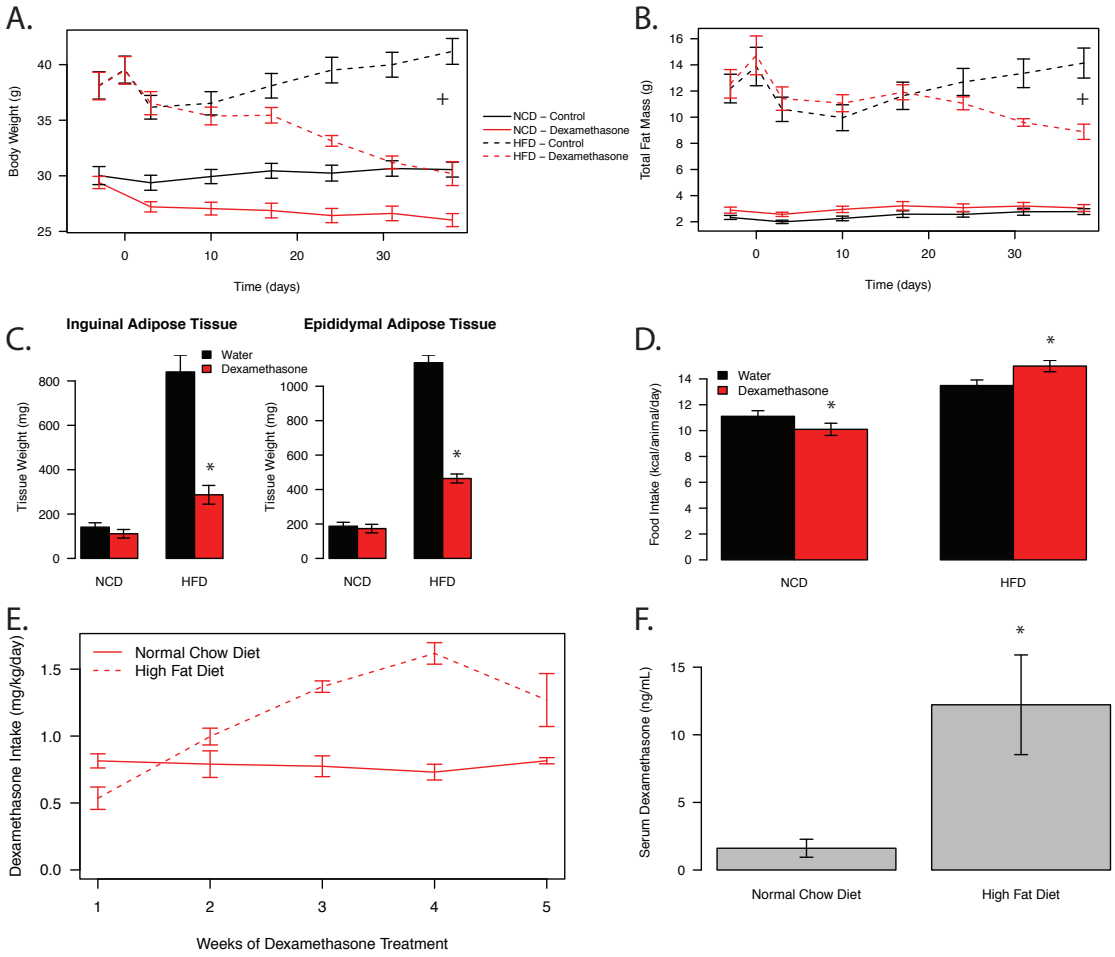

Figure 13: Dexamethasone treatment reduces fat mass in obese mice.
Weekly total body mass (A) and fat mass (B) measures via EchoMRI in mice over the course of treatment (solid lines represent NCD mice and dashed lines represent HFD mice). Adipose tissue weights in 16 hour fasted mice following euthanasia (C). Mice were euthanized at 28 weeks of age following six weeks of dexamethasone (NCD n=8; HFD n=12) or vehicle (NCD n=8; HFD n=22) treatment and 18 weeks of diet. Food consumption measured weekly over the course of treatment (D). Amount of dexamethasone consumed per mouse throughout the study normalized to body weight as determined by volume consumed per cage per week for NCD- (n=12) and HFD-fed (n=20) mice (E). Concentration of dexamethasone in serum of NCD-fed (n=8) and HFD-fed (n=11) at the end of the study as determined by LC-MS (F). (*) indicate $p<0.05$ treatment effect for the pairwise comparison, (+) for interaction effect.

To evaluate glucose homeostasis in more detail we performed hyperinsulinemic-euglycemic clamps in lean and obese mice (11 weeks of HFD) treated with dexamethasone for the final three

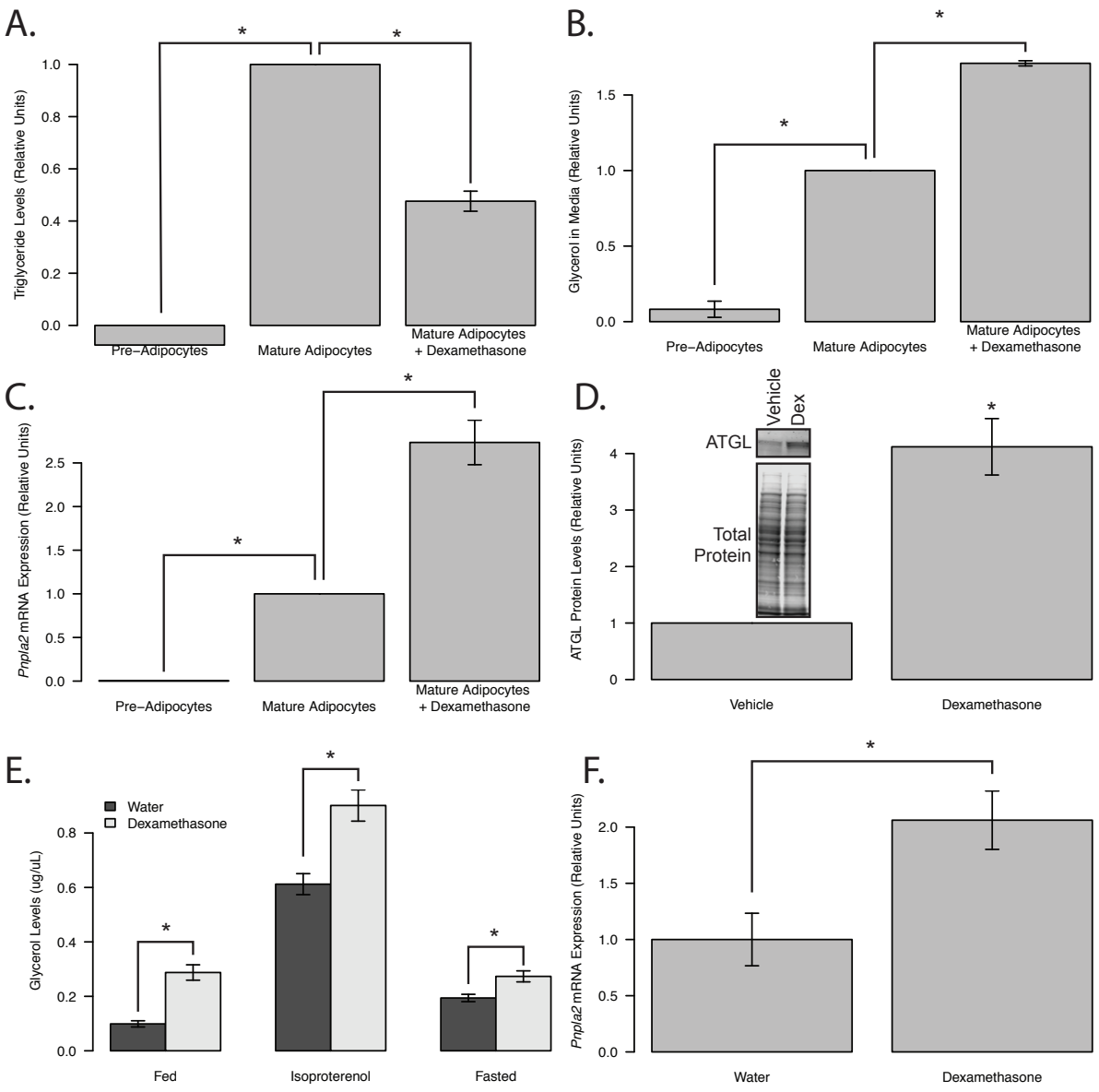

Figure 14: Dexamethasone treatment induces lipolysis *in vivo* and *in vitro*.
Triglyceride levels (A), glycerol released in media (B), qPCR of Pnpla2 transcripts (C), and representative western blot of ATGL (D) from non-differentiated (pre-adipocytes; n=2) or differentiated 3T3-L1 mouse adipocytes (mature adipocytes) following five days of dexamethasone (n=3) or vehicle treatment (n=3). Serum fatty acid and glycerol levels at basal (fed) and following stimulation (10mg/kg isoproterenol or 16hr fast; E) and qPCR of IWAT lipolytic transcripts (F) in 22-week-old, 12-week dexamethasone- (basal and isoproterenol n=7; fasted serum and qPCR n=4) or vehicle- (basal and isoproterenol n=12; fasted serum and qPCR n=11) treated, chow-fed mice with the exception of isoproterenol-stimulated glycerol, which was performed one week prior to euthanasia. Asterisks indicated statistically significant treatment effect for the pairwise comparison.

weeks. This shorter HFD/dexamethasone exposure still caused dramatic insulin resistance, hyperglycemia and reductions in lean mass, but no differences in fat mass (Figure 10A-D). Animals were clamped while conscious and glucose levels during the clamp as well as insulin

turnover rates were significantly different between the treatment groups in lean mice with both variables being lower in the dexamethasone treatment group (glucose $p<0.001$; insulin $p=0.004$; Figure 10E,G), but were similar between groups for the obese mice (Figure 10F,H).

There were no significant differences in glucose infusion rate (GIR; $p=0.991$), hepatic glucose production (basal $p=0.725$; insulin $p=0.474$), insulin suppression of glucose production ($p=0.42$) or glucose turnover ($p=0.517$) between the treatment groups in the lean animals (Figure 11A-D); however, this is likely due to insulin clearance being significantly lower in the dexamethasone treatment group throughout the experiment (Figure 10G). As a result, although the lean mice were given the same dose of insulin, lean mice were effectively exposed to different concentrations of insulin during the clamp, preventing proper interpretation of the clamp results in lean mice. This was not the case for HFD-fed mice, where insulin turnover was similar between control and dexamethasone treated groups.

For the obese animals, during the hyperinsulinemic phase, the GIR was 39% lower in dexamethasone-treated mice when compared to controls indicating insulin resistance at euglycemia (Figure 11E). Basal endogenous glucose production (EGP) was 37% higher in the dexamethasone- treated group ($p=0.026$; Figure 11F). Moreover, in the control group, EGP was reduced to near zero by a high dose of insulin but only reduced 70% in the dexamethasone group ($p=0.0091$) resulting in glucose production being higher during the insulin phase in dexamethasone-treated mice ($p=0.014$) when compared to controls (Figure 11F-G). Glucose turnover was slightly decreased in the presence of insulin ($p=0.141$; Figure 11H). These data suggest that increased glucose production and its impaired suppression by insulin are the likely causes of poor glycemic control in obese, dexamethasone-treated animals.

HFD-Induced Liver Steatosis in Dexamethasone-Treated Mice

Mouse data presented here support the patient findings above of elevated liver enzymes, with H&E staining of hepatic tissue clearly depicting exacerbated lipid levels in the obese, glucocorticoid-treated group when compared to HFD-fed or dexamethasone-treated controls (Figure 12A). In concert with the images, we observe drastically elevated liver triglycerides when compared to all other groups with a significant interaction between drug and diet ($p=0.000068$; Figure 12B).

qPCR was used to measure the expression of genes involved in hepatic *de novo* lipogenesis, *Srebf1* and *Fasn*, in liver lysates (Figure 12 C-D). No synergism in expression levels was observed between HFD and dexamethasone at this time point in the liver. This finding indicates that hepatic lipid accumulation in response to dexamethasone treatment is likely occurring via mechanisms other than accelerated glucocorticoid-dependent activation of *de novo* lipogenesis.

