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The role of glucocorticoid receptor signaling in metabolic disease: a matter of time and sex

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The role of glucocorticoid receptor signaling in metabolic disease: A matter of time and sex

Sheng Li

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The role of glucocorticoid receptor signaling in metabolic disease: A matter of time and sex

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TABLE OF CONTENTS

Chapter 1	**General introduction and outline**	**7**
Chapter 2	**Sexual dimorphism in transcriptional and functional glucocorticoid effects on mouse skeletal muscle**	**35**
Chapter 3	**Out-of-phase treatment with the synthetic glucocorticoid betamethasone causes disturbances in glucose metabolism in mice**	**67**
Chapter 4	**Glucocorticoid receptor antagonism improves glucose metabolism in a mouse model of polycystic ovary syndrome**	**99**
Chapter 5	**General discussion and future perspectives**	**131**
Chapter 6	**English summary**	**149**
	Nederlandse samenvatting	
	List of publications	
	Curriculum Vitae	
	Acknowledgements	

1

General introduction and outline

GENERAL INTRODUCTION AND OUTLINE

Glucocorticoids are steroid hormones that play an essential role in many physiological processes, including the stress response and the maintenance of homeostasis [1-3]. These hormones, primarily cortisol in humans and exclusively corticosterone in mice, are synthesized and secreted by the adrenal glands in response to various stimuli. A factor that strongly affects adrenal glucocorticoid secretion is the circadian rhythm. Glucocorticoid effects are achieved via different signaling pathways, predominantly through binding to the glucocorticoid receptor (GR). The GR is expressed in almost all cell types in the human body. Activation of GRs initiates a cascade of events that alters gene expression and thereby regulates immune responses, metabolism and many other processes [4-8].

Synthetic glucocorticoids are potent anti-inflammatory and immunosuppressive drugs that have been widely used for treating various medical conditions including inflammatory diseases [9-11]. However, chronic glucocorticoid exposure—whether from exogenous sources or prolonged increases in endogenous levels—can result in severe metabolic disturbances, including muscle mass loss, impaired glucose and lipid metabolism, and osteoporosis [12-14]. Understanding the molecular and endocrine effects of glucocorticoids is essential to design appropriate therapeutic strategies and to mitigate the adverse effects when these steroids are used for a long period of time.

In the work presented in thesis, I investigated several metabolic disturbances associated with glucocorticoids, their biological mechanisms, and whether glucocorticoid actions are sexually dimorphic - and if so, whether interactions with sex steroids play a role. In addition, I address the optimal time of treatment with glucocorticoids in relation to reduction of side effects while maintaining therapeutic effects.

1 Glucocorticoid receptor signaling

The hypothalamic-pituitary-adrenal (HPA) axis is the main endocrine system that regulates secretion of glucocorticoids by the adrenal gland. When the HPA-axis is stimulated, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are released by the hypothalamic paraventricular nucleus (PVN) into a portal circulation system that connects the hypothalamus and the pituitary gland [15, 16]. Subsequently, CRH binds to the CRH-R1 receptor in the pituitary gland which leads to the release and secretion of the adrenocorticotrophic hormone (ACTH) into the systemic circulation. ACTH will

in turn increase the synthesis and secretion of cortisol and/or corticosterone from the adrenal glands [17]. At the basal non-stressed level, glucocorticoids are released from the adrenal glands in a circadian and ultradian rhythm. This release pattern is characterized by peak levels preceding and during the early active phase, which is in the morning in humans and at the beginning of the night in mice [18]. Next to the circadian variation, physical and psychological stress is an important stimulus of HPA-axis activation.

The HPA-axis is subject to negative feedback, as elevated circulating levels of glucocorticoids exert inhibition at the hypothalamic and pituitary level, suppressing the synthesis and release of CRH and ACTH respectively [19]. This regulatory mechanism is crucial since it helps in modulating the level of glucocorticoids in the body and balancing the stress response. Dysregulation of the secretion in the HPA-axis can lead to several health-related issues. For instance, long-term stress may lead to the sustained stimulation of the HPA-axis and the constant elevated levels of glucocorticoids in the bloodstream may cause anxiety and depression, immune dysfunction, as well as metabolic diseases [20].

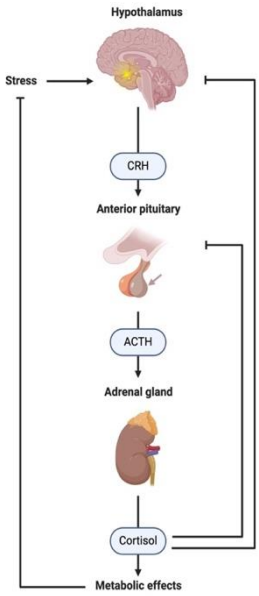

Fig. 1 Hypothalamic–pituitary–adrenal (HPA) axis. The neuroendocrine response to stress involves activation of the HPA axis, beginning with the release of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn triggers the adrenal glands to secrete glucocorticoids-cortisol in humans and corticosterone in rodents. Once in circulation, glucocorticoids exert both peripheral and central effects by binding to mineralocorticoid and/or glucocorticoid receptors in nearly all organs and tissues, including the brain. The hippocampus modulate hypothalamic activity, thereby regulating the HPA axis through feedback mechanisms.

The bioavailability of glucocorticoids is regulated by the balance between active and inactive forms. This process is regulated by two different enzymes that catalyze the turnover between the inactive (analogs of) cortisone or 11-dehydrocorticosterone and the active forms of cortisol or corticosterone. 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) positively affects cortisol

availability, by catalyzing the conversion of cortisone to cortisol, while 11β-HSD2 is responsible for the opposite reaction. 11β-HSD1 is predominantly expressed in metabolic tissues such as the liver and adipose tissue, locally amplifying intracellular glucocorticoid action. Its upregulation is often associated with metabolic dysregulation including insulin resistance, obesity, and dyslipidemia [21-23], emphasizing its role in metabolic homeostasis and the development of metabolic diseases. Conversely, 11β-HSD2 is expressed mainly in aldosterone target organs including the kidney and colon. It prevents cortisol or corticosterone from binding to mineralocorticoid receptors (MR), which allows selectivity for the mineralocorticoid hormone aldosterone. In this way 11β-HSD2 plays a role in the preservation of the levels of electrolytes and blood pressure. Gene mutations or inhibition of 11β-HSD2 are therefore associated with hypertension and abnormal electrolyte levels, demonstrating that 11β-HSD2 action is crucial in cardiovascular and renal functions [24, 25].

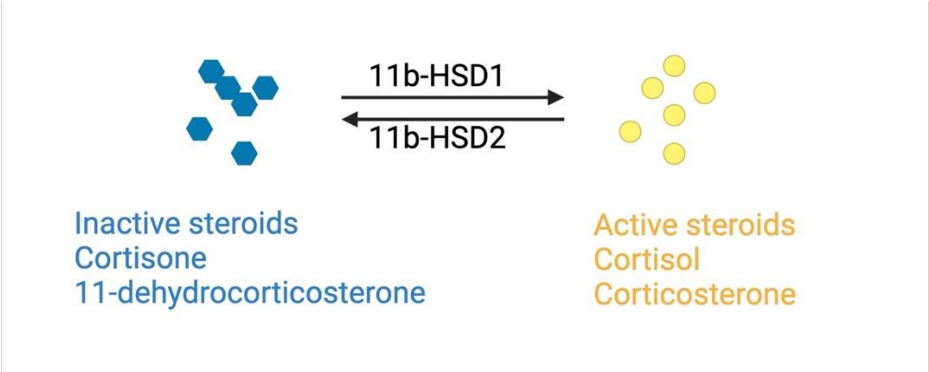

Fig. 2 Interconversion of Inactive and Active Glucocorticoids via 11β-HSD Enzymes. The balance between inactive and active glucocorticoids is regulated by the actions of 11β-HSD1 and 11β-HSD2. 11β-HSD1 converts inactive steroids, such as cortisone in humans and 11-dehydrocorticosterone in rodents, into their active forms, cortisol and corticosterone, respectively. 11β-HSD2 facilitates the reverse process, inactivating these active glucocorticoids to maintain proper signaling and prevent overstimulation.

Corticosterone-binding globulin (CBG) is a glycoprotein synthesized in the liver which modulates glucocorticoid activity. CBG can bind glucocorticoids, thereby limiting their availability in target tissues, and it plays a crucial role in the clearance of glucocorticoids from the circulation. Under basal conditions, 80% of circulating glucocorticoids is bound by CBG, around 15% to albumin and only 5% is available as the free fraction. During stress and inflammation, the concentration of glucocorticoids is increased and can saturate the binding capacity by CBG, which results in increased free glucocorticoids levels and enhanced anti-inflammatory effects [26].

Glucocorticoids can act via two types of receptors: the GR and - in cells that do not express 11β-HSD2 - the MR. These two receptor types are the members of the nuclear receptor (NR) family of intracellular receptors, which also contains the estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR) [27, 28]. Many of these receptors influence various metabolic processes within different tissues. The MR is activated by the endogenous glucocorticoids, while synthetic glucocorticoids do not influence MR activity except at very high doses [29]. In contrast, the GR is activated by cortisol and corticosterone and by synthetic glucocorticoids alike [30]. Upon glucocorticoid binding, GR undergoes conformational changes and translocates into the nucleus where it binds to glucocorticoid response elements (GREs) in the DNA. GR DNA binding is influenced by tissue-specific chromatin accessibility and interactions with coregulators which help regulate transcription. GR signaling is further modulated by cellular variations in receptor isoforms, post-translational modifications, and interactions with other transcriptionally active proteins, which together shape the cell-specific response to glucocorticoid signaling across various tissues [31, 32].

GR is a modular protein comprising of several distinct domains: the N-terminal transactivation domain (NTD), the DNA-binding domain (DBD), the hinge region and the Ligand-Binding Domain (LBD), each contributing to receptor function. The NTD contains an activation function-1 (AF-1) for ligand-independent transcriptional activation [33, 34]. The GR target gene selection depends on the DBD of GR. It contains two zinc finger motifs that allow the receptor to bind to GREs within the DNA. This binding triggers other receptor domains to recruit coactivators, chromatin remodeling complexes and other transcription machinery to the promoter region of the target genes to regulate transcription [35, 36]. These interactions lead to histone modification and nucleosome remodeling, which in turn increases chromatin accessibility and transcriptional activation [37]. Conversely, GR can also repress gene expression by binding to negative GREs (nGREs) or by interacting with other transcription factors such as NF-κB and AP-1 [38, 39]. This repression often involves the recruitment of corepressors and histone deacetylases (HDACs), leading to chromatin condensation and decreased accessibility [40]. The DBD is necessary in the interaction with GREs to regulate the GR in the activation as well as suppression of genes in response to glucocorticoids. The LBD of the GR contains the ligand binding pocket of the receptor, and glucocorticoid binding to this pocket induces a structural (or conformational) change of the receptor. This conformational change forms activation function-2 (AF-2) that is required for transcriptional activation that occurs in the presence of ligand, via recruitment of coactivators and other transcription machinery [41]. Additionally, the LBD is crucial for receptor dimerization required for interaction with many GREs on the DNA [41].

2 Androgens, the HPG-axis and glucocorticoids

Androgens are a group of hormones primarily known for their role in male sexual development and function. Androgen production by the testes is regulated by the Hypothalamus-Pituitary-Gonadal (HPG)-axis [42]. Analogous to the HPA-axis, this process starts in the hypothalamus, which releases gonadotropin-releasing hormone (GnRH) in a pulsatile manner. GnRH stimulates the pituitary gland to secrete two key hormones: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH plays a crucial role in stimulating Leydig cells of the testes to secrete testosterone [43]. Similar to glucocorticoids, the levels of testosterone are also regulated through negative feedback loop in the HPG-axis.

Testosterone is mainly synthesized in the testes in men and in the adrenal glands of both men and women but in lesser amounts. It is involved in the development of the male reproductive tissues, secondary sexual characteristics and is essential to sexual health in both genders [44]. Similar to glucocorticoids, enzymatic modification of androgens is essential for this process. Dihydrotestosterone (DHT) is a potent steroid hormone with androgenic properties involved in several biological processes in human body. This sex hormone is derived from testosterone through the action of the enzyme 5-alpha reductase [45], and its physiological effects are therefore regulated by the expression of this enzyme. DHT exhibits a higher binding affinity to the AR and has increased biological activity in specific tissues that include prostate, skin, and hair follicles [46-48]. The pathways for androgen metabolism are not restricted to the conversion of testosterone to DHT. Aromatase is another essential enzyme which converts testosterone into estradiol, showing that the androgen and estrogen pathways are interrelated. This conversion is crucial in tissues such as adipose tissue, liver and the brain since (testosterone derived) estrogens via ERs regulate important (metabolic) processes in these tissues [49-51]. The equilibrium of these enzymatic conversions is extremely well maintained and loss of this balance has serious consequences for metabolic homeostasis. For instance, the activities of 5α-reductase and aromatase on androgens and estrogens affect muscle mass, body fat distribution, insulin sensitivity, and lipid metabolism [52-56], and dysregulation of these enzymes can lead to metabolic disorders.

The activation of the AR can induce genomic and non-genomic intracellular signaling. For genomic actions, testosterone and DHT diffuse through the cell membrane and bind to intracellular ARs which are present in the cytoplasm. In the nucleus, androgen-AR complexes bind to androgen response elements (AREs) of the regulatory regions of target genes [57]. Its genomic mechanisms of action are similar to those of GR. Expression of AR target genes in turn leads to the synthesis of proteins with various androgenic activities, to e.g. increase

muscle mass and to change the distribution of the body fat [58, 59]. Non-genomic actions involve rapid signaling pathways through membrane-associated AR and secondary messengers including the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the mitogen-activated protein kinase (MAPK) pathway [60]. These pathways lead to rapid cellular responses that do not involve direct changes in gene expression, contribute to a variety of cellular effects, such as increased glucose uptake, enhanced muscle cell contraction, and immediate changes in cellular metabolism [59, 61]. The effects of androgens in adulthood are generally transient. For instance, muscle mass may reduce when androgen concentration is low, but typically restores upon androgen replacement [62]. However, prolonged and excessive exposure to androgens contribute to various deleterious effects as exemplified in patients with polycystic ovary syndrome (PCOS) [63, 64]. Intriguingly, these AR-driven metabolic disorders have several similarities with GR-driven effects, and therefore attenuating GR signaling may provide a novel strategy for some androgen-induced pathologies.

Several studies have shown interactions between glucocorticoids and androgens, *e.g.* with effects of glucocorticoid signaling on the HPG-axis. Elevated glucocorticoid levels as a response to stress inhibit reproductive function to prioritize self-preservation. Glucocorticoid excess suppresses the HPG-axis by inhibition of GnRH and testosterone secretion [65]. The inhibition of testosterone production by glucocorticoids was also found at the level of the testis. In the testis, GR is expressed in various interstitial cell types including Leydig cells, macrophages, fibroblasts, smooth muscle cells [66], and male reproductive accessory tissues including the epididymis and prostate are also GR-enriched [67]. Male patients with Cushing's syndrome, characterized by elevated cortisol levels, show a correlation between high cortisol levels and low plasma testosterone concentrations, illustrating a clinical condition in which glucocorticoids suppress androgen levels [68]. Administration of the synthetic glucocorticoid dexamethasone was shown to suppress testosterone levels [69]. Furthermore, glucocorticoids have been found to inhibit steroidogenesis in the testes, leading to a decrease in testosterone production [70].

In addition to their effects on testosterone levels, glucocorticoids also influence estrogen levels. The ovary, the primary source of estrogens in females, is regulated by glucocorticoids throughout a woman’s reproductive lifespan. Stress-related increases in glucocorticoids negatively affect fertility in women, compromising both ovarian function and uterine function. The GR is present in different ovarian cells including the follicles and corpus luteum and its expression is consistent throughout different stages of the reproductive cycle in rats [71]. Glucocorticoids inhibit LH-induced stimulation of steroidogenesis in ovarian cells, suppressing progesterone synthesis through direct effects on the

enzymes 3β hydroxysteroid dehydrogenase (3β-HSD) and 20α hydroxysteroid dehydrogenase (20α-HSD) [72, 73]. The ovary exhibits tissue-specific regulation of glucocorticoids, including the regulation of 11β-HSD expression during follicular maturation and ovulation [74]. These mechanisms regulate steroidogenesis, oocyte maturation, corpora lutea maintenance, and luteal regression [75, 76]. Although estrogen-glucocorticoid interactions are important, this thesis focuses primarily on androgen and glucocorticoid hormones.

3 Glucocorticoid receptor signaling in metabolic diseases

Metabolic diseases including obesity, type 2 diabetes, steatotic liver disease and cardiovascular diseases have become a global health burden. These conditions cause significant morbidity and mortality and are generally defined by a state of disrupted energy balance, insulin insensitivity and inflammation. Next to its profound effects on inflammatory and autoimmune diseases, GR also emerged as a critical player in the pathophysiology of these metabolic diseases due to its critical role in regulating metabolism, inflammation and the stress response.

GR signaling in various tissues is involved in the pathogenesis of metabolic diseases, including skeletal muscle, adipose tissue and liver [77-79]. However, these pathological effects via metabolic disturbances can differ between endogenous and exogenous glucocorticoids. The effects of synthetic glucocorticoids are often more pronounced due to their higher potency, longer half-life, and their administration may also disturb circadian regulation of endogenous glucocorticoids [80]. Endogenous glucocorticoids are tightly regulated by the body's feedback mechanisms, which (attempt to) mitigate prolonged exposure and its associated risks. In contrast, prolonged or overexposure to synthetic glucocorticoids can overwhelm these regulatory systems and lead to more severe side effects. Moreover, synthetic glucocorticoids are often administered in pharmacological doses that exceed physiological levels, further exacerbating their pathologic potential [80].

Dysregulated GR signaling has a significant impact on whole body metabolism, contributing to different metabolic disturbances. Conditions of glucocorticoid deficiency (e.g. Addison’s disease) can be the result of several causes, including autoimmune disease, genetic defects in glucocorticoids production, or pituitary disease [81]. Symptoms associated with glucocorticoid deficiency include weight loss and low blood sugar levels. In contrast, patients with Cushing's syndrome with excessive cortisol production experience health issues such as central obesity, muscle loss, high blood sugar, fatty liver, high blood pressure, elevated cholesterol, weakened immune system, and insulin resistance [82]. In

patients with metabolic syndrome, elevated glucocorticoids levels are generally found and are often associated with hyperglycemia, insulin resistance and dyslipidemia [83-85]. However, obesity is not typically linked to high systemic glucocorticoid levels, but rather to an increase in local glucocorticoid effects that contribute to the development of metabolic syndrome [86].

One of the most concerning outcomes of chronic glucocorticoid exposure is muscle atrophy, a condition characterized by the loss of muscle mass and strength. This is particularly relevant in metabolic diseases where glucocorticoid levels are persistently high. Glucocorticoids stimulate the ubiquitin-proteasome pathway and the autophagy-lysosome system which degrades proteins from the skeletal muscles into amino acids [87, 88]. This catabolic effect is achieved through the upregulation of muscle-specific E3 ubiquitin ligases including muscle RING finger 1 (MuRF1) and atrogin-1 [89, 90]. As a result, glucocorticoids reduced muscle mass and function through this process of catabolism of muscle proteins, thereby resulting in muscle wasting and weakness [91]. GR signaling additionally impairs insulin signaling pathways in muscle tissue, leading to insulin resistance [92]. Glucocorticoids are involved in the activity of insulin receptor substrate (IRS) and phosphoinositide 3-kinase (PI3K)/Akt signaling which is crucial in muscle cells for glucose uptake and glycogen synthesis [87]. Decrease in the uptake and utilization of glucose in muscles contributes elevated blood glucose levels that lead to hyperglycemia in metabolic disorders.

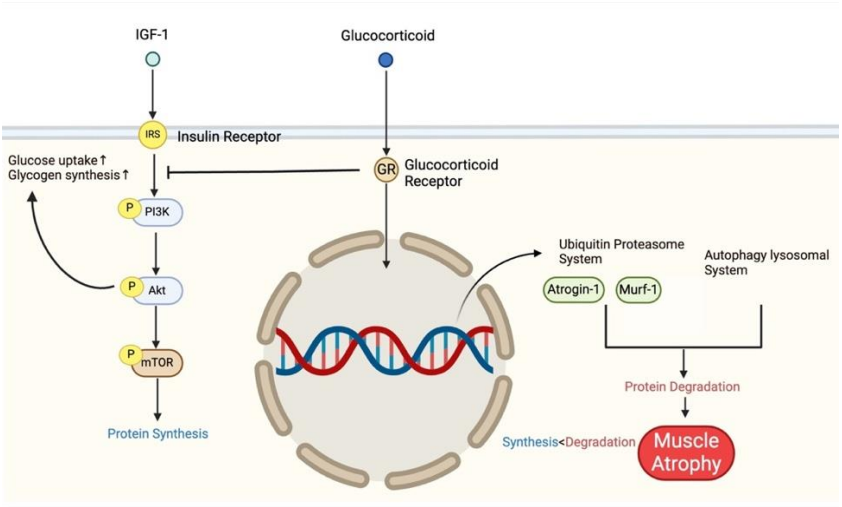

Fig. 3 Glucocorticoid-induced regulation of muscle protein synthesis and degradation pathways. Insulin-like growth factor 1 (IGF-1) signaling through the insulin receptor (IRS) activates the PI3K-Akt-mTOR pathway, promoting glucose uptake, glycogen synthesis, and protein synthesis. Conversely, glucocorticoids bind to the glucocorticoid receptor (GR) and modulate transcriptional activity in muscle cells, leading to the activation of catabolic

pathways, including the ubiquitin-proteasome system (UPS) and autophagy-lysosomal system. Key markers such as Atrogin-1 and Murf-1 facilitate protein degradation, tipping the balance towards muscle atrophy when protein degradation exceeds protein synthesis. The interplay between these anabolic and catabolic pathways highlights the impact of glucocorticoid signaling on muscle homeostasis.

GR signaling pathways also significantly influence the function of adipose tissues. Chronic activation of GR signaling can result in obesity and adipocyte hypertrophy [93]. This includes the differentiation of preadipocytes into adipocytes and the expansion of these cells due to increased lipid accumulation [93, 94]. The overexpression of key adipogenic transcription factors such as PPARγ and C/EBPα is driven by GR activation, and contributes to the expansion of adipose tissue mass, particularly in visceral fat depots [95]. The hypertrophic adipocytes become dysfunctional with reduced ability to store lipids, and with altered secretion of adipokines that in turn exacerbate the metabolic disturbances. The expanded visceral adipose tissue is metabolically active, secreting high levels pro-inflammatory cytokines, adipokines and free fatty acids, resulting in insulin resistance and cardiovascular disease [96, 97]. Moreover, overexpression of 11β-HSD1 in adipose tissues or liver is also associated with metabolic diseases. 11β-HSD1 increases local glucocorticoid levels and influences receptor activation in tissues, thereby affecting processes such as fatty acid metabolism and all other aspects mentioned above [98].

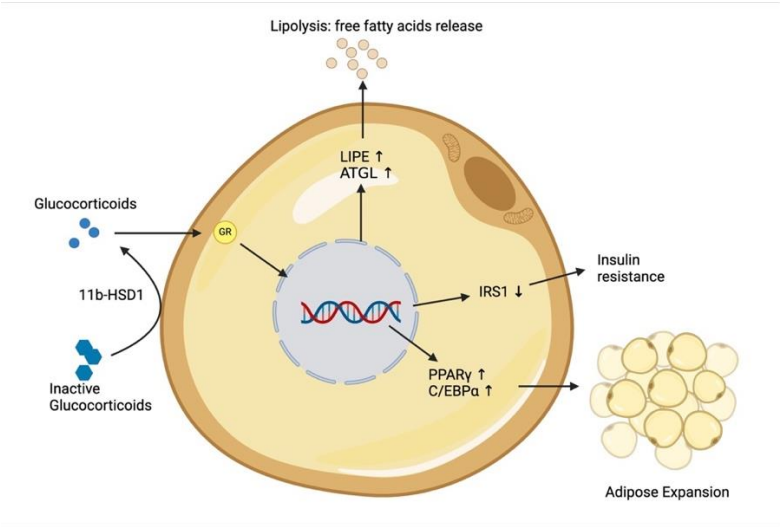

Fig. 4 Glucocorticoids signaling in Adipocytes. Glucocorticoids are activated locally within adipose tissue by 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), converting inactive GCs into their active forms. Glucocorticoid activation of GRs leads to increased lipolysis, mediated by the upregulation of lipolytic enzymes such as adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (LIPE), resulting in the release of free fatty acids (FFAs) into circulation. This contributes to systemic metabolic changes. Concurrently, glucocorticoids

impair insulin signaling by reducing Insulin Receptor Substrate 1 (IRS1) activity, promoting insulin resistance. Additionally, Glucocorticoids enhance the expression of transcription factors, including peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα), which drive adipocyte differentiation, hypertrophy, and hyperplasia. These combined processes contribute to adipose tissue remodeling and expansion, further exacerbating obesity-related metabolic dysfunctions.

The liver is a central organ in glucose homeostasis and GR signaling significantly affects hepatic glucose metabolism. Glucocorticoids increase gluconeogenesis through the upregulation of key enzymes expression in the hepatic gluconeogenic pathway such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [99]. In Metabolic dysfunction-Associated Fatty Liver Disease (MAFLD), the chronic activation of GR signaling stimulates the hepatic gluconeogenesis and thus causes hyperglycemia and impaired glucose tolerance [100, 101]. In addition, prolonged activation of GR exacerbate hyperglycemia by disrupting glycogenolysis in states of fasting and stress [101]. Moreover, glucocorticoids affect lipid fluxes in the body, which may also contribute to obesity and metabolic disease [102, 103].

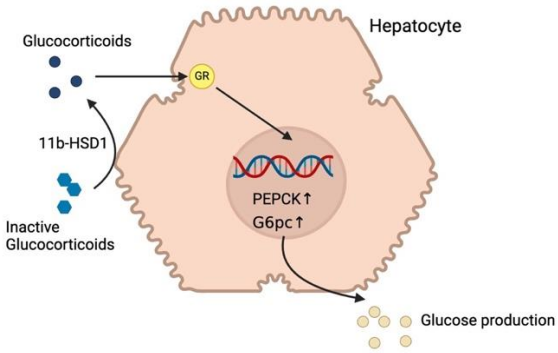

Fig. 5 Glucocorticoid-Regulated Hepatic Gluconeogenesis. Within the liver, inactive glucocorticoids are enzymatically converted into their active forms by 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), thereby enhancing their local bioavailability. The GR signaling cascade upregulates the expression of key gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6pc), which are critical for the synthesis and release of glucose from non-carbohydrate substrates.

Given the central role of GR in metabolic diseases, intervention of GR signaling could be a potential therapeutic approach. Furthermore, novel and targetable biochemical pathways can be discovered by understanding the tissue-specific effects of GR and the molecular mechanisms behind its interactions with other metabolic regulators.

3.1 Androgen and glucocorticoid signaling crosstalk in metabolic tissues and sexual dimorphism of glucocorticoid effects

Sexually dimorphic effects of glucocorticoids have been observed in metabolic processes including inflammation and glucose metabolism. Males and females exhibit sex differences in transcriptional regulation in response to glucocorticoid treatment, involving differential regulation of signaling pathways such as apoptosis in thymocytes [104] and circadian rhythm of skeletal muscle, liver, adipose tissues, kidney [105-108]. In addition, chronic glucocorticoid exposure-induced metabolic alterations differ between sexes, in which male mice show increased blood glucose levels, insulin resistance, insulinemia, adiposity and hyperlipidemia as compared to female mice [109]. These findings suggest that males are more susceptible to the adverse metabolic effects of glucocorticoid exposure.

In skeletal muscle, previous studies suggest steroid hormone interaction between androgens and glucocorticoids. Dexamethasone treatment decreased muscle weight in male rats, which was prevented by concurrent administration of testosterone [110, 111]. Androgen administration thus protects against glucocorticoid-induced muscle atrophy, and this is likely mediated via downregulation of muscle specific ubiquitin ligases atrogin-1 and Murf1, which are known to be involved in glucocorticoid-induced protein degradation and muscle wasting [112]. These findings suggest direct crosstalk between glucocorticoids and androgens in skeletal muscle. In this tissue, the two steroids tend to have opposite (anabolic and catabolic) effects.

In white adipose tissue and liver, glucocorticoid-induced gene expression is in part dependent on AR signaling [113]. This suggests that AR activity can – in contrast to effects in skeletal muscle - increase GR-induced transcription in various peripheral tissues and this is possibly related to metabolic outcomes. In male but not in female mice, chronic exposure to glucocorticoids inhibits thermogenic activity in brown adipose tissue. [79, 114], indicating a sexual dimorphism that is possible related to differences in androgen and/or estrogen signaling. Excess corticosterone leads to lipid accumulation and a white adipose tissue-like appearance of brown adipose tissue in male mice, which is reversed by orchiectomy and restored with DHT administration [79]. Furthermore, DHT treatment potentiates GR signaling in brown adipose tissue in intact male mice [79]. In contrast, female mice are inherently more resistant to glucocorticoid-induced effects and exhibit lipid accumulation in brown adipose tissue following AR activation with DHT [115]. Altogether, many metabolic effects of glucocorticoids, including insulin resistance, seem to be androgen-dependent in mice.

Glucocorticoids and androgens exhibit different interactions in various tissues via different potential mechanisms. This crosstalk may involve competitive binding to shared response elements and possible coordination in the process of transcription. GR DNA binding is dependent on chromatin pre-accessibility [116], but can be influenced by AR-mediated chromatin opening [117]. In addition, various modulatory coactivators and the chaperone protein FKBP51 can affect GR signaling and are also associated with AR signaling, contributing to the complex crosstalk between glucocorticoids and androgens [118]. Other mechanisms potentially involve a negative androgen response element (nARE) in the GR promoter, overlapping cistromes of GRs and ARs, and potential cooperative transcriptional regulation through assisted loading [119]. This interference can result in mutual repression or modulation of target gene expression, affecting metabolic pathways regulated by both receptors. Besides direct interaction, AR activity can induce 11β-HSD1, influencing the local balance of GR and AR activation [120]. In addition, cytochrome P450 Enzymes (CYPs) are involved in the metabolism of both glucocorticoids and androgens. Regulation of CYP enzymes by GR and AR can affect the clearance and activity of these hormones, influencing their overall effects on metabolism [121].

The main focus of this thesis is crosstalk between glucocorticoids and androgens, but it should be noted that estrogens can also interact with glucocorticoids at the endocrine and molecular level. Estrogen signaling can contribute to the sexually dimorphic effects of glucocorticoids, and molecular interactions between these hormone systems play a crucial role in shaping metabolic processes and inflammatory responses.

3.2 The role of circadian glucocorticoid signaling in metabolic health

The daily oscillation of glucocorticoids is controlled by the central clock and the adrenal clock. In the suprachiasmatic nucleus of the hypothalamus, the central clock controls the circadian rhythm by regulating the activity of HPA-axis and the sympathetic innervation of the adrenal gland [122]. This regulation concerns the release of CRH and ACTH in response to environmental stimuli [123]. In addition, the adrenal gland also has an intrinsic clock that controls the steroid production and its response to ACTH. This peripheral clock is synchronized by the central clock and forms part of the regulation of this rhythm by controlling the adrenal's capability to secrete glucocorticoids [124]. This regulation is crucial for optimizing physiological processes and behavior at the right time of day [125]. The circadian rhythm of adrenal glucocorticoids is an important 'zeitgeber' mechanism for many cells in the body, and has significant implications for human health and disease.

The circadian secretion of glucocorticoids plays a vital role in regulating energy balance by increasing glucocorticoids levels before the active period [126]. Imbalances in glucocorticoid rhythms are associated with metabolic disorders like obesity, diabetes, dyslipidemia, and atherosclerosis [127]. Pathological excess or glucocorticoid insufficiency can lead to symptoms affecting metabolic functions, but loss of rhythmicity is often intrinsic to these situations, and disrupted circadian glucocorticoid rhythms are also linked to metabolic disorders [128]. The circadian aspect may well play a role in the onset or progression of conditions like obesity, type 2 diabetes, dyslipidemia, and atherosclerosis.

The strategies of chronotherapy in medicine have gained attention in the recent years, with studies demonstrating that the timing of medication administration may influence therapeutic outcomes [129, 130]. Recent findings suggest that whether or not the timing of glucocorticoid administration aligns with body's endogenous circadian rhythms may significantly influence their metabolic effects [130, 131]. In the clinic, morning compared to evening administration of glucocorticoids, when given in a pattern consistent with the endogenous rhythm of cortisol, improved glycemic control and reduced insulin resistance [132, 133]. The GR itself is also subjected to circadian regulation with variations in its expression and responsiveness during the day. Administration glucocorticoids when endogenous glucocorticoids levels are high can potentially minimize the negative effects including insulin insensitivity and dyslipidemia development caused by the prolonged exposure. However, the underlying mechanisms and clinical implications of these findings remain to be fully elucidated [134].

OUTLINE OF THIS THESIS

In this thesis we investigated how sex (hormones) and time can influence the functional and transcriptional response of glucocorticoid signaling, with a particular focus on metabolic processes in different peripheral tissues and under different pathological conditions.

In chapter 2, we investigated the potential sex differences in the effects of chronic corticosterone exposure and synthetic glucocorticoid treatment on muscle atrophy and dysfunction in mice. This revealed robust sex differences in muscle function and transcriptome in response to glucocorticoid exposure. Increased corticosterone exposure reduced grip strength specifically in female mice, while muscle mass decreased in both sexes. On skeletal muscle transcriptome, we observed that male mice exhibited more pronounced transcriptional variations in response to corticosterone treatment compared to

female mice. Altogether these findings help to outline the influence of sex on the skeletal muscle response to glucocorticoids.

In chapter 3, we evaluated whether the timing of synthetic glucocorticoid treatment affects the development of (metabolic) side effects. We found that out-of-phase but not in-phase treatment of synthetic glucocorticoid betamethasone induced insulin resistance and hyperinsulinemia. In the context of glucose metabolism, in-phase treatment generally caused less side effects compared to the out-of-phase treatment. The time of treatment in relation to the circadian variation in endogenous glucocorticoid levels should be considered when measuring glucocorticoid response.

In chapter 4, we investigated the role of GR signaling in the metabolic symptoms of polycystic ovary syndrome (PCOS) using a mouse model of prolonged DHT exposure. We observed that *Nr3c1* (GR) and *Hsd11b1* mRNA expression were upregulated various tissues of DHT-treated mice, suggesting a deregulated GR signaling. We evaluated the importance of GR signaling by performing treatment with a selective GR antagonist and found that this alleviated DHT-induced hyperglycemia and restored glucose tolerance. Given the similarities in metabolic symptoms between PCOS and excess glucocorticoid exposure, our results suggest that GR signaling may contribute to the metabolic symptoms observed in PCOS, but further research is required to determine the relevance of these findings to humans. To conclude, results of these studies and indications for human applications are discussed in chapter 5.

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2

Sexual dimorphism in transcriptional and functional glucocorticoid effects on skeletal muscle

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ABSTRACT

Muscle atrophy is a common problem in patients with increased glucocorticoid exposure, but it is unclear whether this response differs between males and females, and if so, why. In this study, we evaluated glucocorticoid-induced muscle atrophy in mouse models of increased corticosterone exposure and synthetic glucocorticoid treatment. We found that increased corticosterone exposure specifically reduced female grip strength, but that muscle mass was suppressed in both sexes. The skeletal muscle transcriptional responses to elevated corticosterone were generally much more pronounced and widespread in male mice. Upon synthetic glucocorticoid treatment, we found a reduction in grip strength for both male and female mice, but muscle atrophy in female mice was more sensitive compared to males. To evaluate the role of androgens, we repeated synthetic glucocorticoid treatment in chemically-castrated male mice. We found that androgen depletion and glucocorticoid treatment additively reduce muscle mass, but no interaction effects. Altogether, we show sex differences in response to glucocorticoids in skeletal muscle, and although differences in androgen levels may in part contribute to this. Further studies are warranted to fully delineate the mechanism behind these sex-specific effects. We believe that this study will contribute to a better understanding of the sex differences in muscle atrophy in patients with elevated glucocorticoid exposure.

INTRODUCTION

Muscle atrophy is the wasting or loss of muscle tissue and significantly reduces the quality of life and increases mortality [1-3]. Muscle atrophy is observed in multiple diseases including cancer, diabetes, sepsis and renal failure, but also upon synthetic glucocorticoid (GC) treatment. GC-induced muscle atrophy is prevalent, and is mostly the result of high dose and the sustained usage of GCs or increased endogenous GC levels [4, 5]. Muscle atrophy and reduced muscle function were observed at different doses and treatment regimens with the synthetic glucocorticoids dexamethasone and prednisolone [6-9]. Another commonly used synthetic glucocorticoid is betamethasone [10], which is prescribed for a range of inflammatory diseases at a wide range of doses [11].

Skeletal muscle is composed of different types of muscle fibers, including the slow/oxidative type 1 fibers and fast/glycolytic type 2 fibers [12, 13]. Type 1 fibers have an oxidative capacity and contain more myoglobin and mitochondria, are important primarily to muscle endurance, and have higher resistance to fatigue as compared with type 2 fibers [14]. Type 2 fibers are predominantly glycolytic and can be subdivided in several subtypes including type 2A and type 2B fibers. Type 2A fibers have a fast contraction velocity and are less prone to fatigue compared to type 2B fibers. Type 2B fibers are the largest fiber type and generate ATP by anaerobic metabolic processes when maximum power is required [15]. Muscle types are characterized by a distinct mixture of fiber types [16], and a changes in muscle function and atrophy generally often also involve a re-distribution in muscle fiber type composition [17].

Total muscle mass is regulated by many endocrine factors, including anabolic factors such as androgens and catabolic factors such as GCs [18, 19]. GCs negatively regulate muscle mass directly and via interference with anabolic pathways, and this results in a loss of protein and a reduction of muscle fiber number and density [20, 21]. Upon GC exposure, the ubiquitin-proteasome system is activated in skeletal muscle, which plays a major role in myofibrillar protein degradation [22]. Muscle atrophy F-box (*atrogin-1*) and muscle ring finger 1 (*MurF-1*) are two muscle-specific ubiquitin ligases of which expression is increased under atrophy-inducing conditions, and these so-called atrogenes play a critical role in muscle atrophy [23, 24]. Krüppel-like transcription factor (*Klf15*) is a pivotal factor in skeletal muscle, and was shown to directly regulate the expression of the *atrogin-1* and *MurF-1* atrogenes [25] but is also involved in muscle endurance [26].

Many processes that are influenced by GC exposure are known to be sexually dimorphic [27, 28], possibly explained by differences in sex hormone levels. Synthetic glucocorticoid treatment influences sex hormone levels, i.e. lowers the testosterone level in male rats, while it increases testosterone levels in female

rats [29]. In humans, glucocorticoids response in muscle function can be sexually dimorphic, but it is unclear to what extent androgens play a role in such effects. In this study, we investigated the effects of corticosterone and synthetic glucocorticoid treatment on muscle atrophy and function in male and female mice. We found that male and female muscle responded differently to glucocorticoid exposure at a transcriptomic and functional level, and that androgen signaling may in part contribute to these differences.

METHODS

Animals

All animal experiments were approved by the ethical committee of Leiden University Medical Center (functional cohorts) or Erasmus MC (RNA-sequencing cohort). Mice were purchased from Charles Rivers Laboratories and group housed in conventional cages with a 12-hour:12-hour light:dark cycle and had ad libitum access to water and RM3 chow diet (Special Diet Services, Essex, UK). Male and female C57BL/6J mice aged 8-10 weeks were used.

Animal experiments

To test muscle sensitivity to corticosterone treatment, male (N=8/group) and female mice (N=8/group) were implanted subcutaneously with either a corticosterone-pellet (20 mg corticosterone and 80 mg cholesterol) or a vehicle-pellet (100 mg cholesterol) in the neck region [30, 31], and mice were followed for 14 days. Corticosterone and vehicle pellets were synthesized at Leiden University Medical Center.

To study sex differences in sensitivity to synthetic glucocorticoid betamethasone treatment, male (N=4/group) and female mice (N=6/group) were intraperitoneally injected with 3 mg/kg betamethasone, 25 mg/kg betamethasone, or PBS (vehicle) daily for 14 days. The dose of betamethasone was based on previous muscle atrophy studies with dexamethasone [32], which has approximately the same potency as compared to betamethasone.

To investigate the role of androgen signaling in glucocorticoid-induced muscle atrophy, male mice were chemically castrated using a subcutaneous injection with 25 mg/kg degarelix (MedChemExpress), which is a GnRH antagonist that blocks LH and FSH release and results in diminished testosterone levels [33]. Intact and chemically-castrated mice were intraperitoneally injected daily with 3 mg/kg betamethasone or vehicle (PBS) for 14 days (N=8/group).

Body Weight, Body Composition, Grip Strength and Grid Hanging Measurement

All cohorts were subjected to several functional tests and measurements to assess body weight, body composition and muscle function. Body weight, body composition (EchoMRI-100-analyzer) and grip strength were measured twice a week and grid hanging was measured once a week, and all functional measurements were performed between 3-6 hours after lights-on. Grip strength of the forelimb was measured using a grid attached to an isometric force transducer (Chatillon, Columbus Instruments 080529). The force transducer records the maximum force that is required to break the mouse's grip from the mesh surface. In total, we recorded five sets of measurements, each consisting of three pulls and with a resting period of at least one minute between them. The three highest values obtained were averaged. Overall muscle function was assessed with the four limbs hanging test, the mouse was placed on a grid, which was turned upside down, 15 cm above a cage filled with soft bedding. This test was performed weekly with a maximum of three attempts per session from which the best performance was used. Maximum allowed hanging time was 600 seconds. At the end of the experiments, mice were killed by CO2 asphyxiation (between 3-6 hours after lights-on) and several muscle types were isolated, weighed and frozen in liquid nitrogen for further processing.

RNA Isolation and RT-qPCR Analysis

Total RNA was isolated by using Tripure (Roche) according to the manufacturer's instructions. RNA concentration was measured by NanoDrop spectrophotometer (Thermo Fisher). Total RNA was diluted into 1 µg for reverse transcription using M-MLV reverse-transcriptase (Promega). cDNA (4 ng) was used per 10 µl RT-qPCR reaction, and each qPCR reaction contained 1 µl primers (0.5 µl forward and 0.5 µl reverse of each) and 5 µl SYBR green supermix (Bio-Rad) using a Bio-Rad CFX96. GAPDH was used as housekeeping gene. Primer sequences: MurF-1 Fwd: TGTGCAAGGAACAGAAGAC; Rev: CCAGCATGGAGATGCAGTTA; Atrogin-1 Fwd: TTGGATGAGAAAAGCGGCAG; Rev: TACAGTATCCATGGCGCTCC; Klf15 Fwd: AAATGCACTTTCCCAGGCTG; Rev: CGGTGCCTTGACAACTCATC; Gapdh Fwd: GGGGCTGGCATTGCTCTCAA; Rev: TTGCTCAGTGTCCTTGCTGGGG.

RNA Sequencing

To study the corticosterone-induced transcriptome in quadriceps muscle, male and female mice were subcutaneously implanted in the neck region with slow-release pellets containing corticosterone (50 mg corticosterone and 50 mg cholesterol; N=6 per sex) or vehicle (100 mg cholesterol; N=6 per sex) (corticosterone and vehicle pellets were synthesized at Leiden University Medical Center). After 14 days, mice were fasted for 5 hours and killed by cardiac puncture under isoflurane anaesthesia (28). Quadriceps muscle was collected and homogenized in Tripure using a Kimble pellet pestle followed by a phase-

separation with chloroform. Total RNA was isolated using the RNeasy kit according to manufacturer's instructions (Qiagen 74104). RNA quality was ensured (RNA Integrity number > 7.0 and 28/18s ratio > 1.0) using the RNA 6000 Nano kit bioanalyser (Agilent). Stranded mRNA libraries were constructed and 100bp paired-end bulk RNA-sequencing was performed at BGI Genomics (Hong Kong, China) on the DNBseq platform. Over 20 million reads were sequenced per sample. RNA sequencing data has been deposited in NCBI's Gene Expression Omnibus (GEO series accession number GSE202787).

RNA Sequencing Data Analysis

The RNA-seq pipeline (version 4.1.0), published as part of BioWDL, was used for read quality control, alignment and quantification. BioWDL contains the main sequencing analysis pipelines and workflows developed at Leiden University Medical Center by the sequencing analysis support core with code being accessible at https://biowdl.github.io/.

Quality control was performed using FastQC and MultiQC. Reads were aligned to Mus Musculus genome version 10 (mm10) using STAR (version 2.7.3a). Tool settings used were: '–runThreadN' '4' '–outSAMunmapped' 'Within KeepPairs' '–twopassMode' 'Basic'. The gene-read quantification was performed using HTSeq-count (version 0.12.4). Tool settings used were: '–order' 'pos' '–stranded' 'reverse'. Uniquely assigned reads were mapped to known genes based on Ensembl release 97 of mm10. HTSeq-count output files were merged into a count matrix per experiment as input for differential gene expression analysis.

DEseq2 (version 1.29.4) was used for normalization of the count data (median of ratio's method) and identification of differentially expressed genes. For the differential expression analysis, all genes which were expressed in a minimum of four replicates with >20 normalized counts for at least one of the groups were selected. This resulted in 13,049 genes that were included in the analysis. Pair-wise comparisons of groups within experiments were analysed and a false discovery rate adjusted p-value of 0.01 and a log2FC <-1 or >1 was used as a cut-off for detection of differential gene expression. Principal component analysis was performed using DEseq2 and heatmaps of scaled, normalized counts were made with pheatmap (version 1.0.12). Gene ontology (GO) term enrichment analysis was performed with the ViSEAGO package (version 1.4.0), using fisher's exact test with 0.01 as a significance cut-off.

Histology and Immunofluorescence Microscopy

Muscles were isolated and frozen in liquid nitrogen-cooled isopentane. Samples were stored at -80°C until further processing. Gastrocnemius tissue was cryo-sectioned (8 µm thick) using a cryostat (Leica CM3050S). Cryosections were first stained with rabbit anti-laminin (1:100, Abcam) for 3 hours. After washing with

PBS/Tween, sections were stained with secondary goat-anti-rabbit antibodies conjugated to Alexa Fluor-647 (1:1000, Abcam). Sections were incubated overnight at 4°C with a mixture of the following fiber-type specific fluorophore-conjugated primary antibodies (Molecular Probes, Life Technologies): BA-D5 conjugated to Alexa Fluor 350 (1:400; type 1), SC-71 conjugated to Alexa Fluor 488 (1:800; type 2B), and BF-F3 conjugated to Alexa Fluor 594 (1:600; type 2A). A Zeiss Axio Observer A1 microscope was used for imaging. Area quantification and representative pictures were acquired via ZEN 2 software.

Image Quantification

Carl Zeiss Image format were converted to multichannel TIFF files, and image processing was performed in Fiji. Mean fluorescence intensity (MFI) was recorded from each myofiber object using a modified Muscle J macro [34]. In brief, tissue masks from the laminin staining were created to determine muscle regions for quantification. The masks were then manually corrected, removing technical artifacts such as tissue folds. To improve the myofiber segmentation outputs, a classifier was trained in the Ilastik pixel classification algorithm [35]. All the masked laminin images were processed followed by myofiber segmentations defined as the region of interest. Mean MFI and geometrical properties were recorded for each myofiber. As the laminin segmentation was automated, non-myofiber objects were removed by implementing a percentile filtering for the pixel-classification on the object boundary, pixel-classification in the interior of the object, cross-sectional area and the circularity values. After the filtering step, MFI values for each of the three MyHC isoforms were scaled per myofiber as previously described [36]. MFI values for each vehicle group were normalized as 1 transformed (natural logarithm) and a myofiber-based MFI analysis was carried out in R (version 3.5.1).

Statistical Analysis

Statistical analyses were performed with SPSS (version 25) and GraphPad Prism version 9.0.1. The following statistical analyses were used: ANOVA with Turkey multi-comparison according to different variables (including 1-way ANOVA for one variable, 2-way ANOVA for two variables) and unpaired Student t-test. All data are presented as means ± SEM.

RESULTS

Elevated corticosterone exposure causes muscle atrophy in male and female mice, but specifically decreases grip strength in female mice

To identify the effects of elevated corticosterone exposure, male and female mice were subcutaneously implanted with a vehicle or corticosterone slow-release pellet. Total body weight was not altered in male and female mice up to two weeks during chronic corticosterone exposure (**Fig. 1A**). Elevated corticosterone exposure did significantly decrease total lean mass (**Fig. 1B**) and increased fat mass in both male and female mice (**Fig. 1C**). To investigate the effect of this excess corticosterone exposure on muscle function, we performed grip strength and grid hanging measurements. We found that upon excessive corticosterone exposure grip strength was only decreased in female mice, but not in male mice (**Fig. 1D**). Grid hanging time was not influenced in neither male nor female mice (**Fig. 1E**).

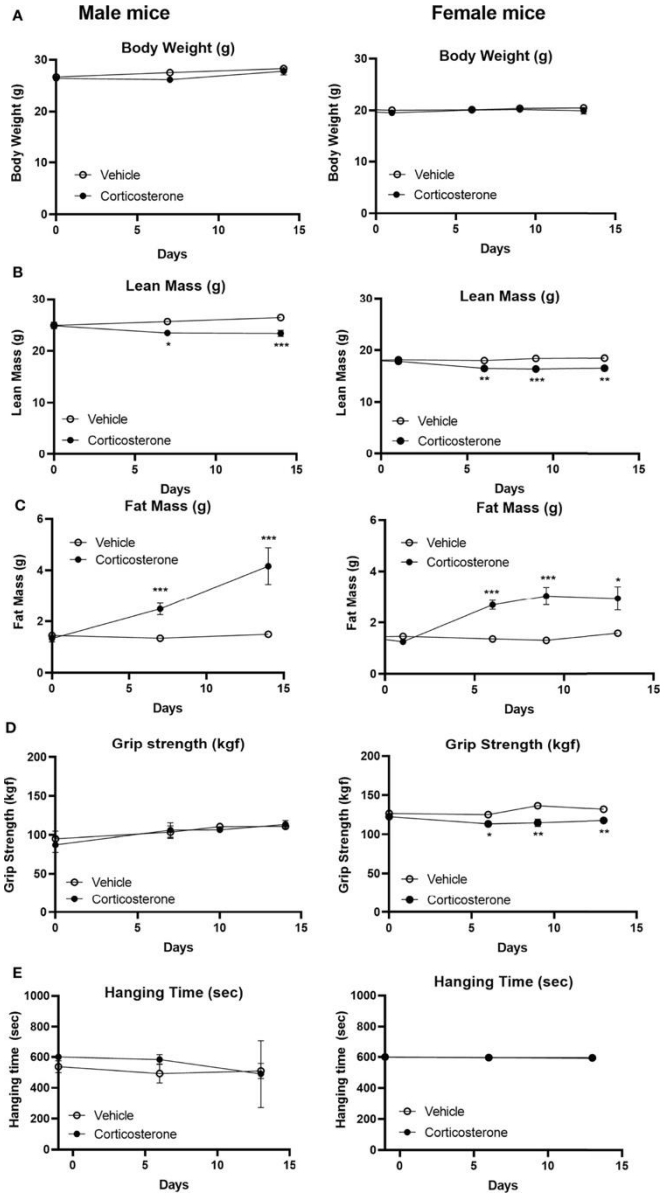

Fig. 1 Corticosterone treatment specifically decreases muscle function in female mice. The effect of corticosterone treatment on (A) total body mass, (B) lean mass, (C) fat mass, (D) fore limb grip strength and (E) grid hanging in male and female C57BL/6J mice. N=8 mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle. Statistical significance was calculated using a two-way ANOVA.

To investigate muscle atrophy, we collected 3 different muscle types in male and female mice upon elevated corticosterone exposure. We found reduced muscle weights of gastrocnemius (**Fig. 2A**), extensor digitorum longus (EDL) (**Fig. 2B**) and tibialis anterior (TA) (**Fig. 2C**) upon excess corticosterone exposure, similarly in male and female mice albeit not significant for male EDL, indicative of muscle atrophy in both sexes. Overall, the results show that excessive

corticosterone exposure has sexual dimorphic effects on grip strength, but not on other functional parameters.

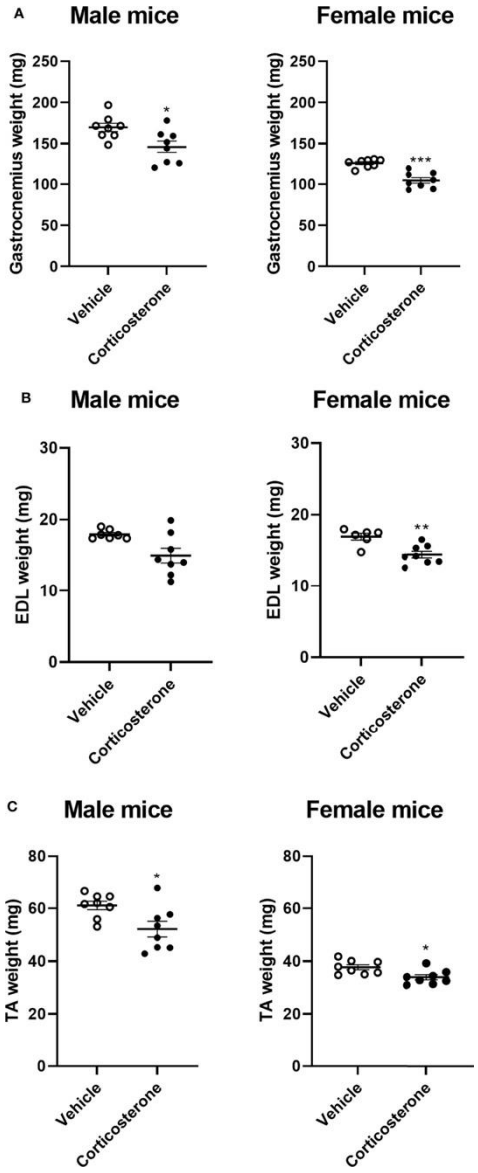

Fig. 2 Corticosterone treatment causes muscle atrophy in male and female mice. The effect of corticosterone treatment on tissue weight of (A) gastrocnemius, (B) extensor digitorum longus (EDL) and (C) tibialis anterior (TA) of male and female C57BL/6J mice. N=8 mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle. Statistical significance was calculated using an unpaired Student's t-test.

Elevated corticosterone exposure has sexually dimorphic effects on transcription in quadriceps muscle

We next investigated the transcriptional effects of elevated corticosterone exposure in both male and female mice. We choose the quadriceps muscle for RNA-sequencing analysis, as this muscle type is representative for human

muscle [37] and showed muscle atrophy in mice after corticosterone exposure (data not shown). We identified 1817 differentially expressed genes upon corticosterone exposure that were shared between male and female mice, while 3576 genes were specifically regulated in male mice and 2002 genes were specifically regulated in female mice (**Fig. 3A**). Principal component analysis showed that biological replicates of corticosterone-treated male mice clustered closely together, while female mice exhibited considerable variation in muscle transcriptome as response to corticosterone exposure (**Fig. 3B**). Further scrutiny confirmed the considerable overlap in corticosterone-regulated genes between sexes as well as the sex-specific effects (**Fig. 3C**). Heatmap representation generated 4 clusters of corticosterone-regulated genes, comprising of genes similarly regulated between sexes (upper two gene clusters) and sex-specific effects (lower two gene clusters) (**Fig. 3D**).

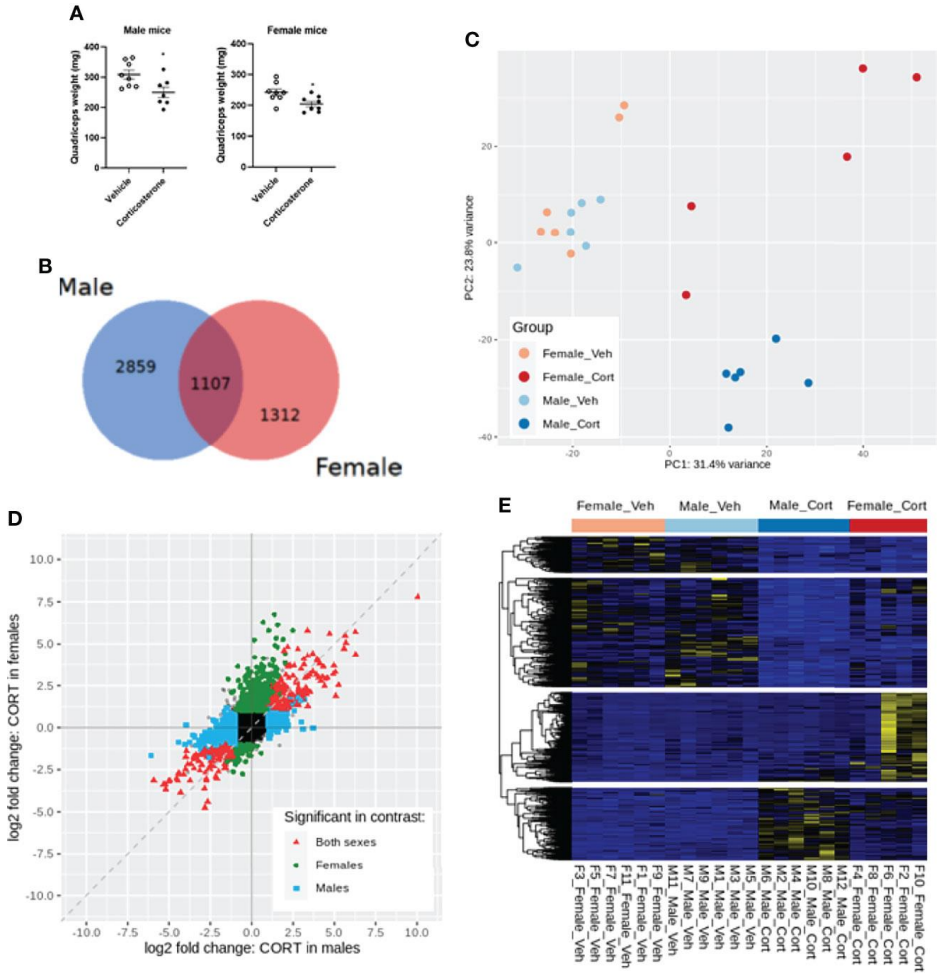

Fig. 3 Transcriptome profiling of the quadriceps in male and female mice after corticosterone treatment. (A) The effect of corticosterone treatment on muscle weight of the quadriceps. (B) Venn-diagram representing sex-specific and shared differentially expressed genes upon corticosterone treatment. (C) Principal component analysis of vehicle- and corticosterone-treated male and female C57BL/6J mice. (D) Fold change-fold change plot comparing significant changes in corticosterone-treated male and female mice. Male-specific differentially expressed genes are shown in blue, female-specific genes in green, and genes differentially expressed in both sexes in red. © Heatmap showing all genes regulated by corticosterone. A blue color code represents low expression, a yellow color code high expression. N=6 mice/group for all groups except female-Cort (N=5).

When evaluating expression of specific genes, we first plotted expression of genes that encode for the superfamily of nuclear steroid receptors. We found that the *Nr3c1* gene (encoding for the glucocorticoid receptor) was significantly downregulated by corticosterone exposure in female but not male mice, while *Nr3c2* (mineralocorticoid receptor) was downregulated in both male and female quadriceps muscle, albeit only significant in male mice (**Suppl. Table 1** and **Suppl. Fig. 1A**). *Nr3c3* (progesterone receptor) and *Esr2* (estrogen receptor-β) were not detected in quadriceps muscle, while the *Nr3c4* (androgen receptor) was not significantly changed by corticosterone exposure in both male and female mice and *Esr1* (estrogen receptor-α) was strongly downregulated in the quadriceps muscle of both male and female mice (**Suppl. Table 1** and **Suppl. Fig. 1A**). We next looked at classical GR target genes, including *Fkbp5, Gilz* (*Tsc22d3*), *Per1*, *Sgk1* and *Zbtb16,* as proxies for GR activity. All evaluated GR target genes were upregulated stronger in male mice as compared to female mice (**Suppl. Table 2** and **Suppl. Fig. 1B**). In an attempt to better understand the sex-specific effect of corticosterone exposure on muscle function (grip strength), we performed a go-term analysis. Comparison of differentially expressed genes after corticosterone showed many pathways that were specifically regulated in male mice, including the muscle atrophy pathway [25, 38], with 37.5% genes differentially expressed in male mice ($p>0.01$) and 0% in female mice ($p=1.00$) (**Suppl. Table 3**). Male-specific regulated genes involved in atrophy included Klf15 and its downstream ubiquitin ligases *MurF-1* (*Trim63*) and *atrogin-1* (*Fbxo32*) (**Suppl. Table 4** and **Suppl. Fig. 1C**). Other factors involved in ubiquitination, including *UBC*, *Ube4b* and *Usp14*, were not influenced by corticosterone exposure in male and female mice. Other noteworthy sex differences in corticosterone response was the stronger upregulation of the *FoxO1, -3* and *-4* transcription factors in male as compared to female mice. There were no clear effects of corticosterone on several proteasome subunits, and factors related to autophagy (*Bnip3, LC3*) were similarly upregulated in male and female mice (**Suppl. Table 4** and **Suppl. Fig. 1C**). Collectively our data show many transcriptomic similarities and differences between male and female quadriceps muscle in response to excess corticosterone, with several noteworthy sexually dimorphic effects including atrophy-related genes.