Dexamethasone Causes Decreased Fat Mass in Obese Mice

To understand the how dexamethasone effects body composition in these animals, we determined total fat mass. We observed reductions in body weight and fat mass in the HFD-fed dexamethasone-treated group (weight, $p=0.001$ and fat, $p<0.001$ for the interaction of diet and treatment; Figure 13A-B). These reductions do not appear to be depot-specific, as we observed reductions in both inguinal WAT (65% reduced) and epididymal WAT mass (59% reduced; Figure 3C) in the obese, dexamethasone-treated mice. There were no significant differences in fat mass, either by MRI or gross tissue weights of iWAT or eWAT depots in response to

dexamethasone treatment in the chow-fed groups (Figure 13C). To determine if changes in body composition could be explained by altered caloric consumption, we compared food intake among the groups (Figure 13D). Lean dexamethasone-treated mice ate significantly less than lean controls (9% reduction; p=0.006), as previously reported (Haber and Weinstein 1992; Roussel et al. 2003). Surprisingly, we found that the obese dexamethasone-treated mice ate slightly more food (11% increase, p=0.032), even though they lost both fat and fat-free mass. These data suggest that the weight loss in obese animals provided dexamethasone is not due to reductions in food intake and may be due to increased energy expenditure.

Over the course of the experiment, obese dexamethasone-treated mice consumed more water, starting at a lower amount, which then increased over the duration of the experiment (Figure 13E). Overall this corresponded to a 47% increase when normalized to the animal's body weight, 92% increase when not normalized to body weight. By the end of the study, this increased intake resulted in a 7.6-fold increase in serum dexamethasone concentration in the obese dexamethasone-treated mice when compared to lean dexamethasone-treated mice (Figure 13F; p=0.031).

Dexamethasone Treatment Results in Increased Lipolysis

Lipolysis has previously been associated with insulin resistance (Edgerton et al. 2017; Rebrin et al. 1996), is known to be elevated in patients with NAFLD (Gastaldelli et al. 2009), and has been shown to increase with high levels of glucocorticoids (Djurhuus et al. 2002; Hochberg, Harvey, et al. 2015; Djurhuus et al. 2004; Kršek et al. 2005). To assess whether dexamethasone directly

affected the lipid content in adipose tissue, we measured markers of lipolysis in cultured

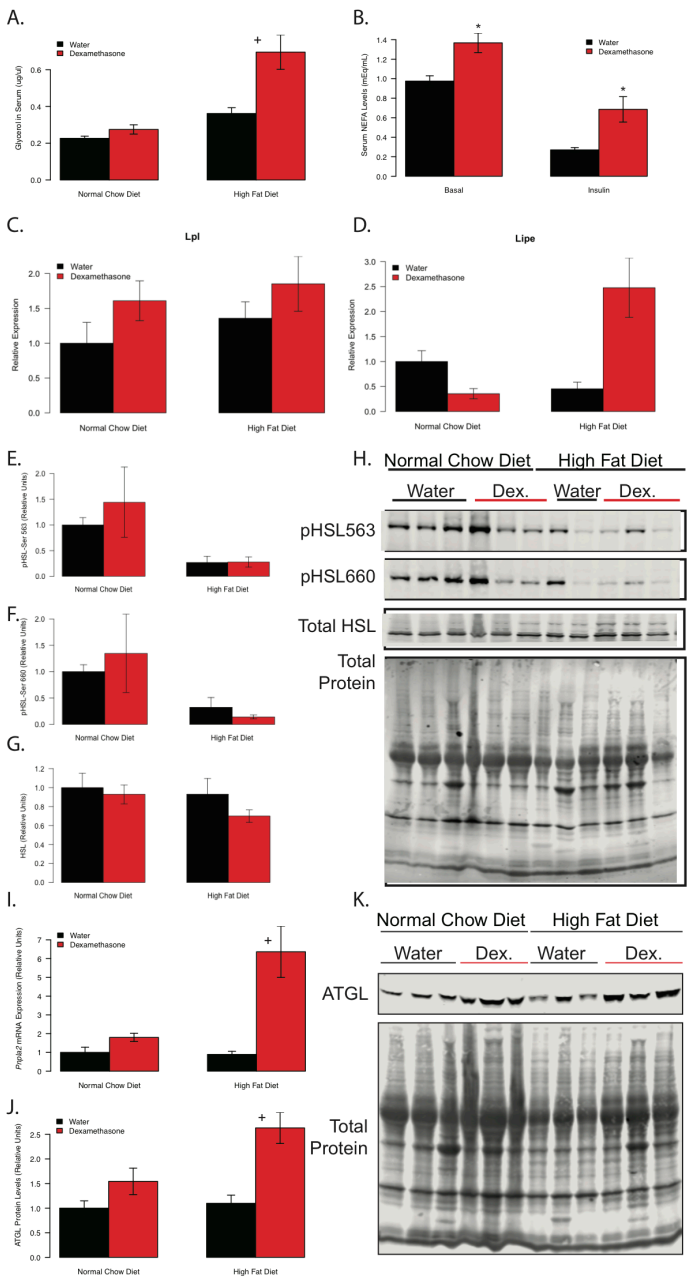

Figure 15: Obesity exacerbates dexamethasone-induced lipolysis.
Serum glycerol (A) following 16 hour fast, serum NEFA in obese dexamethasone treated (n=14) or control (n=11) mice following a 5 hour fast, before and after insulin during hyperinsulinemic euglycemic clamp (B), qPCR of Lpl and Lipe transcripts from IWAT (C-D). Western blot quantification (E-G) and image (H) of HSL phosphorylated and total protein from IWAT. qPCR of Pnpla2 transcripts (I) and western blot quantification (J) and image (K) of ATGL protein from IWAT. Mice euthanized at 28 weeks of age following six weeks of dexamethasone (NCD n=8; HFD n=10) or vehicle (NCD n=8; HFD n=10) treatment. Mice from A and C-J were euthanized at 28 weeks of age following six weeks of dexamethasone (NCD n=8; HFD n=10) or vehicle (NCD n=8; HFD n=10) treatment. Mice from B were fasted for 5 hours prior to euglycemic clamp following 3 weeks of dexamethasone (n=14) or vehicle (n=11) treatment and 11 weeks of HFD. For clamp experiments, insulin was infused at 8 mU/kg/min following a prime continuous infusion of 40mU/kg bolus. Crosses indicate a significant interaction between diet and treatment. Asterisks indicate a statistically significant treatment effect for the pairwise comparison.

adipocytes. 3T3-L1 fibroblasts were undifferentiated (pre-adipocytes); or differentiated and treated with vehicle or dexamethasone following differentiation. Dexamethasone treatment following differentiation led to decreased lipid content (52.4% reduction, p=0.005; Figure 14A) and a 71% increase in the amount of glycerol in the media (p=0.001; Figure 14B), suggesting increased lipolysis. In order to identify a potential GR-dependent lipolytic target, we evaluated the levels of ATGL, the rate limiting enzyme in lipolysis. Expression of ATGL (encoded by the *Pnpla2* gene) was enhanced following dexamethasone treatment in 3T3-L1 cells at the transcript (2.7 fold, p=0.002; Figure 14C) and protein (4.2 fold, p=0.025; Figure 14D) levels. These data show that glucocorticoids elevate ATGL levels and metabolites of lipolysis in cultured adipocytes.

To measure the effects of glucocorticoid-induced lipolysis *in vivo,* we quantified glycerol levels in animals chronically exposed to dexamethasone in basal and stimulated conditions (Figure 14E). Stimulation of lipolysis was achieved via isoproterenol, a ß-adrenergic receptor agonist, or by a 16-hour fast. Dexamethasone treatment led to increases in glycerol in the fed (2.9 fold), fasted (1.5 fold) and isoproterenol-stimulated (1.4 fold) conditions ($p<0.05$ for all pairwise comparisons), indicating that dexamethasone enhances basal and stimulated lipolysis *in vivo* in chow-fed mice. Consistent with these findings, mRNA analysis from iWAT of these mice showed an upregulation of *Pnpla2* transcripts in the dexamethasone-treated mice compared to controls (2.1 fold, p=0.016; Figure 14F).

To understand how diet-induced obesity alters dexamethasone-induced lipolysis, we quantified serum glycerol concentrations in lean and obese mice following an overnight fast (Figure 15A).

We observed a nearly two-fold increase in serum glycerol levels by dexamethasone treatment in the obese animals, compared with only a 18% increase in lean mice ($p=0.017$ for the interaction between diet and dexamethasone). Under hyperinsulinemic euglycemic clamp conditions, in the obese mice there was a 40% elevation in serum basal non-esterified fatty acids (NEFA's) in response to three weeks of dexamethasone treatment ($p=0.004$; Figure 15B). During the insulin phase, dexamethasone treatment attenuated the ability of insulin to suppress serum NEFA levels with insulin leading to a 71% reduction in controls compared to only a 48% reduction in dexamethasone-treated mice ($p=0.058$). These findings suggest that in the obese setting, dexamethasone elevates lipolysis to a greater extent and attenuates the effects of insulin.

To investigate the molecular basis for this synergistic increase in lipolysis, we quantified mRNA expression for multiple genes involved in lipolysis including *Lpl, Lipe* (encodes HSL) and *Pnpla2*, as well as protein expression of HSL and ATGL in the iWAT of these mice (Figure 15C-K). There were no significant interactions of diet and treatment with *Lpl,* or *Lipe* expression of HSL phosphorylation or levels that would explain the synergistic elevations in glycerol and NEFA due to the combination of dexamethasone and obesity. In fact, phosphorylation of PKA sites on HSL tended to be lower in obese mice when compared to lean, as has been reported previously (Gaidhu et al. 2010). Consistent with the hypothesis that ATGL activation could drive increased lipolysis in obese dexamethasone-treated mice, expression of ATGL was elevated in both dexamethasone-treated groups, with a significant synergistic effect of dexamethasone and obesity at the transcript ($p=0.02$) and protein ($p=0.043$) levels. These data support the hypothesis that glucocorticoid-stimulated lipolysis is augmented in the context of obesity, potentially via increased transactivation of *Pnpla2*/ATGL.

Discussion

Here we have described a transcriptional signature in adipose tissue from subjects with Cushing's disease and verified several of these changes using a mouse model of glucocorticoid treatment. We have identified several pathways that are significantly changed in response to chronic glucocorticoid exposure. Broadly, these changes reflect a shift towards more rapid metabolism of glucose through glycolysis and the TCA cycle, and shifting of glucose and protein metabolites towards lipogenic pathways in adipose tissue. This is indicated by significant increases in glycolytic (*ALDOC, ENO1, IDH1, ME1, GALM* and *GAPDH)*, proteolytic (*PSMD1/14*) and lipogenic (*ACACA, FASN, ACSL1, ELOVL5, GPAM* and *DGAT2*) transcripts in human adipose tissue, with similar transcript expression changes seen in mouse adipose and muscle tissue when treated with dexamethasone. A limitation of our human data is the difference in age between non-secreting adenoma and Cushing's disease subjects. Cushing's disease is diagnosed and treated much more rapidly than non-secreting tumors, which leads to these differences. I therefore confirmed many of our human findings in a mouse model of excessive glucocorticoid treatment, wherein the mice were treated under more controlled conditions. Studies using a *Hsd11b1* knockout mouse showed similar findings to our data including increased fat mass and decreased lean mass and strength, along with reduced insulin sensitivity (S. A. Morgan et al. 2014). In our study, we observed induction of fatty acid synthesis genes in both humans and mice (Figure 4A/F), which was not observed in the Morgan *et al*. study. Three differences could potentially explain these discrepancies. One is that in our case, dexamethasone is already active and cannot be further activated by 11β-HSD1, whereas in their study corticosterone can be both inactivated by 11β-HSD2 and reactivated by 11β-HSD1. Another key difference is the duration of treatment, which for my study was three months and for the Morgan *et al*. study was just over

one month. Finally, they determined mRNA levels from gonadal adipose tissue, not subcutaneous adipose tissue, as I did in my work.