Daily betamethasone treatment similarly decreases grip strength in both sexes, while female mice are more sensitive to muscle atrophy

To evaluate the effects of synthetic glucocorticoid treatment on muscle function, male and female mice were injected daily with 3 or 25 mg/kg betamethasone for a period of 2 weeks. In both sexes, body weight was non-significantly decreased by daily betamethasone treatment (**Fig. 4A**). Treatment with 25 mg/kg betamethasone significantly decreased lean mass of male mice, while treatment with both 3 and 25 mg/kg betamethasone decreased lean mass in female mice (**Fig. 4B**). Fat mass was similarly increased in both male and female mice (**Fig. 4C**). In both male and female mice, treatment with 25 mg/kg betamethasone significantly reduced grip strength (**Fig. 4D**). Treatment with 3 mg/kg betamethasone transiently decreased grip strength in male mice, while reduced grip strength in female mice was only observed after 14 days of treatment (**Fig. 4D**). Grid hanging performance was not significantly altered in male nor female mice after betamethasone treatment, although male mice tended to perform better upon treatment with 25 mg/kg betamethasone (**Fig. 4E**).

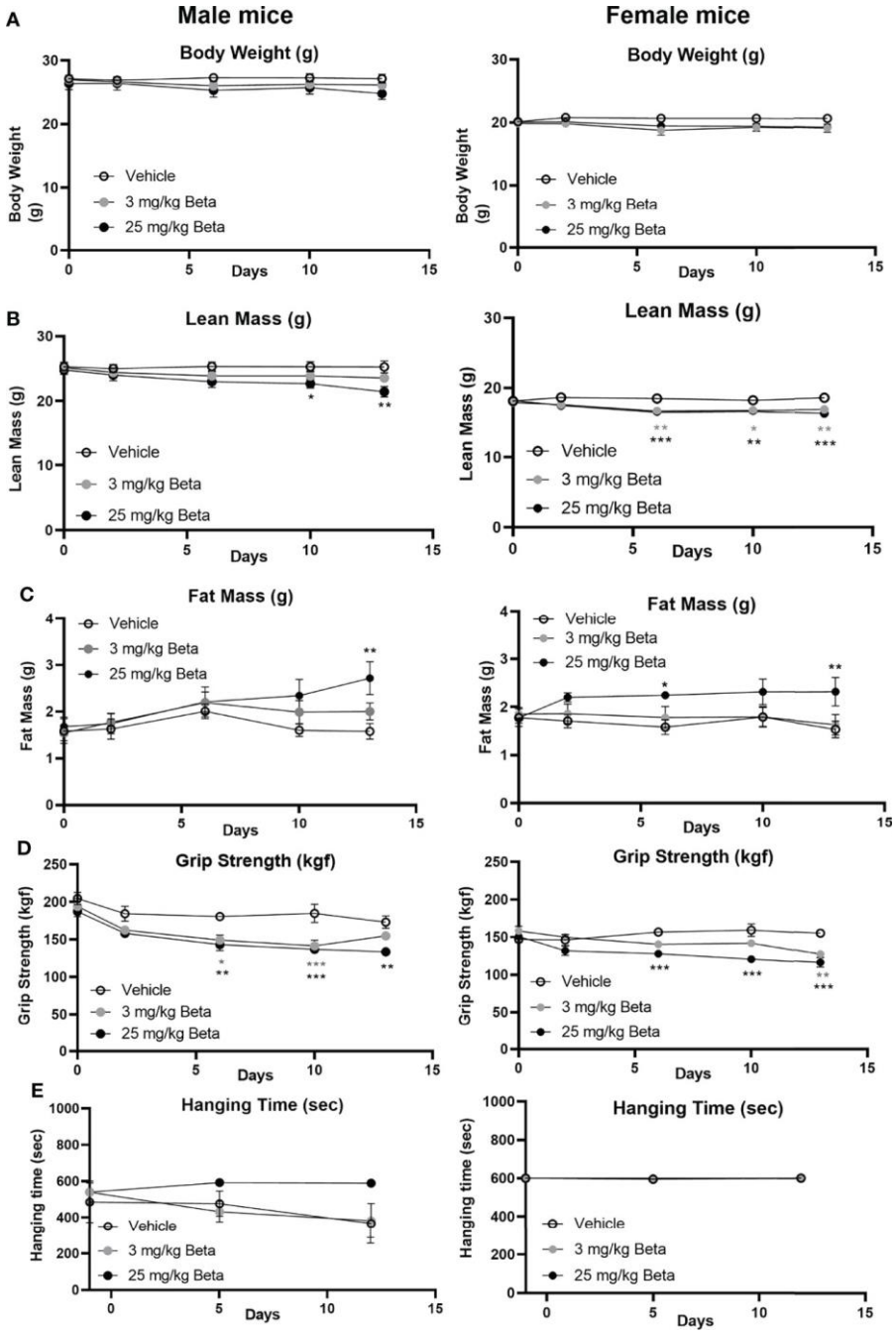

Fig. 4 Daily betamethasone treatment decreases muscle function in male and female mice. The effect of daily treatment with 3 or 25 mg/kg betamethasone on (A) total body mass, (B) lean mass, (C) fat mass, (D) fore limb grip strength, and © grid hanging time in male and female C57BL/6J mice. N=4 male mice/group, N=6 female mice/group. *p<0.05 vs.

Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle. Statistical significance was calculated using a one-way ANOVA.

To investigate the effect of daily betamethasone treatment on muscle atrophy, we collected 5 different muscle types. Gastrocnemius weight of female mice was significantly decreased after 3 and 25 mg/kg betamethasone treatment, whereas male mice only showed a decrease in muscle weight upon 25 mg/kg betamethasone treatment (**Fig. 5A**). Similar patterns were observed for the muscle weights of EDL (**Fig. 5B**) and TA (**Fig. 5C**). In the glucocorticoid-resistant soleus muscle, we did not observe any significant effect of betamethasone treatment on muscle weight in both male and female mice (**Fig. 5D**). Consistent with the patterns observed on muscle weight, gene expression analysis of atrophy-related genes in the gastrocnemius muscle revealed a possible decrease of *Klf15*, *atrogin-1* and *MurF-1* in male mice treated with 3 mg/kg betamethasone, but increased expression after 25 mg/kg betamethasone expression (**Fig. 5E-G**). Female mice showed an upregulation of *atrogin-1* and *MurF-1* in gastrocnemius muscle after 25 mg/kg betamethasone treatment (**Fig. 5F-G**).

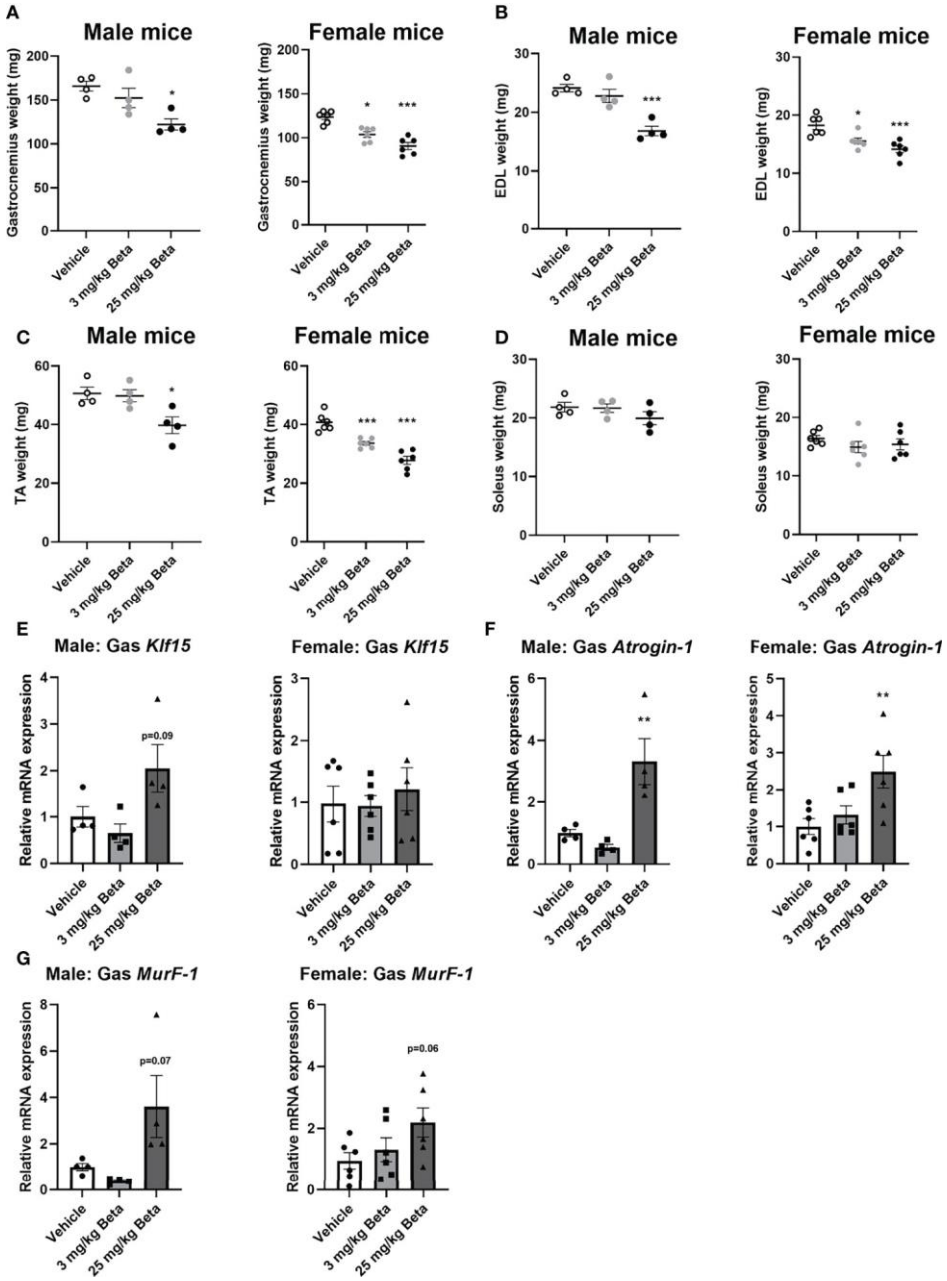

Fig. 5 Female mice exhibit muscle atrophy at lower doses of daily betamethasone treatment as compared to male mice. The effect of daily treatment with 3 or 25 mg/kg betamethasone on tissue weight of (A) gastrocnemius, (B) extensor digitorum longus (EDL), (C) tibialis anterior (TA) and (D) soleus. The effect of 3 or 25 mg/kg betamethasone treatment on gene expression in gastrocnemius muscle of © Klf15, (F) atrogin-1 and (G) MurF-1. N=4

male mice/group, N=6 female mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle. Statistical significance was calculated using a one-way ANOVA.

To further investigate the effect of betamethasone treatment on muscle in male and female mice, we analysed gastrocnemius myofiber composition. As expected, gastrocnemius muscles of vehicle-treated male mice were comprised of relatively little type 1 fibers and type 2A fibers, and relatively many type 2B fibers in (Fig. 6A-B). In contrast, vehicle-treated female mice had relatively many type 2A fibers (Fig. 6C-D). Betamethasone treatment of male mice significantly increased type 2A myofibers (Fig. 6B). In female mice, type 1 and type 2A myofiber composition was unaffected by betamethasone treatment, while type 2B tended to be decreased after daily betamethasone treatment (Fig. 6D). Collectively, these functional data suggest that female mice are more sensitive to betamethasone-induced muscle atrophy, with similar effects on grip strength between sexes.

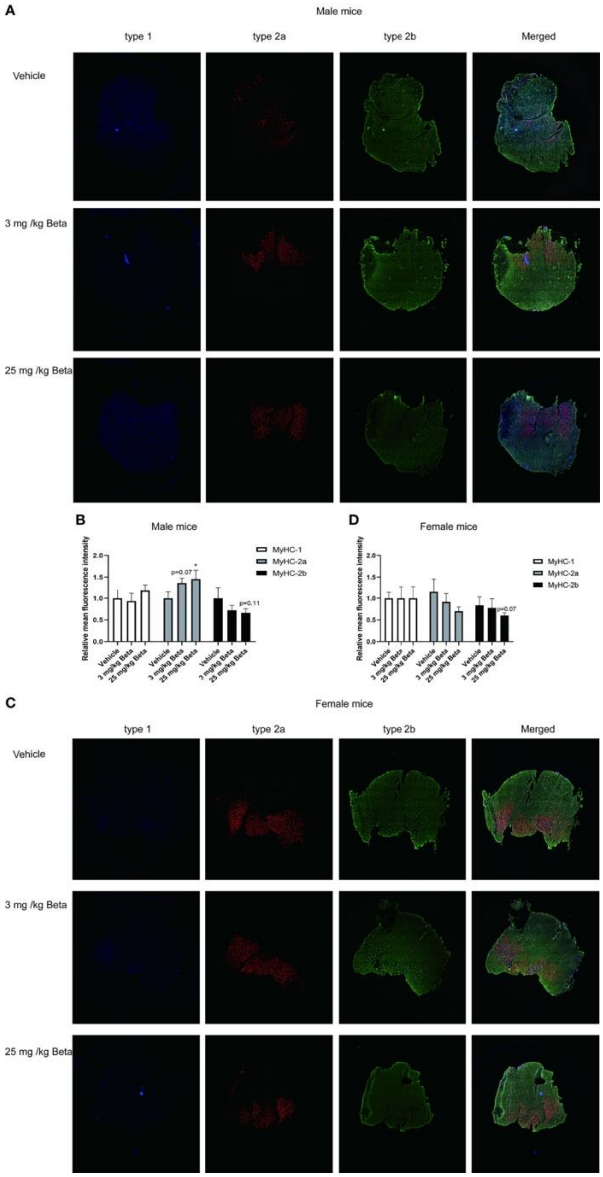

Fig. 6 Daily betamethasone treatment increases abundance of type 2A myofibers in male mice. Histological analysis of gastrocnemius muscle for type 1, type 2A and type 2B myofibers. (A) Representative images of myofiber staining in gastrocnemius muscle of male mice and (B) Relative mean fluorescence intensity for individual myofiber isoforms. N=3 mice/group. (C) Representative images of myofiber staining in gastrocnemius muscle of female mice and (D) Relative mean fluorescence intensity for individual myofiber isoforms. N=3 mice/group. Type-1=Blue; Type-2a=Red; Type-2b=Green. *p<0.05 vs. Vehicle.

Daily betamethasone treatment and chemical castration similarly and additively cause muscle atrophy

To explore the underlying mechanism of male-female differences in glucocorticoid response in muscle function, we investigated a possible contribution of androgen signalling [30]. To this end, we chemically castrated male mice using the GnRH antagonist degarelix, and intact and chemically-castrated male mice were subsequently injected daily with 3 mg/kg betamethasone for 2 weeks. During this treatment period, chemical castration on itself did not significantly influence total body weight and lean body mass, but seemed to potentiate the effect of betamethasone treatment on body weight and lean mass (**Fig. 7A-B**). Fat mass appeared to transiently decrease upon chemical castration, but was not influenced by betamethasone treatment (**Fig. 7C**). Chemical castration on its own decreased grip strength, and additional betamethasone treatment did not further influence this (**Fig. 7D**).

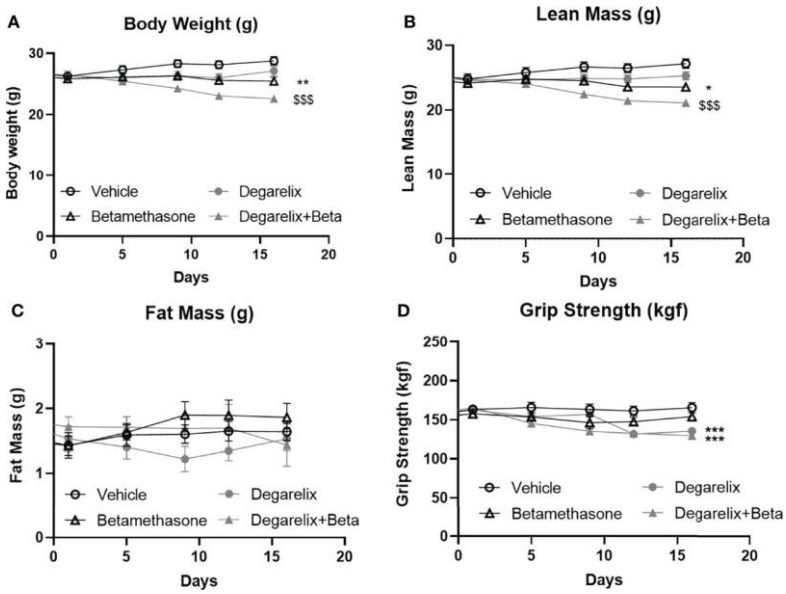

Fig. 7 Daily betamethasone treatment reduces total body weight and lean mass in intact and chemically-castrated male mice. The effect of daily treatment with 3 mg/kg betamethasone in chemically-castrated mice and intact mice on (A) total body mass, (B) lean mass, (C) fat mass, and (D) fore limb grip strength. N=8 mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle, $$$ p<0.001 vs degarelix. Statistical significance was calculated using a two-way ANOVA.

As expected, post-mortem analysis of glucocorticoid and androgen-responsive tissues showed decreased adrenal weight after betamethasone treatment but no effect of chemical castration (Fig. 8A), and diminished seminal vesicle weight after chemical castration (Fig. 8B). Analysis of muscle tissue showed that

chemical castration alone significantly decreased gastrocnemius, EDL and TA, but not soleus weight (Fig. 8C-F). Treatment with 3 mg/kg daily betamethasone reduced gastrocnemius weight in intact male mice, and further reduced muscle weight in chemically-castrated mice (Fig. 8D). Similar observations were found for EDL and TA muscle weight, for which betamethasone treatment further decreased muscle weight of chemically-castrated mice (Fig. 8E-F). Expression analysis revealed that in gastrocnemius muscle, degarelix treatment induced the expression of *Klf15*, an effect that was lowered by betamethasone treatment (Fig. 8G). In line with our previous experiment, 3 mg/kg betamethasone treatment lowered *MurF-1* expression, which was unaffected by chemical castration (Fig. 8H). Similarly as in gastrocnemius, degarelix treatment induced *Klf15* in tibialis anterior muscle, while *MurF-1* expression was unaltered (Fig. 8I-J). Collectively, these data suggest that androgen depletion and glucocorticoid treatment both have separate effects on muscle atrophy, and that combined intervention has additive effects.

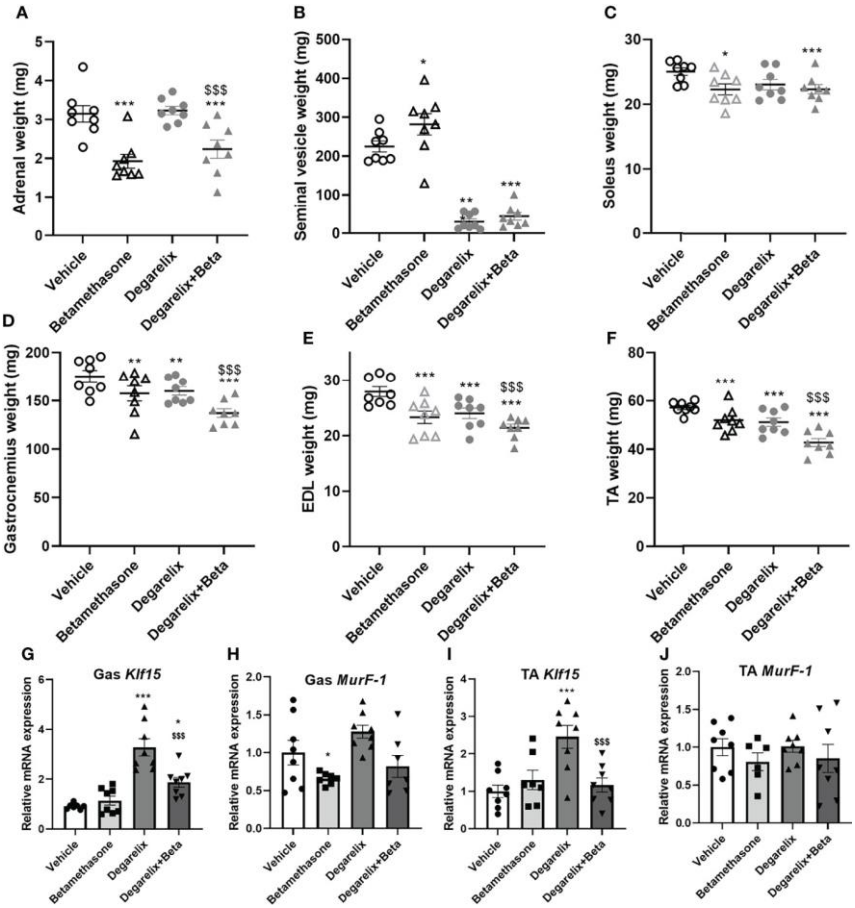

Fig. 8 Chemical castration and daily betamethasone treatment additively decrease muscle weight in male mice. The effect of daily treatment with 3 mg/kg betamethasone on intact and chemically-castrated male mice on weight of the (A) adrenal gland, (B) seminal vesicle, (C) soleus, (D) gastrocnemius muscle, (E) extensor digitorum longus (EDL) and (F) tibialis anterior (TA). The effect of daily treatment with 3 mg/kg betamethasone on intact and chemically-castrated male mice on expression of (G) Klf15 in gastrocnemius, (H) MurF-1 in gastrocnemius, (I) Klf15 expression in TA, and (J) MurF-1 expression in TA. N=8 mice/group. *p<0.05 vs. Vehicle, **p<0.01 vs. Vehicle, ***p<0.001 vs. Vehicle, $$$ p<0.001 vs degarelix. Statistical significance was calculated using a two-way ANOVA.

DISCUSSION

In this study, we set out to investigate sexual dimorphism in glucocorticoid-induced muscle dysfunction. Muscle dysfunction as a result of elevated glucocorticoid exposure is common in patients with hypercortisolism but is also frequently observed during synthetic glucocorticoid treatment regimens. Although the magnitude of this problem in clinical practice is evident, to our knowledge no studies exist to study sex differences and the role of androgen sex hormones in glucocorticoid-induced muscle dysfunction. In our present study, we found that elevated corticosterone exposure similarly causes muscle atrophy in male and female mice, based on the analysis of five different types of skeletal muscle. Despite similar atrophy-inducing effects in both sexes by corticosterone, only female mice exhibited a decreased grip strength, while male mice were unaffected by this. We performed an extensive transcriptomic analysis of male and female quadriceps muscle after corticosterone exposure in an attempt to capture the similarities and differences between sexes. Overall, we observed more differentially expressed genes in male mice as compared to female mice (3576 male-specific genes versus 2002 female-specific genes after corticosterone treatment). This finding was also evident when looking at several classic GR-target genes (e.g. *Gilz*, *Per1* and *Sgk1*) that were found stronger regulated in male mice as compared to female mice. It is interesting to note that in contrary to the results above, the response to fasting shows greater induction of GR-regulated genes in female gastrocnemius muscle as compared to male [39]. The response to glucocorticoid/GR-induced transcription thus appears context-dependent but likely also muscle fiber type-dependent. In corticosterone-treated female mice, we found a large in-group variation in transcriptomic response (as represented in the PCA analysis and heatmap), possibly related to different stages of the estrous cycle at which tissues were collected for which we did not stratify.

Gene ontology analysis revealed muscle atrophy amongst the main sexually dimorphic pathways, and indeed male-specific upregulations of atrophy-related genes were found for *Klf15*, *atrogin-1* and *MurF-1*, amongst several others. Despite that the transcriptional response to corticosterone in quadriceps muscle was thus stronger in male mice (including genes related to muscle atrophy), this did not yield stronger atrophy-induction in males, and decreased grip strength was even specific for female mice. It is likely that different pathways contribute to muscle atrophy in male and female muscle, and in addition to direct catabolic effects also antagonism of anabolic pathways can underlie decreased muscle mass by glucocorticoids. It should also be noted that the transcriptomic analysis was performed after 14 days of elevated corticosterone exposure – a timeframe that allows adaptation in tissue response - and transcriptional effects after acute corticosterone treatment may differ. We also observed that reduced muscle

mass does not necessarily influence muscle function (grip strength), but it should be noted that we analyzed muscle weight in the back limps, while the functional test evaluated forelimb muscle strength. We do expect that the muscle atrophy upon elevated corticosterone that we observed in back limps is representative for most muscles in the mouse. A notable exception to this is the soleus muscle – previously reported to be largely resistant to glucocorticoid-induced atrophy [25]. Also in our studies the soleus was largely unaffected by synthetic glucocorticoid treatment in both male and female mice, and this lack of response in the soleus muscle is likely attributed to low GR expression levels.

For synthetic glucocorticoid treatment with betamethasone, we show that both male and female mice exhibited reduced grip strength – with both doses that were tested in this study. We found that male mice are less sensitive to betamethasone-induced muscle atrophy – and treatment with 25 mg/kg/day was required to induce atrophy in male mice while 3 mg/kg/day betamethasone induced this in female mice. Differences in sensitivity are not explained by GR expression levels, as these were reported to be similar in male and female gastrocnemius muscle [39]. The analysis of atrophy-related gene expression [40] in gastrocnemius revealed that *Klf15*, *atrogin-1* and *MurF-1* expression were only upregulated after 25 mg/kg betamethasone in male mice, while 3 mg/kg even seemed to reduce expression of these atrogenes. However, also female mice were mostly responsive to the 25 mg/kg dose, while the 3 mg/kg dose had little effect on the expression of the tested atrogenes. We observed that betamethasone induced a transformation of muscle fibers in gastrocnemius muscle [17, 41, 42], with increased type 2A and decreased type 2B fibers in male mice after betamethasone treatment. Such a shift of muscle fiber isoforms was previously associated with a reduction of muscle strength [43, 44]. In female mice, we did not observe a distinct change in type 1 fibers, consistent with the lack of effect on muscle endurance during the grid hanging test.

We postulated that the sex difference in glucocorticoid effects on muscle may be related to the relative androgen levels. Androgens are well-known anabolic factors that are involved in muscle physiology, and increased anabolic signaling may protect from glucocorticoid-induced muscle atrophy and dysfunction. To test to what extent androgen signaling contributes to the sex differences, we chemically castrated male mice using GnRH antagonist degarelix. Androgen depletion on its own strongly reduced forelimb grip strength, but betamethasone treatment did not further influence this. Muscle weight of several muscle types was reduced after androgen depletion – likely due to reduced anabolic signaling. In addition, chemical castration of male mice seemed to potentiate the atrophy-inducing effects of low dose betamethasone treatment, but for many muscle types both effects were additive rather than synergistic. Our findings thus cannot rule out separate anabolic and catabolic signaling

pathways, and do not provide direct evidence for crosstalk between these pathways.

Sex-based differences in skeletal muscle physiology are known in humans, including differences in fiber type prevalence which translates to altered performance, endurance and recovery of skeletal muscles [45]. Also in response to glucocorticoids, humans show differences between sexes. In patients with subclinical hypercortisolism, women exhibited lower skeletal muscle mass but men do not [46]. In addition to this, differential expression of 11β-HSD1 is likely to influence local glucocorticoid turnover, and in fact higher 11β-HSD1 expression in women is associated with reduced grip strength [47]. It should be noted that while 11β-HSD1 influences (local) levels of endogenous glucocorticoids, many synthetic glucocorticoids are not a substrate for such enzymatic (in)activation and their activity is thus unlikely to be affected. Also at the receptor level, differences between men and women exist, and polymorphisms in the GR gene were shown to reduce grip strength in male patients with overt hypercortisolism (Cushing's syndrome) [48]. Altogether, clearly many aspects of glucocorticoid response in skeletal muscle are different between men and women. Our studies in mice provide suitable models to further study the mechanisms that underlie these sex-specific effects, and propose that differential androgen levels may in part contribute to these discrepancies.

Acknowledgements

We acknowledge Hetty Sips and Amanda Pronk for their excellent technical assistance.