Cushing's disease patients have a significant change in fat distribution (Mayo-Smith et al. 1989), and higher lipogenesis, as measured by conversion of glucose to neutral lipid *ex vivo* in subcutaneous adipose tissue from Cushing's disease patients compared to obese controls (Galton and Wilson 1972). Higher triglyceride synthesis has also been found in animal models of Cushing's disease, including CRH overproducing mice, which also have elevated glucocorticoid levels (Harris et al. 2013) and in dexamethasone treated mice (Roohk et al. 2013). These findings are consistent with our observed elevations of lipogenic mRNA transcripts in human and mouse subcutaneous adipose tissue. Key transcripts in this category found to be significantly upregulated include Acetyl-CoA carboxylase alpha (*ACACA*), responsible for the first step of lipogenesis (the irreversible conversion of acetyl-CoA to malonyl-CoA) and Glycerol-3-phospahte acyltransferase (*GPAM*) is responsible for the first step in the synthesis of glycerolipids. In addition to a shift towards lipid storage, we also observed elevated expression of glycogen synthesis mRNA transcripts in the Cushing's disease patients. Most notably of these are significantly elevated mRNA transcripts Glycogen synthase 2 (*GYS2*) and UDP-glucose pyrophosphorylase 2 (*UGP2*), both of which are required for glycogen synthesis.

Muscle wasting is a well-recognized adverse event of excess glucocorticoids caused by both increased muscle proteolysis and decreased protein synthesis (Deng et al. 2004; Menconi et al. 2007). Exposure of rats to glucocorticoids activates the muscle ubiquitin-proteasome system (Price et al. 1994; Wing and Goldberg 1993), increasing muscle expression of proteases

(cathepsins B and D, calpain) and components of the ubiquitin-proteasome pathway (D. Dardevet et al. 1995) along with inhibition of muscle protein synthesis (Long, Wei, and Barrett 2001). A study in healthy humans also found that prednisone (a synthetic corticosteroid) increases leucine oxidation supporting our observation of elevated amino acid catabolic genes (Beaufrere et al. 1989). We found higher expression of both proteasomal and the amino acid degradation pathways in adipose tissue, suggesting that a similar induction occurs in muscle tissue from our Cushing's disease subjects. We also observe elevations in lysosomal genes, though these changes appear to be restricted to obese Cushing's disease patients. The metabolic relevance of activated proteolysis in adipose tissue has not been widely explored and warrants further study. The effect of elevated glucocorticoids on muscle will be discussed in Chapter 4.

There are several limitations to our evaluation of insulin sensitivity in the human study. One aspect is that two of the three patients with Cushing's syndrome and diabetes were treated with antidiabetic medications. Secondly, it is possible that insulin resistance in these subjects/mice is mainly due to muscle or liver insulin resistance and that adipose tissue may respond to insulin in a relatively normal fashion. Glucocorticoid-induced insulin resistance is thought to be mostly secondary to the increase in free fatty acids caused by the induction of lipolysis (Geer, Islam, and Buettner 2014). Results from a recent study imply that glucocorticoids do not induce insulin resistance in subcutaneous adipose tissue *in vivo* in healthy subjects (Hazlehurst et al. 2013), suggesting that peripheral insulin resistance may not occur in adipocytes and that whole-body insulin resistance may primarily occur in muscle and liver tissues. This is consistent with our observations of a lack of changes in proximal insulin signaling transcripts (Figure 7A) or ceramides in our subcutaneous adipose tissue lysates (Figure 7B).

Another limitation in our human study is the small sample size, especially the number of biological replicates in Cushing's group (n=5). Adding a covariate such as BMI or age in the model further reduces the sample size to 2 or 3 replicates. Although this sample size is small, it is reasonable for a rare disease such as Cushing's. Realizing our limitation, we chose DESeq2 as the statistical method for our RNAseq data. DESeq2 overcomes the small sample size problem by pooling information across genes. Maximum likelihood estimation is applied to estimate the dispersion or variance of a gene across all replicates in a group. An empirical Bayes approach is then used to get maximum a *posterior* as the final dispersion estimate. This method utilizes the available data to the maximum extent; therefore, helps avoiding potential false positives (Love, Huber, and Anders 2014).

Chronic glucocorticoid elevations are associated with co-morbidities such as increased fat mass (Hochberg, Harvey, et al. 2015; Abad et al. 2001; Geer et al. 2010), decreased muscle mass (Hochberg, Harvey, et al. 2015; Dominique Dardevet et al. 1995; Schakman et al. 2013), insulin resistance and NAFLD (Paredes and Ribeiro 2014). Many of these adverse effects are similar to those seen in obesity; however, the combination of chronically elevated glucocorticoids in the context of pre-existing obesity has not been assessed. Though limited, our human findings provided preliminary evidence to suggest that obesity does modify the impact of glucocorticoids on overall metabolic health. The aforementioned limitations are what led me to investigate glucocorticoid-induced metabolic disease in the context of obesity in mice. Here, we show that the effects of glucocorticoid-induced insulin resistance and NAFLD are exacerbated when paired with obesity.

With the appreciation that glucocorticoids directly affect many other tissues, such as muscle, liver and the pancreas that may also influence insulin sensitivity, it is evident that adipose tissue plays a key role in glucocorticoid-induced metabolic disease. In support of a central role of adipocytes, several studies demonstrate that adipocyte-specific reductions in glucocorticoid signaling being associated with improved metabolic profiles (Mueller et al. 2017; Shen et al. 2017; S. A. Morgan et al. 2014; Y. Wang et al. 2014). I hypothesize that adipose tissue lipolysis plays a pivotal role in dexamethasone-induced insulin resistance and hepatic steatosis, especially in the case of obesity.

Excess adiposity, such is seen in obesity, has been associated with increased insulin resistance. We show increased fat mass following 12 weeks of dexamethasone treatment in lean mice, in accordance with what others have reported (Burke et al. 2017), as well as reduced insulin sensitivity. However, to our surprise, the glucocorticoid treatment in obese mice led to a lipodystrophic phenotype, which indicates the disturbances in glucose homeostasis are not a result of increased fat mass. The loss in fat mass observed in the obese, dexamethasone treated mice was not due to reduced food intake, in fact these mice ate significantly more calories per day than obese controls. This suggests a potential increase in energy expenditure with the combination of obesity and dexamethasone treatment over time. This study evaluated glucocorticoid treatment in the context of diet-induced obesity; however, Riddell and colleagues have reported similar findings when providing HFD and glucocorticoids in concert to rats, prior to the onset of obesity (D'souza et al. 2012; Beaudry et al. 2013; Shpilberg et al. 2012). It is not clear whether diet or obesity status have similar mechanisms causing exacerbated metabolic risk, but these interactions deserve further evaluation.

Lipolysis has been linked to increased gluconeogenesis by several studies (Nurjahan Nurjhan, Consoli, and Gerich 1992; N Nurjhan et al. 1986; Perry et al. 2017, 2015; Williamson, Kreisberg, and Felts 1966). Glucocorticoids are known to stimulate lipolysis (Djurhuus et al. 2002; Hochberg, Harvey, et al. 2015; Djurhuus et al. 2004; Kršek et al. 2005), possibly as a way to promote gluconeogenesis to maintain blood glucose levels. Lipolysis has also been implicated in insulin resistance (Edgerton et al. 2017; Rebrin et al. 1996) and NAFLD (Gastaldelli et al. 2009). We found synergistic elevations in glycerol, indicative of enhanced lipolysis, as well as in hepatic fat accumulation in the HFD-fed, dexamethasone-treated mice, but no data supporting enhanced hepatic *de novo* lipogenesis. Therefore, we propose the dexamethasone-induced increase in hepatic steatosis in the obese mice is primarily due to enhanced lipolysis observed in these animals.

There is some debate as to which genes glucocorticoids act on to promote lipolysis. Downregulation of *Pde3b* (Xu et al. 2009) and upregulation of β-adrenergic receptors (Lacasa, Agli, and Giudicelli 1988) and ATGL transcripts (Campbell et al. 2011; Serr, Suh, and Lee 2011; Shen et al. 2017) have been proposed as possible mechanisms, though we only found evidence supporting the third mechanism. We show that ATGL, the rate limiting enzyme for adipose triglyceride lipolysis, is synergistically activated by obesity and glucocorticoid-treatment. These findings bear a resemblance to elevations in glycerol levels in obese, dexamethasone-treated mice when compared to diet or glucocorticoids alone. There were no significant differences in the effects of diet or treatment on HSL phosphorylation. Interestingly, obesity and dexamethasone treatment appeared to slightly decrease HSL phosphorylation, consistent with

previous reports (Gaidhu et al. 2010). Given these results, we attribute enhanced lipolysis seen in obese dexamethasone-treated mice in part to upregulated ATGL. The mechanisms by which obesity and glucocorticoids synergize to activate ATGL expression are not clear at this time, nor are the relative contributions of other glucocorticoid receptor-dependent targets.

The obese, dexamethasone treated animals consumed increasing amounts of dexamethasone as the study progressed resulting in increased serum dexamethasone at sacrifice. This was unexpected and may be due to the increased urination, and water requirement in severely diabetic animals, as has been documented previously (S. M. Lee and Bressler 1981). This is an important limitation to our study, although we note that several phenotypes including fasting glucose, liver triglycerides, hepatic lipogenic gene expression, and adipose tissue mass changed in different directions in lean and obese animals, and therefore are unlikely to be due to an increased dose of dexamethasone. For example, dexamethasone reduced fasting blood glucose levels in lean mice, but led to hyperglycemia in obese mice. The dose of dexamethasone received was within the clinical range administered to human patients (Tyrrell et al. 1986; Fleseriu et al. 2012), corresponding to approximately 5 mg/day in an averaged sized human. Circulating concentrations of dexamethasone were similar to those observed following therapeutic doses of glucocorticoids (Ballard PL, Granberg P 1975; P. L. Ballard et al. 1980; Weijtens et al. 1998) and in Cushing's syndrome patients (Martin et al. 2006; Papanicolaou et al. 2009) even after accounting for dexamethasone's higher potency, and similar to other studies investigating glucocorticoid-induced metabolic effects in rodent models (Beaudry et al. 2013).

In summary, glucocorticoids are commonly prescribed drugs used to treat a multitude of health issues, but are known to induce a variety of adverse metabolic effects. The data presented in this chapter provide a variety of novel transcriptional changes that may be causative of the co-morbidities associated with Cushing's disease. Glucocorticoid actions in persons with obesity are not yet clear, even though there is a significant number of individuals with obesity routinely taking prescription glucocorticoids. I am the first to show that diet-induced obesity in mice exacerbates several co-morbidities associated with chronically elevated glucocorticoids. These effects may be considered by physicians when determining glucocorticoid treatment options for patients with obesity. Further studies in animals and cells using knockout or overexpression of specific transcripts may verify which of the changes is crucial in the metabolic effects of glucocorticoid effects in adipose tissue in lean and obese individuals.

Chapter 3: Evaluation of glucocorticoid signaling in adipose tissue

Introduction

Adipose tissue development and function are critical for proper glucose homeostasis. Disruption of normal adipose function can lead to the onset and progression of insulin resistance. This is evident in obesity as well as lipodystrophy, opposite phenomena leading to the same result, decreased insulin sensitivity and ultimately reduced glucose uptake. In the case of obesity, one hypothesis is that adipose tissue expansion, specifically the hypertrophy of individual adipocytes exhibit enhanced lipolysis (Laurencikiene et al. 2011) leading to ectopic fat deposition and insulin resistance; indeed, there is evidence that basal lipolysis is elevated in obesity (Langin et al. 2005). Whereas in the case of lipodystrophy, there is little to no adipose tissue to store lipids; therefore, promoting excess circulating lipids and storage in other tissues, thus resulting in insulin resistance (J. K. Kim et al. 2000), similar to what is seen in elevated lipolysis.

Glucocorticoids have been implicated in both in increased fat mass and adipocyte lipolysis (Hochberg, Harvey, et al. 2015; Harvey et al. 2018), with both processes potentially playing a role in glucocorticoid-induced insulin resistance (Boden et al. 1995; Kahn and Flier 2000); however, glucocorticoids have been shown to promote adipogenesis in vitro, a process which is thought to alleviate insulin resistance (Sandouk, Reda, and Hofmann 1993). Therefore, it is important to understand the role of adipocyte GR signaling in the context of metabolic

syndrome. In this chapter I will describe studies on isolated adipocytes and pre-adipocytes, as well as inducible, tissue specific knockout of the adipocyte glucocorticoid receptor.

Adipogenesis and the Role of Glucocorticoids

Glucocorticoids are required for adipogenesis in culture and there is evidence that chronically elevated glucocorticoids lead to increased fat mass in mice (Hochberg, Harvey, et al. 2015) and are associated with elevated adiposity in humans (Abad et al. 2001; Geer et al. 2010). However, the genes involved in glucocorticoid-induced adipogenesis are unclear. Here I investigated several potential GR targets to determine if they are directly regulated by glucocorticoids early in the adipogenic process. 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). The C/EBP proteins (α, β *and* δ), along with PPARγ, make up the master regulators required for early adipogenesis.

Among other Kruppel-like family members, the transcription factor, Kruppel-like factor 5*(Klf5)*, had been proposed to be involved in adipogenesis (Wu and Wang 2013). Oishi and colleagues show that KLF5 overexpression leads to spontaneous adipocyte differentiation in MEF cells and that KLF5 works in concert with C/EBPδ to activate *Pparg* transcription (Oishi et al. 2005). Furthermore, this group reports that heterozygote *Klf5* knockout mice have reduced WAT and are resistant to diet-induced obesity (Oishi et al. 2005, 2008). There is evidence that *Klf5* has GREs upstream of its promotor region; based on ENCODE data (The ENCODE Project

Consortium 2012; Mouse ENCODE Consortium 2014). However, there had been no studies to date that have evaluated KLF5's involvement in glucocorticoid-induced adipogenesis.

Models of Adipocyte GR action

It is clear that elevated fat mass is not the primary cause of glucocorticoid-induced insulin resistance, and that adipogenesis alone cannot explain glucocorticoid-associated insulin resistance. First, elevated adipogenesis is broadly causal of *improved* insulin sensitivity (Sandouk, Reda, and Hofmann 1993; Souza et al. 2001). Second, as I showed in the previous chapter, obese, dexamethasone treated mice displayed a *loss* in fat mass along with insulin resistance. In terms of mechanism, we and others have demonstrated that glucocorticoids lead to increased adipose tissue lipolysis (Hochberg, Harvey, et al. 2015; Harvey et al. 2018; Djurhuus et al. 2002, 2004; Kršek et al. 2005). The previous chapter described that dexamethasone-induced lipolysis along with ATGL expression is further exacerbated in the presence of obesity (Harvey et al. 2018). Elevated lipolysis likely influences both the disruption of glucose homeostasis, as well as promotes hepatic lipid accumulation. This is supported by previous findings that glucocorticoid signal depletion in adipose tissue results in the reduction of lipolysis, improves overall glucose homeostasis and reduces hepatic lipid content (Mueller et al. 2017; Shen et al. 2017; S. A. Morgan et al. 2014). Based on this work I hypothesized that blocking glucocorticoid signaling in the adipose tissue would alleviate the exacerbations in systemic insulin resistance and NAFLD caused by the combination of excess glucocorticoids and obesity that I observed in Chapter 2.

Methods

Animal Housing and Procedures

Floxed Glucocorticoid receptor alleles, B6.Cg-*Nr3c1*$^{tm.1.1Jda}$/J (stock #021021), targeting exon 3 in the *Nr3c1* gene (responsible for transcribing the glucocorticoid receptor) and B6.129-Tg(Adipoq-cre/Esr1*)1Evdr/J (stock #024671) mice were purchased from Jackson Laboratory. Adipoq-ER-Cre male transgenic mice were crossed with *Nr3c1* homozygous-floxed (*fl/fl*) female mice yielding all pups that were heterozygous for both transgenes. The generated mice were then bred with each other to yield wild-type (+/+) pups, with or without Cre; *Nr3c1* heterozygote-floxed (*fl*/+) pups with or without Cre and homozygous *Nr3c1* (*fl/fl*) with or without Cre. Homozygous *fl/fl* mice were then crossed with heterozygous Cre mice to generate all homozygous *Nr3c1 fl/fl* mice with or without the Cre to be used in the experiments.
Mice were placed on standard chow (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) at ten weeks of age and weights and body composition were monitored monthly for three months. At 22 weeks of age both genotypes were transferred into biocontainment housing (same building; kept in the same conditions) IP injected with 150mg/kg/day of tamoxifen or vehicle (corn oil) for five consecutive days; this process served to monitor any effects of the transgene or tamoxifen alone. True knockouts were animals expressing both the Adipoq-ER-Cre and floxed *Nr3c1* alleles injected with tamoxifen to create adipocyte specific, tamoxifen-inducible *Nr3c1* knockout mice. After one week in biocontainment, mice were transferred back to their regular housing and treated with approximately 1mg/kg/d dexamethasone or vehicle.

Water intake was measured during the treatment period and all mice were provided with access to food and water *ad libitum* throughout the study, unless otherwise noted. Mice were kept on a light dark cycle of 12/12 h and were housed at 22 ºC in groups of up to five per cage. For all cohorts, weight and body composition (via EchoMRI 2100) were measured weekly. Following three weeks of treatment, mice were fasted for 16 hours 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, tissues were carefully excised, iWAT, eWAT and quadriceps were weighed and all collected tissues were snap frozen in liquid nitrogen and stored at -80 °C for further analysis. Additionally, small pieces of liver tissues were fixed in 10% phosphate-buffered formalin for histology. Animal procedures were approved by the University of Michigan Institutional Animal Care and Use Committees.

Assessment of Insulin Sensitivity via ITT

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 and glucose was determined using a One Touch Ultra Glucometer (Lifescan).

Lipolysis

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.

Liver Histology

Sections of the left lateral lobe of the liver 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 40x objective of an EVOS XL digital inverted microscope.

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. For differentiation and ChIP experiments, cells were switched to full adipogenic cocktail (250nM dexamethasone, 500uM 3-isobutyl-1-methylxanthine and 1ug/mL insulin in 10% fetal bovine serum, in 4.5g/L glucose DMEM with PSG), vehicle (95% ethanol) or some combination of these drugs at two days post confluence for up to four days (Chiang S,

Chang L 2006). For full differentiation experiments, differentiation media was replaced with media (DMEM, serum and PSG) containing only insulin for an additional three days. For the following three days, cells were given fresh media with no additional treatment. Cells used for these experiments were not cultured beyond 22 passages.

For assessment of genes involved in early adipogenesis experiments, cells were treated with full differentiation cocktail, only IBMX and insulin, or dexamethasone alone for 0, 2, 4, 6, 24 and 48 hrs to assess mRNA. For experiments investigating dexamethasone treatment following differentiation, cells we differentiated normally for ten days and then treated with dexamethasone or vehicle for five days with media being refreshed on day three. For assessments of GR binding, cells were treated with full differentiation cocktail, only IBMX and insulin, vehicle, or dexamethasone alone for two hours. Following these time points, cells were lysed in TRIzol for qPCR analysis.

qPCR

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 level was

normalized to *Actb* and analyzed using the ΔΔCt method after evaluation of several reference genes. Primer sequences for the qPCR experiments are listed in Table 5.

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-ATGL) and 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).

Chromatin Immunoprecipitation

Crosslinking of protein-DNA complexes was achieved by incubating with 1% formalin for 10 minutes at 37° C and 125mM Glycine was added to halt crosslinking. Cells were placed on ice and washed with PBS and lysed with Farnham Lysis Buffer (FLB; reagents). Lysates were centrifuged for 5 min at 2000 RPM and 4° C. Pellets were suspended in FLB and homogenized using an 18G needle and syringe for 10 repetitions and spun again at the same settings. Pellets

were resuspended in 1mL RIPA (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) and sonicated on ice using a Sonics VCX-130 FSJ VibraCell Ultrasonic Liquid Processor with a Power Output 5 watts 6 times for 30 seconds each. Sonicated DNA was centrifuged at 4°C and 14000 RPM for 15 minutes and then snap frozen in liquid nitrogen and stored at -80°C until further analysis. For immunoprecipitation, the Millipore EZ ChIP protocol was followed using SantaCruz anti-GR and IgG antibodies and magnetic g-protein beads (Thermo Fisher Scientific; catalog #88848). All reagents and buffers were used at 4°C except glycine and formalin, which were kept at room temperature. Primers (Table 4) were designed using DNA sequences taken from ChIP sequencing peaks from ENCODE or found by searching putative GRE sites in genes of interest. Quantitative PCR was used to determine percent binding relative to input, methods as described above.

Statistics

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 when appropriate. 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 on excel or using the R software package version 3.30.

Results

Table 4: List of qPCR and ChIP Primers used in Chapter 3.

Gene	Forward 5'-3' Sequence	Reverse 5'-3' Sequence
Pnpla2	CCACTCACATCTACGGAGC	GATGCAGAGGACCCAGGAAC
Pparg	TTGCTGTGGGGATGTCTCA	AACAGCTTCTCCTTCTCGGC
Cebpa	GCGGGAACGCAACAACAT	GTCACTGGTCAACTCCAGCAC
Cebpb	ATGCACCGCCTGCTG	TGGCCACTTCCATGGGTCTA
Cebpd	AGAACCCGCGGCCTTCTAC	AATGTAGGCGCTGAAGTCGAT
Hsd11b1	AGCATTGCCGTCATCTCCT	TTGCTTGCAGAGTAGGGAGC
Klf5	TGGAGAAGCGACGTATCC	AGGTGCACTTGTAGGGCTTC
Klf5_GRE1	TGCCTACAAGGAAGTTTCC	CCACCTCATCAGCGTCACAA
Klf5_GRE2	TAGACGCCTGGGTCTCACT	TTGCTTCACTGCCTCAGAGC
Klf5_GRE3	GAGTAAGGCCGTGTAATG	AAAGCCTCCATGCACCATGA
Adig	AGACCATCTGAGTGTGGCT	CCAGTGTTCTCCCTCCATCC
Rora	GGCGCAGGCAGAGCTA	CCTTGCAGCCTTCACACGTA
Smad6	ATTCTCGGCTGTCTCCTCC	GTGGCCTCGGTTTCAGTGTA
Runx1t1	GGCGCAGGCAGAGCTA	GGTTGGCCTTCAAAAACGGG
Mmp11	GAACCCAGCGAGTGGACA	TCCAGTAGAGATGGCCACGA

Computational Analysis Reveals Several Potential Genes Involved in Glucocorticoid-Induced Adipogenesis

Glucocorticoids are known to be required for adipocyte differentiation *in vitro*, as previously shown (Hartman et al. 2018; Chapman, Knight, and Ringold 1985). To confirm these findings in my model of dexamethasone-induced adipogenesis, I treated confluent 3T3-L1 cells with