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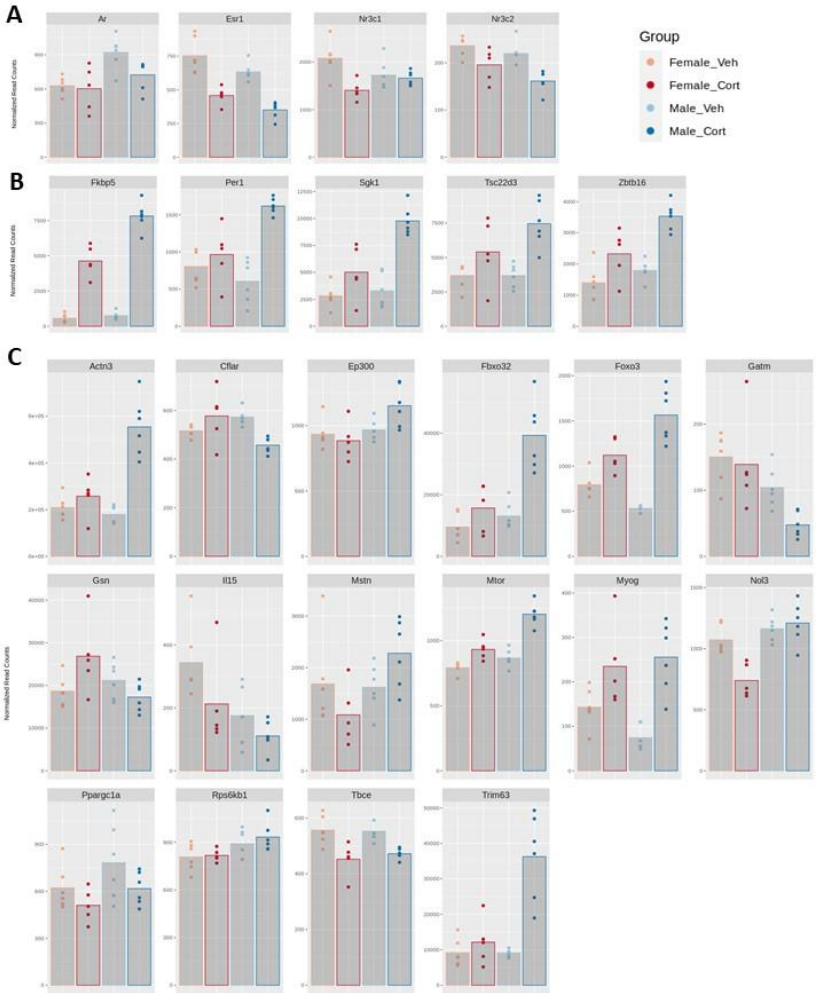
SUPPLEMENT

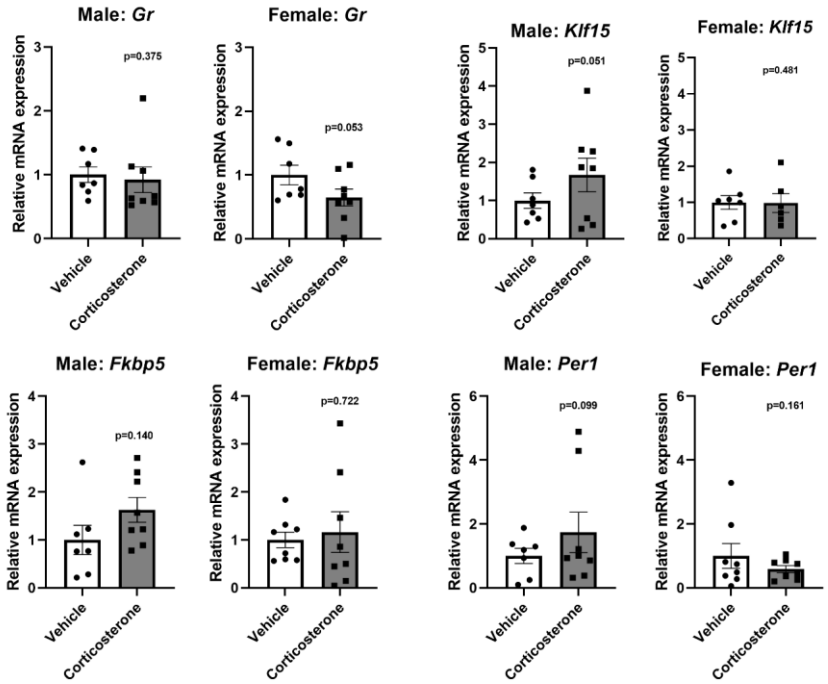
Suppl. Figure 1



Supplementary Fig. 1

Quadriceps RNA sequencing data of selected genes. (A) Normalized read counts for (A) genes encoding for steroid nuclear receptors, (B) classical glucocorticoid receptor-target genes, and (C) genes included in the gene ontology term muscle atrophy.

Suppl. Figure 2



Supplementary Fig. 2

Quadriceps RT-PCR analysis of selected genes. Relative mRNA expression of Gr, Klf15, Fkbp5 and Per1 in quadriceps muscle of male and female mice after corticosterone treatment (20 mg). N=6-8 per group. Statistical significance was calculated using an unpaired students t-test.

3

Out-of-phase treatment with the synthetic glucocorticoid betamethasone disturbs glucose metabolism in mice

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ABSTRACT

Objective

Endogenous glucocorticoid levels display a strong circadian rhythm, which is often not considered when synthetic glucocorticoids are prescribed as anti-inflammatory drugs. In this study we evaluated the effect timing of glucocorticoid administration, i.e. in-phase (administered when endogenous glucocorticoid levels are high) versus out-of-phase (administered when endogenous glucocorticoid levels are low). We investigated the synthetic glucocorticoid betamethasone – which is extensively used in the clinic - and monitored the development of common metabolic side effects in mice upon prolonged treatment, with a particular focus on glucose metabolism.

Methods

Male and female C57BL/6J mice were treated with the synthetic glucocorticoid betamethasone in-phase and out-of-phase, and the development of metabolic side effects was monitored.

Results

We observed that, compared with in-phase treatment, out-of-phase treatment with betamethasone results in hyperinsulinemia in both male and female C57BL/6J mice. We additionally found that out-of-phase betamethasone treatment strongly reduced insulin sensitivity as compared to in-phase administration during morning measurements. Our study shows that the adverse effects of betamethasone are dependent on the time of treatment with generally less side effects on glucose metabolism with in-phase treatment.

Conclusions

This study highlights differences in glucocorticoid outcome based on the time of measurement, advocating that potential circadian variation should be taken into account when studying glucocorticoid biology.

INTRODUCTION

Glucocorticoids play a crucial role in various physiological processes in the body, including glucose metabolism, immune responses, and the stress response [1-3]. Endogenous glucocorticoid secretion is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, and this hormonal axis is tightly regulated by negative feedback on the level of the pituitary and hypothalamus [4]. The HPA axis is aligned with the light-dark cycle through signals originating from the suprachiasmatic nucleus, which is known as the central pacemaker of the circadian timing system [2, 5]. This ensures that endogenous glucocorticoid levels are highest in preparation for the body's daily activities[6]. In humans the peak in cortisol is in the early morning and levels gradually decrease during the active period of the day. As mice are nocturnal animals the glucocorticoid rhythm is reversed with a peak in corticosterone levels at the beginning of the active dark-phase. The circadian rhythm in glucocorticoids appears to be critical for various aspects of lipid and glucose metabolism [7, 8] by mediating metabolic activity of several tissues including adipose tissue [9, 10] , the liver [11] and skeletal muscle [12, 13].

Synthetic glucocorticoids are commonly prescribed for anti-inflammatory diseases [14]. It is known that its long term and/or excessive use is associated with pronounced metabolic side effects including osteoporosis, muscle atrophy and altered glucose metabolism[15-19]. Betamethasone is an extensively used synthetic glucocorticoid, with high selectivity for its therapeutic target, the glucocorticoid receptor [20]. Earlier, we evaluated its effects on skeletal muscle [21]. In this study, we evaluated how the timing of betamethasone treatment influences its adverse effects in mice, with a particular focus on glucose metabolism. We found that out-of-phase treatment with betamethasone, when endogenous glucocorticoid levels are low, generally results in more severe adverse effects on glucose metabolism compared to in-phase treatment, when endogenous glucocorticoid levels are high. We also found that these effects are largely similar between male and female mice after prolonged betamethasone treatment.

METHODS

Animals

Animal experiments were approved by the institutional ethics committee of Leiden University Medical Center and executed under a license granted by the central authority for scientific procedures on animals (CCD), in accordance with the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU. Eight

to ten week-old male and female C57BL/6J mice were obtained from Charles River Laboratories and group housed in conventional cages. Mice were on a 12-hour light/12-hour dark cycle with clock time 07h00 as lights-on, and 19h00 as lights-off. Mice had ad libitum access to water and RM3 chow diet (Special Diet Services, Essex, UK), unless otherwise specified. We evaluated the effects of treatment timing with betamethasone in two separate experiments, in which we compared out-of-phase with in-phase betamethasone treatment.

In experiment 1, we investigated the effect of out-of-phase versus in-phase betamethasone treatment on the development of glucocorticoid-associated side effects in male (N = 18 in total) and female mice (N = 18 in total). Mice were intraperitoneally injected with PBS (vehicle, 100 µl) at zeitgeber time (ZT)-2, with 3.0 mg/kg betamethasone (dissolved in PBS in a volume of 100 µl) at ZT2 ('out-of-phase') or with 3.0 mg/kg betamethasone at ZT10 ('in-phase') by daily injections for a total of 30 days (N = 6 mice per group per sex). The dosage of betamethasone was based on a previous study [21].

In experiment 2, we focused on the effect of betamethasone treatment timing on glucose metabolism in male mice (N = 48). Mice were intraperitoneally injected with PBS (vehicle) at ZT2, with 3.0 mg/kg/day betamethasone at ZT2 ('out-of-phase'), with PBS (vehicle) at ZT10 or with 3.0 mg/kg/day betamethasone at ZT10 ('in-phase') for a total of 14 days. We included two parallel cohorts of mice to perform functional measurements at two different timepoints (N = 6 mice per treatment group for morning-afternoon measurements between ZT2–7 and N = 6 mice per treatment group for evening measurements between ZT8–15. The exact time range of the functional measurements including body mass and composition, grip strength, glucose tolerance, insulin tolerance and lipid/glucose uptake is detailed below and summarized in Table 1).

Table 1. Timepoints of functional measurements.

Empty Cell	**Measurement**		**Time since last treatment (h)**	
	Morning	**Evening**	**Morning**	**Evening**
Body weight and composition	ZT3	ZT11	1 & 16	9 & 3
Grip Strength	ZT4	ZT12	2 & 16	10 & 2
OGTT	ZT7-9	ZT15-17	5 & 21	14 & 5
ITT	ZT7-9	ZT15-17	5 & 21	14 & 5
Plasma biochemistry	ZT7	ZT15	5 & 21	14 & 5
Organ uptake	ZT7-9	ZT15-17	5 & 21	14 & 5

Body mass and body composition measurement

In experiment 1, body mass and composition (lean and fat mass) were determined weekly at ZT3 by using an EchoMRI-100 analyzer (EchoMRI, Houston, TX, USA). In experiment 2, body mass and composition were evaluated weekly at ZT3 for the morning measurement and ZT11 for the evening measurement.

Grip strength test

In experiment 1, grip strength was measured at ZT4. In experiment 2, grip strength was measured weekly at ZT4 for morning measurements and at ZT12 for evening measurements.

Oral glucose tolerance test

In experiment 2, mice were fasted for 6 h from ZT1-7 (morning measurement) or from ZT9-15 (evening measurement) on day 11 to perform an oral glucose tolerance test (OGTT) at ZT7-9 and ZT15-17, respectively. Blood glucose was measured with an Accu-Chek glucometer (Roche) in blood collected from the tail vein at $t = 0$ and subsequently 2 g/kg of glucose was administered via oral gavage and blood glucose was measured at $t = 15, 30, 60, 120$ min. Data is shown

as absolute glucose values, as absolute change from baseline (incremental), and as fold change from the $t = 0$ value (normalized).

Insulin tolerance test

In experiment 2, mice were fasted for 6 h from ZT1-7 (morning measurement) or from ZT9-15 (evening measurement) on day 14 to perform an insulin tolerance test (ITT) at respectively ZT7-9 and ZT15-17. Blood glucose was measured using an Accu-Chek glucometer (Roche) at $t = 0$ and mice were subsequently intraperitoneally injected with 0.75 U/kg human insulin (Sigma I9278) and blood glucose levels were measured at $t = 15, 30, 60, 120$ min. Mice that became hypoglycemic after insulin injection were rescued by an intraperitoneal injection with 300 µl 10 % glucose solution, and glucose levels of these mice were recorded as the lower limit of 2 mM for the rest of ITT measurement. Data is shown as absolute glucose values and as fold change from the $t = 0$ value (normalized).

Plasma biochemistry

In experiment 1 mice were fasted for 6 h on day 14 and 30 from ZT1-7 and in experiment 2 mice were fasted for 6 h on day 14 from ZT1-7 (morning measurements) or ZT9-15 (evening measurements). After fasting, blood was collected in heparin-coated capillaries from the tail vein via a small nick and blood was centrifuged 8000 RMP for 5 min to isolate plasma for biochemistry analysis including insulin (Crystal Chem), glucose, triglycerides, cholesterol (colorimetric kits from Roche Diagnostics) and C-peptide (Crystal Chem Catalog #90050). Heart puncture blood was collected in EDTA-coated tubes directly after euthanasia to isolate plasma for measurements of P1NP (Immunodiagnostic Systems) and osteocalcin (Abcam).

Organ uptake of radiolabeled triglyceride-derived fatty acids and deoxy-glucose

In experiment 2, mice were fasted for 6 h from ZT1-7 (morning measurements) or ZT9-15 (evening measurements). An experiment was performed to evaluate the tissue uptake of [^{3}H]oleate derived from glycerol [^{3}H]oleate-labeled lipoprotein-like emulsion particles (average size 80 nm) and [^{14}C]deoxy-glucose [22], after intravenous injection into the tail vein (in 200 µl PBS per mouse). Mice were euthanized after 15 min using CO_2 asphyxiation and perfused with ice-cold PBS for 5 min. Various tissues were collected and the tissue pieces (max. 50 mg) were dissolved in 500 µl Solvable (Perkin Elmer) overnight at 56 °C. ^{3}H and ^{14}C activity were measured using scintillation counting solution (Ultima Gold XR, Perkin Elmer).

Gene expression analysis

Total RNA was extracted from snap-frozen tissues using Tripure RNA isolation reagent (Roche). Complementary DNA was generated using M-MLV reverse-transcriptase (Promega). Quantitative reverse transcriptase-PCR was performed on a CFX96 PCR machine (Bio-Rad, Veenendaal, the Netherlands), and expression levels were normalized to the housekeeping gene *Gapdh*. Primer sequences: *Atrogin-1* Fwd: TTCAGCAGCCTGAACTACGA; Rev.: GGATGGCAGTCGAGAAGTCC; *Gapdh* Fwd: GGGGCTGGCATTGCTCTCAA; Rev.: TTGCTCAGTGTCCTTGCTGGGG*;* *G6pc* Fwd: CTTAAAGAGACTGTGGGCATCAA; Rev.: ATTACGGGCGTTGTCCAAAC; *Hk2* Fwd: GATCGCCGGATTGGAACAGA; Rev.: GTCTAGCTGCTTAGCGTCCC; *MurF-1* Fwd: TGTGCAAGGAACAGAAGAC; Rev.: CCAGCATGGAGATGCAGTTA; *Pepck* Fwd: ATCTTTGGTGGCCGTAGACCT; Rev.: GCCAGTGGGCCAGGTATTT; *Slc2a4* Fwd: GGCTCTGACGTAAGGATGGG; Rev.: AAACTGAAGGGAGCCAAGCA.

Statistical analysis

Statistical analyses were performed with SPSS (version 27) and GraphPad Prism version 10.0.3. ANOVA with Turkey multi-comparison was used according to number of variables i.e. including 1-way ANOVA for one variable and 2-way ANOVA for two variables. 2-way ANOVA interaction terms are shown in Supplementary Table 1. All data are presented as means ± SEM.

RESULTS

Out-of-phase and in-phase betamethasone treatment similarly restrain gain of body weight and lean mass in both male and female mice

We evaluated the metabolic effects of out-of-phase (ZT2) versus in-phase (ZT10) treatment with the synthetic glucocorticoid betamethasone by daily intraperitoneal injection of male and female C57BL/6J mice with 3.0 mg/kg betamethasone for 30 consecutive days. We found that both ZT2 and ZT10 betamethasone treatment similarly attenuated the gain in body weight and lean mass in both male and female mice (Fig. 1A-F, Supplementary Fig. 1). Total fat mass was not significantly altered in male nor female mice upon ZT2 and ZT10 betamethasone treatment (Fig. 1D and F). When evaluating the wet weight of tissues, we did not observe any significant effect of ZT2 or ZT10 betamethasone treatment on the total weight of the liver, gonadal white adipose tissue weight (gWAT) or interscapular brown adipose tissue (iBAT) weight in either sex (Supplementary Fig. 2A-F).

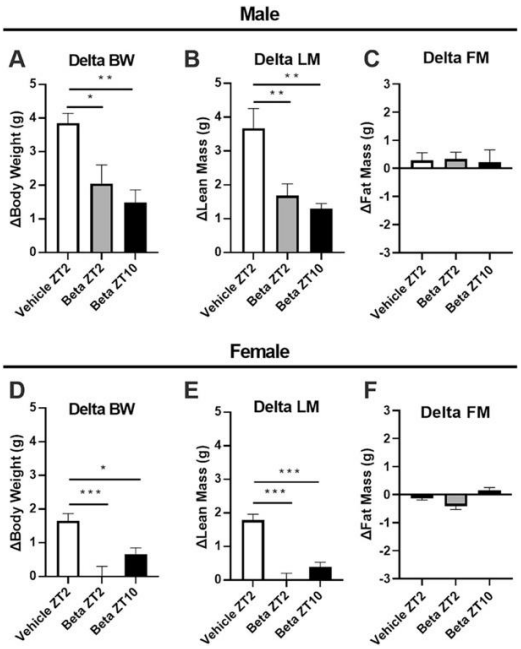

Fig. 1. Out-of-phase and in-phase betamethasone treatment similarly attenuate total body weight and lean mass gain in male and female mice. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on (A) delta body weight (BW), (B) delta lean mass (LM) and (C) delta fat mass (FM) in male mice; and (D) delta BW, (E) delta LM and (F) delta FM in female mice. N = 6 mice/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical significance was calculated using a one-way ANOVA.

Out-of-phase betamethasone treatment increases plasma insulin levels in both male and female mice

To evaluate the effect of timing of betamethasone administration on glucose metabolism, we measured plasma insulin and glucose levels after a 6 h fast from ZT1-7, during which the ZT2 treatment was given. Daily betamethasone administration at ZT2 (out-of-phase) but not at ZT10 (in-phase) significantly increased plasma insulin levels in male and female mice at day 14, while plasma glucose was significantly lowered in male but not female mice upon ZT2 betamethasone treatment (Fig. 2A-D). The effect of ZT2 betamethasone administration on plasma insulin was slightly less pronounced after 30 days of treatment, although still significantly elevated in female mice (Suppplementary Fig. 3A-D). We measured C-peptide levels as a readout for pancreatic insulin release, which were elevated upon ZT2 treatment in both male and female mice (Fig. 2E and G). C-peptide levels mirrored the effects on insulin in both male and female mice and both at 14 and 30 days, with the exception of ZT10 betamethasone treatment in female mice at day 30 (Supplementary Fig. 3E and G). Consistent with the elevated insulin levels, ZT2 but not ZT10 betamethasone treatment resulted in a higher HOMA-IR in male and female mice at day 14 (Fig. 2F and H) and in female mice at day 30 (Supplementary Fig. 3F and H). To investigate how morning betamethasone administration causes hyperinsulinemia, we measured expression levels in tissues collected at ZT3–5 of genes involved in glucose metabolism including *Pepck* and *G6pc* in liver and *Hk2* and *Slc2a4* (encoding Glut4) in skeletal muscle. Neither ZT2 nor ZT10 betamethasone treatment affected the expression of these genes in either male or female mice (Supplementary Fig. 4A-H). We additionally measured markers for other synthetic glucocorticoid-related side effects. With regard to lipid metabolism, we did not observe changes in plasma triglyceride or total cholesterol levels after a 6 h fast from ZT1-7 on day 14 (Supplementary Fig. 5A-D). Similarly, we did not observe any effects of ZT2 or ZT10 betamethasone treatment on bone turnover markers P1NP and osteocalcin on day 30 (Supplementary Fig. 5E-H). Daily treatment with betamethasone at ZT2 or ZT10 also did not influence the weight of the triceps, gastrocnemius, soleus and tibialis anterior (TA) muscles at day 30, and we did not observe any major effects on forelimb grip strength and muscle atrophy-related genes *Atrogin-1* and *Murf-1* in gastrocnemius and triceps muscle (Supplementary Fig. 6A-R). Altogether we show that out-of-phase treatment with 3.0 mg/kg betamethasone at ZT2 markedly influences glucose metabolism but no other markers for glucocorticoid-associated side effects.

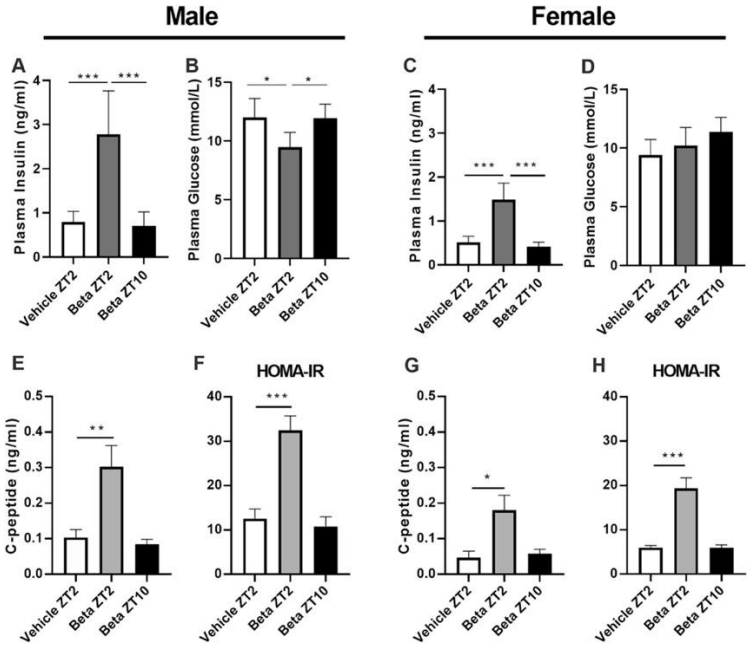

Fig. 2. Out-of-phase but not in-phase betamethasone treatment increases plasma insulin levels in both male and female mice. Plasma biochemistry measured at ZT7 upon treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) after a 6 h fast on day 14. (A) Insulin and (B) glucose in male mice, (C) insulin and (D) glucose in female mice, (E) plasma c-peptide and (F) HOMA-IR in male mice, (G) plasma c-peptide, and (H) HOMA-IR in female mice. N = 6 mice/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical significance was calculated using a one-way ANOVA.

Out-of-phase and in-phase betamethasone treatment similarly restrain total body weight and lean mass gain independent on time of measurement

In a subsequent experiment we further focused on the effects of out-of-phase (ZT2) and in-phase (ZT10) treatment with betamethasone on glucose metabolism. We investigated a 14 day treatment regimen in two parallel cohorts to be able to perform functional measurements on two different times (morning measurement and evening measurement; Table 1). This was done in order to establish if effects of betamethasone treatment are dependent on time of measurement. In line with our previous experiment, we found that both ZT2 and ZT10 betamethasone treatment similarly attenuated total body weight gain and reduced lean mass, and we observed that these effects were independent of the time of measurement (Fig. 3A-F, Supplementary Fig. 7). On fat mass we did not observe significant effects in the morning measurement while we found minor effects of betamethasone treatment in the evening measurement (Fig. 3C and F, Supplementary Fig. 7). In both morning and evening measurements, we did not observe any effect of ZT2 or ZT10 betamethasone treatment on the wet weight of the liver, gWAT, subcutaneous white adipose tissue (sWAT) and iBAT

(Supplementary Fig. 8A-H). Consistent with our previous experiment, treatment with betamethasone at ZT2 or ZT10 did not alter forelimb grip strength during either morning or evening measurement (Supplementary Fig. 8I-J).

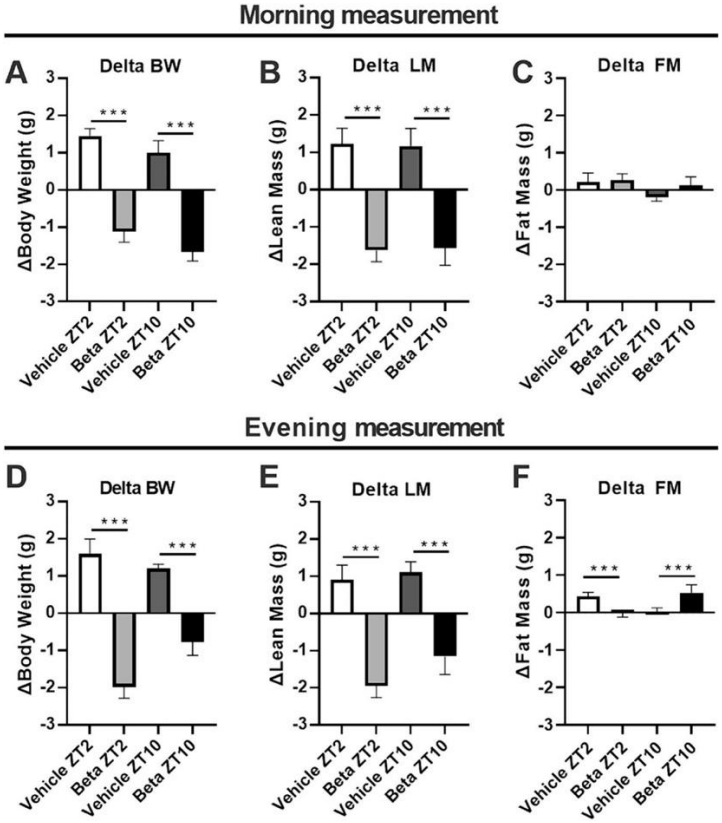

Fig. 3. Out-of-phase and in-phase betamethasone treatment similarly attenuate total body weight and lean mass gain independent on time of measurement. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on (A) delta body weight (BW), (B) delta lean mass (LM) and (C) delta fat mass (FM) in male mice during the morning measurement. (D) Delta BW, (E) delta LM and (F) delta FM during evening measurement. N = 6 mice/group. ***$p < 0.001$. Statistical significance was calculated using a one-way ANOVA.

Out-of-phase and in-phase betamethasone treatment differentially influence insulin sensitivity, which is dependent on time of measurement

To further investigate effects on glucose metabolism, we performed an OGTT at day 11. The absolute glucose levels and corresponding area under the curve (AUC) are shown in Supplementary Fig. 9A-D. When evaluating incremental and normalized glucose levels, we observed a nonsignificant increase in glucose and the total glucose exposure (AUC) upon ZT2 betamethasone treatment in the morning measurement (Fig. 4A-B, Supplementary Fig. 9E-F). These measures were strongly dependent on time of measurement, as evening OGTT

measurement showed a sharp reduction in total glucose exposure with betamethasone treatment independent of the time of administration (Fig. 4C-D, Supplementary Fig. 9G-H). On day 14, we performed an ITT for which the absolute glucose levels and corresponding AUC are shown in Supplementary Fig. 9I-L. After insulin injection, we observed that ZT2 and ZT10 vehicle-treated mice were highly insulin sensitive, with declining blood glucose levels during both morning and evening measurements (Fig. 4E-H, Supplementary Fig. 9I-L). In sharp contrast, ZT2 betamethasone treatment completely abolished insulin sensitivity in the morning, with no meaningful reduction in blood glucose during the morning ITT measurement, while ZT10 treatment only modestly reduced insulin sensitivity (Fig. 4E-F). During the evening ITT measurement, both ZT2 and ZT10 betamethasone similarly diminished insulin sensitivity (Fig. 4G-H). On day 15, we evaluated plasma biochemistry after a 6 h fast and found that ZT2 betamethasone treatment reduced plasma glucose in both morning and evening measurements while ZT10 administration only influenced evening glucose levels (Supplementary Fig. 10A-B). We observed a modest and nonsignificant increase in plasma insulin upon ZT2 betamehasone administration in the morning measurement with no effects in the evening measurements (Supplementary Fig. 10C-D).

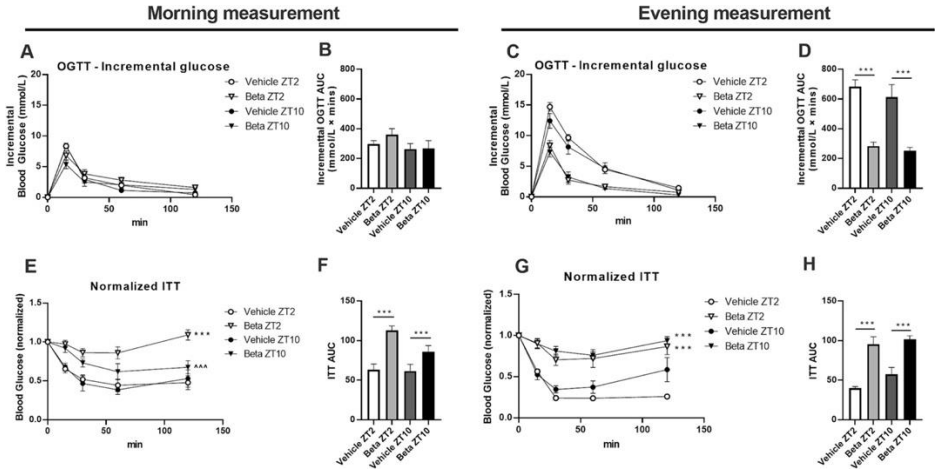

Fig. 4. Out-of-phase betamethasone treatment impairs glucose tolerance and insulin sensitivity which is dependent on time of measurement. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on glucose metabolism in male mice. (A) Incremental glucose levels during an oral glucose tolerance test (OGTT) in the morning (ZT7–9) and (B) area under the curve (AUC), and (C) during an OGTT in the evening (ZT15–17) and (D) AUC. (E) Normalized glucose levels during an insulin tolerance test (ITT) in the morning (ZT7–9) and (F) AUC, and (G) during an ITT in the evening (ZT15–17), and (H) AUC. N = 6 mice/group. *** and ^^^ $p < 0.001$ vs. respective vehicle groups. Statistical significance was calculated using a two-way ANOVA.