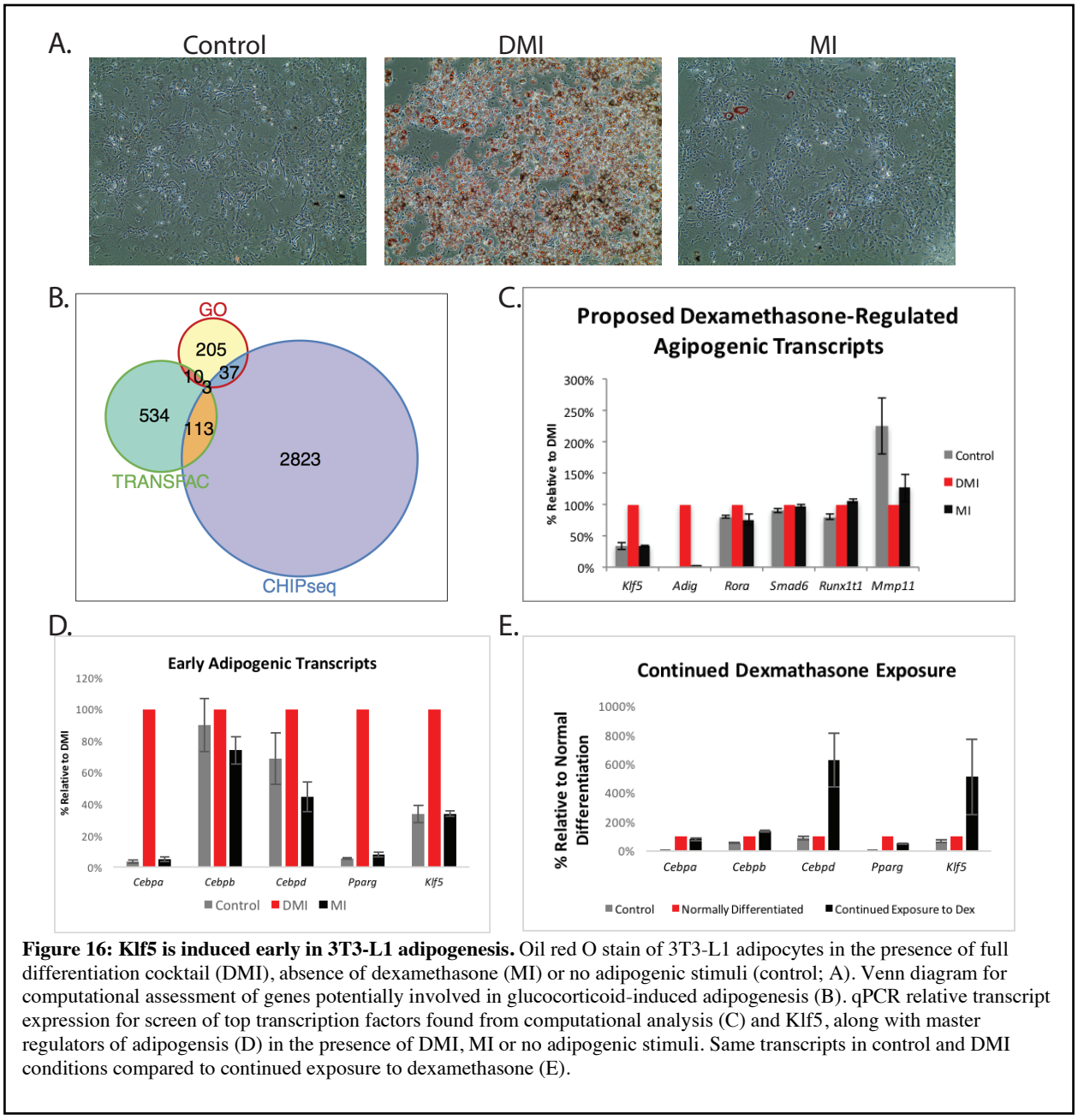

Figure 16: Klf5 is induced early in 3T3-L1 adipogenesis. Oil red O stain of 3T3-L1 adipocytes in the presence of full differentiation cocktail (DMI), absence of dexamethasone (MI) or no adipogenic stimuli (control; A). Venn diagram for computational assessment of genes potentially involved in glucocorticoid-induced adipogenesis (B). qPCR relative transcript expression for screen of top transcription factors found from computational analysis (C) and Klf5, along with master regulators of adipogensis (D) in the presence of DMI, MI or no adipogenic stimuli. Same transcripts in control and DMI conditions compared to continued exposure to dexamethasone (E).

adipogenic cocktail with (DMI) or without (control, no stimuli; MI, insulin and IBMX only) dexamethasone and assessed lipid accumulation and adipogenic markers of differentiation. Figure 16A shows Oil Red O, a hydrophobic dye that accumulates in the presence of lipid, accumulation only in the presence of DMI and confirms that dexamethasone is required for pre-adipocytes to mature. To determine which genes may be involved in glucocorticoid-induced adipogenesis, we performed a computational analysis on publicly available datasets (Figure 16B)

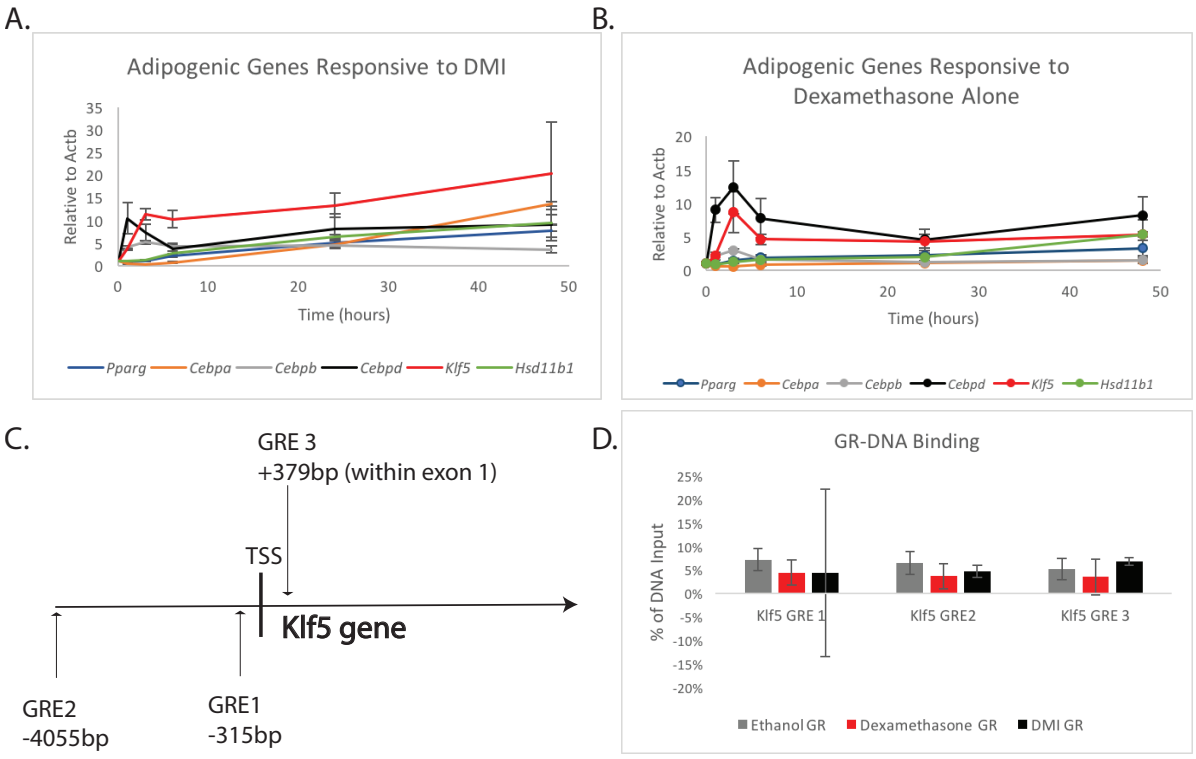

Figure 17: Klf5 is responsive to dexamethasone alone but GR-Klf5 binding is not enhanced. Time course of adipogenic transcript expression following full differentiation cocktail (DMI; A) and dexamethasone only (B) in 3T3-L1 pre-adipocytes. Schematic of putative GREs located in and around the Klf5 gene (C). ChIP experiment showing GR-binding to putative Klf5 GREs in response to 2 hours of ethanol, dexamethasone or DMI relative to total DNA input (D).

of genes that were involved in adipocyte differentiation (GO), genes that were known transcription factors with putative GREs (TRANSFAC) and genes that were positive for GR binding in response to dexamethasone (CHIPseq). There were 255 genes involved in differentiation, 660 genes with GRE's and 13 which overlapped.

Based on the data in Reddy et al. we identified 2976 genes associated with GR CHIP peaks (Reddy et al. 2009). We found that 40 of these genes overlapped with the GO category for adipocyte differentiation. We focused our initial studies on genes in this overlapping dataset using 3T3-L1 pre-adipocyte differentiation as a model system.

Klf5 mRNA levels are Induced Early in Adipocyte Differentiation by Dexamethasone

I assessed several transcription factors that we predicted to be glucocorticoid responsive and involved in adipogenesis according to our computational analysis. Of these, *Klf5*, one of the three genes found to be glucocorticoid-responsive, involved in differentiation *and* contain a putative GRE, was most increased in the presence of full differentiation cocktail (DMI), when compared to other potential adipogenic genes at day 10 of differentiation (Figure 16C). This effect was reversed when dexamethasone was removed from the differentiation cocktail, with DMI leading to a near-significant induction of *Klf5* when compared to MI ($p=0.05$). *Adig*, the gene encoding adipogenin responded similarly to *Klf5*, but adipogenin is upregulated later in the differentiation process (Hong et al. 2005), so I chose to focus on *Klf5* for further experiments since early induction is more likely to be a characteristic of a functional inducer of adipogenesis.

The extent of upregulation in *Klf5* is similar to what is seen with other well-known regulators of early adipogenesis, *Pparg* and *Cebpa* (Figure 16D; (Lowe, O'Rahilly, and Rochford 2011)). Furthermore, when dexamethasone remains in the media throughout differentiation, when it is normally removed, both *Cebpd* and *Klf5* are increased well above DMI levels (Figure 16E),

though due to variability of response this was not a statistically significant finding ($p>0.05$ for both). This suggests that *Cebpd* and *Klf5* may be responsive directly to glucocorticoids, and not just secondary to other differentiation related processes. To assess when *Klf5* is induced in the differentiation process in relation to known regulators of early adipogenesis, a series of time course experiments with 3T3-L1 cells in the differentiation cocktail were performed (Figure 17A). These data indicate that both *Cepbd* and *Klf5* are induced prior to the induction of *Pparg, Cebpb* and *Cebpa,* consistent with previous findings (Oishi et al. 2005).

To understand whether glucocorticoids could affect *Klf5* induction in the absence of other pro-adpogenic stimuli, cells were treated with dexamethasone alone and assessed *Klf5* transcription over a similar time series (Figure 17B). Again, upregulation of *Klf5* was observed, similar to the response of *Cebpd*. Under these conditions there is minimal induction of *Pparg* and *Cebpa/b*. These data suggest that dexamethasone regulates transcription of *Klf5* and *Cebpd* early in the adipogenic process, potentially upstream of PPARγ.

The Glucocorticoid Receptor is Bound to Sites on the *Klf5* Promoter

To determine if the GR was bound to specific GRE sites on the *Klf5* promoter, a ChIP assay was performed on 3T3-L1 preadipocytes testing multiple putative GRE sites identified from ENCODE data that showed potential ChIP peaks of GR-*Klf5* binding in A549 human lung cells in response to dexamethasone (Figure 17C). There was no clear evidence that dexamethasone treatment led to enhanced binding of the GR to GRE sites surrounding the *Klf5* promoter (Figure

17D). Future studies will be needed to identify if, and where GR binds to the *Klf5* promoter and the functional significance of the loss of GR induction of KLF5.

Generation of Adipocyte Specific *Nr3c1* Knockout Mice

To understand the physiological role of adipocyte GR knockout on dexamethasone function in lean and obese mice we engineered tamoxifen-inducible knockouts of *Nr3c1* the gene that encodes the glucocorticoid receptor. This approach targets the essential third exon of GR, resulting in a nonfunctional protein, and this allele has been used extensively in other tissues (Mittelstadt, Monteiro, and Ashwell 2012; Bauerle et al. 2018; Bose, Hutson, and Harris 2016; Guo et al. 2014; Kugler et al. 2013; Li et al. 2015; Mittelstadt, Taves, and Ashwell 2018; J. S. Park et al. 2014; Roh et al. 2018; Pierce et al. 2017).