Out-of-phase and in-phase betamethasone treatment similarly increase deoxyglucose uptake in the liver and gWAT but differentially affect sWAT and iBAT

To investigate whether the timing of betamethasone treatment influences lipid and glucose uptake we intravenously injected triglyceride-rich lipoprotein-like emulsion particles labeled with glycerol tri[^{3}H]oleate, and [^{14}C]deoxyglucose added to the emulsion. We observed that both ZT2 and ZT10 betamethasone treatment enhanced the uptake of triglyceride-derived fatty acids and [^{14}C]deoxyglucose by liver and gWAT specifically in the evening measurement, potentially explaining the rapid reduction in blood glucose during the OGTT at this timepoint (Fig. 5A-D; Supplementary Fig. 11A-D). We did not observe significant effects in the uptake of triglyceride-derived fatty acids and [^{14}C]deoxyglucose by liver and gWAT in the morning measurement (Fig. 5A-D; Supplementary Fig. 11A-D). In sWAT, we found both ZT2 and ZT10 betamethasone treatment reduced [^{14}C]deoxyglucose uptake in the morning, while only ZT10 treatment reduced [^{14}C]deoxyglucose uptake in the evening measurement (Fig. 5E-F). We did not observe any effects of ZT2 or ZT10 betamethasone treatment on [^{3}H]oleate updateby sWAT (Supplementary Fig. 11E-F). In iBAT, we observed treatment timing-specific effects, as ZT10 treatment increased [^{14}C]deoxyglucose uptake in the morning measurement while ZT2 treatment decreased this in the evening measurement (Fig. 5G-H). ZT10 betamethasone treatment also reduced triglyceride-derived fatty acid uptake in iBAT in the evening measurement but not in the morning measurement (Supplementary Fig. 11G-H).

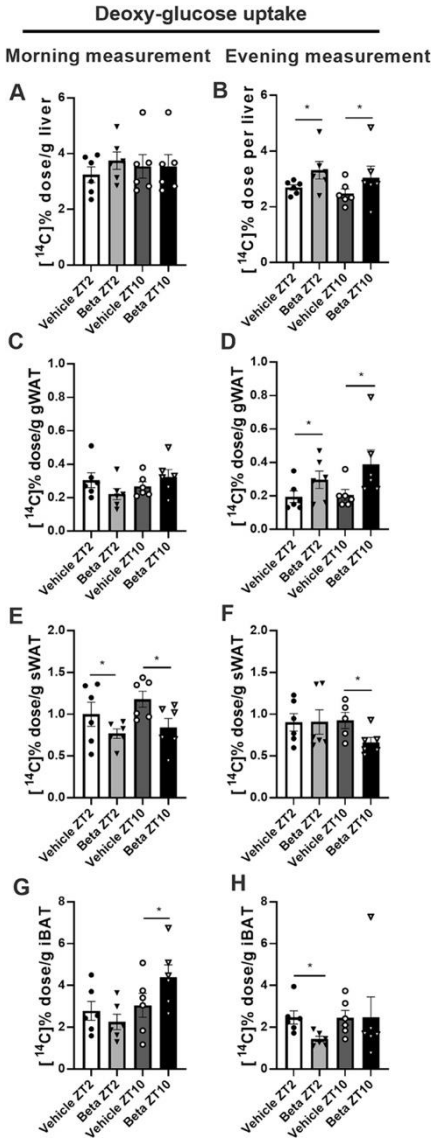

Fig. 5 Out-of-phase and in-phase betamethasone treatment similarly increase deoxyglucose uptake in the liver and gWAT but differentially affect sWAT and iBAT. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on [^{14}C]deoxyglucose uptake in (A-B) liver, (C-D) gonadal white adipose tissue (gWAT), (E-F) subcutaneous white adipose tissue (sWAT) and (G-H) interscapular brown adipose tissue (iBAT) in the morning (ZT7–9) and evening (ZT15–17) after a 6 h fast. N = 6 mice/group. *$p < 0.05$. Statistical significance was calculated using a two-way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DISCUSSION

Synthetic glucocorticoid treatment is widely used in the clinic for a range of applications including inflammatory diseases and autoimmune diseases and as an antiemetic during cancer chemotherapy. In our study we found that out-of-phase treatment with the synthetic glucocorticoid betamethasone causes a clear disturbance of glucose metabolism, while the effects of in-phase betamethasone treatment are less pronounced.

The effects of glucocorticoids are influenced by time of day. It is known that the endogenous steroids cortisol and corticosterone exhibit a strong diurnal rhythm and glucocorticoid replacement treatment for adrenal insufficiency often intends to mimic this circadian fluctuation in glucocorticoid exposure. Evening treatment (in rodents) is thus aligned with the circadian rhythm of endogenous glucocorticoids [23, 24], and this might lead to fewer side effects [25, 26]. Many treatment regimens do not necessarily take circadian variation in glucocorticoid levels into account and are typically only given at one time of the day, albeit more often in the morning during the endogenous glucocorticoid peak. For example in rheumatoid arthritis symptoms like stiffness and pain are more severe in the morning and glucocorticoid is therefore administered during this period [27, 28]. It is however unclear if the side effects that develop as a consequence of glucocorticoid therapy are also dependent on timing. We therefore compared the effect of time of treatment with the potent synthetic glucocorticoid betamethasone to evaluate how this influences various side effects, with a particular focus on glucose metabolism.

We first evaluated timing effects of betamethasone with a particular focus on potential sex differences in glucocorticoid effects, given the different responses between males and females previously reported by others and ourselves [21, 29-31]. We compared 'out-of-phase' betamethasone treatment at ZT2 (during the endogenous corticosterone trough in mice) with 'in-phase' treatment at ZT10 (during the endogenous corticosterone peak in mice). Despite baseline differences between male and female mice in total body weight and lean mass, the reduction in body weight gain and lean mass in response to ZT2 and ZT10 betamethasone treatment was largely similar between sexes. We did not find effects of either ZT2 or ZT10 betamethasone treatment on many other known glucocorticoid-associated side effects, including muscle function, adiposity, lipid metabolism and bone turnover. This is not fully consistent with a previous study in which 3.0 mg/kg daily betamethasone 'in-phase' (ZT9) resulted in modest effects on body composition and muscle function [21]. It is also worthwhile to note that in the current experiment we only observed modest sex differences in glucocorticoid response, while other studies show more pronounced sex differences in response to excess corticosterone [29, 31].

A significant effect on fasted plasma insulin levels was observed after 14 days of ZT2 betamethasone treatment in both sexes albeit more pronounced in male as compared to female mice. This hyperinsulinemia was explained by enhanced pancreatic insulin release as evidenced by elevated C-peptide levels. Given the robust effects on plasma insulin (release) in this study, our second experiment was designed to study glucose metabolism specifically. The effects of ZT2 and ZT10 betamethasone on total body weight and fat mass were consistent with the previous experiment and with literature [32, 33], and the effects were independent on the time of measurement (morning vs evening measurement; Table 1). We included three functional tests to measure glucose metabolism, namely an OGTT, an ITT and the uptake of radiolabeled deoxy-glucose. For the

OGTT, the effects of betamethasone were highly dependent on the time of measurement, with a possible increase in glucose excursion the morning after ZT2 betamethasone treatment, but with a very steep decrease in glucose excursion upon ZT2 and ZT10 betamethasone treatment. This reduction during the evening is potentially explained by betamethasone-induced uptake of deoxy-glucose by liver and gWAT. It is worthwhile to note that there were baseline differences in blood glucose between morning and evening measurements in vehicle-treated mice, skewing incremental glucose values. We observed decreased plasma glucose after 14 days of ZT2 betamethasone treatment, which is counterintuitive but suggests that these mice are in transition towards overt insulin resistance and are still partially responsive to (increased) insulin.

It is known that glucocorticoids influence pancreas β-cell function and insulin secretion [34], and we therefore performed an ITT in the morning and evening [35, 36] and again observed differences between time of measurements. During the morning measurement, we found that betamethasone markedly decreased insulin sensitivity but that this effect was much more pronounced upon ZT2 treatment compared to ZT10 treatment. During the evening measurement we found that betamethasone treatment strongly decreased insulin sensitivity independent of timing of treatment. In the second experiment the effect of ZT2 betamethasone treatment on fasted insulin was much less pronounced as compared to the previous experiment, but it has to be noted that these results may be confounded by the ITT test that was performed shortly before blood collection for plasma biochemistry measurements. When evaluating deoxy-glucose uptake by different tissues we again found differences between time of measurement, e.g. with an increase of glucose uptake in the liver and gWAT during the evening but not morning measurement. For brown adipose tissue we found an increase upon ZT10 betamethasone in the morning and a decrease in glucose uptake upon ZT2 treatment measured during the evening. Taken all those findings together, we can conclude the effect of glucocorticoid treatment on glucose metabolism is dynamic and that outcome differs throughout the time of day.

One limitation of our study is that time of measurements was not symmetric for all functional measurements, with different time between ZT2 and ZT10 betamethasone treatment and functional measurements (Table 1). We expect that this may influence some of the analyses, although the OGTT, ITT and organ uptake study were all performed with comparable time after ZT2 treatment until morning measurement and ZT10 treatment until evening measurement. It is reassuring that some of the timed glucocorticoid effects like the effect of ZT2 betamethasone treatment on insulin sensitivity during the ITT were observed during both the morning and evening test, excluding that the time since last treatment is a confounding factor for this readout. The glucocorticoid effects that we observed may be mediated via disturbance endogenous glucocorticoid levels as well as overexposure to synthetic glucocorticoids [37, 38]. Our study setup does not allow us to dissect the exact contribution, but both scenarios

share the aspect of GR activation at the wrong time of day. Finally, it should be noted that species differences (e.g. between rodents and humans) exist for glucocorticoids, so it is unclear to what extent these findings translate to humans [39].

In addition to glucose metabolism, we also measured other possible glucocorticoid-associated side effects. We did not observe strong effects on muscle mass or function, besides a transient reduction of muscle strength in female mice. We previously found that female mice were more sensitive to betamethasone-induced muscle dysfunction after 14 days of treatment [21], but we did not observe strong effects in this study (up to 30 days). We also monitored markers for glucocorticoid-induced osteoporosis [40], including plasma osteocalcin and P1NP, but these were not significantly influenced by neither ZT2 nor ZT10 betamethasone treatment in both male and female mice. Hyperinsulinemia can be caused by different processes in various tissues [41-43]. We could not attribute the effects of betamethasone on one tissue per se, as we did not find transcriptional changes in the liver and skeletal muscle. It is noted that we performed expression analysis after 30 days and that the effect on plasma insulin was attenuated as compared to after 14 days of treatment.

In summary, our study compared the effects of morning and evening daily betamethasone treatment on the development of glucocorticoid-associated side effects. Even if the magnitude of side effects in our study was overall modest, we found that out-of-phase betamethasone administration generally caused more adverse effects on glucose metabolism. Given the differences we observed between different times of measurement we urge the importance to carefully consider the time of measurements in studies focusing on glucocorticoid-induced side effects, at least in relation to glucose metabolism.

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Credit authorship contribution statement

Sheng Li: Writing – original draft, Project administration, Investigation, Formal analysis, Data curation. **Sen Zhang:** Investigation. **Patrick C.N. Rensen:** Writing – review & editing, Resources, Methodology. **Onno C. Meijer:** Writing – review & editing, Supervision. **Sander Kooijman:** Writing – review & editing, Conceptualization. **Jan Kroon:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization.

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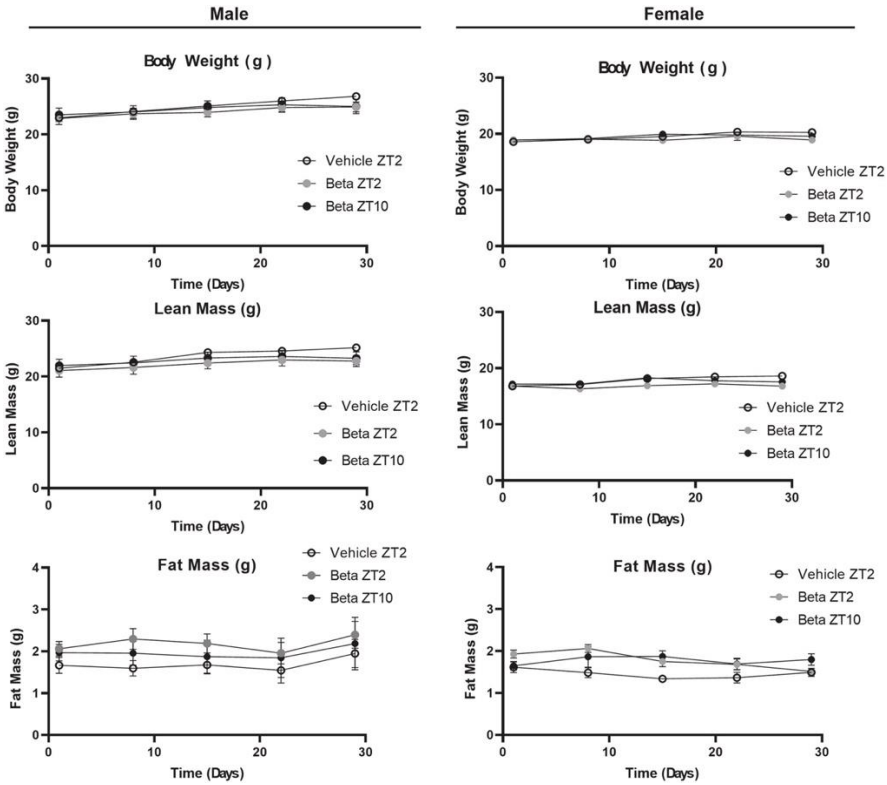
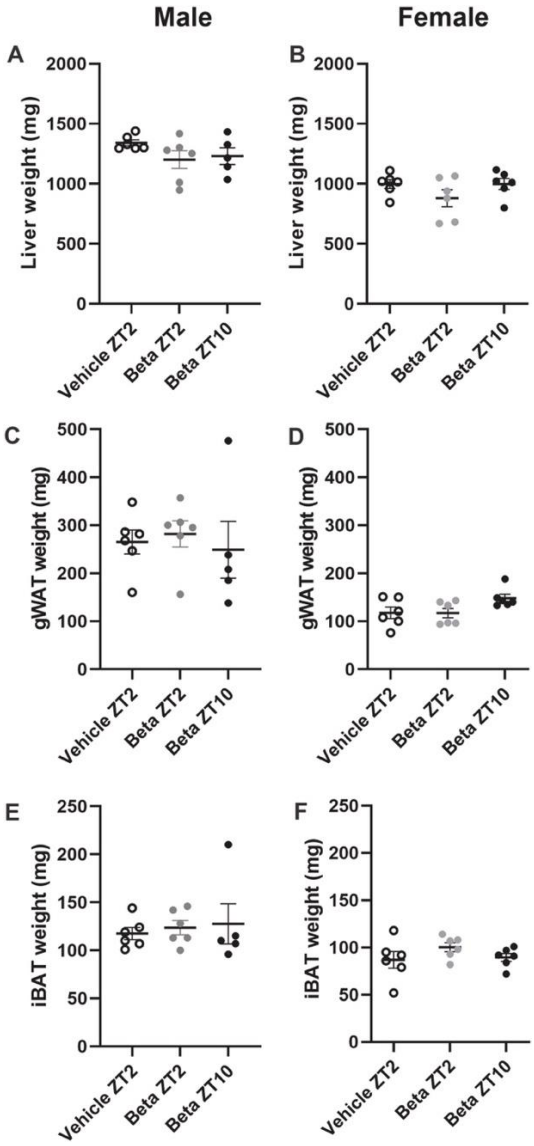
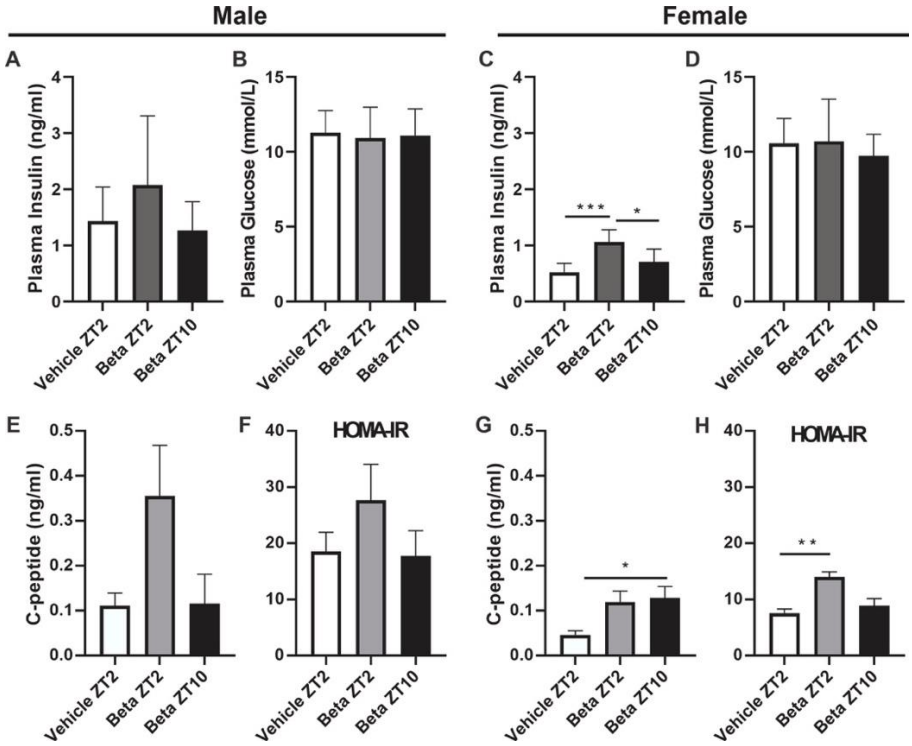
SUPPLEMENT



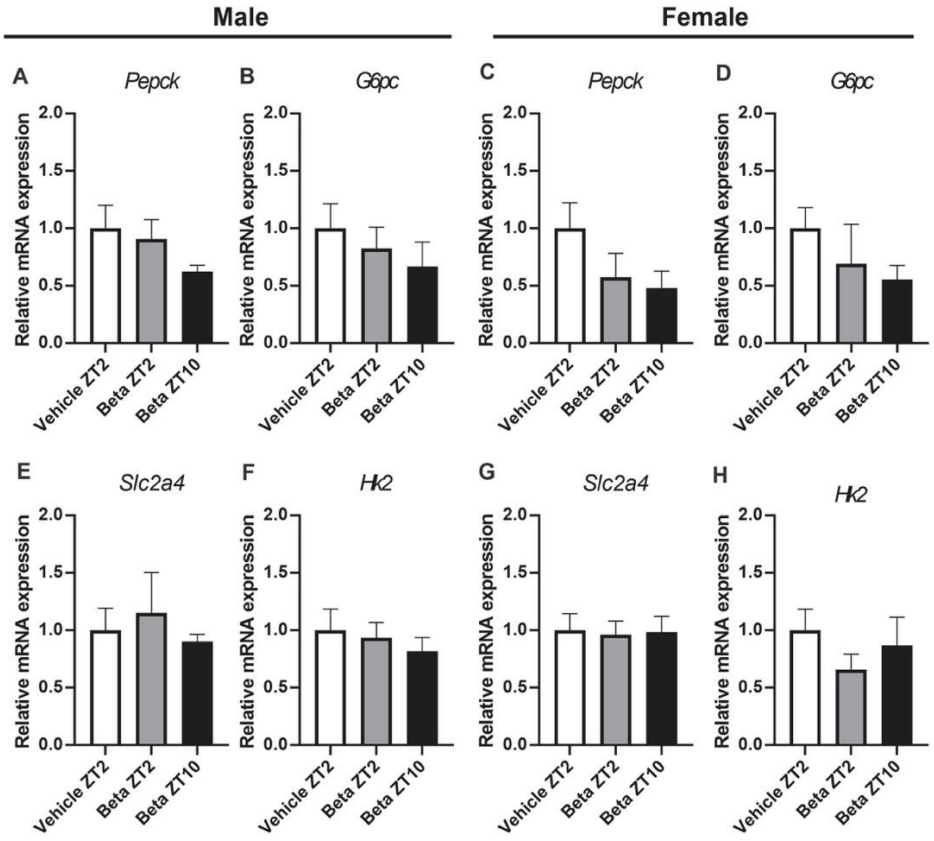
Supplementary Fig. 1. The effect of out-of-phase and in-phase betamethasone treatment on body weight and composition in male and female mice. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on (A) body weight, (B) lean mass and (C) fat mass of male and female mice over time. N = 6 mice/group.



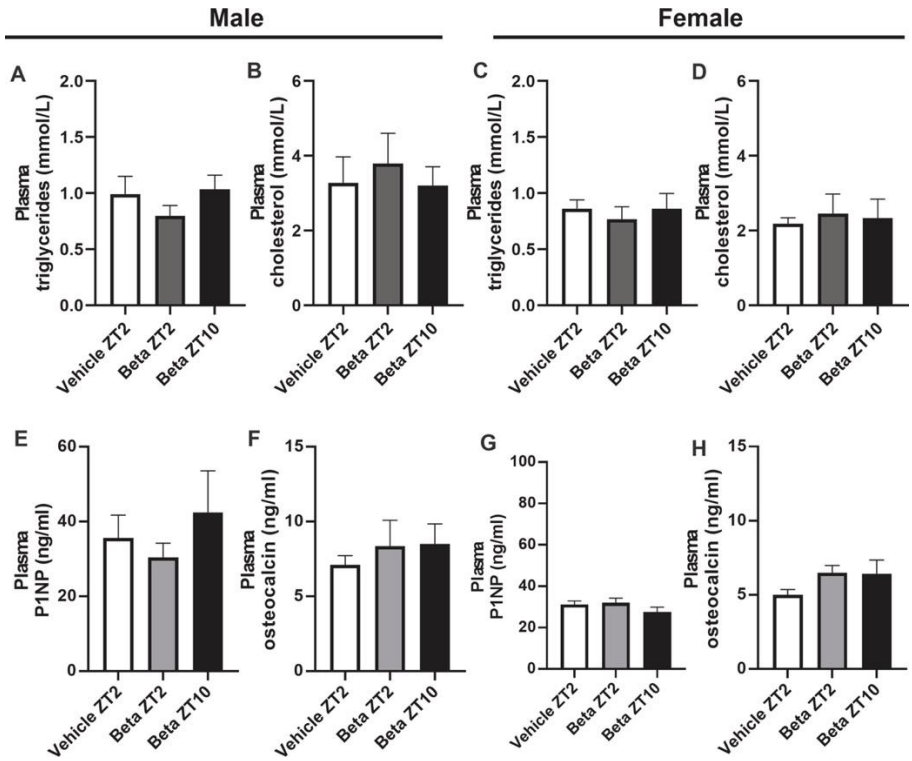
Supplementary Fig. 2 The effect of out-of-phase and in-phase betamethasone treatment on metabolic tissue weights. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on tissue weights of male and female mice. (A) Liver weight, (B) gonadal white adipose tissue (gWAT) weight, (C) interscapular brown adipose tissue (iBAT) weight. N = 6 mice/group. Statistical significance was calculated using a one- way ANOVA.



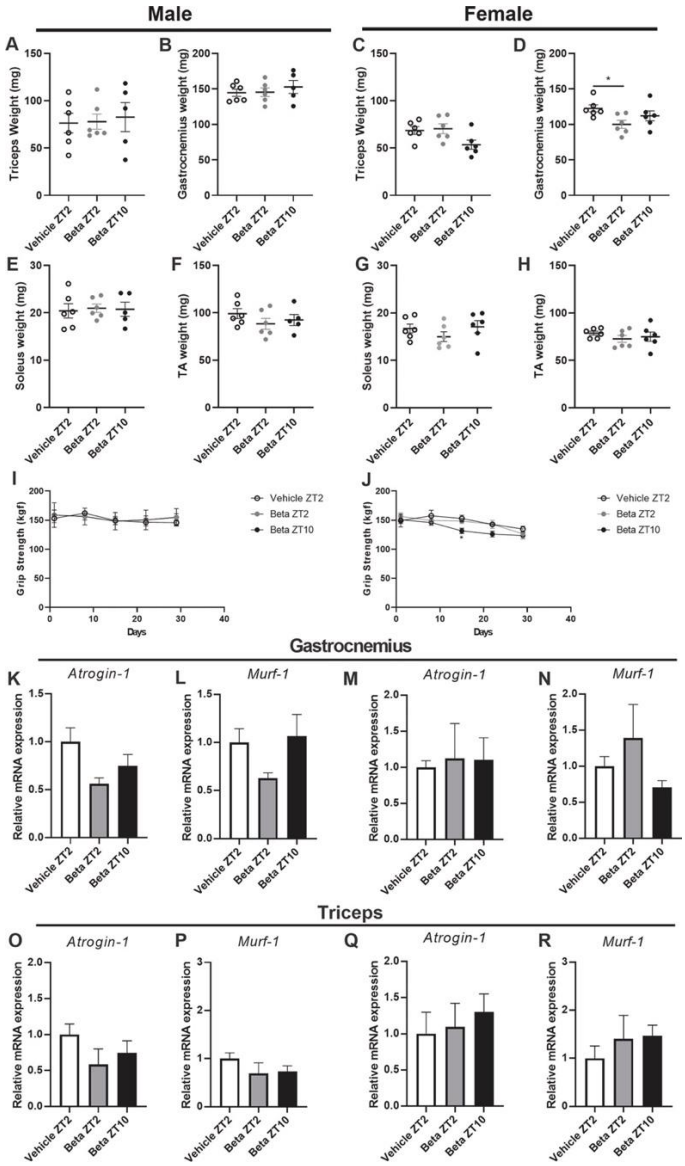
Supplementary Fig. 3 The effect out-of-phase and in-phase betamethasone treatment on plasma insulin at day 30. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on plasma biochemistry after 6 h fast on day 30. (A) Insulin, (B) and glucose in male mice, (C) insulin, (D) and glucose in female mice, (E) Plasma c-peptide, (F) and HOMA-IR in male mice, (G) plasma c-peptide, (H) and HOMA-IR in female mice. N = 6 mice/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical significance was calculated using a one-way ANOVA.



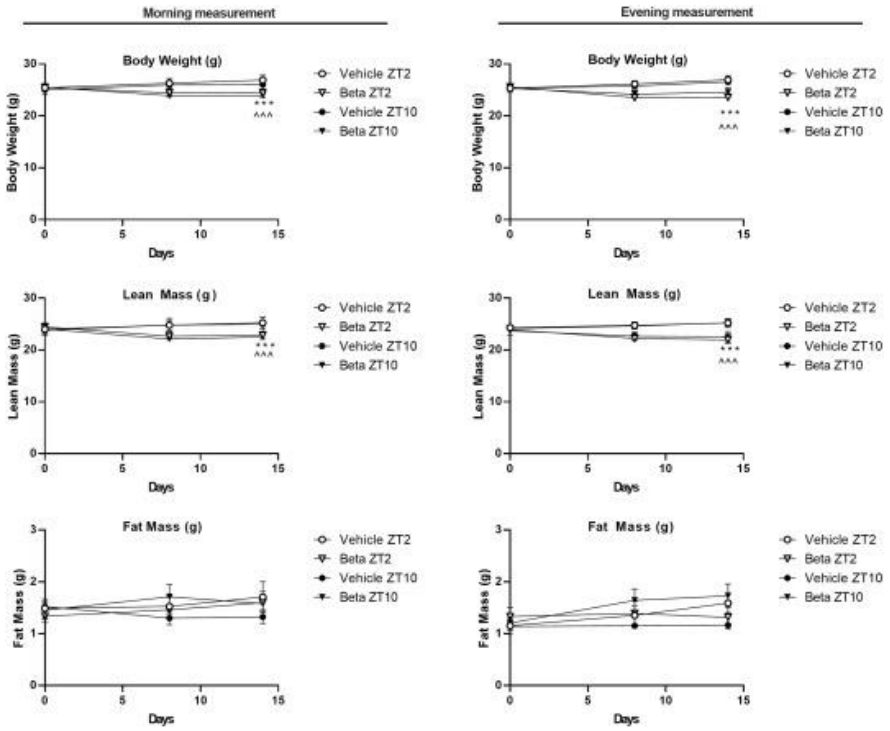
Supplementary Fig. 4 The effect out-of-phase and in-phase betamethasone treatment on gene expression in liver and gastrocnemius muscle. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on gene expression in the liver: (A) Pepck, and (B) G6pc in male mice, (C) Pepck, and (D) G6pc in female mice; and in gastrocnemius muscle: (E) Slc2a4, and (F) Hk2 in male mice, and (G) Slc2a4, and (H) Hk2 in female mice. N = 6 mice/group. Statistical significance was calculated using a one-way ANOVA.



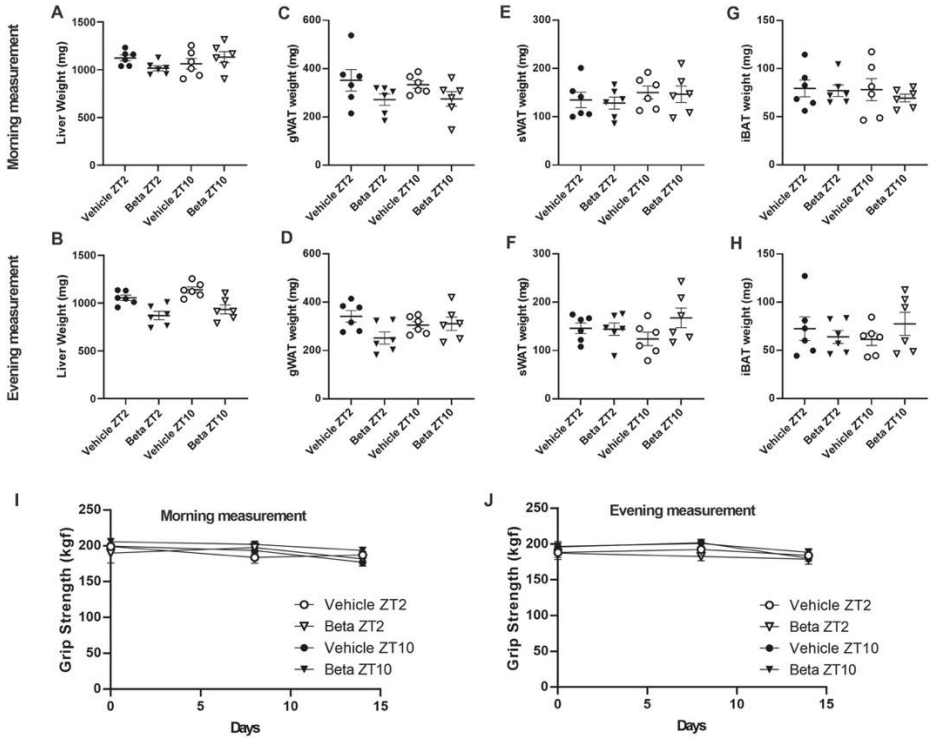
Supplementary Fig. 5 The effect out-of-phase and in-phase betamethasone treatment on plasma lipids and bone turnover markers. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on plasma biochemistry after 6 h fasting on day 14 of (A) triglycerides, and (B) cholesterol in male mice, (C) triglycerides, and (D) cholesterol in female mice. Plasma levels on day 30 of (E) P1NP, and (F) osteocalcin in male mice, (G) P1NP, and (H) osteocalcin in female mice. Statistical significance was calculated using a one-way ANOVA.