For this study, mice were bred and fed either normal chow or a high fat diet (beginning at 70 days) for 12 weeks prior to induction of the knockout. This was done in order to allow for obesity to develop *with* normal glucocorticoid function, and mitigated potential adipocyte-GR dependent effects on adipose mass accumulation (Mueller et al. 2017). After 12 weeks of feeding, knockouts were induced by tamoxifen injections, and then after recovery animals were randomized into with either glucocorticoid or control treated mice (Figure 18A). Both male and female mice were evaluated in this study.

Changes in Weight and Adiposity Due to Adipocyte *Nr3c1* Ablation

As expected, prior to the induction of the knockout mice bearing or lacking the AdipoQ-ER-Cre transgene were similar in terms of fat and fat-free mass (data not shown). This indicates that

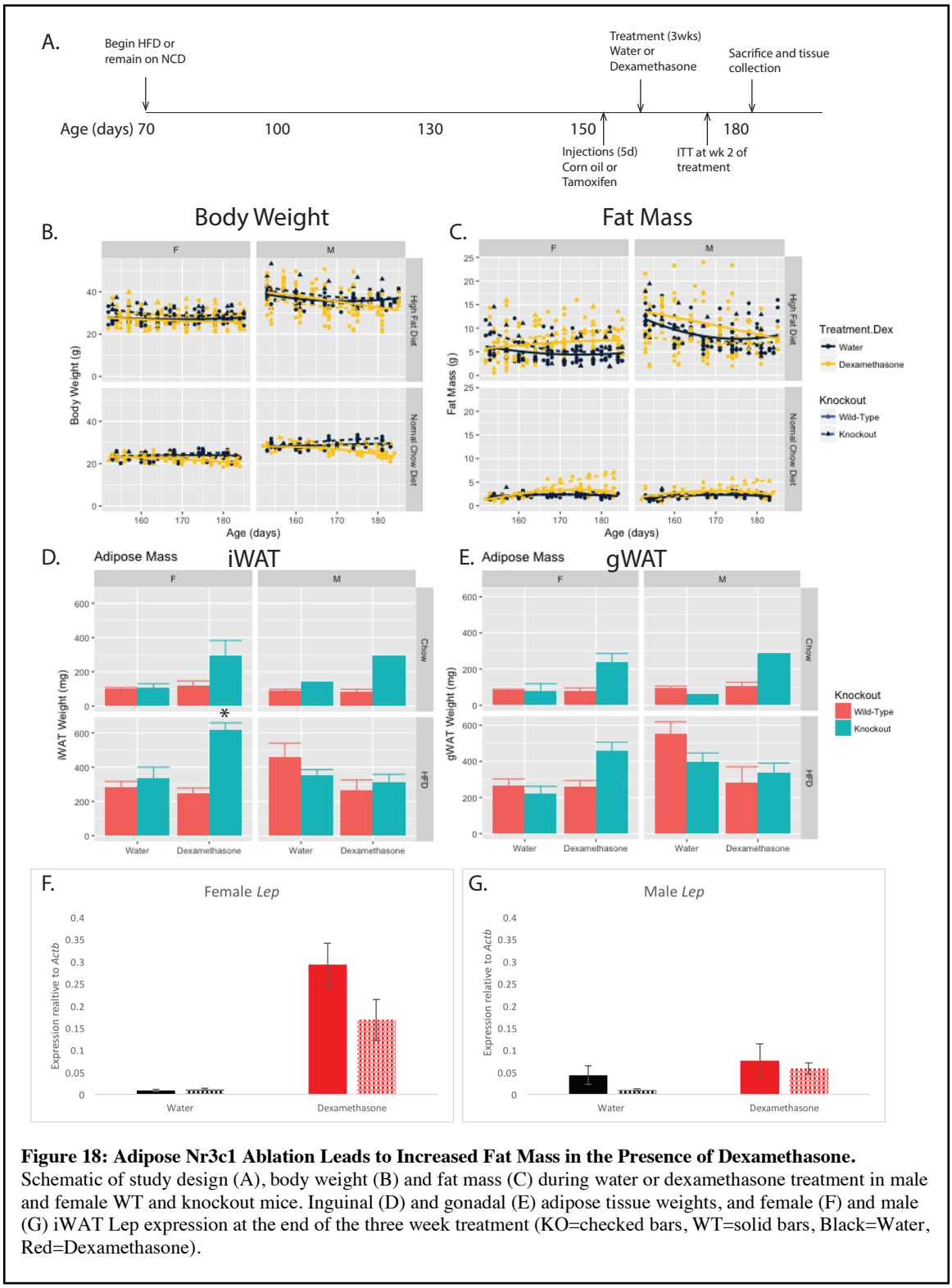

Figure 18: Adipose Nr3c1 Ablation Leads to Increased Fat Mass in the Presence of Dexamethasone. Schematic of study design (A), body weight (B) and fat mass (C) during water or dexamethasone treatment in male and female WT and knockout mice. Inguinal (D) and gonadal (E) adipose tissue weights, and female (F) and male (G) iWAT Lep expression at the end of the three week treatment (KO=checked bars, WT=solid bars, Black=Water, Red=Dexamethasone).

prior to knockout the groups were roughly comparable in terms of body composition; however, we will continue to monitor this throughout the study. There was no significant effect of knockout on body weight throughout the study when comparing genotype and treatment within each sex (Figure 18B). Similarly, we observed no significant effects of adipocyte *Nr3c1* knockout on changes in fat mass (Figure 18C). We did observe statistically significant moderating effects of diet and sex on dexamethasone-induced body weight, fat mass and lean mass loss ($p<0.0001$ for each individually as well as their interaction, with male sex and HFD diet resulting in more dexamethasone-induced loss).

Adipocyte GR Knockout Results in Fat Mass Gain after Dexamethasone Treatment

Over the course of a three-week dexamethasone treatment in wild-type animals, we typically have not observed increases in fat mass in NCD of HFD (see Figure 18C, and Figures 10 and 13 in Chapter 2), though prolonged dexamethasone administration did result in increased adiposity (Figure 2B in Chapter 2). Interestingly, dexamethasone treatment led to an increase in fat mass in most knockout animal groups (Figure 18C-E), with the exception the obese male knockout mice. These findings were not significant (males, $p=0.232$; females, $p=0.11$) at the time of writing of this dissertation. Similarly, upon sacrifice, we observed elevated mass of inguinal (Figure 18D; 84 mg increase, $p=0.024$ after adjusting for sex, treatment, diet and the moderating effects of diet on dexamethasone treatment) fat pads of the knockouts. A slight, nonsignificant increase was also observed in knockout mouse gondal fat pad weight (Figure 18E, 26 mg increase, $p=0.46$). Taken together these data indicate that the ablation of the glucocorticoid

receptor in adipose tissue may increase adipose tissue expansion, though the mechanism for this is unclear at this time.

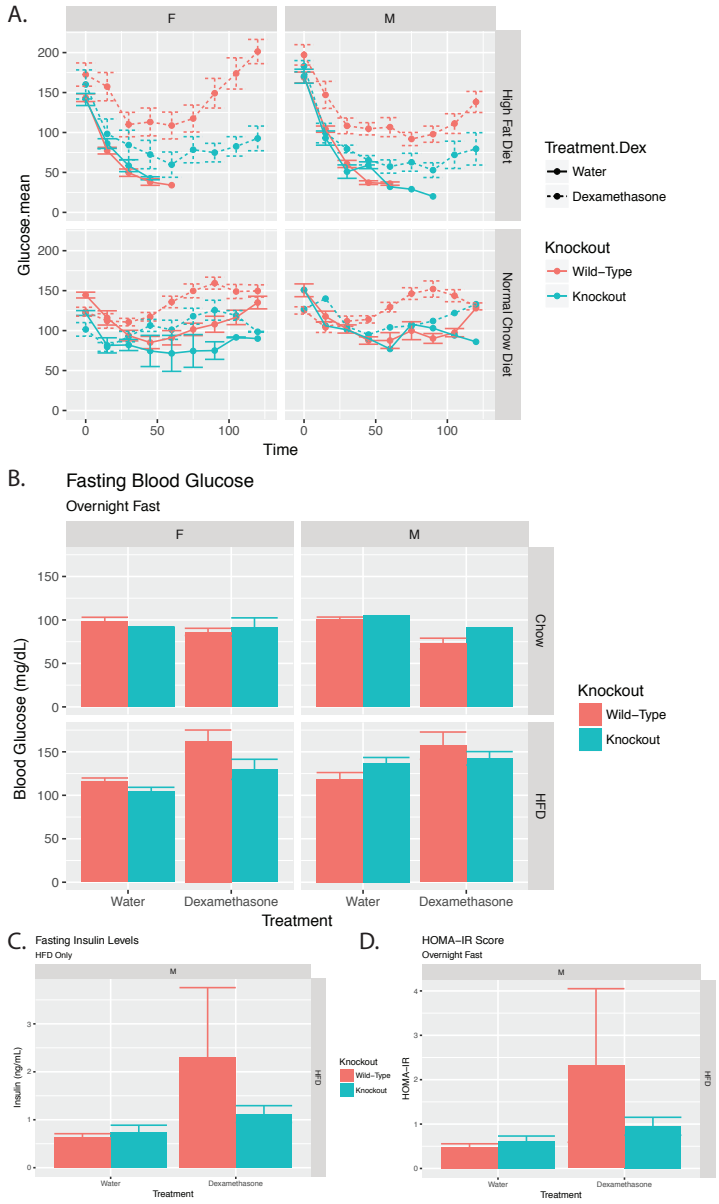

Figure 19: Adipose Nr3c1 Ablation Partially Rescues Glucocorticoid-Induced Insulin Resistance. Glucose values during an ITT for lean and obese WT and knockout mice following 2 weeks of dexamethasone or water treatment (A). Lean mice were given 1U/kg lean and obese mice were given 5U/kg lean of insulin via IP injection. Overnight (16hr) fasting blood glucose (B) and insulin (C; Obese males only) were used to calculate HOMA-IR Score (D) for the same mice as described above.

Based on this *Nr3c1*-specific increase in dexamethasone-induced fat mass gain, we considered this finding further. Since these mice were not separated by genotype when they were housed, I was unable to determine whether there was a drug and genotype effect on food consumption or energy expenditure. One hypothesis that there is an adipocyte glucocorticoid-dependent factor that would normally temper gains in adiposity, and that the loss of GR signaling in adipose tissue may result in increased fat mass accumulation. One candidate is Leptin, the adipocyte-derived anorexic signal, that has

previously been shown to be glucocorticoid-responsive (Masuzaki et al. 1997; Berneis, Vosmeer, and Keller 1996; Pralong et al. 2015; Halleux et al. 1998), and participates in a feedback loop with glucocorticoids (Zakrzewska et al. 1997; Rosmond et al. 2000). We tested the expression of *Lep*, the Leptin gene in the adipose tissue of the obese mice following a 16-hour fast. In obese females, dexamethasone led to a dramatic induction of *Lep,* an effect that was slightly attenuated in the knockout mice (Figure 18F); however, these findings did not reach statistical significance ($p=0.121$). There was no effect of genotype in the water treated groups, again supporting a dexamethasone-responsive modulation of Leptin levels. In the males, dexamethasone led to an insignificant induction of *Lep* that was not resolved with the knockout ($p=0.685$; Figure 18G). Decreased leptin would be predicted to cause reduced satiety and increased appetite. One potential explanation for reduced leptin is reduced fat mass, which is not the case in our model (Figure 18C). Therefore, these preliminary data suggest that adipocyte GR signaling to leptin may link HPA activity to suppression food intake, potentially in a sex-dependent manner. This hypothesis, if true would have important implications for our understanding of the mechanisms by which stress, obesity and appetite inter-relate.