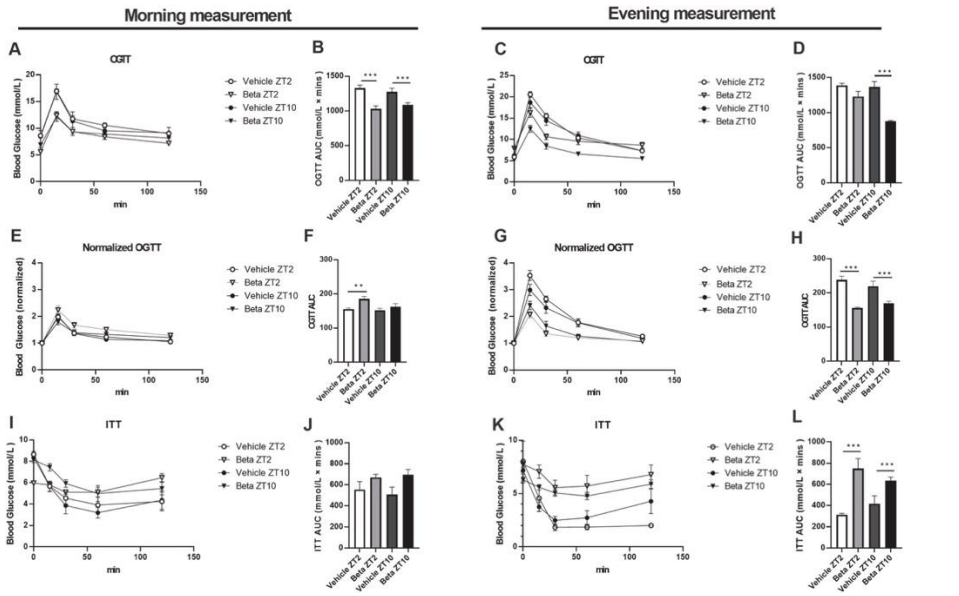
Supplementary Fig. 6 The effect out-of-phase and in-phase betamethasone treatment on muscle function. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on weight of (A) triceps, (B) gastrocnemius weight in male mice, (C) triceps, and (D) gastrocnemius in female mice, (E) soleus, and (F) tibialis anterior (TA) in male mice, (G) soleus, and (H) TA in female mice. Forelimb grip strength in (I) male mice and (J) female mice. Gene expression in the gastrocnemius muscle of (K) *Atrogin-1*, and (L) *Murf-1* in male mice, (M) *Atrogin-1*, and (N) *Murf-1* in female mice. Gene expression in triceps muscle of (O) *Atrogin-1*, and (P) *Murf-1* in male mice, (Q) *Atrogin-1*, and (R) *Murf-1* in female mice. $N = 6$ mice/group. $*p < 0.05$ vs. Vehicle. Statistical significance was calculated using a one-way ANOVA.



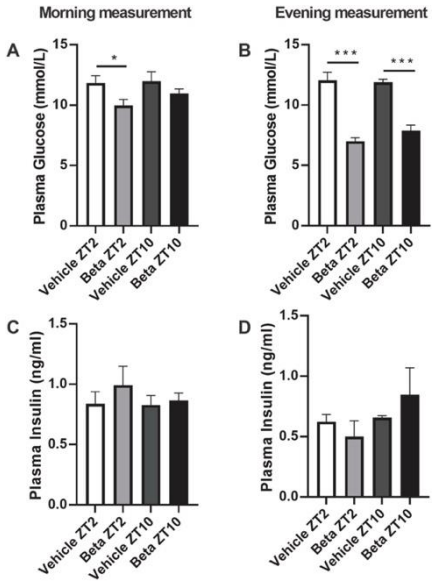
Supplementary Fig. 7 The effect of out-of-phase and in-phase betamethasone treatment on body weight and composition in male mice during morning and evening measurements. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) of male mice on (A) body weight, (B) lean mass and (C) fat mass during morning and evening measurements. N = 6 mice/group. *** and ^^^ $p < 0.001$ vs respective vehicle groups. Statistical significance was calculated using a two-way ANOVA.



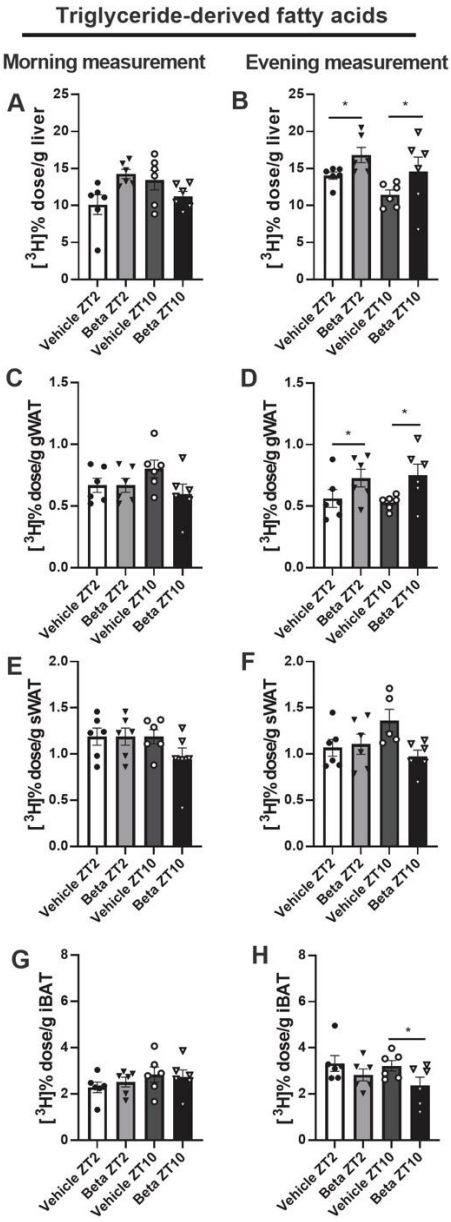
Supplementary Fig. 8 The effect of out-of-phase and in-phase betamethasone treatment on metabolic tissue weights. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) of male mice on the wet weight of (A-B) liver, (C-D) gWAT, (*E*-F) sWAT, and (G-H) iBAT in the morning or in the evening. Forelimb grip strength during (I) morning and (J) evening measurements. N = 6 mice/group. Statistical significance was calculated using a two-way ANOVA.



Supplementary Fig. 9 The effect of out-of-phase and in-phase betamethasone treatment on glucose and insulin tolerance. Absolute glucose levels during an oral glucose tolerance test (OGTT) in (A) the morning (ZT7–9) and (B) the area under the curve (AUC); and (C) in the evening (ZT15–17), and (D) the AUC. (E) Normalized glucose levels during an OGTT in the morning, and (F) the AUC; and (G) in the evening, and (H) the AUC. Absolute glucose levels during an insulin tolerance test (ITT) in (I) the morning, and (J) the AUC, and (K) in the evening, and (L) the AUC. $N = 6$ mice/group. *** and ^^^ $p < 0.001$ vs. respective vehicle groups. Statistical significance was calculated using a two-way ANOVA.



Supplementary Fig. 10 The effect of out-of-phase and in-phase betamethasone treatment on plasma biochemistry. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) of male mice after a 6 h fast at day 14 on (A) glucose in the morning (ZT7), (B) glucose in the evening (ZT15), (C) insulin in the morning (ZT7), and (D) insulin in the evening (ZT15). N = 6 mice/group. *p < 0.05 vs. Vehicle, ***p < 0.001 vs. Vehicle. Statistical significance was calculated using a two-way ANOVA.



Supplementary Fig. 11 Out-of-phase and in-phase betamethasone treatment similarly increase triglyceride-derived fatty acids uptake in the liver and gWAT. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on glycerol tri[3H]oleate derived [3H]oleate uptake by (A-B) liver, (C-D) gonadal white adipose tissue (gWAT), (E-F) subcutaneous white adipose tissue (sWAT) and (G-H) interscapular brown adipose tissue (iBAT) in the morning (ZT7–9) and evening (ZT15–17) after a 6 h fast. N = 6 mice/group. *$p < 0.05$. Statistical significance was calculated using a two-way ANOVA.

4

Glucocorticoid receptor antagonism improves glucose metabolism in a mouse model of polycystic ovary syndrome

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ABSTRACT

Context

Polycystic ovary syndrome (PCOS) is a complex metabolic disorder associated with obesity, insulin resistance, and dyslipidemia. Hyperandrogenism is a major characteristic of PCOS. Increased androgen exposure is believed to deregulate metabolic processes in various tissues as part of the PCOS pathogenesis, predominantly through the androgen receptor (AR). Notably, various metabolic features in PCOS are similar to those observed after excess glucocorticoid exposure.

Objective

We hypothesized that glucocorticoid receptor (GR) signaling is involved in the metabolic symptoms of PCOS.

Methods

In a PCOS model of chronic dihydrotestosterone (DHT) exposure in female mice, we investigated whether GR signaling machinery was (de)regulated, and if treatment with a selective GR antagonist alleviated the metabolic symptoms.

Results

We observed an upregulation of GR messenger RNA expression in the liver after DHT exposure. In white adipose tissues and liver we found that DHT upregulated *Hsd11b1*, which encodes for the enzyme that converts inactive into active glucocorticoids. We found that preventive but not therapeutic administration of a GR antagonist alleviated DHT-induced hyperglycemia and restored glucose tolerance. We did not observe strong effects of GR antagonism in DHT-exposed mice on other features like total fat mass and lipid accumulation in various tissues.

Conclusion

We conclude that GR activation may play a role in glucose metabolism in DHT-exposed mice.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common hormonal disorder in women leading to infertility and is estimated to have a global prevalence of 6% to 20% [1]. In PCOS, long-term and continuous exposure to elevated levels of androgens are considered the major driving force of the clinical features [2]. In addition to infertility, PCOS is associated with metabolic symptoms such as obesity, insulin resistance, and dyslipidemia [1, 3, 4]. Insulin resistance can also induce elevated androgen levels by reducing sex hormone binding globulin and thereby resulting in increased free androgen levels and increased androgen signaling [5, 6]. At the molecular level, androgens primarily exert their effects through the androgen receptor (AR), and preclinical studies in AR knockout mice have shown that AR signaling is crucial in the development of PCOS-related symptoms [7, 8]. The AR undergoes a conformational change on ligand binding and translocates to the nucleus, where it exerts its transcriptional effects [9, 10]. Besides the involvement of androgen signaling, the underlying mechanisms of how metabolic symptoms in PCOS develop still remain largely unknown. We previously found in male mice that androgen signaling can strongly influence the outcome of glucocorticoid signaling [11]. Glucocorticoid receptor (GR) signaling is known to play a major role in various metabolic process [12, 13], including lipid metabolism [14] and glucose metabolism [15-17]. Notably, many of the clinical features of PCOS overlap with those of excess glucocorticoid exposure [18].

Glucocorticoid signaling is regulated at several levels. At an enzymatic level active glucocorticoid levels are controlled by 11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) [19], an enzyme that converts inactive glucocorticoids into active glucocorticoids, and that is known to play a role in the development of obesity [20]. It has been shown that the androgen dihydrotestosterone (DHT) increases the expression of 11β-HSD1 in mouse and human adipose tissue, thereby influencing local turnover of corticosterone/cortisol [21, 22]. The enzyme 11 beta-hydroxysteroid dehydrogenase type 2 inactivates glucocorticoids, and its expression is more restricted [18]. In addition to enzymatic regulation, the outcome of GR signaling is dependent on interaction with coregulatory proteins such as nuclear receptor coactivator 1 (NCOA1/SRC1) and nuclear receptor coactivator 2 (NCOA2/SRC2) [23, 24]. NCOA1 and NCOA2 were shown to play an important role in metabolic homeostasis [25-28]. It is important to note that many of these coregulatory proteins are also involved in AR signaling [29]. Given the similarities in metabolic symptoms in PCOS and upon excess glucocorticoid exposure, we hypothesized that glucocorticoid signaling may contribute to the metabolic symptoms observed in PCOS [30, 31]. In this study, we evaluated if GR machinery is altered in female mice on DHT exposure, and explored whether GR

antagonism can be used to alleviate DHT-induced metabolic symptoms. For this, we made use of the recently developed GR antagonist CORT125134 (relacorilant), that—in contrast to classic GR antagonist RU486—lacks cross-reactivity with the AR and the progesterone receptor (PR) [32, 33].

MATERIALS AND METHODS

Cell Culture and Reporter Assay

Human HEK293T cells were seeded at 80000 cells per well in 24-well plates in Dulbecco's modified Eagle's medium + GlutaMAX with 10% charcoal-stripped fetal bovine serum supplemented with penicillin/streptomycin. The next day cells were transfected using one of the following mixtures: I) 25 µL OPTIMEM, 10 ng human GR, 25 ng TAT1-luciferase, 1 ng CMV-renilla, 265 ng pcDNA, and 1.25 µL Fugene HD transfection reagent (Promega); II) 25 µL OPTIMEM, 10 ng human PR, 25 ng TAT3-luciferase, 1 ng CMV-renilla, 265 ng pcDNA, and 1.25 µL Fugene HD transfection reagent; and III) 25 µL OPTIMEM, 10 ng human AR, 25 ng TAT1-luciferase, 1 ng CMV-renilla, 265 ng pcDNA, and 1.25 µL Fugene HD transfection reagent. Cells were pretreated for 1 hour with 0.1 to 1000 nM CORT125134 (relacorilant) (for GR signaling assays) or 10 to 1000 nM CORT125134 (for PR and AR signaling assays), and were subsequently treated with agonists for the GR (3 nM dexamethasone), PR (10 nM progesterone), and AR (100 nM DHT). After 24 hours, cells were harvested and firefly- and renilla-luciferase signals were measured using a dual-luciferase assay (Promega). Data are normalized to agonist treatment and half maximal inhibitory concentration values were calculated using nonlinear regression. All conditions were performed in technical triplicate.

Animals

This animal study was approved by the ethics committee of Leiden University Medical Center. Female C57BL6/J mice were purchased from Charles Rivers Laboratories and group-housed in conventional cages with a 12-hour:12-hour light/dark environment and had ad libitum access to water and a synthetic low-fat diet for 90 days.

Animal Experiment

We used the androgen DHT to induce PCOS-like characteristics in female mice [34]. Female mice aged between 4 and 5 weeks were implanted subcutaneously under isoflurane anesthesia with either a blank 1-cm Silastic tube (inner diameter, 1.58 mm; outer diameter, 2.41 mm) or with a tube containing 10 mg DHT. Silastic implants were made in house and are known to provide a steady-

state steroid hormone release for a period up to 6 months [35]. As a quality control, the presence of DHT powder was confirmed post euthanasia (12 weeks after implantation of silastic tubes).

We compared female mice with blank vs DHT implants to investigate the expression of GR-related factors in diverse metabolic tissues. We examined the role of GR signaling in the development of DHT-induced symptoms by feeding mice either a low-fat diet or low-fat diet supplemented with the selective GR antagonist CORT125134 (relacorilant) for a period of 90 days (500 mg per kg diet, resulting in an estimated dose of 60 mg/kg/day; "preventive" group). In parallel, we investigated GR antagonism in mice with an established PCOS-like metabolic phenotype as a result of DHT exposure, by administering 60 mg/kg/day CORT125134 or solvent (10% dimethyl sulfoxide, 0.5% Tween-80, 0.5% hydroxypropyl-methylcellulose in phosphate-buffered saline [PBS]) via daily oral gavage during weeks 9 to 12 (for a total of 21 days; "therapeutic" group). Overall, we evaluated the following groups: 1) control (N = 5), 2) control + preventive GR antagonism, N = 6), 3) control + therapeutic GR antagonism, 4) DHT (N = 6), 5) DHT + preventive GR antagonism (N = 6), and 6) DHT + therapeutic GR antagonism (N = 6).

Body Mass and Body Composition Measurement

Body mass and total lean and fat mass were determined weekly by using an EchoMRI-100 analyzer.

Plasma Biochemistry Measurements

At the end of week 12, blood plasma was collected from the tail vein from 6-hour fasted mice and these samples were used to measure plasma levels of insulin (Crystal Chem), glucose, triglycerides, and cholesterol (enzymatic kits from Roche Diagnostics).

Oral Glucose Tolerance Test

In week 11, mice were fasted for 6 hours before a baseline blood glucose measurement was performed (t = 0). After this, 2 g/kg glucose was administered via oral gavage and blood glucose concentration was then measured at t = 15, 30, 60, 90, 120 minutes using an Accu-Check glucometer (Roche).

Organ Uptake of Radiolabeled Triglyceride-derived Fatty Acids and Deoxyglucose

Triglyceride-rich lipoprotein–like emulsion particles (average size 80 nm) radiolabeled with glycerol tri[^{3}H]oleate were prepared as previously described [36, 37]. Mice were fasted for 4 hours and injected intravenously in the tail vein with particles containing 1.0 mg triglyceride in combination with

[^{14}C]deoxyglucose in 200 µL PBS. After 15 minutes, mice were killed by CO_2 inhalation and perfused with ice-cold PBS for 5 minutes before tissues were isolated to determine the ^{3}H and ^{14}C activity in various tissues. Tissue pieces were dissolved in 500 µL of Solvable (Perkin Elmer) overnight at 56 °C, and the ^{3}H and ^{14}C activity was determined using scintillation counting solution (Ultima Gold XR, Perkin Elmer).

Histology

Ovaries, interscapular brown adipose tissue (iBAT) and gonadal white adipose tissue (gWAT) were fixed in 4% paraformaldehyde for 24 hours and stored in 70% ethanol before processing. Tissues were dehydrated, embedded in paraffin, cut into 5-µm sections and then stained with hematoxylin and eosin as previously described [38]. Lipid content in iBAT and average adipocyte cell size in gWAT were quantified using Image J software (version 1.48). In the ovaries, total number of corpora lutea (identified with consistent luteinized follicles and visible in serial sections) were quantified using a Zeiss Axio Observer A1 microscope. Large antral follicles were identified with a single large antrum. Follicles were assessed only in the sections where the oocyte's nucleolus were visible. Large antral follicles were categorized as unhealthy if they included a degenerate oocyte, and/or more than 5% of the granulosa cells were pyknotic in appearance, the percentage of unhealthy follicles per ovary was calculated. All large antral follicles were assessed for granulosa layer thickness and theca layer thickness using ImageJ software (version 1.48), as previously described [34]. One ovary could obtain more than one antral follicle. Several samples were lost during tissue processing, yielding ovaries of only N = 3 per group for analysis, and as we were thus underpowered we decided to not perform statistical analysis.

Gene Expression Analysis

Total RNA was extracted from snap-frozen tissues using Tripure RNA isolation reagent (Roche). Complementary DNA was generated using M-MLV reverse-transcriptase (Promega). Quantitative reverse transcription–polymerase chain reaction was performed on a CFX96 PCR machine (Bio-Rad), and expression levels were normalized to the housekeeping gene *GAPDH*. Primer sequences: *Gapdh* Fwd: GGGGCTGGCATTGCTCTCAA; Rev: TTGCTCAGTGTCCTTGCTGGGG; *Gr* Fwd: CCCTCCCATCTAACCATCCT; Rev: ACATAAGCGCCACCTTTCTG; *Ar* Fwd: GCCTCCGAACTGTGGTATCC; Rev: CCTGGTACTGTCCAAACGCA; *Ncoa1* Fwd: GCGAGTCAAAGGGTGCAGTT; Rev: CCAGCCCGAAGCACATACA; *Ncoa2* Fwd: CGTCACCAACTGAGAAGCCA; Rev: GGACGGGTCAGAGGTGTTGTTTT; *Hsd11b1*: Fwd: AGTACACCTCGCTTTTGCGT; Rev: CTCTCTGTGTCCTTGGCCTC. *Hsd11b2*: Fwd: CACTCGAGGGGACGTATTGT;

Rev: CGTTTCTCCCAGAGGTTCAC. Baseline expression (CT-values) for each gene is shown in Supplementary Table S1.

Statistical Analysis

Statistical analyses were performed with SPSS (version 25) and GraphPad Prism version 8.0.2. The following statistical analyses were used: 2-way analysis of variance with least significant difference post hoc test, unpaired *t* test, and linear mixed models. Data with 2 factors and multiple time points were analyzed using linear mixed models analysis that included independent variables as fixed factors. All data are presented as means ± SEM. *P* values of main effects and interactions of the analysis of variance are depicted in Supplementary Table S2.

RESULTS

Dihydrotestosterone Treatment Increased the Expression of Glucocorticoid Receptor Signaling Factors

We first set up to investigate the effect of DHT treatment on the expression of factors related to GR signaling in a range of tissues including gWAT, subcutaneous white adipose tissue (sWAT), iBAT, subscapular brown adipose tissue (sBAT), and liver. GR messenger RNA (mRNA) expression was elevated in the liver on DHT exposure, but was not significantly changed in other tissues (Fig. 1A-E, first column). *Ar* expression was not changed after DHT exposure in any tissue (see Fig. 1A-E, second column). Analysis of GR coactivators showed that *Ncoa1* expression in sBAT and liver was lower on DHT treatment, while *Ncoa2* expression was significantly increased in liver on DHT treatment (see Fig. 1A-E, third and fourth column). Tissue-specific 11β-HSD1 activity determines the local active glucocorticoid level, and we found that DHT exposure significantly upregulated *Hsd11b1* mRNA in gWAT and liver, while a similar pattern was observed in sWAT (see Fig. 1A-E, fifth column). *Hsd11b2*, the gene encoding for the enzyme 11β-HSD2 that inactivates glucocorticoids, was not expressed in any of these tissues, in neither vehicle condition, or on DHT exposure (see Supplementary Table S1).

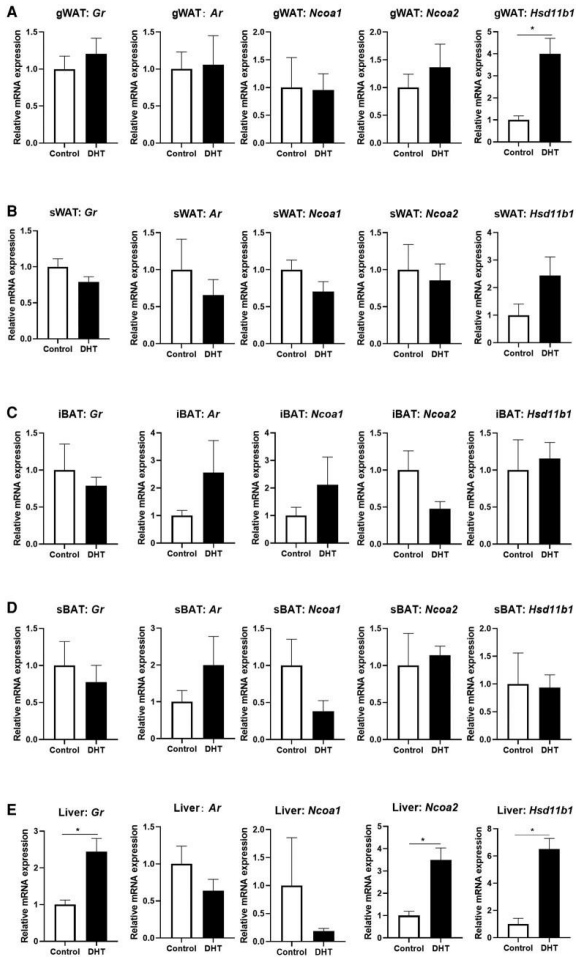

Fig. 1 Effect of dihydrotestosterone (DHT) treatment on the expression of the glucocorticoid receptor (GR) and GR-related signaling factors. The messenger RNA (mRNA) expression of Gr, Ar, Ncoa1, Ncoa2, and Hsd11b1 in A, gWAT; B, sWAT; C, iBAT; D, sBAT; and E, liver. Data are shown as mean ± SEM. N = 5 for the control group and N = 6 for the DHT group. Statistical significance is calculated using unpaired t test. *P less than .05 vs control.

Glucocorticoid Receptor Antagonism Does Not Influence Dihydrotestosterone-induced Features in the Ovary

We first confirmed the specificity of our GR antagonist CORT125134. Pretreatment with CORT125134 did not influence progesterone-induced PR signaling and DHT-induced AR signaling in human HEK293T cells, while GR signaling was potently antagonized with a half maximal inhibitory concentration of 2.8 nM (Fig. 2A). To investigate if GR antagonism influences the DHT-induced symptoms, we administered the GR-specific antagonist both to control and DHT-treated mice in a preventive and therapeutic treatment regimen (Fig. 2B).

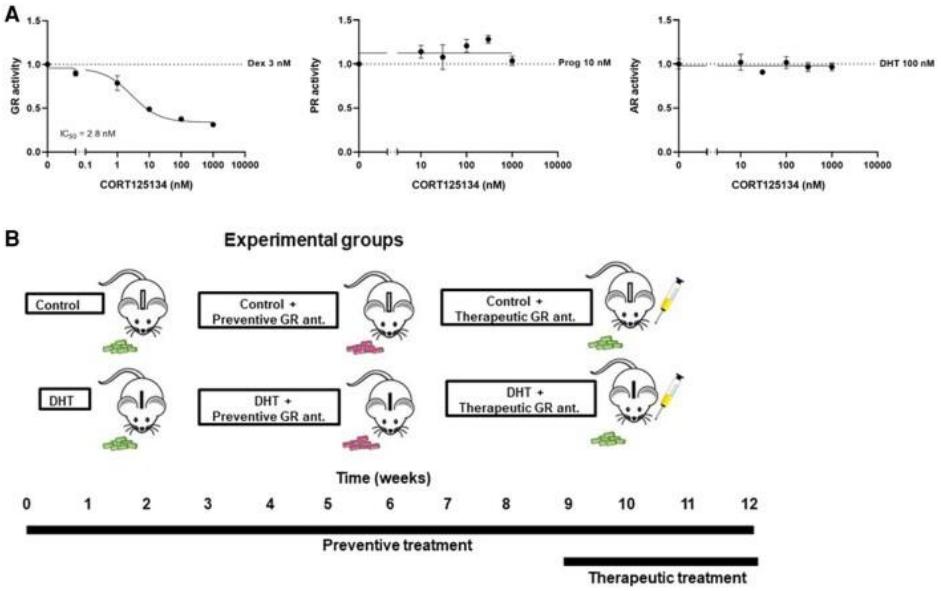

Fig. 2 Experimental design to determine the effects of selective glucocorticoid receptor (GR) antagonism in a mouse model of elevated dihydrotestosterone (DHT) exposure. A, The effect of GR antagonist CORT125134 on GR, progesterone receptor (PR), and androgen receptor (AR) signaling in human HEK293T cells. B, Female mice were exposed to control or DHT-silica implants for 12 weeks. Mice were treated with a GR antagonist for 12 weeks via diet supplementation ("preventive treatment") and during the last 3 weeks via oral gavage administration ("therapeutic treatment"). Body weight and composition were determined weekly; an oral glucose tolerance test was performed at week 11, and blood and tissues were collected after a 6-hour fast at the end of week 12.

We first confirmed the PCOS-like features by histological analysis of the ovary. As expected, DHT treatment induced an increase in percentage of unhealthy antral follicles, a decrease in the number of corpora lutea, and a decrease in granulosa layer thickness and an increase in theca layer thickness, as compared to control mice (Fig. 3A-E). GR antagonism, either in a preventive or therapeutic setting, did not seem to alter any of these DHT-induced features in the ovary (see Fig. 3A-E).

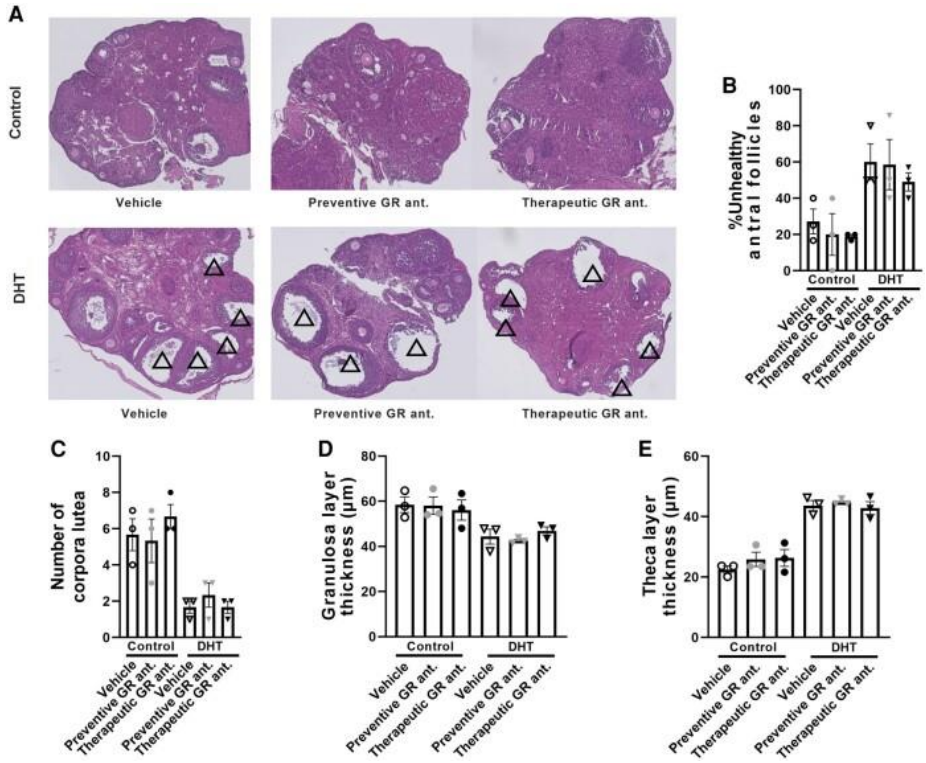

Fig. 3 Dihydrotestosterone (DHT) exposure induces polycystic ovary syndrome (PCOS)-associated features in the mouse ovary. A, Histological sections of the ovary of control mice and DHT-exposed mice on preventive or therapeutic treatment with a glucocorticoid receptor (GR) antagonist. The PCOS-related features in the ovary are defined by the presence of multiple arrested large antral follicles (indicated with triangles). B, Proportion of unhealthy large antral follicles per ovary, and C, the number of corpora lutea. N = 3 per group. D, Average thickness of granulosa cell layer and E, theca layer, confirming PCOS-related features. Multiple follicles were averaged per mouse, as one ovary could contain multiple follicles. N = 3 mice per group. Data are shown as mean ± SEM.

Preventive Glucocorticoid Receptor Antagonism Reduced Body Weight and Lean Mass Both in Control and Dihydrotestosterone-treated Mice

We next investigated body weight and composition in control and DHT mice on preventive or therapeutic treatment with a GR antagonist. We observed that DHT exposure increased body weight as compared to control mice, and that preventive GR antagonism decreased total body weight in control and DHT-treated mice (Fig. 4A). On initiation of therapeutic treatment with the GR antagonist, we observed a reduction of body weight both in the control mice and the DHT-treated mice, although the effect appeared stronger in control than DHT-treated mice (Fig. 4B). When evaluating lean mass, we observed a significant increase in DHT-treated mice compared to control mice (Fig. 4C).

Preventive treatment with the GR antagonist resulted in a decrease in lean mass both in control and DHT-treated mice (see Fig. 4C), while therapeutic GR antagonism reduced lean mass in control mice only (Fig. 4D). Both preventive and therapeutic treatment resulted in significant reduction in fat mass in control mice (Fig. 4E and 4F). DHT exposure increased fat mass, which was not further affected by preventive or therapeutic GR antagonism (see Fig. 4E and 4F).

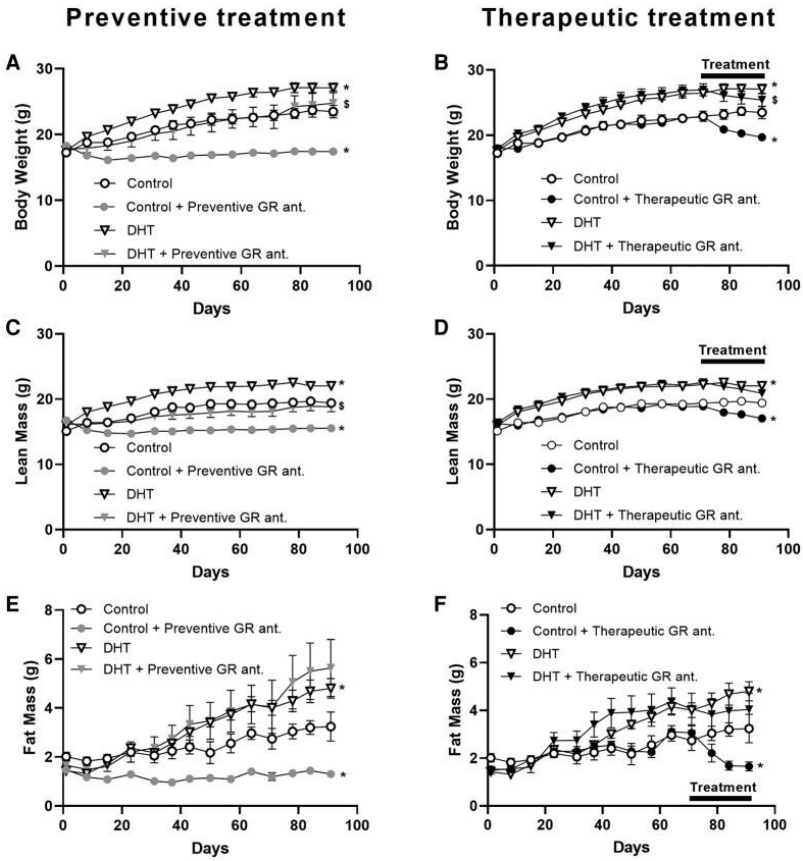

Fig. 4 The effect of preventive and therapeutic glucocorticoid receptor (GR) antagonism on body weight, lean mass, and fat mass of control and dihydrotestosterone (DHT)-exposed mice. A and B, Body weight; C and D, lean mass; and E and F, fat mass. Data are shown as mean ± SEM. N = 5/6 per group. The control and DHT groups were plotted both in the preventive and the therapeutic graphs for clarity. Statistical significance was calculated using a linear mixed-model analysis with Bonferroni multiple comparisons. *P less than .05 vs control, $P less than .05 vs DHT.

When evaluating the wet weight of different metabolic tissues, we found that DHT increased the weight of iBAT, sBAT, gWAT, and sWAT as compared to control mice, but that neither preventive nor therapeutic GR antagonism further influenced this (significant main effect of DHT but not of treatment; no statistical

interaction; Fig. 5A-5D). In line with these findings, histological analysis of iBAT and gWAT showed increased iBAT lipid content and average adipocyte cell size in gWAT on DHT treatment, but no further effect by GR antagonism (significant main effects of DHT but not of treatment; no statistical interaction; Fig. 5E-5H).

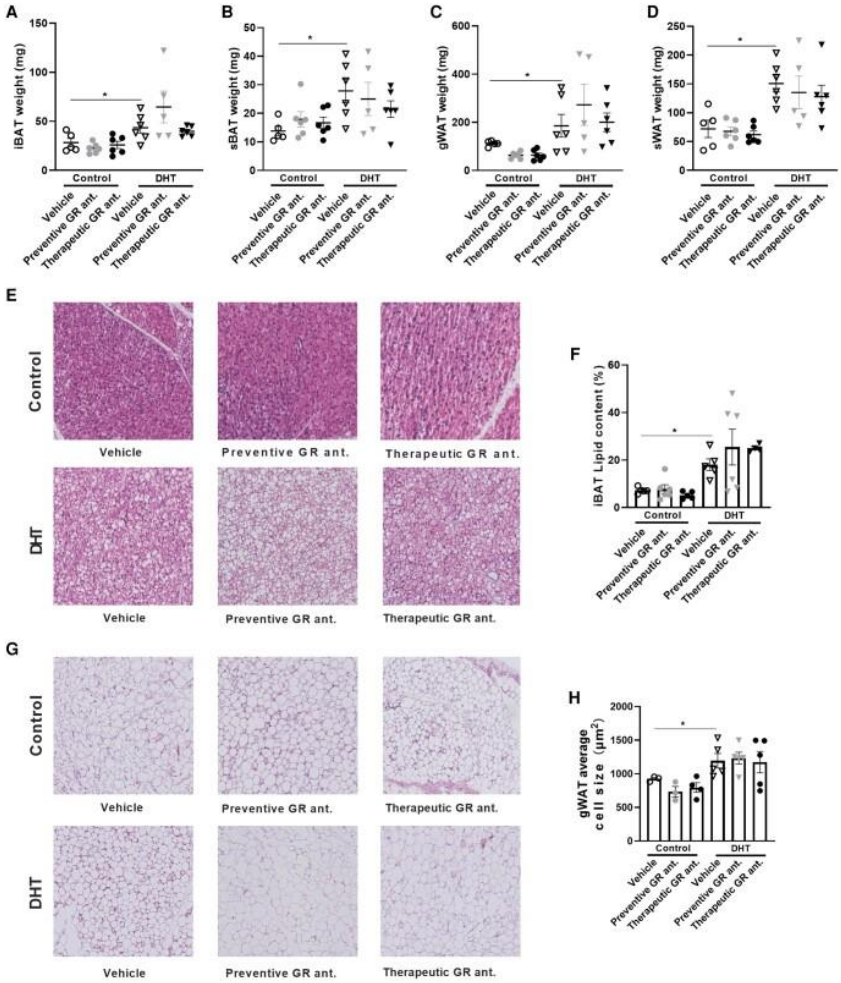

Fig. 5 The effect of preventive and therapeutic glucocorticoid receptor (GR) antagonism on adipose tissue weight and lipid content of control and dihydrotestosterone (DHT)-exposed mice. The effect of preventive or therapeutic GR antagonism in control mice and DHT-exposed mice on A, iBAT weight; B, sBAT weight; C, gWAT weight; and D, sWAT weight. E, Representative histological images of hematoxylin and eosin–stained iBAT. F, iBAT lipid content. G, Representative histological images of hematoxylin and eosin–stained gWAT. H, Average adipocyte cell size. A to D, N = 5/6 per group; E and F, N = 4/5/6 per group; G and H, N = 3/4/5 per group. Statistical significance is calculated using 2-way analysis of variance followed by least significant difference post hoc test. *P less than .05 vs control.

Preventive Glucocorticoid Receptor Antagonism Alleviates Hyperglycemia and Improves Glucose Tolerance in Dihydrotestosterone-exposed Mice

Analysis of plasma biochemistry showed that DHT exposure caused an increase in plasma insulin, glucose, and total cholesterol (main effects of DHT: $P < .0006$, $P < .003$, and $P = .051$, respectively), but no significant effect on triglyceride levels (Fig. 6A-6D). In control mice, we found that therapeutic GR antagonism significantly increased plasma insulin (see Fig. 6A). We observed that preventive treatment alleviated the DHT-induced increase in glucose (interaction effect $P = .03$; see Fig. 6B). Therapeutic GR antagonism increased plasma total cholesterol levels in control mice, with no clear effects in DHT-treated mice (see Fig. 6B). We performed an oral glucose tolerance test (OGTT) experiment in week 11 of the study to investigate glucose tolerance. DHT-treated mice exhibited a significant increase in plasma glucose levels as compared to control mice, both at 15 minutes and 120 minutes post glucose-bolus and on total glucose exposure (area under the curve) (Fig. 6E-6G). In DHT-treated mice, preventive GR antagonism resulted in a reduction in plasma glucose levels at 15 minutes (see Fig. 6E), while therapeutic treatment did not decrease plasma glucose level in control mice nor DHT-treated mice (see Fig. 6F). Preventive treatment with the GR antagonist readily lowered total glucose exposure in DHT-exposed mice (see Fig. 6G).

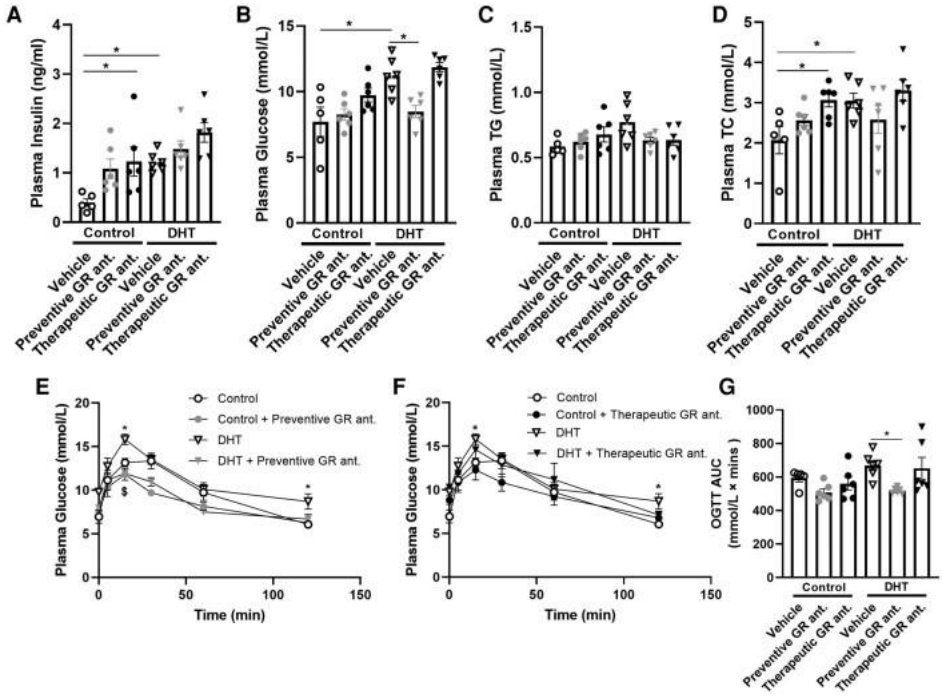

Fig. 6 The effect of preventive and therapeutic glucocorticoid receptor (GR) antagonism on biochemistry and glucose tolerance of control and dihydrotestosterone (DHT)-exposed mice. Plasma levels after a 6-hour fast of A, insulin; B, glucose; C, triglycerides (TG); and D, total cholesterol (TC). E and F, Plasma glucose levels during an oral glucose tolerance test (OGTT) performed after a 6-hour fast in week 11. G, Area under the curve of glucose during OGTT. N = 5/6 per group. Statistical significance is calculated using 2-way analysis of variance followed by least significant difference post hoc test. *P less than .05 vs control, $P less than .05 vs DHT.

Glucocorticoid Receptor Antagonism Increased Triglyceride-derived Fatty Acid Uptake in Adipose Tissues of Control Mice, Which Was Blunted in Dihydrotestosterone-exposed Mice

Both DHT and GR antagonist treatment affected triglyceride-derived fatty acid uptake by different tissues (Supplementary Table S2). We found in control mice that therapeutic GR antagonism seemed to increase [^{3}H] activity in gWAT and significantly increased uptake in sWAT (Fig. 7A and 7B). Preventive treatment increased [^{3}H] activity in iBAT and nonsignificantly in sBAT (Fig. 7C and 7D). DHT exposure in itself reduced [^{3}H] activity in gWAT, sWAT, iBAT, sBAT, and liver as compared to control (significant main effects of DHT for all tissues; see Fig. 7A-7E). In DHT-treated mice, we observed that the effect of both preventive and therapeutic treatment with the GR antagonist on triglyceride-derived fatty acid uptake was completely blunted (see Fig. 7A-7C). When evaluating the uptake of [^{14}C]-labeled deoxyglucose, DHT exposure reduced the uptake in sWAT and iBAT (significant main effects of DHT; Fig. 7G and 7H). We did not observe any other major effects on [^{14}C] activity of GR antagonism (Fig. 7F-7J).

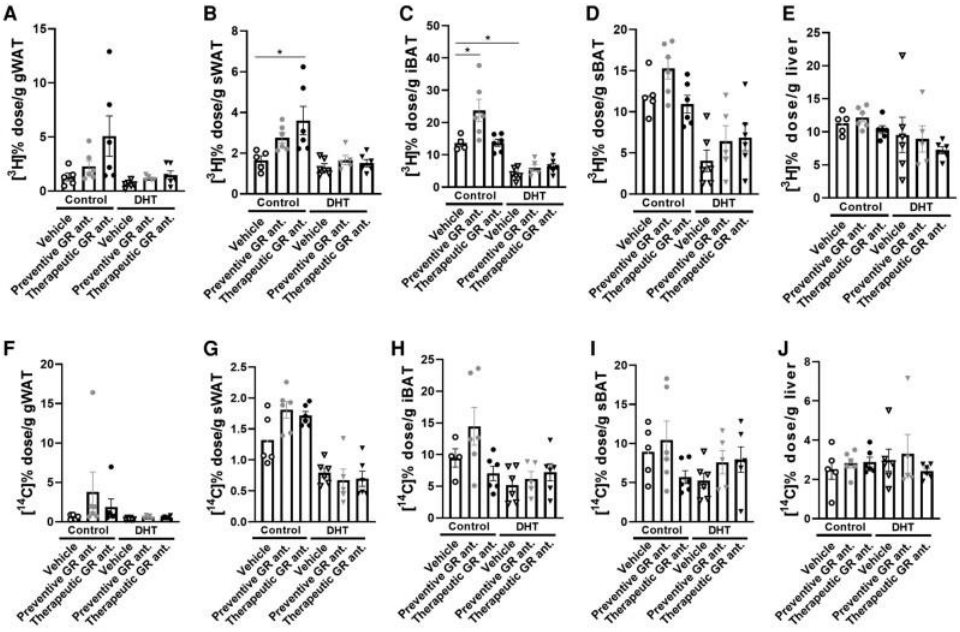

Fig. 7 The effect of preventive and therapeutic glucocorticoid receptor (GR) antagonism on uptake of triglyceride-derived [3H]-labeled fatty acids and [14C]-labeled deoxyglucose in control and DHT-exposed mice. [3H] activity in A, gWAT; B, sWAT; C, iBAT; D, sBAT; and E, liver. [14C] activity in F, gWAT; G, sWAT; H, iBAT; I, sBAT; and J, liver. N = 5/6 per group. Statistical significance is calculated using 2-way way analysis of variance followed by least significant difference post hoc test. *P less than .05 vs control.

4

DISCUSSION

Women with PCOS have a higher risk of developing obesity and other metabolic disorders, and women with obesity show increased prevalence of PCOS [39, 40]. Many of the complications in PCOS are believed to be driven by hyperandrogenism, and previous studies show that a global loss of AR signaling fully protects female mice from the development of PCOS-like metabolic traits on excess androgen exposure [41]. However, many of the clinical features in PCOS are also characteristics of metabolic disorders driven by deregulated glucocorticoid signaling (eg, in Cushing syndrome) [42-44]. In addition to this, we previously observed (functional) crosstalk between glucocorticoid and androgen signaling [11] and we therefore investigated a possible role of GR signaling in (DHT-induced) metabolic features of PCOS capitalizing on the availability of the selective GR antagonist CORT125134 [33]. We confirmed the PCOS-associated features in our model of DHT exposure, including altered ovarian morphology with an increased number of unhealthy antral follicles and a decrease in granulosa cell layer thickness [45, 46]. GR antagonism did not influence any of these DHT-induced effects in the ovary, although our

histological analysis of the ovary was underpowered, prohibiting any formal conclusion. It is important to note that while our model recapitulated many features of PCOS/prolonged DHT exposure, DHT-exposed mice did not develop liver steatosis (Supplementary Fig. S1), in contrast to previously reported data using a similar model of DHT exposure that was accompanied by overt liver steatosis [47]. Of note, our observation was based on biochemical measurements of triglycerides and total cholesterol, while histological determination of steatosis was not performed.

We observed that, after prolonged DHT exposure, the expression of many factors related to GR signaling were changed at the mRNA level. This includes hepatic expression of *Nr3c1* (coding for GR), *Hsd11b1*, and *Ncoa2*, which were all upregulated in DHT-treated mice. This shows that different aspects of glucocorticoid signaling, from receptor and coregulator expression to prereceptor metabolism of the ligand, are regulated by androgens. This is consistent with the previous findings that DHT treatment enhanced local concentrations of corticosterone in the liver [48]. Of note, we did not measure (hepatic) corticosterone levels directly in this study. In other tissues, we found little evidence of altered GR signaling in DHT-exposed mice, with the exception of gWAT and possibly sWAT, in which expression of *Hsd11b1* was increased, potentially resulting in increased local glucocorticoid (re)activation. The regulation of glucocorticoid-related factors like *Hsd11b1* by androgens could also play a role in metabolic physiology in women with PCOS, and likely results in elevated glucocorticoid turnover in tissues like WAT and liver. This could in turn (partially) contribute to the metabolic features observed in PCOS, including deregulated glucose metabolism. It is important to note that our mouse model of PCOS results in supraphysiological exposure of DHT, and the regulation of *Hsd11b1* expression and activity under more physiological androgen exposure requires further investigation. It was previously found that *HSD11B1* expression was elevated in ovaries of women with PCOS, as compared to non-PCOS patients [49]. This, in addition to the increased in hepatic and adipose expression of *Hsd11b1* observed in our mouse study, could contribute to elevated cortisol/corticosterone exposure in patients with PCOS. Indeed, patients with PCOS showed increased hair cortisol concentrations as compared to healthy women [50], possibly mediated via androgen regulation of *HSD11B1* expression.

Given the deregulated glucocorticoid signaling in the liver, we evaluated whether GR antagonism can prevent or alleviate DHT-induced metabolic features. For this we used a GR-specific antagonist, either administered continuously via diet-supplementation for a total period of 12 weeks (during the whole period of DHT treatment), or administered daily via oral gavage in mice with established DHT-induced metabolic symptoms. We found that preventive treatment with a GR antagonist alleviated the glucose levels during an OGTT, with significantly lower glucose exposure as compared to vehicle-treated DHT-exposed animals. On the other hand, we did not observe such improvement on

therapeutic treatment with the GR antagonist. The differential effects of preventive and therapeutic GR antagonism may suggest that GR signaling is involved in the development of metabolic symptoms on DHT exposure, but that this does not necessarily provide a suitable therapeutic target when symptoms are fully established. We cannot exclude that a longer treatment duration with a GR antagonist may provide metabolic benefit. Importantly, the mode of administration of the GR antagonist was different between the preventive and therapeutic treatment groups, disallowing direct comparison as this may have resulted in differences in bioavailability and kinetics between the two treatment groups. We were unable to directly determine drug concentrations in plasma or target tissues, and it is therefore uncertain whether we approximated steady-state levels in the animals that received the compound via oral gavage, and if so, at what level.

For many other metabolic features that were observed in DHT-treated animals, neither preventive nor therapeutic GR antagonism showed improvement. We observed that DHT exposure induced lipid accumulation in adipose tissues, in line with previous findings in PCOS mouse models [46, 51, 52]. However, GR antagonism did not alter adipose tissue weight and lipid accumulation in DHT-treated mice. In other models for metabolic disease, benefits on metabolic health were observed on treatment with GR antagonists [53-57]. It thus seems that many activities of GR antagonists are lost in PCOS, possibly due to elevated androgen exposure that potentially takes over (part of) glucocorticoid effects. We indeed found that the uptake of triglyceride-derived fatty acids in adipose tissues was readily induced on GR antagonism in control mice, but that this was completely blunted in DHT-treated mice. Previous transcriptome studies showed that the large majority of GR-responsive genes are also regulated by AR [58], suggesting that GR-responsive transcripts can also be AR responsive. Furthermore, enhanced 5α-reductase activity has been reported in women with PCOS, leading to glucocorticoid degradation [59-61], and this may also result in abolished responsiveness to GR antagonists.

In summary, we found that GR antagonism improved glucose metabolism, but not other metabolic features, in a mouse model of elevated androgen exposure. The effects of GR antagonism on tissue uptake of triglyceride-derived fatty acids were lost in DHT-treated mice, showing that responsiveness to a GR antagonist may strongly depend on disease stage and context.

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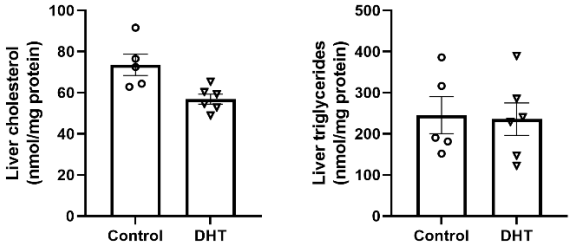
SUPPLEMENT



Supplementary Fig. S1 DHT-exposed mice did not develop liver steatosis. Total cholesterol and triglycerides were determined in the liver. N = 5 for the control group and N = 6 for the DHT group. Statistical significance is calculated using unpaired t test. *P less than .05 vs control.

CT values (related to Figure 1)				
Tissue	Gene	Group	Avg	SD
Liver	*Gapdh*	*Vehicle*	17.5	0.1
		DHT	17.7	0.3
	Gr	*Vehicle*	25.5	0.4
		DHT	24.4	0.5
	Hsd11b1	*Vehicle*	24.8	2.6
		DHT	21.3	0.5
	Hsd11b2	*Vehicle*	ND	
		DHT	ND	
	Ncoa1	*Vehicle*	32.5	2.6
		DHT	33.1	0.8
	Ncoa2	*Vehicle*	29.1	0.4
		DHT	27.3	0.6
	Ar	*Vehicle*	25	0.8
		DHT	25.9	0.5
iBAT	*Gapdh*	*Vehicle*	17	0.7
		DHT	17.3	0.7
	Gr	*Vehicle*	30.2	0.8
		DHT	29.9	1
	Hsd11b1	*Vehicle*	29.9	1.2

		DHT	28.8	1.2
	Hsd11b2	*Vehicle*	ND	
		DHT	ND	
	Ncoa1	*Vehicle*	25	0.8
		DHT	25.9	0.5
	Ncoa2	*Vehicle*	33	0.7
		DHT	33.7	1.8
	Ar	*Vehicle*	33.5	0.4
		DHT	32.7	1.5
sBAT	*Gapdh*	*Vehicle*	21.1	0.9
		DHT	19.7	2.3
	Gr	*Vehicle*	30.5	2.3
		DHT	28.7	1.5
	Hsd11b1	*Vehicle*	30.8	2.7
		DHT	28.5	2
	Hsd11b2	*Vehicle*	ND	
		DHT	ND	
	Ncoa1	*Vehicle*	31.8	1
		DHT	31.9	1.5
	Ncoa2	*Vehicle*	33.5	1.6
		DHT	31.5	2.3
	Ar	*Vehicle*	33.1	1.8
		DHT	30.6	1.4
gWAT	*Gapdh*	*Vehicle*	21.9	1.2
		DHT	22	1.5
	Gr	*Vehicle*	29.8	0.5
		DHT	29.6	0.7
	Hsd11b1	*Vehicle*	31.8	1.3
		DHT	29.9	0.9
	Hsd11b2	*Vehicle*	ND	
		DHT	ND	
	Ncoa1	*Vehicle*	33.5	1.6
		DHT	33.3	1.1
	Ncoa2	*Vehicle*	33.5	1
		DHT	33.3	1.3

	Ar	*Vehicle*	28.9	0.6
		DHT	29.8	1.3
sWAT	*Gapdh*	*Vehicle*	23.1	2.1
		DHT	23.2	0.8
	Gr	*Vehicle*	30.5	2.1
		DHT	30.9	0.7
	Hsd11b1	*Vehicle*	32.6	1.9
		DHT	31.1	6
	Hsd11b2	*Vehicle*	ND	
		DHT	ND	
	Ncoa1	*Vehicle*	31	2.3
		DHT	32.8	1.3
	Ncoa2	*Vehicle*	34.5	1
		DHT	35	0.6
	Ar	*Vehicle*	32	0.9
		DHT	31.7	0.9

Supplementary Table S1 The CT values of control group and DHT treatment on the expression of the glucocorticoid receptor (GR) and GR-related signaling factors.

Figure 4A	**BW preventive**	**P-value**
	Interaction	**<0,0001**
	Time	**<0,0001**
	Treatment+DHT	**<0,0001**
Figure 4B	**BW therapeutic**	**P-value**
	Interaction	**<0,0001**
	Time	**<0,0001**
	Treatment+DHT	**<0,0001**
Figure 4C	**Lean Mass preventive**	**P-value**
	Interaction	**<0,0001**
	Time	**<0,0001**
	Treatment+DHT	**<0,0001**
Figure 4D	**Lean Mass therapeutic**	**P-value**
	Interaction	**<0,0001**
	Time	**<0,0001**
	Treatment+DHT	**<0,0001**
Figure 4E	**Fat Mass preventive**	**P-value**
	Interaction	**0.0005**
	Time	**<0,0001**
	Treatment+DHT	**<0,0001**
Figure 4F	**Fat Mass therapeutic**	**P-value**
	Interaction	**<0,0001**
	Time	**<0,0001**
	Treatment+DHT	**<0,0001**
Figure 5A	**iBAT weight**	**P-value**
	Interaction	0.0827
	DHT	0.0002
	Treatment	0.2778
Figure 5B	**sBAT weight**	**P-value**
	Interaction	0.381
	DHT	0.0043
	Treatment	0.766
Figure 5C	**gWAT weight**	**P-value**
	Interaction	0.2738
	DHT	0.0002
	Treatment	0.6729

Figure 5D	**sWAT weight**	**P-value**
	Interaction	0.9135
	DHT	<0,0001
	Treatment	0.5997
Figure 5F	**iBAT lipid content**	**P-value**
	Interaction	0.5124
	DHT	<0,0001
	Treatment	0.5828
Figure 5H	**gWAT average cell size**	**P-value**
	Interaction	0.6263
	DHT	0.0007
	Treatment	0.7577
Figure 6A	**Plasma insulin**	**P-value**
	Interaction	0.5559
	DHT	0.0006
	Treatment	0.0031
Figure 6B	**Plasma glucose**	**P-value**
	Interaction	0.0307
	DHT	0.0003
	Treatment	0.001
Figure 6C	**Plasma TG**	**P-value**
	Interaction	0.04
	DHT	0.1555
	Treatment	0.5115
Figure 6D	**Plasma TC**	**P-value**
	Interaction	0.1537
	DHT	0.0513
	Treatment	0.0253
Figure 6E	**Plasma glucose OGTT**	**P-value**
	Interaction	0.1235
	Time	<0,0001
	Treatment+DHT	<0,0001
Figure 6F	**Plasma glucose OGTT**	**P-value**
	Interaction	0.7696
	Time	<0,0001
	Treatment+DHT	0.0006

Figure 6G	**OGTT AUC**	**P-value**
	Interaction	0.5023
	DHT	0.0581
	Treatment	0.0081
Figure 7A	**3H gWAT**	**P-value**
	Interaction	0.205
	DHT	0.0188
	Treatment	0.0381
Figure 7B	**3H sWAT**	**P-value**
	Interaction	0.0603
	DHT	0.0004
	Treatment	0.0175
Figure 7C	**3H iBAT**	**P-value**
	Interaction	0.0159
	DHT	<0,0001
	Treatment	0.0074
Figure 7D	**3H sBAT**	**P-value**
	Interaction	0.2016
	DHT	<0,0001
	Treatment	0.1495
Figure 7E	**3H Liver**	**P-value**
	Interaction	0.8557
	DHT	0.0312
	Treatment	0.3919
Figure 7F	**14C gWAT**	**P-value**
	Interaction	0.4765
	DHT	0.1076
	Treatment	0.3822
Figure 7G	**14C sWAT**	**P-value**
	Interaction	0.0641
	DHT	<0,0001
	Treatment	0.3452
Figure 7H	**14C iBAT**	**P-value**
	Interaction	0.0609
	DHT	0.0078
	Treatment	0.1427

Figure 7I	**14C sBAT**	**P-value**
	Interaction	0.1387
	DHT	0.2774
	Treatment	0.337
Figure 7J	**14C Liver**	**P-value**
	Interaction	0.5408
	DHT	0.6834
	Treatment	0.6841

Supplementary Table S2 The p value of preventive and therapeutic glucocorticoid receptor (GR) antagonism of control and dihydrotestosterone (DHT)-exposed mice outcomes.