Effects of Adipocyte *Nr3c1* Knockout on Glucose Homeostasis

To understand how adipocyte GR signaling relates to insulin sensitivity, I performed insulin tolerance tests and assessed fasting glucose and insulin to determine HOMA-IR score. As predicted, dexamethasone led to insulin resistance in the WT animals when compared to the water group (Figure 19A). Dexamethasone-induced insulin resistance is nearly fully rescued in the *Nr3c1* knockout lean males (bottom right quadrant of Figure 19A). We observed a partial

rescue of insulin sensitivity in the obese *Nr3c1* knockout dexamethasone-treated mice. Overall the knockout significantly attenuated insulin resistance in male mice (p=0.005). Due to severe insulin resistance observed in obese dexamethasone-treated mice described in the previous chapter, obese mice were challenged with an exceptionally large dose of insulin (5U/kg lean mass), because of this many of the water-treated controls became hypoglycemic, requiring a bolus of glucose, thereby being removed from the analysis at that point. Therefore, it was not possible to assess insulin sensitivity of obese mice, in the absence of glucocorticoid treatment. Additionally, I am unable to directly compare lean (with an insulin dose of only 1U/kg lean mass) and obese animals to each other for the ITT experiment given the different doses used.

In for females, there appears to be a genotype dependent-effect regardless of glucocorticoid exposure in the lean group, as we observe increased insulin sensitivity *both* in the presence and absence of dexamethasone treatment. In the obese, female group we observed a partial rescue of dexamethasone-induced insulin resistance and hyperglycemia in the *Nr3c1* knockout mice when compared to the WT mice. Similar to the males, we observed significant increases in insulin sensitivity in knockout females (p=0.018). Again, the actual amount of rescue is difficult to determine due to the high dose of insulin. There were no significant effects of sex on insulin tolerance outcomes findings (p=0.346)

Glucose levels following an overnight fast were similar to what was observed in the previous chapter, where fasting glucose is lower in the lean dexamethasone-treated WT mice when compared to WT controls; whereas fasting glucose in the obese dexamethasone treated WT animals was higher than their water-treated counterparts for both sexes (Figure 19B). In the

obese females, the knockout almost fully rescued the dexamethasone-induced increase in blood glucose and partially rescued this effect in obese the males. There is not enough data at this time to assess what the effects of genotype and treatment are in the lean animals. In the lean and obese males, there was no effect of genotype on fasting blood glucose or HOMA-IR Score (Figure 19C-D).

Dexamethasone and Obesity-Induced Liver Triglyceride Accumulation is Blocked in *Nr3c1* Mice

As expected from previous findings (Chapter 2 and Harvey et al. 2018), dexamethasone treatment led to excessive lipid accumulation in the obese male wild type mice when compared to vehicle controls (Figure 20A), this effect was alleviated by the ablation of the adipocyte GR. Furthermore, obesity-induced hepatic lipid accumulation also appears to be reduced in the water-treated knockout animals. These data support the hypothesis that obesity and dexamethasone-induced fatty liver requires adipocyte GR function. While we have not determined the biochemical levels of hepatic triglycerides in this system, our histology also supports a role for adipocyte GR-dependent functions in obesity-associated NAFLD, even in the absence of dexamethasone treatment.

The Effect of *Nr3c1* Ablation on Dexamethasone-Induced Lipolysis

To assess whether obesity- and dexamethasone-induced lipolysis was modulated in *Nr3c1* mice, we measured serum glycerol from overnight fasted animals. The knockout led to lower glycerol

levels overall and completely protected obese female mice from dexamethasone-induced increases in glycerol (p=0.026; Figure 20D-E). There was no effect of the knockout in the water-treated obese females (p=0.424).

Potentially due to the high levels of variation and the limited number of mice that had completed this ongoing study at the time of writing, I did not observe an effect of dexamethasone treatment on serum glycerol (Figure 20B) in the males, nor was there a reduction in *Nr3c1* knockout mice

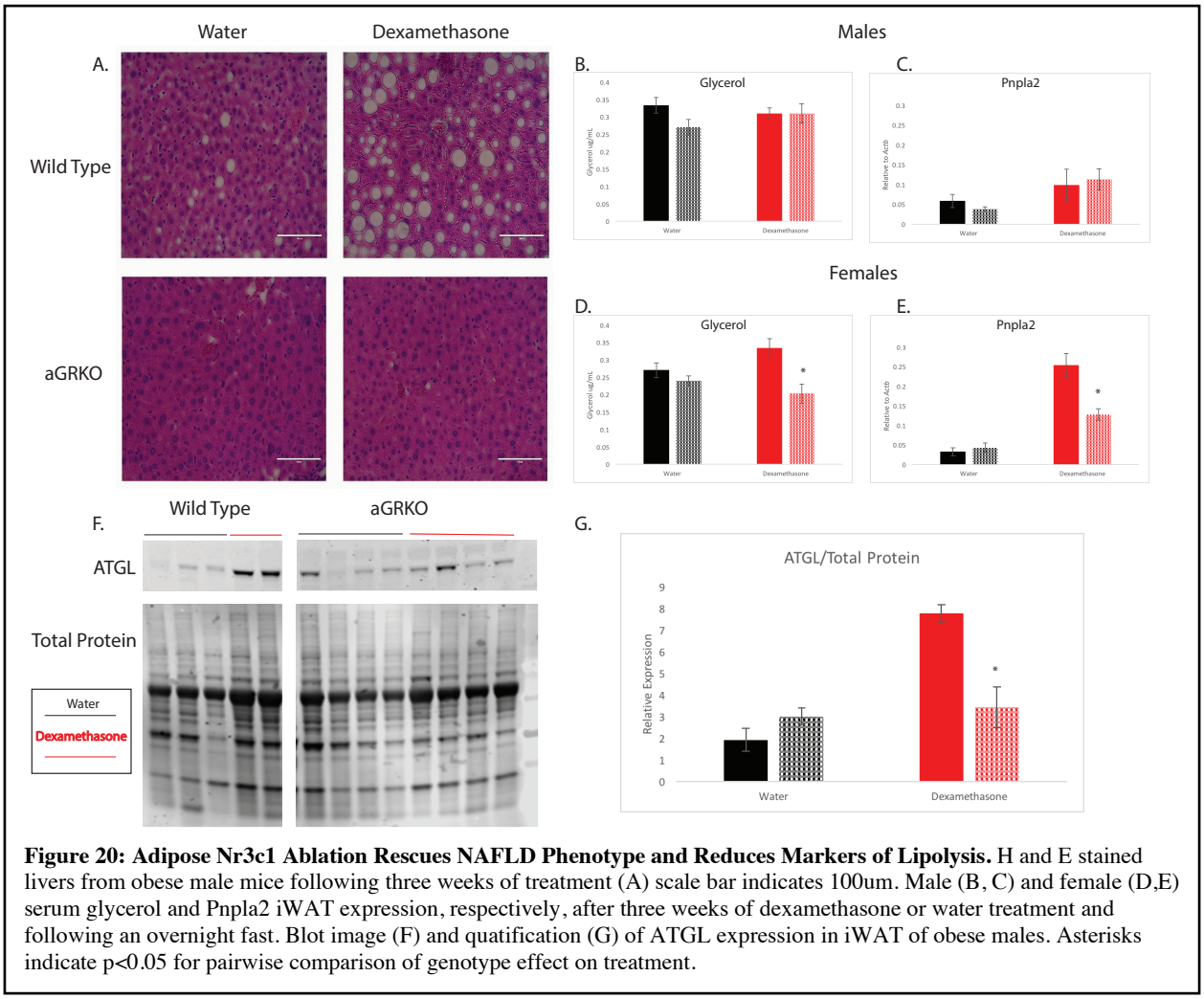

Figure 20: Adipose Nr3c1 Ablation Rescues NAFLD Phenotype and Reduces Markers of Lipolysis. H and E stained livers from obese male mice following three weeks of treatment (A) scale bar indicates 100um. Male (B, C) and female (D,E) serum glycerol and Pnpla2 iWAT expression, respectively, after three weeks of dexamethasone or water treatment and following an overnight fast. Blot image (F) and quatification (G) of ATGL expression in iWAT of obese males. Asterisks indicate p<0.05 for pairwise comparison of genotype effect on treatment.

(p=0.983). Vehicle-treated knockout males did show a slight non-significant reduction in serum glycerol when compared to the vehicle treated wild type mice (p=0.110). The lack of increase in

glycerol following dexamethasone treatment was unexpected, as I have shown dexamethasone-induced elevations of serum glycerol following six weeks of treatment (Harvey et al. 2018) and data from other groups support the finding of elevated glycerol following glucocorticoid treatment *in vitro* (Xu et al. 2009) and *in vivo* (Campbell et al. 2011; Samra et al. 1998). It is likely a larger group sizes may reveal the expected induction of lipolysis that we have observed previously in male mice.

Similarly, to the data presented in Chapter 2, evidence of increased ATGL in obese dexamethasone-treated male mice was observed when compared to water treatment in the WT animals (Figure 20F-G) which is significantly attenuated by GR ablation ($p=0.0148$); though this is not seen at the transcript level in males ($p=0.784$; Figure 20H). In obese female WT mice there is an upregulation of *Pnpla2* in adipose tissue following dexamethasone treatment and this is significantly attenuated in the knockouts ($p=0.011$; Figure 20I), consistent with the lower glycerol observed in this group. Overall, these ongoing studies using the inducible adipocyte-GR knockout mice have revealed that adipocyte glucocorticoid signaling is required for dexamethasone-induced insulin resistance, NAFLD and attenuation of fat mass gains. It is important to note that these results are preliminary findings from an ongoing study and alterations of the knockout on lipolysis will continue to be monitored as more mice come through.

Discussion

Adipose tissue function is paramount to metabolic health. Dysfunction can lead to a variety of metabolic disturbances including insulin resistance and NAFLD and can occur through excess adiposity as well as lipodystrophy. Though opposite in definition, both excess and depletion of adiposity can result in elevated circulating free fatty acids and ectopic fat deposition, similar to what is observed with elevated lipolysis, which can contribute to metabolic disease (Ravussin and Smith 2002). Interestingly, glucocorticoids have been shown to regulate both adipogenesis and adipocyte lipolysis, though the underlying mechanisms have yet to be fully elucidated. In this chapter I have assessed the role of glucocorticoid signaling in adipose tissue as it relates to adipogenesis, lipolysis and metabolic disease.

Glucocorticoids have long been known to be required for adipogenesis *in vitro*, as the removal of glucocorticoid signaling prevents the accumulation of lipid, thereby preventing differentiation (M. J. Lee and Fried 2014). However, whether glucocorticoids induce adipogenesis *in vivo* is still up for debate; as several groups have shown that GR signaling may not be required for adipogenesis (Y.-K. Park and Ge 2017; Bauerle et al. 2018), but it is agreed to at least enhance the process. It has also been suggested that the mineralcorticoid receptor (MR), not the GR, is responsible for adipogenesis (M. Caprio et al. 2007; Massimiliano Caprio et al. 2011). It is difficult to adequately assess the involvement of glucocorticoid-induced adipogenesis *in vivo* as there is not to date an acceptably specific preadipocyte Cre that would be expressed early enough in the adipogenic process. Several genes have been tested for their involvement in glucocorticoid-induced adipogenesis *in vitro*, such as *Nfil3, Dexras1, Ccar1 and Cebpd* (Y. Yang et al. 2017; Cha et al. 2013; Ou et al. 2014; Cao, Umek, and McKnight 1991), but the exact

mechanisms governing glucocorticoid-induced adipogenesis have not been fully elucidated. Here I investigated the role of the transcription factor KLF5 in glucocorticoid-dependent adipogenesis.

Oishi and colleagues showed that the heterozygosity of *Klf5* led to decreased fat mass in mice at birth; however, this was not maintained at 4 weeks of age (Oishi et al. 2005). This paper also demonstrated reduced adipogenesis in response to siRNA-mediated depletion of *Klf5*. Adipogenesis-associated pathophysiologies such as markers of lipodystrophy were not assessed. Additionally, this group showed that overexpression of Klf5 in MEF cells led to partial adipocyte differentiation (absent of insulin, dexamethasone and IBMX); however, they did not evaluate Klf5-related differentiation in the context of glucocorticoids. In further support of glucocorticoid regulation of Klf5, ChIP-seq studies have shown that glucocorticoid stimulation leads to GR binding on putative GRE sites located upstream of the *Klf5* promoter. Here I show that KLF5 transcripts are induced by dexamethasone in 3T3-L1 cells very early in adipogenesis, consistent with a role as an early modulator of adipogenesis. At the time of writing, I was unable to demonstrate of GR binding to any of the putative GRE sites on *Klf5* in response to dexamethasone or full differentiation cocktail above control levels. Further studies, including ChIP and luciferase reporter studies using the KLF5 promoter are needed to determine glucocorticoid regulation of KLF5 in adipogenesis and to clarify whether *in vivo* KLF5 is a MR or GR target.