5

General discussion and perspective

GENERAL DISCUSSION

This thesis focused on the complex role of glucocorticoid signaling in metabolic diseases, emphasizing novel insights into sex-specific responses, circadian influences, and therapeutic interventions. Glucocorticoid signaling plays a pivotal role in the regulation of various physiological processes. However, long lasting or excess exposure to glucocorticoids may lead to a range of metabolic side effects including hyperglycemia, insulin resistance, obesity, muscle loss and osteoporosis (1-4). In fact, very recent data indicate a degree of hypercortisolemia in over 20% of difficult-to-treat type 2 diabetes patients (5). Understanding the role of the glucocorticoid receptor (GR) in metabolic disease is also essential for improving the management of patients receiving chronic glucocorticoid therapy, as these individuals are at increased risk for developing metabolic complications. Therefore, elucidating the mechanisms by which the GR regulates metabolic homeostasis, and how these mechanisms are altered in various disease states, remains a crucial area of research in endocrinology.

Summary of the findings

In **chapter 2**, we investigated the effects of treatment with the synthetic glucocorticoid betamethasone and excess exposure to the endogenous glucocorticoid corticosterone on muscle function and atrophy in both male and female mice. We directly compared male and female mice, allowing us to identify sex differences in the glucocorticoid response in muscle. Corticosterone treatment led to reduced grip strength specifically in female mice, while muscle mass being decreased in both sexes. By performing RNA-sequencing, we observed that male mice exhibited more pronounced transcriptional responses to corticosterone as compared to female mice. We thus found stronger functional consequences in female mice, but more transcriptomic effects in male mice. The sex-difference following a synthetic glucocorticoid treatment regimen were somewhat different: we found that betamethasone administration reduced grip strength in both sexes, but that female mice were more sensitive to glucocorticoid-induced muscle atrophy. In an attempt to understand the sexually dimorphic glucocorticoid effects, we addressed the contribution of androgen signaling in male mice and found that part of the glucocorticoid responses in skeletal muscle were influenced by androgen deprivation. This finding did not suggest that glucocorticoid-induced muscle atrophy is completely androgen-dependent, as both sexes experienced atrophy and androgen signaling might only partly contribute to the differences.

In addition to sex differences in the glucocorticoid response, we found that the time of glucocorticoid administration influences its adverse effects. We describe the comparison of morning versus evening betamethasone administration in **chapter 3**. Morning (out of phase) betamethasone treatment significantly reduced insulin sensitivity and caused more potent effects on glucose

metabolism, compared to evening administration. We additionally found that the outcome of glucocorticoid treatment was dependent on the time of measurement. In general, circadian rhythm should thus be taken into account in research on glucocorticoids.

In **chapter 4**, we explored the role of glucocorticoid signaling in mouse model of androgen-induced polycystic ovary syndrome (PCOS). We observed that treatment with a GR antagonist only had limited effects for most of the metabolic features associated with PCOS/elevated androgen exposure. Nevertheless, we found that GR antagonism during the development of metabolic symptoms can result in improved glucose metabolism, with no strong effects on other DHT-exposed features including lipid metabolism.

Sex-Specific Responses to Glucocorticoid Treatment and Therapeutic Implications

Across all chapters, we observed consistent evidence of glucocorticoid-induced muscle atrophy, hyperglycemia, and insulin resistance. Given the widespread metabolic effects of glucocorticoid signaling, it is critically important to determine how these effects may differ between sexes, particularly those of muscle atrophy and metabolic outcomes described in **chapter 2**. We generally found that glucocorticoid treatment promotes muscle atrophy in male and female mice, but some glucocorticoid effects on muscle were sex-dependent. Male mice displayed a more extensive transcriptional response, including the upregulation of key atrogenes such as *Klf15*, *MurF-1* (*Trim63*), and *atrogin-1* (*Fbxo32*). Interestingly, despite the stronger atrophic gene response in males, functional impairment including grip strength was more pronounced in females. This discrepancy may indicate the involvement of additional, possibly non-genomic, pathways in the sex-specific responses to glucocorticoids. Crosstalk of glucocorticoid signaling with androgen signaling was previously described in the liver and other tissues (6), and may thus also play an important role in skeletal muscle function. The dominant androgen testosterone plays a dual role in muscle metabolism. On the one hand, it has anabolic effects, promoting muscle protein synthesis (7,8), but it also mitigates glucocorticoid-induced muscle atrophy. Androgens may not be able to fully prevent the activation of catabolic pathways including the upregulation of *Murf-1* and *atrogin-1* under conditions of high glucocorticoid exposure (9). In addition, prolonged dexamethasone treatment leads to significant decrease of androgen receptor (AR) mRNA expression in skeletal muscle and plasma androgen levels (10), which potentially further reduces the inhibitory effect of androgens on glucocorticoid-induced muscle atrophy.

To further explore the role of androgens in glucocorticoid-induced muscle atrophy, we chemically castrated male mice using degarelix, a GnRH antagonist that has several advantages over other castration procedures. GnRH agonists

initially evoke a surge of LH and FSH (and thus testosterone) before leading to a downregulation of GnRH receptors. Degarelix directly and irreversibly blocks the GnRH receptors without this initial surge (11,12). Androgen depletion alone already led to significant muscle atrophy and reduced grip strength. When combined with betamethasone treatment, an additive effect on muscle atrophy was observed, in particular on the gastrocnemius, EDL, and TA muscles. This points toward a protective role of androgens in muscle maintenance through their actions, which are likely counteractive against the glucocorticoid-driven catabolic processes. The activation of the Akt/mTOR pathway critical for muscle protein synthesis can be reduced with androgen deprivation (7). Synthetic glucocorticoids further downregulate this pathway, leading to more pronounced anabolic processes (13). These muscle changes were accompanied by a shift in the composition of fiber types, increasing the proportion of type 2A fibers at the expense of type 2B fibers. This shift is consistent with previous findings that glucocorticoid treatment can drive a transition from fast-twitch glycolytic fibers to more oxidative fiber types, potentially contributing to the observed reductions in muscle strength (14). This fiber type shift upon betamethasone treatment was less pronounced in female mice, but it should be noted that females have more slow-twitch (type 1) fibers than males (15). These fibers are oxidative and more resistant to fatigue and are generally less prone to atrophy under stress. Under prolonged glucocorticoid treatment regimens, the oxidative capacity of such slow-twitch fibers could become compromised, leading to muscle wasting (16).

Given the crucial role of androgens in muscle function and other metabolic processes, we also investigated the interplay between glucocorticoid and androgen signaling in the metabolic features of PCOS. Many of the clinical features of PCOS including insulin resistance and adiposity overlap with those observed in conditions of excess glucocorticoid exposure such as Cushing's syndrome (17,18). This overlap suggests that GR signaling may be an important modulator of PCOS-associated metabolic symptoms. The availability of a selective GR antagonist without affinity for the androgen and progesterone receptors allowed us to test this hypothesis (19). In **chapter 4**, we demonstrate that prolonged DHT exposure upregulates GR signaling machinery, particularly in key metabolic tissues like the liver and adipose tissue. Specifically, DHT increased the expression of GR mRNA and the enzyme 11β-HSD1 in the liver and gWAT. This is supported by earlier studies indicating that androgen signaling can modulate glucocorticoid metabolism, with DHT enhancing local glucocorticoid concentrations by activating 11β-HSD1 (6). This suggests that in PCOS, androgens may enhance local glucocorticoid activation, thereby amplifying glucocorticoid-mediated metabolic disturbances. These findings are consistent with the previous report showing that androgen exposure enhances the concentrations of corticosterone in the liver and adipose tissues (20). The increased expression of 11β-HSD1 may further stimulate the GR signaling, exacerbating insulin resistance and fat deposition. This mechanistic insight highlights the androgen-glucocorticoid interplay in the pathogenesis of PCOS

and underlines the potential of targeting GR signaling as a therapeutic strategy (21).

The estrogen receptor (ER) plays a critical role in maintaining metabolic and reproductive functions particularly in females. Estrogens typically exert protective effects against metabolic dysfunction through the regulation of glucose homeostasis by enhancing insulin sensitivity in the adipose tissue and skeletal muscle (22). Despite the potent effect of estrogens on maintenance of muscle mass, females are more sensitive to disuse atrophy (as a consequence of muscle inactivity) (23,24). Estrogen supplementation or activation of ER was shown to mitigate atrophy in male mice (25,26) but not in female rats (27). Besides, the lower levels of androgens in females could render them more sensitive to glucocorticoid-induced atrophy as the protective effects of androgens are attenuated. This discrepancy indicates that aromatase could play a role in maintaining muscle mass and reducing atrophy. Studies performed in ArKO mice reveal that muscle mass is significantly reduced and mice are more vulnerable to muscular atrophy in the absence of estrogens (28). This highlights the importance of estrogen signaling in male muscle maintenance. Moreover, it is likely that testosterone acts to maintain muscle protein synthesis and muscle function through its conversion into estradiol via aromatization and subsequent activation of ERs (29,30). Estrogen signaling can alleviate the degradation of type 1 fibers (31,32), although it may not be sufficient to fully prevent glucocorticoid-induced atrophy. In contrast, androgens have more pronounced effect on type 2 fibers, promoting muscle hypertrophy and strength (33). In PCOS, this could shift the balance due to the excess of androgen toward dampening beneficial effects from ER signaling and further promoting metabolic dysregulation via the dominant AR and GR pathways (34).

Therapeutic strategies targeting glucocorticoid signaling may hold potential in conditions like PCOS, given the upregulation of GR machinery by excess androgen exposure. In **chapter 4**, we show that preventive GR antagonism (during disease progression) improves glucose metabolism in DHT-exposed mice, indicating that early intervention by GR blockade can mitigate the metabolic consequences of androgen excess. Similarly, GR antagonism with RU486 was able to mitigate high-fructose-induced insulin resistance and lipid accumulation both in adipose tissues and liver (35). However, GR antagonism does not always result in improved metabolic outcomes, and despite the clear advantage of improving insulin sensitivity, the overall metabolic benefit of GR antagonism may be highly dependent on the disease model and the timing of intervention (36). This is illustrated by the fact that while preventive GR antagonism showed clear benefits in our study, GR antagonism that started after the onset of metabolic dysfunction did not yield similar improvements. It is possible that GR signaling mainly plays a role in the development of metabolic disturbances, or that compensatory pathways such as enhanced androgen signaling may dominate the metabolic dysregulation. One study found that androgens can modulate GR activity in adipose tissue and the liver, enhancing

insulin resistance and exacerbating fat accumulation in male mice (37). This suggests that androgen signaling can aggravate GR-mediated metabolic dysfunction. Moreover, once metabolic consequences are fully established, the compensatory mechanisms including heightened androgen signaling and mitochondrial dysfunction might minimize the influence of GR signaling (38,39). Thus, early intervention targeting GR signaling might be more effective in preventing the progression of hyperandrogenism-driven metabolic complications (39).

We observed a differential impact of GR antagonism on body composition and lipid metabolism. Compared with control mice, DHT exposure increased fat mass as well as lean mass. Preventive GR antagonism significantly decreased body weight and lean mass in mice by comparison with vehicle or DHT treatment alone. This was distinct from other models of metabolic disease in which GR antagonism resulted in reduced fat mass and improved lipid metabolism (40). However, in DHT-treated mice we found that neither preventive nor therapeutic GR antagonism altered the increased fat mass or adipose tissue weight. It is known that AR agonism can amplify GR transcriptional responses in white and brown adipose tissue, while AR antagonism attenuates these effects (6). This suggests that the role of GR antagonism on adiposity is blunted under conditions of hyperandrogenism and thus points out a complex interplay of glucocorticoid-androgen signaling in regulating adipose tissue function. Interestingly, GR antagonism enhanced triglyceride-derived fatty acid uptake in adipose tissues of control mice but not in DHT-treated mice. This blunted response in the DHT-exposed mice might be a consequence of the excessively potent influence of the androgen signaling that overrides the metabolic consequences of GR antagonism. Indeed, GR-responsive genes were previously reported to be subject to regulation by AR, and such a shared regulatory network might underlie why GR antagonism fails to correct disturbances in lipid metabolism in the context of elevated androgen levels (6).

Circadian Rhythm and Glucocorticoid Treatment

In **chapter 3**, we explored the effect of glucocorticoid administration at different times of the day. Our study provides insights into how out-of-phase administration of the synthetic glucocorticoid betamethasone, i.e. administered during the inactive phase when endogenous glucocorticoid levels are low leads to more pronounced disturbances in glucose metabolism compared to in-phase treatment (when glucocorticoid administrations aligns with the natural peak of endogenous glucocorticoids).

We found that out-of-phase betamethasone treatment significantly impairs glucose metabolism. The glucocorticoid-mediated suppression of insulin signaling pathways was reflected by reduced insulin sensitivity and impaired glucose uptake. In humans, circadian misalignment, whether due to disrupted

sleep patterns or shift work, has been associated with insulin resistance and impaired glucose tolerance (41,42), but it is unclear if disturbed glucocorticoid rhythm is involved in these effects. Similarly, circadian misalignment (12-hour behavioral cycle inversion) such as shift work impairs glucose tolerance via separate mechanisms related to insulin secretion and insulin sensitivity in human (43). Our study extends these findings by exploring that both in-phase and out-of-phase synthetic glucocorticoid administration, providing a distinct insight of how exogenous glucocorticoids such as betamethasone disrupt glucose metabolism.

We observed higher plasma insulin levels (i.e. hyperinsulinemia) in response to out-of-phase treatment, reflecting the body's attempt to compensate for the reduced insulin sensitivity by increasing insulin release (which we confirmed by c-peptide measurements). However, the increase in insulin seemed insufficient to maintain normal glucose metabolism, leading to impaired glucose clearance. In addition, out-of-phase treatment increased glucose and triglyceride uptake patterns in some tissues such as gonadal white adipose tissue (gWAT) and liver. This suggests an adaptive response to hyperinsulinemia, but with long-term detrimental effects on glucose handling and insulin sensitivity. (44,45).

It is important to note that the time of measurement greatly influences the measured values for glucose metabolism and insulin levels. This is illustrated by our morning measurements (ZT7) that showed a sharp increase in insulin resistance following out-of-phase betamethasone treatment, while evening measurements (ZT15) revealed different patterns of glucose tolerance and insulin sensitivity. The decision to use different time points for oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) allows for a nuanced understanding of how betamethasone affects glucose metabolism differently at various points in the circadian cycle.

The rhythmic synchronization of glucocorticoid signaling generally allows the body to maintain better glucose homeostasis with relatively higher insulin sensitivity across metabolic tissues, limiting the extent of metabolic disruption (46,47). We found that in-phase (ZT10) betamethasone treatment generally induced less pronounced metabolic disturbances, as compared to out-of-phase administration. Similarly in humans, administrated hydrocortisone caused more potent metabolic effects including elevated glucose and insulin in the evening (when endogenous glucocorticoid levels are low in humans) rather than in the morning (48). In-phase hydrocortisone treatment still caused a reduction in insulin sensitivity, but this effect was milder compared to out-of-phase treatment, altogether in line with our study in mice.

Beyond glucose metabolism, betamethasone treatment also had potent effects on body composition. Our study showed that both in-phase and out-of-phase treatments similarly reduced lean body mass, potentially reflecting glucocorticoid-induced muscle protein breakdown (49). It is noted that hindlimb muscles, especially in male mice, might be less sensitive to the

catabolic action of glucocorticoids than other muscle types (50,51). Glucocorticoid-induced muscle atrophy is not uniform in all muscle types and are influenced by multiple factors such as timing of treatment, sex, and muscle-specific properties (15,52).

In our study, males exhibit greater metabolic disturbances following out-of-phase betamethasone treatment, possibly due to the interaction between testosterone and glucocorticoid. Consistently, deprivation of androgen increases glucocorticoid-induced insulin resistance and fat accumulation in male mice (37), highlighting the intersection of sex hormones and glucocorticoid signaling in metabolic outcomes. Moreover, time of glucocorticoid administration has prominent effects on lipid metabolism and behavioral resultants in rats (53). Although we did not observe significant difference between in-phase and out-of-phase betamethasone treatment on lipid metabolism, which could be partly attributed to the species difference and variation of specific glucocorticoids, our findings are in line with the observation that in-phase delivery of glucocorticoids causes fewer metabolic disturbances, suggesting that timing-based strategy may alleviate certain side effects of chronic glucocorticoid exposure.

Conclusion and Future Directions

In this thesis, we expand knowledge of glucocorticoid signaling in metabolic diseases by pointing out differences between sexes, the function of circadian rhythms, and investigation into the therapeutic potential of modulation of GR. Indeed, all of our studies uniformly showed that exposure to glucocorticoids results in muscle atrophy and metabolic dysfunction, but outcomes were highly divergent between males and females. The stronger transcriptional response in male mice and more severe functional impairments in female mice underlines the necessity for further investigation that govern these sex-specific responses. Androgen signaling was shown to provide some protective effects against glucocorticoid-induced muscle wasting in males, whereas the relatively weaker influence of estrogen on muscle maintenance in females suggests that sex hormones play distinct modulatory roles in the effects of glucocorticoids.

Further studies will be necessary to investigate the molecular pathways underlying sex differences in glucocorticoid response. Exploring how androgen and estrogen receptors interact with the GR in different tissues is an important requirement for developing sex-specific therapies. Moreover, further study is required to delineate non-genomic pathways, epigenetic regulation, and tissue-specific receptor dynamics that mediate these differences, and many of these aspects were not explored or discussed in detail in this thesis.

In **chapter 3**, we investigated the timing of glucocorticoid administration to explored the complexity in glucocorticoid therapy. Glucocorticoids

administered during the active phase of the circadian cycle in mice (morning) resulted in less metabolic disturbance compared to glucocorticoid treatment in the inactive phase of the circadian cycle (evening). This finding points toward a role of circadian biology in the outcome of glucocorticoid therapy, particularly with regard to glucose metabolism. These data suggest that optimization of the timing of glucocorticoid administration might reduce side effects such as insulin resistance and hyperglycemia, which are common in patients undergoing long-term glucocorticoid treatment. Future clinical research requires to translate these preclinical observations into humans for identification of optimum treatment schedules to reduce metabolic adverse effects.

Our study focused on relatively short-term glucocorticoid exposure, and the long-term impact of chronic glucocorticoid use on muscle function, glucose metabolism and general metabolic health are poorly understood. Longitudinal studies which follow glucocorticoid treatment over an extended period of time especially in aging populations are needed, to determine if early metabolic disturbances lead to irreversible changes in humans or the body could adapt over time. This might also elucidate how intermittent versus continuous glucocorticoid therapy affects long-term metabolic outcomes.

Targeting glucocorticoid signaling is also a potential therapeutic strategy for metabolic disorders. In **Chapter 4,** the role of GR antagonism in the PCOS model was discussed. Although GR antagonism had only minor effects on lipid accumulation, it did improve glucose metabolism through early administration in disease development. These findings suggest that timely intervention in hyperandrogenic states may prevent or attenuate many of the metabolic disturbances that occur as a result of excess androgen and glucocorticoid signaling. However, the observation that therapeutic GR antagonism was less effective after metabolic dysfunction had fully developed underlines the early diagnosis and intervention in metabolic diseases is important. It also suggests that other compensatory pathways including androgen signaling may predominate at the late-stage disease, rendering GR antagonism less effective. This now raises questions about combination therapies that target both GR and androgen receptors in disorders such as PCOS in which both pathways are dysregulated.

Personalization of glucocorticoid therapy was one of the important takeaways from this thesis. The dosage of glucocorticoid treatment should consider sex, circadian rhythms, and hormonal status. Biomarkers predicting individual susceptibility to glucocorticoid-induced side effects may be explored in future research. For instance, measuring androgen or estrogen levels and assessing circadian rhythm markers could help clinicians identify the ideal timing of glucocorticoid administration for each patient. Although this thesis investigated glucocorticoid signaling in animal models, it is crucial to translate these findings to human contexts. Future studies should explore whether sex differences and circadian influences on glucocorticoid metabolism affect human physiology or

behavior. Clinical trials incorporating both sex and timing in glucocorticoid treatment regimens will be essential to optimizing therapeutic outcomes across populations.

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6

English Summary

Samenvatting

List of publications

Curriculum vitae

Acknowledgement

ENGLISH SUMMARY

Glucocorticoids are essential modulators of the stress response and metabolic homeostasis. However, their long-term elevation can lead to many adverse effects, including muscle atrophy, glucose intolerance and obesity. This dissertation addresses differential aspects of glucocorticoid signaling in metabolic diseases, focusing on how sex differences and circadian rhythm influence glucocorticoid effects. We focused on how GR signaling differs between sexes and how circadian timing modulates glucocorticoid-induced metabolic dysregulation.

In **Chapter 1,** a general introduction to the physiological importance of glucocorticoid signaling and the pathological outcome arising from its dysregulation was given. This chapter points out how widely synthetic glucocorticoids are used in the medical treatment of a wide range of inflammatory and autoimmune diseases and that their long-term use causes metabolic side effects. It also outlines that understanding the involvement of sex-specific differences and the circadian rhythm may improve therapeutic strategies.

In **Chapter 2**, we investigate sex differences in glucocorticoid-induced muscle atrophy using a mouse model. Muscle atrophy has been well documented in patients with prolonged use of synthetic glucocorticoids and Cushing Syndrome. We found that female mice show greater loss in muscle function after chronic exposure to corticosterone as compared to male mice, while both sexes exhibit similar muscle mass loss. Transcriptomic analysis reveals that male skeletal muscle undergoes more pronounced transcriptional changes as compared to female muscle, suggesting that sex hormones including androgens may play a protective role. These findings highlight the importance of sex in glucocorticoid-induced muscle atrophy.

The circadian rhythm refers to the biological processes that occur within a cycle of 24 hours. In humans, glucocorticoids levels peak in the early morning, and the timing of administration has significant biological consequences. **Chapter 3** highlights that the circadian rhythm affects the outcome of synthetic glucocorticoid treatment. We found that administering synthetic glucocorticoids at te wrong time of the day (misaligned with the circadian rhythm) exacerbated insulin resistance and hyperinsulinemia. In contrast, the administration of glucocorticoids aligned with the endogenous circadian rhythm results in fewer metabolic side effects. These findings suggest that glucocorticoid chronotherapy can potentially minimize metabolic complications and thereby improving treatment outcomes.

Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder in women, which is characterized by increased levels of androgens like testosterone in women, and by symptoms including insulin resistance and obesity. In **Chapter 4**, we investigated if glucocorticoid signaling plays a role in the metabolic symptoms associated with PCOS. We found that chronic exposure of female mice to androgens resulted in hyperglycemia and glucose intolerance. Androgens also upregulate the glucocorticoid receptor, and other factors involved in glucocorticoid signaling. For this reason we investigated if blockade of the glucocorticoid receptor is a promising approach for PCOS, and we found that preventive but not therapeutic treatment mitigated the metabolic abnormalities associated with PCOS. This study supports recent findings indicating significant overlap between androgen and glucocorticoid signaling pathways in various metabolic tissues and shows a possible role of glucocorticoids in PCOS

Chapter 5 is the general discussion in which the implications of the studies in this thesis are discussed. This chapter underlines the importance of considering sex and timing in glucocorticoid therapies to minimize adversity and maximize therapeutic efficacy. Further studies will be necessary for the molecular mechanisms which determine sex differences in the glucocorticoid sensitivity in metabolic tissues. This thesis contributes to our understanding if the complex interactions between glucocorticoid signaling, sex steroids, and circadian rhythm. These studies highlight the sexually dimorphic responses and the critical role of treatment timing, offering valuable insights for the optimizing glucocorticoid therapies and provide important guidance for developing novel strategies in the management of metabolic diseases.

SAMENVATTING

Glucocorticoïden zijn essentiële modulatoren van de stressrespons en metabole homeostase. Langdurige verhoging van glucocorticoïdconcentratie kan echter leiden tot veel nadelige effecten, waaronder spieratrofie, (pre)diabetes en obesitas. Dit proefschrift behandelt verschillende aspecten van glucocorticoïdsignalering in metabole ziekten, met een focus op hoe sekseverschillen en het circadiane ritme de effecten van glucocorticoïden beïnvloeden.

In **hoofdstuk 1** wordt een algemene inleiding gegeven over het fysiologische belang van glucocorticoïdsignalering en over de pathologische gevolgen van ontregeling van glucocorticoïdsignalering. Synthetische glucocorticoïden worden veel gebruikt voor de behandeling van ontstekings- en auto-immuunziekten. Het is echter zo dat het langdurige gebruik van glucocorticoïden veel metabole bijwerkingen kan geven, waaronder spieratrofie, insulineresistentie en het metabool syndroom.

In **hoofdstuk 2** onderzoeken we sekseverschillen in door glucocorticoïden veroorzaakte spieratrofie met behulp van een muismodel. Spieratrofie komt veel voor bij langdurig gebruik van synthetische glucocorticoïden, en in patiënten met het syndroom van Cushing. De resultaten van dit onderzoek tonen aan dat vrouwelijke muizen een groter verlies in spierfunctie vertonen na chronische blootstelling aan corticosteron, terwijl beide geslachten een vergelijkbaar verlies van spiermassa vertonen. Analyses van genexpressie laten zien dat mannelijk spierweefsel na blootstelling aan glucocorticoïden meer uitgesproken transcriptieverschillen vertoont in vergelijking met vrouwelijk spierweefsel. Dit suggereert dat geslachtshormonen zoals androgenen mogelijk een beschermende rol spelen in dit proces. Deze bevindingen onderstrepen het belang van sekse-specifieke behandelingen bij spieratrofie veroorzaakt door glucocorticoïden.

Het circadiane ritme verwijst naar de biologische processen die plaatsvinden binnen een cyclus van 24 uur. Bij mensen pieken de glucocorticoïdspiegels in de vroege ochtend, en het tijdstip van toediening heeft aanzienlijke biologische gevolgen. **Hoofdstuk 3** belicht bevindingen over hoe het circadiane ritme de resultaten van glucocorticoïdbehandelingen beïnvloeden. We ontdekten dat de toediening van synthetische glucocorticoïden op verschillende tijdstippen van de dag het glucosemetabolisme verstoorde, wat leidde tot insulineresistentie, voornamelijk wanneer de behandeling werd gegeven tijdens de inactieve periode. Daarentegen resulteerde toediening van glucocorticoïden afgestemd op het endogene circadiane ritme in minder metabole bijwerkingen. Deze bevindingen suggereren dat chronotherapie de metabole complicaties van glucocorticoïdbehandeling kan minimaliseren.

Polycysteus ovarium syndroom of PCOS is een veelvoorkomende endocriene aandoening bij vrouwen, die functioneel wordt gekenmerkt door een verhoogde androgeen productie en door insulineresistentie en obesitas. In **hoofdstuk 4** wordt de rol van glucocorticoïden onderzocht in de metabole symptomen van PCOS. We ontdekten dat chronische blootstelling aan androgenen resulteerde in verhoogde expressie van de glucocorticoïd receptor, en andere factoren betrokken in glucocorticoïd signalering. Remming van glucocorticoïd signalering in een muismodel gaf een verbetering op een aantal metabole symptomen van PCOS, met name glucosemetabolisme. Deze studie ondersteunt recente bevindingen die wijzen op overlap tussen de signaleringsroutes van androgenen en glucocorticoïden in verschillende metabole weefsels. Daarnaast bieden deze bevindingen een nieuw perspectief op hoe glucocorticoïden betrokken kunnen zijn bij de metabole symptomen bij vrouwen met PCOS.

Hoofdstuk 5 is de algemene discussie, waarin de bevindingen uit de voorgaande hoofdstukken worden samengevat en hun implicaties voor verder onderzoek en therapieën worden besproken. Dit hoofdstuk benadrukt het belang van het in overweging nemen van sekse en timing bij glucocorticoïdtherapieën, met name om bijwerkingen te minimaliseren en de therapeutische effectiviteit te maximaliseren. Verdere studies naar de moleculaire mechanismen die sekseverschillen in glucocorticoïdgevoeligheid in metabole weefsels bepalen zullen noodzakelijk zijn. Dit proefschrift draagt dus bij aan ons begrip van de complexe interacties tussen glucocorticoïdsignalering, geslachtshormonen en het circadiaan ritme.

LIST OF PUBLICATIONS

1. **Sheng Li**, Sen Zhang, Patrick C.N. Rensen, Onno C. Meijer, Sander Kooijman, Jan Kroon. Out-of-phase treatment with the synthetic glucocorticoid betamethasone disturbs glucose metabolism in mice. **Life Sciences 2024**; 357: 123080.
2. **Sheng Li**, Zhixiong Ying, Max Gentenaar, Patrick C.N. Rensen, Sander Kooijman, Jenny A. Visser, Onno C. Meijer, Jan Kroon. Glucocorticoid Receptor Antagonism Improves Glucose Metabolism in a Mouse Model of Polycystic Ovary Syndrome. **J Endocr Soc 2024**; 8:1.
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CURRICULUM VITAE

Sheng Li was born on April 21, 1992, in Ulanhot, Inner Mongolia, China. In June 2014, he earned his BSc degree in Animal Science from Jilin University. After graduation, he worked as a technician at Charoen Pokphand Group in Shenyang, China. In December 2014, he was promoted to Technical Director, where he was responsible for providing technical support to breeders and conducting internal training sessions. Three months later, he resigned to prepare for postgraduate entrance exams.

In September 2016, Sheng began a three-year master's program in Animal Nutrition and Feed Science at Jilin University in Changchun, China. His research, titled "Identification and Analysis of Long Non-Coding RNAs by Whole-Transcriptome Sequencing in Porcine Preadipocytes Induced by BMP2 (Bone Morphogenetic Protein 2)," was supervised by Prof. Dr. Jiabao Zhang and Prof. Dr. Chengzhen Chen. He published two first-author scientific articles and earned his MSc degree in 2019. That same year, Sheng was awarded a scholarship from the China Scholarship Council (CSC) and joined the research group of Prof. Dr. Onno Meijer in the Department of Internal Medicine, Division of Endocrinology, at Leiden University Medical Center.

In February 2020, Sheng began his PhD program titled "Glucocorticoid Signaling in Metabolic Diseases" under the supervision of Prof. Dr. Onno Meijer and Dr. Jan Kroon. His research focused on the differential impact of glucocorticoid receptor (GR) signaling between sexes and the role of circadian timing in mitigating glucocorticoid-associated metabolic dysregulation. He also explored the potential therapeutic role of GR in polycystic ovary syndrome (PCOS) using rodent models. His findings offer new insights into how GR modulation may improve metabolic outcomes in patients. The results of his research are presented in this thesis.

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