In the previous chapter I showed that glucocorticoid-induced metabolic disease was exacerbated in the presence of diet-induced obesity and that similar synergistic elevations were seen in markers of lipolysis. Those results suggested but did not demonstrate a key role of adipocyte GR

action in dexamethasone and obesity-associated NAFLD and insulin resistance (Harvey et al. 2018). In this chapter, I found that ablation of the GR in adipocytes rescued or at least partially rescued glucocorticoid-induced insulin resistance and NAFLD in mice, even in the context of obesity and that this was true for both sexes. Within the recent literature, these findings consistent with data from constitutive adipocyte GR and *Hsd11b1* ablation studies. Most (Mueller et al. 2017; Shen et al. 2017; Kotelevtsev et al. 1997; S. A. Morgan et al. 2014) but not all (Desarzens and Faresse 2016) of which demonstrate that adipocyte GR action is essential to glucocorticoid-induced insulin resistance and NAFLD. Our results advance those papers in several important ways, including the incorporation of obesity-moderating effects and the evaluation of female mice. Our inducible knockout approach also reduces potential developmentally-associated adaptations to loss of adipocyte GR function.

An unexpected but intriguing finding that is worth further study is the *increased* adiposity in the dexamethasone-treated GR knockout mice. Several previous studies have suggested feedback looks between leptin and the HPA axis (Zakrzewska et al. 1997; Rosmond et al. 2000; Hochberg, Harvey, et al. 2015). Glucocorticoids promote leptin production, which should suppress food intake. This has been observed in cells, mice and in humans with Cushing's disease including our data, described earlier (See Chapter 2, Figure 3C as well as Masuzaki et al. 1997; Berneis, Vosmeer, and Keller 1996; Pralong et al. 2015; Halleux et al. 1998). To our knowledge this is the first functional association of adipocyte-specific GR action with leptin mRNA levels *in vivo* and an association with weight gain. This circuit may be important for understanding how glucocorticoids regulate appetite in relationship to stress, and how leptin resistance may play a role moderating activity the HPA axis. While our studies have focused on exogenous

administration of glucocorticoids, a far more common situation may be *endogenous* overproduction of glucocorticoids in the obese (Jessop et al. 2001; Andrew, David, and Walker 1998), which we would predict would have similarly worsened metabolic outcomes.

There are several minor differences between this ongoing study and the published work in Chapter 2. We did not observe the same level of obesity- and dexamethasone-induced hyperglycemia. This could be due to several modifications in the protocol, including duration of dexamethasone exposure (six weeks in the previous study versus only three weeks here) or strain differences (inbred C57BL/6J compared to these mice on a mixed C57BL/6 background). Similar to the previous study there was a dexamethasone-dependent decrease in fasting glucose in lean animals that was not influenced by the knockout, indicating adipose GR signaling is not involved in glucose reduction following a six-hour fast. These differences may be resolved as this study is completed and the planned 8 animals per group are assessed.

Obesity and glucocorticoids are known to promote NAFLD. One potential mechanism is by direct action on hepatocytes but our data supports a key role of adipose tissue in this process. Here, and in Chapter 2, I showed that obesity led to hepatic lipid accumulation and that this was exacerbated in the presence of elevated glucocorticoids. Ablation of adipocyte glucocorticoid signaling provided almost a complete rescue of the diet- and treatment-induced hepatic lipid content. Currently there are no established pharmaceutical treatments for NAFLD and drug development has been focused on targeting the liver. Since in our case the knockout was introduced following the onset of obesity and hepatic lipid accumulation, I propose that depletion of GR signaling in the adipose tissue may lead to removal of hepatic triglycerides. The data put

forth here provide promising evidence to support that targeting the adipose tissue for NAFLD treatment would be beneficial.

Several limitations exist in this chapter. First, currently there is not a good Cre available to investigate the role of early adipogenic genes as adiponectin, the staple adipocyte-specific promoter-driven Cre, is not transcribed until late in the adipocyte differentiation process. Therefore, I was only able to investigate the role of *Klf5* in glucocorticoid-induced adipogenesis *in vitro*. Second, performing ChIP studies in adipocytes proved challenging and collaborating with someone with ChIP expertise in this cell type may prove beneficial for obtaining more reliable data on GR-*Klf5* binding. Third, we used a tamoxifen inducible model. Tamoxifen has been reported to cause adipose tissue atrophy and possibly promote adipogenesis (Liu et al. 2015; Ye et al. 2015). As such, I utilized controls (WT +/- tamoxifen) to account for the effects of tamoxifen, though this model may not be not optimal. Finally, this is an ongoing study dependent on breeding transgenic mice and there were insufficient *Nr3c1* knockout mice enrolled in the lean experimental group, thereby preventing full analysis of the findings presented here.

As expected, dexamethasone led to an increased in serum glycerol in the WT females, which was attenuated in the dexamethasone-treated KO mice. Surprisingly, I did not observe dexamethasone-induced lipolysis as measured by serum glycerol in WT males; however, I did observe reductions in ATGL protein, the rate limiting enzyme in adipocyte lipolysis. At this time, it is unclear why glycerol was not elevated in the WT dexamethasone-treated male mice. This is an ongoing study and we plan to measure serum NEFA levels to improve our grasp on

what is occurring in terms of lipolysis. Future studies within the Bridges lab will focus in on whether adipocyte lipolysis is the essential link between glucocorticoid action and insulin resistance.

Chapter 4: The effects of elevated glucocorticoids on muscle physiology

Introduction

The data described here are partially adapted from (Hochberg, Harvey, et al. 2015). While the previous chapters have focused on glucocorticoid-dependent changes on adipose tissue, there are also dramatic and metabolically relevant changes to muscle tissue. Glucocorticoid-dependent muscle atrophy is a major contraindication of prescription glucocorticoids, and the loss of muscle mass alone is predicted to have dramatic effect on post-prandial glucose disposal. Physiologically, under chronic stress glucocorticoids activate proteasomal degradation of proteins, especially in muscle tissue and the released amino acids can feed hepatic gluconeogenesis to maintain blood glucose levels. In this chapter I will describe studies I have performed that investigate how glucocorticoids and obesity affect muscle function.

Developmentally, the consequences of excess glucocorticoids in children, including how this relates to health later in life, is largely unknown. Identifying whether exposure to glucocorticoids early in life predisposes one to metabolic disturbances in adulthood is important, as many children must undergo glucocorticoid treatment for conditions such as asthma or cancer; whereas others may have elevated glucocorticoids due to stressful home environments. There have been a few studies performed using a combination of chart review and prospective assessment in children undergoing glucocorticoid treatment for various cancers, with Acute Lymphoblastic

Leukemia being the most common (Wilson et al. 2015; Chow et al. 2007; Van Dongen-Melman et al. 1995). These longitudinal studies in pediatric patients from multiple children's hospitals using a variety of treatment protocols were assessed with treatment durations ranging from five weeks to three years. Type of therapy (radiation, chemotherapy, or a combination of these; surgery was not accounted for among these analyses), area and dose of radiation, type and dose of drug, and duration of treatment were stratified at different levels in each study to investigate individual treatment effects. Ages ranged from 0-20 years at diagnosis and patients were followed anywhere from 1-40 years after cessation of treatment.

The overall findings from the above studies report pediatric patients given glucocorticoids have increases in relative risk ranging from 1.21 to 2.18 of developing obesity later in life compared to those not given glucocorticoids. This remains true after other treatment related factors and existing relative risk for obesity in the otherwise healthy control populations used in these studies are considered. Additionally, other retrospective studies involving children exposed to high-stress living conditions also found an increased likelihood of obesity in adulthood (Felitti 2002; Gundersen et al. 2011; Tamayo, Christian, and Rathmann 2010).

Though the above studies include other factors that may confound the acquired data, these findings suggest that exposure to glucocorticoids during childhood leads to increased risk for obesity later in life. Further investigation in a more controlled setting is needed in this area as many children across the world are exposed to high levels of glucocorticoids due to increased stress or treatment for other health problems such as asthma and cancer. Additionally, the effects

of juvenile and adult elevated glucocorticoids on muscle and overall metabolic health warrant further investigation.

Muscle is one of the most metabolically active tissues in the body and is extremely important for overall health as well as growth and development. Under normal circumstances, muscle tissue is in a constant flux of degradation and regeneration via protein breakdown (or proteolysis) and synthesis of amino acids, respectively; however, when glucocorticoids are elevated for prolonged instances this process is dysregulated. Muscle wasting is a common side effect of excess glucocorticoids and is caused by both increased muscle proteolysis and decreased protein synthesis (Deng et al. 2004; Menconi et al. 2007). Glucocorticoids exposure has been shown to activate the muscle ubiquitin-proteasome system in rats (Price et al. 1994; Wing and Goldberg 1993), increasing muscle expression of proteases (cathepsins B and D, calpain) and elements of the ubiquitin-proteasome pathway (D. Dardevet et al. 1995) along with inhibition of muscle protein synthesis (Long, Wei, and Barrett 2001). A study in healthy humans also found that prednisone increases leucine oxidation (Beaufrere et al. 1989). Moreover there is evidence to suggest that glucocorticoid treatment leads to reduced muscle strength and cardiorespiratory fitness in juveniles (Blom et al. 2017) and adults (Barry and Gallagher 2003).

Aside from being extremely important for mobility, strength, posture and overall health and fitness, muscle tissue is the primary site for insulin-stimulated glucose uptake (Baron et al. 1988). Glucocorticoids have also been shown to affect muscle metabolic health, as shown by impairments in muscle insulin signaling following dexamethasone treatment (Weinstein et al. 1995). Indeed, insulin resistance and Type-2 Diabetes are common in individuals with

chronically elevated glucocorticoids, as previously shown in Chapter 2 (Hochberg, Harvey, et al. 2015; Harvey et al. 2018). Although there is no evidence to suggest that muscle atrophy affects insulin signaling in the muscle, insulin resistance in the muscle has been shown to promote muscle atrophy (Xiaonan Wang et al. 2006; Dirks et al. 2016). These findings are consistent with insulin inhibiting muscle proteolysis (Chen et al. 2011; O. L. K. Smith 1988). Therefore, muscle insulin signaling is essential in the maintenance of muscle health, especially in individuals who are already at risk for muscle atrophy.

Similar to chronically elevated glucocorticoids, obesity has been associated with decreased muscle strength and function across variables of age and sex (Maffiuletti et al. 2007; Blimkie, Sale, and Bar-Or 1990; Hulens et al. 2001; Zoico et al. 2004), as well as decreased muscle mass when compared to lean humans (Wood et al. 1996). Systemic insulin resistance is a well-known co-morbidity of obesity; however, the exact mechanisms governing obesity-induced insulin resistance remain up for debate, muscle has been suggested as a key tissue in this process. Both insulin and glucocorticoids regulate FOXO-dependent transcription involved in the processes of insulin signaling and muscle atrophy, respectively (X Wang et al. 2017; Tzivion, Dobson, and Ramakrishnan 2011; Waddell et al. 2008). Though much evidence exists for the catabolic effects of glucocorticoid in muscle and how this relates to glucocorticoid-induced insulin resistance, the underlying mechanisms are not yet fully understood. Furthermore, the modifying effects of obesity on glucocorticoid-induced muscle atrophy and insulin resistance has yet to be investigated. Given their similarities, I hypothesized that the combination of chronically elevated glucocorticoids and obesity would lead to exacerbated perturbations in muscle insulin signaling.